Cell cycle-dependent modification of Pot1 and its effects on telomere function

Vitaliy Kuznetsov

Thesis submitted towards the degree of Doctor of Philosophy

University College of London

Department of Biology

London, WC1E 6BT

The program of research was carried out

at Cancer Research U.K. Laboratory of Telomere Biology

44 Lincoln's Inn Fields, London, U.K. WC2A 3PX

Supervisor: Dr Julia Promisel Cooper

September 2008

I, Vitaliy Kuznetsov, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Telomere functions are tightly controlled throughout the cell cycle to allow telomerase access while suppressing a *bona fide* DNA damage response (DDR) at linear chromosome ends. However, the mechanisms that link cell cycle progression with telomere functions are largely unknown. Here we show that a key S-phase kinase, DDK (Dbf4-dependent protein kinase), phosphorylates the telomere binding protein Pot1, and that this phosphorylation is crucial for DNA damage checkpoint inactivation, the suppression of homologous recombination (HR) at telomeres, and the prevention of telomere loss. DDK phosphorylates Pot1 in a very conserved region of its most amino-terminal-proximal OB fold, suggesting that this regulation of telomere function may be widely conserved.

Mutation of Pot1 phosphorylation sites leads to telomerase independent telomere maintenance through constant HR, as well as a dependence of telomere maintenance proteins involved in checkpoint activation and HR. These results uncover a novel and important link between DDR suppression and telomere maintenance. The failure in Pot1 phosphorylation and DDR inactivation could potentially lead to uncontrolled cell proliferation without a requirement for telomerase by switching cells to HR dependent telomere homeostasis. In mammals this could result in ALT (Alternative Lengthening of Telomeres), a recombination dependent mode of telomere maintenance, uncontrolled cell proliferation and cancer.

CONTENTS

ACKNOWLEDGEMENTS

There too many people to thank for making this work possible. I would like to thank to all my lab mates for making our laboratory the best place in the world for my PhD. The supportive atmosphere there was keeping me constantly inspired.

And of course thank to Julie, for having faith in me, for teaching me how to think and for giving us the example of how to be a real scientist and just a great person. With Julie I understood that science is not just a result and PhD is not just a thesis, but more the state of your personal believes. Julie was able to keep in me the ability to wonder while thinking about scientific question. To do science is nothing else, but being able to wonder and being motivated by selfish desire to know - is what I've studied with Julie.

And of course all these wouldn't be possible without constant support of my family. I thank for Lesia and Andrey for their love and encouragement, for being with me and giving my live a real sense and meaning. I also thank to my mum and granny for bringing me up as who I am now.

ABBREVIATIONS

a.a.: amino acids
ATP: adenosine 5′-triphosphate
BSA: bovine serum albumin
bp: base pairs
Ccq1: coiled-coil quantitatively enriched protein 1. Component of *S. pombe* Pot1 complex
CDK: cycling dependent kinase
DDK: Dbf4 dependent kinase
DDR: DNA damage response
DAPI: 4′-6-diamidino-2-phenylindole
DIC: differential interference contrast
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
ssDNA: single stranded DNA
dsDNA: double stranded DNA
DSBs: double strand breaks
DTT: dithiothreitol
2D-PGE: two-dimensional protein gel electrophoresis
dATP: deoxyadenosine 5′-triphosphate
dCTP: deoxycytosine 5′-triphosphate
ddH_2O: double distilled water
dNTPs: deoxynucleotide 5′-triphosphate
EDTA: ethylenediaminetetraacetic acid
EMM: Edinburgh minimal media
EtOH: ethanol
G_1: gap phase one
G_2: gap phase two
GFP: green fluorescent protein
HA: hemagglutin epitop
HR: homologous recombination
hrs: hours
HU: hydroxyurea
IP: immunoprecipitation
kan: kanamyacin
kb: kilobases
kDa: kilodalton
mg: milligram
min: minutes
ml: millilitre
µl: microliter
µM: micromolar
MMS: methylmethane sulfonate
ng: nanogram
NHEJ: nonhomologous end-joining
OB fold: oligonucleotide/oligosaccharide binding fold

O.D.: optical density
ORF: open reading frame
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEG: polyethyleneglycol
PFGE: pulsed field gel electrophoresis
PMSF: phenilmethylsulphonyl fluoride
Pot1: protection of telomeres protein 1
Poz1: Pot1-associated in *Schizosaccharomyces pombe*. Component of *S. pombe* Pot1 complex
PVDF: polyvinylidene fluoride membrane
Rad3: fission yeast homolog of ATR
rDNA: ribosomal deoxyribonucleic acid
RNA: ribonucleic acid
rt: room temp
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide electrophoresis
Tel1: fission yeast homolog of ATM
Tpz1: TPP1 homolog in *Schizosaccharomyces pombe*. Component of *S. pombe* Pot1 complex
S-phase: synthesis phase
SSC: saline-sodium citrate buffer
TBS: tris buffered saline
TBS-T: tris buffered saline with Tween
TCA: trichloroacetic acid
ts: temperature sensitive
wt: wild type
YES: yeast extract supplements media
°C: degrees Celsius

LIST OF TABLES AND FIGURES

1 Introduction

The telomere is a specific region at the end of a linear chromosome. The existence of telomeres as some kind of structure at the ends of eukaryotic chromosomes first was only a theoretical idea. This idea came from two completely independent observations; one was the discovery of the genetic stability of natural chromosome ends, and the other arose from consideration of the chromosome end replication problem – the inability of known DNA polymerases to fully copy the ends of linear DNA molecules. These two chromosome end puzzles have been resolved by proposing that there is a specific structure on the natural chromosome ends that ensures chromosome stability and allows them to fully replicate in every cell division cycle.

After the identification of DNA structure it became clear that replication of the end of the DNA lagging strand would not be an easy task. In 1972, Watson mentioned that there is no simple way for 3′ to 5′ lagging strand growth to reach the 3′ end of its template (Watson, 1972). He correctly predicted that the lagging strand of linear chromosomes copied by the semi-conservative replication machinery would not be fully replicated, because of the removal of the RNA primer of the most distal Okazaki fragment and subsequent loss of genetic material every cell doubling. In other words, he described a so called “end-replication problem” (Watson, 1972). At the same time, A. M. Olovnikov proposed the “marginotomy theory of ageing”, suggesting that “telogenes” located at opposite ends of DNA molecules carry no genetic information and fulfil a buffer function to solve an end replication

problem; these ends could be shortened with each round of replication without any consequences for the cell until this shortening reaches an essential gene (Olovnikov, 1972, 1973). He stated that these telogenes are stochastically shortened during each mitotic cycle, providing a mechanism for ageing. Thus the telomere theory of aging was born. It was used to describe why human cells derived from embryonic tissues can only divide about 50 times. According to Olovnikov, it was determined by the length of the telomeres and the rate of telomere shortening; this has later been proved experimentally (Harley et al., 1990; Lundblad and Szostak, 1989). But final proof that telomere length was rate-limiting for indefinite cell proliferation was shown much later in experiments where the inducible elongation of telomeres in senescent cells induced indefinite life span (Bodnar et al., 1998).

The problem of chromosome ends stability was realised even earlier than the end replication problem. Muller and McClintock, working with Drosophila and maize independently, came to the conclusion that natural chromosome ends should differ from X-ray-induced chromosome breaks. After subjecting fruit flies and maize to X-rays, different products of modified chromosomes were recovered: inversions, translocations, duplications, formation of circular chromosomes, and other anomalies. The appearance of these genetic rearrangements was explained as the result of rejoining two broken chromosome ends, which by becoming joined in aberrant arrangements, created the rearranged chromosomes. These observations open a new question. Why are the natural chromosome ends stable and not involved in such rearrangements, in contrast to chromosome ends generated

from chromosome breakage? Muller called these natural chromosome ends telomeres –specific genes that cannot be lost and that determine the unique stability of natural ends (Muller, 1938). McClintock also explained chromosome rearrangements in maize as a natural feature of broken ends, differentiating them from natural chromosome ends which were protected from such events (McClintock and Hill, 1931). Moreover, McClintock reported that a broken end can lose its tendency to fuse with other broken ends, and she concluded that the broken end can be permanently "healed", becoming as stable as normal chromosome ends (McClintock, 1939). Thus, she first described the idea of *de novo* telomere addition to chromosome breaks.

After these observations, it becomes obvious that there is a specific structure at the ends of the chromosomes that is very important for genomic stability. Nonetheless, the specific nature of this protective structure remained unknown until the telomere sequence was determined. The first telomere sequence was defined from amplified rDNA-containing minichromosomes from the somatic nucleus of the ciliate *Tetrahymena thermophila* (Blackburn and Gall, 1978). This protozoan organism contains about 10,000 21-kb linear minichromosomes that greatly facilitate the purification of telomeres and determination of their sequence. The telomere sequence of *Tetrahymena thermophila* is composed of tandem GGGGTT repeats with the G-rich DNA strand ending with a 3′-OH. Subsequently, similar telomere sequences were determined from other organisms.

The next milestone in understanding telomere biology came from discovery of the telomeric DNA synthesizing activity, which was first identified

in *Tetrahymena thermophila* mated cells (Greider and Blackburn, 1985). The enzyme responsible for this activity was called telomerase. It was shown that telomerase contains a RNA component and uses this RNA as a template for the synthesis of telomeric repeats (Greider and Blackburn, 1987, 1989).

At the same time, the catalytic reverse transcriptase component of telomerase, Est2p, was isolated from *S. cerevisiae* in a genetic screen for mutants that would abolish telomerase activity (Lundblad and Szostak, 1989), but it would be several years before the realization that Est2p was indeed the catalytic subunit. This realization awaited the biochemical purification of telomerase from the ciliate *Euplotes aediculatis* (Lingner and Cech, 1996). These investigators found that the purified protein contained homology to Est2p, and that, as predicted by the existence of the telomerase RNA subunit, both *Euplotes* telomerase and Est2p contained reverse transcriptase domains. Telomerase deletion in yeast leads to a progressive decrease in telomere length as well as an increased frequency of chromosome loss. The mutants were not immediately inviable; instead, they had a senescence phenotype, due to the gradual loss of sequences essential for telomere function, leading to a progressive decrease in chromosomal stability and subsequent cell death. This discovery finally confirms that Muller and McClintock were absolutely right – the telomere is an essential component chromosome structure that makes it different from the chromosome breaks. Telomere loss in telomerase negative cells (ie, most human somatic cells) leads to chromosome de-protection, genetic instability and cell death.

1.1 Telomere protects natural chromosome ends from DNA damage response.

Telomeres are the specific chromatin structures that protect chromosome ends from being recognised as damage induced DNA double strand breaks (DSBs). Telomeres suppress a number of mechanisms that would normally be connected with processing of DSBs. Telomeres accomplish this anti-DNA damage response function by recruiting specific telomeric proteins that directly or indirectly interact with telomere DNA sequences. Telomere DNA comprises short repetitive sequences with a protruding 3′- single stranded overhang at the end of the chromosomes. Placing a stretch of telomere repeats in close proximity to an induced DSB suppresses the ability of that DSB to confer a *bona fide* DNA damage response and checkpoint activation. Thus the maintenance of telomere repetitive sequence at the chromosomes ends is crucial to ensure genome stability of organisms with linear chromosomes.

1.1.1 Telomeric proteins and their complexes

The telomeric repeat sequences are essential for many of the key biological features of telomeres because of their ability to recruit telomere-binding factors. A six-protein complex is thought to protect the telomeres of human chromosomes and a very similar complex is found at *S. pombe* telomeres. Mammalian TRF1 and TRF2 and their *S. pombe* homolog Taz1 directly bind double stranded telomeric DNA (Broccoli et al., 1997; Chong et al., 1995; Cooper et al., 1997), and both Taz1 and TRF2 recruit Rap1

(Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Li et al., 2000). POT1 directly binds the single-stranded 3' overhang at the very chromosome end (Baumann and Cech, 2001; Lei et al., 2003; Loayza and De Lange, 2003) and forms a complex with TPP1/Tpz1 (Houghtaling et al., 2004; Liu et al., 2004; Miyoshi et al., 2008; Ye et al., 2004). The TRFs/Taz1 and Pot1 complexes interact with each other *via* the linking proteins TIN2 and Poz1: mammalian TIN2 interacts with TPP1 and TRF1/2 (Kim et al., 2004; O'Connor et al., 2006) and *S. pombe* Poz1 brings the Taz1/Rap1 complex and the Tpz1/Pot1 complex together (Miyoshi et al., 2008).

In both *S. pombe* and mammals, Pot1 form a complex with TPP1/Tpz1 protein (formerly named PTOP/PIP1/TINT1) (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004). This complex appears to be very conserved throughout evolution and important for Pot1 function and recruitment to telomeres. Pot1/TPP1 closely resembles the ciliate TEBPα/TEBPβ complex (Xin et al., 2007), which binds to the single stranded overhangs of *Oxytricha nova* telomeres (Gottschling and Zakian, 1986). Pot1 and TEBPα have similar domain structures, with multiple OB folds organized in a similar way; likewise, TPP1 and TEBPβ have similar crystal structures (Lei et al., 2003; Wang et al., 2007).

The telomere protein complex and its integrity are important for the ability of telomeres to suppress DNA damage response. Deletion of different components of telomere protein complex leads to recognition of chromosome ends as a breaks and activation of checkpoint and DNA repair pathways. In *S. pombe*, deletion of the gene encoding Taz1 leads to de-protection of the

telomeres. *taz1Δ* cells experience DNA ligase IV dependent telomere fusions in G_1, formed by the nonhomologous end-joining repair pathway (NHEJ) (Ferreira and Cooper, 2001). Taz1 is also important for telomere capping in G_2, in which its loss results in increased levels of homologous recombination (HR) at telomeres. Surprisingly, *taz1Δ* cells fail to activate the checkpoint response, even though *taz1Δ* telomeres are clearly recognized as DSBs. This means that different facets of the DNA damage response, like DNA damage checkpoint activation and the DNA repair pathways (HR and NHEJ), are suppressed by different mechanism at *S. pombe* telomeres.

Deletion of one of the Taz1 homologs, TRF2, from mouse cells or its inhibition with a dominant negative allele in human cells results in a robust DNA damage signal that is mediated by the ATM kinase (Celli and de Lange, 2005; Denchi and de Lange, 2007; Karlseder et al., 1999). In TFR2 -/- cells the telomeres are processed by the non-homologous end-joining pathway. Non-homologous end joining of telomeres can be abrogated in DNA ligase IV-deficient cells, but telomeres are still recognized as sites of DNA damage, as they accumulate the DNA damage response factors 53BP1 and gamma-H2AX and activate the ATM kinase. The DNA damage signal generated by telomeres lacking TRF2 is completely abrogated when ATM is absent (Denchi and de Lange, 2007). This suggests that even in the absence of telomere fusions, TFR2 -/- telomeres are recognized as DSBs. TRF2 could also play a general role in DDR suppression, because overexpression of TRF2 can suppress the activation of the ATM kinase, even at nontelomeric sites of DNA damage (Karlseder et al., 2004).

The 3′ overhang binding proteins, like *S. pombe* and mammalian Pot1 and *S. cerevisiae* Cdc13, also have crucial roles in protecting chromosome ends. These factors bind single-stranded DNA through a conserved OB (oligonucleotide/oligosaccharide binding) fold domain (Mitton-Fry et al. 2002; Lei et al. 2003) and believed to play crucial roles in preventing the inappropriate triggering of the DDR by the telomere. Indeed, in *S. cerevisiae* lacking functional Cdc13, the CA-rich telomeric strand is rapidly degraded, leading to RAD9-dependent cell-cycle arrest (Garvik et al. 1995; see below).

DNA damage response at telomeres also can be induced by deletion of POT1 (Denchi and de Lange, 2007; He et al., 2006; Hockemeyer et al., 2005; Wu et al., 2006). Simultaneous deletion of both POT1 paralogs from mouse cells leads to the accumulation of 53BP1 and gamma-H2AX foci as well as cell cycle arrest (Churikov et al., 2006; Jacob et al., 2007). This response is dependent on ATR and subsequent phosphorylation of the Chk1, and Chk2 downstream kinases. The ability of POT1 to repress the ATR kinase-dependent DNA damage response depends on its association with TPP1 (Hockemeyer et al., 2007). TPP1 functions to recruit POT1 to telomeres (Wang et al., 2007). Inhibition of TPP1 gives rise to a DNA damage response at telomeres that is indistinguishable from the response to POT1 deletion (Denchi and de Lange, 2007; Xin et al., 2007). Likewise, the interaction between Pot1 and Tpz1/TPP1 in *S. pombe* is required for telomere protection, and its disruption leads to the *pot1Δ* phenotype (Miyoshi et al., 2008).

Telomere proteins that connect the double strand telomere-binding complex with telomere proteins on the single stranded 3′-overhang telomere region are also important for suppression of DDR. Cells with diminished TIN2 function activate DDR similarly to TRF2 deficient cells (Kim et al., 2004). TIN2 may play this role by stabilizing TRF1 and TRF2 at telomeres, or by promoting Pot1/Tpp1 complex recruitment to telomeres.

Although the integrity of the telomere protein complex is clearly important for DDR suppression at telomeres, how this suppression occurs is not completely understood. Telomere proteins could promote the formation of a specific chromatin structure at the end of chromosomes that could hide the DNA end from DNA damage response factors like RPA. The Pot1/TPP1 complex could potentially compete with RPA for telomere 3′-overhang binding. Although RPA is much more abundant than POT1/TPP1, the latter has greater affinity and sequence specificity for telomeric DNA, which may confer efficient competition with RPA for telomere binding. In addition, other components of the telomere complex may stabilize or recruit POT1/TPP1 to telomeres, or may be involved in the formation of high order telomere chromatin structure.

1.1.2 Special structures at chromosome ends that could suppress DNA damage response

In addition to binding the proteins described above, there is evidence that telomeric DNA may adopt an unusual and specific structure, the so-called T-loop. In this structure, the very end of the chromosome is folded back and the single-stranded telomeric 3-overhang is tucked into a portion of the double-stranded telomeric DNA, resulting in a three-stranded structure (Griffith et al., 1999). This conformation has been suggested to prevent telomeric ends from being recognized as DNA damage and triggering the DDR. The T-loop was proposed to be a conserved telomere structure, and T-loops have been described in trypanosomes, ciliates, plants, *Caenorhabditis elegans* and *Kluyveromyces lactis* (Cesare et al., 2008; Cesare et al., 2003; Munoz-Jordan et al., 2001; Murti and Prescott, 1999; Raices et al., 2008; Stansel et al., 2001). Nevertheless, it is still unclear whether the structure exists *in vivo* and whether it is protective or is rather an intermediate arising from inappropriate repair reactions at the telomere.

1.2 Telomere homeostasis

The inability of the conventional DNA replication machinery to fully replicate linear DNA molecules leads to progressive telomere shortening at each cell division. Telomere shortening below a critical threshold eventually will result in loss of the telomere protection function. At this point, the chromosome end is recognized as a DSB and triggers a DNA damage

response. Thus, the maintenance of telomere length homeostasis becomes a crucial task for protecting the genome from genome instability caused by dysfunctional critically short telomeres.

Two telomere-lengthening processes must be controlled to confer telomere length homeostasis. One requires telomerase, a cellular reverse transcriptase, which uses an internal RNA component as a template for the synthesis of telomere repeats. Telomerase elongates the 3′ ends of chromosomes, whereas the complementary strand is filled in by conventional DNA polymerases. Net telomere synthesis can also occur through certain recombination pathways (eg break-induced replication). Recombination based telomere maintenance occurs in the absence of telomerase and is characteristic of some telomerase negative yeast survivors and so called ALT (**a**lternative **l**engthening of **t**elomeres) surviving mammalian cells.

1.2.1 Telomerase dependent telomere maintenance

Elongation of telomeres by telomerase is controlled by two levels of regulation. First, telomerase acts at chromosome ends only during S phase. This restriction may be enabled through cell-dependent changes in telomere structure and the cell cycle-restricted assembly of active telomerase. The second level of telomere length homeostasis control involves mechanisms to measure telomere length in *cis*, which promote more efficient elongation of short telomeres by telomerase or which promote activities to shorten preferentially long telomeres.

1.2.1.1 Telomerase association with telomeres is cell cycle regulated

Telomere addition by telomerase occurs at S-phase and is coupled with semiconservative telomere replication. In *S. cerevisiae*, telomere elongation of an artificially shortened telomere coincides with semiconservative telomere replication, which occurs late in S phase, suggesting coupling between the two processes. No elongation occurs in G1 of the cell cycle or in nocodazole-arrested cells (Marcand et al., 2000). The latter finding contrasts with telomere addition to a double-stranded DNA break adjacent to a short telomeric DNA tract, which can occur in nocodazole-arrested cells in M-phase (Diede and Gottschling, 1999) and depends on functional DNA polymerases α and δ (Diede and Gottschling, 1999). Mutations in DNA polymerase α cause telomere lengthening in *S. cerevisiae* and in mouse cells (Adams and Holm, 1996; Nakamura et al., 2005). Moreover, The B subunit of DNA polymerase α physically and genetically interacts with the Cdc13p-interacting protein Stn1 (Grossi et al., 2004). In addition, Cdc13p interacts with the catalytic subunit of DNA polymerase α (Qi and Zakian, 2000).

Not only is telomerase activity restricted to late S-phase, but also the association between telomerase and telomeres is cell cycle-restricted. In *S. cerevisiae*, two separate pathways of telomerase recruitment appear to exist. Cdc13 binds the telomerase associated regulation subunit Est1, and this interaction is essential for telomerase activity (Pennock et al., 2001). Est1 preferential associates with telomeres during S-phase, thus correlating well

with the time of telomerase action (Schramke et al., 2004) (Taggart et al., 2002). A second telomerase recruitment mechanism in *S. cerevisiae* is provided in G_1 through the interaction of the telomere-binding protein Ku with telomerase (Fisher et al., 2004). Ku recruits telomerase through its specific binding of a stem-loop in TLC1 telomerase RNA (Stellwagen et al., 2003). The Ku-mediated recruitment of telomerase in G_1 may increase the local concentration of telomerase near its substrate, thus favoring the assembly of telomerase with Cdc13 and the telomere 3′ end in S phase.

In fission and budding yeast, the ATM and ATR-related homologs, Tel1 and Mec1, have also been implicated in the assembly of telomerase during S phase. Simultaneous deletion of Tel1 and Mec1 in fission or budding yeast gives a senescence phenotype (Naito et al., 1998; Ritchie et al., 1999). Mec1 is recruited to telomeres during late S phase, whereas Tel1 associates with telomeres in G_1 (Takata et al., 2004). It was also shown that Tel1 was highly enriched at short telomeres from early S through G2 phase. Tel1 binding was required for the preferential binding of telomerase to short telomeres. These data suggest that Tel1 targets telomerase to the DNA ends most in need of extension (Arneric and Lingner, 2007; Sabourin et al., 2007).

In humans, semiconservative DNA replication of telomeres occurs throughout S phase (Hagen et al., 1990; Wright et al., 1999). Based on *in situ* hybridization studies with oligonucleotide probes, human telomeres are more accessible in S phase (Jady et al., 2006) and they seem to lose part of their protective structure at the end of S phase, becoming transiently recognized as DNA damage in G_2 (Verdun et al., 2005).

Cytological analyses indicate S-phase-specific assembly of human telomerase with telomeres. (Jady et al., 2006; Tomlinson et al., 2006). Human telomerase RNA (hTR) was detected during interphase in Cajal-bodies, dynamic structures involved in the biogenesis of small ribonucleoprotein complexes (RNPs). Human TERT (hTERT), on the other hand, was present in nucleoplasmic foci of unknown composition (Tomlinson et al., 2006). During S phase, hTERT and hTR both localized to foci adjacent to Cajal bodies. Furthermore, some Cajal bodies, hTERT and hTR were also found in association with telomeres during S phase. These experiments suggest cell cycle-dependent assembly of active telomerase and cell cycle-dependent association of telomerase with telomeres.

1.2.1.2 Counting mechanism of telomere length homeostasis

Evidence for telomere length regulation in *cis* became apparent in experiments in which a linear plasmid containing terminal telomeric repeat sequences from *Tetrahymena* was transformed and maintained in *S. cerevisiae* cells (Szostak and Blackburn, 1982). Approximately 200 bp of new yeast telomeric sequence was added *de novo* to the end of the linear molecule, thus giving a telomere length of natural *S. cerevisiae* chromosomes (Shampay et al. 1984).

The telomere length homeostasis was explained by the so-called protein counting mechanism (Marcand et al., 1997). This model suggests that telomere double stranded binding complexes have an inhibitory effect on the ability of

telomerase to elongate telomeres. Telomere length depends on the number of targeted molecules, consistent with a feedback mechanism of telomere length regulation. This model was first confirmed in *S. cerevisiae*. The involvement of the Rap1 telomere-binding protein for *cis*-regulation of telomere length was demonstrated by targeting different numbers of Rap1 carboxyl termini *via* a heterologous DNA-binding domain to a model telomere (Marcand et al., 1997).

This protein counting mechanism for telomere length regulation was also recapitulated in human cells with TRF1. Moreover, subtelomeric tethering experiments showed that TRF1 indeed acts in *cis* (Ancelin et al., 2002). As predicted by the model, increasing the amount of telomere bound TRF1 leads to progressive telomere shortening, whereas a dominant-negative form of TRF1 that removes the endogenous TRF1 from telomeres induces telomere elongation (van Steensel and de Lange, 1997). Similarly, mammalian TIN2, TPP1, TRF2, Rap1 and POT1 behave as negative regulators of telomerase-mediated telomere elongation (Houghtaling et al., 2004; Kim et al., 2004; Liu et al., 2004; Smogorzewska et al., 2000; Ye et al., 2004).

An assay that allows analysis of the elongation of single telomere molecules in yeast demonstrates that telomerase does not act on every telomere in each cell cycle, but rather exhibits an increasing preference for telomeres as their lengths decline (Teixeira et al., 2004). This analysis therefore suggests that telomere length homeostasis is achieved *via* a switch between 'extendible' and 'nonextendible' states. These states could be regulated by Tel1 kinase. It was also shown that Tel1 was highly enriched at short telomeres from

early S through G2 phase. Tel1 binding was required for preferential binding of telomerase to short telomeres. These data suggest that Tel1 targets telomerase to the DNA ends most in need of extension (Arneric and Lingner, 2007; Sabourin et al., 2007).

1.2.2 Telomere maintenance without telomerase

The majority of cells die in the absence of telomerase after telomere shortening leads to senescence. However, a small subpopulation of *S. cerevisiae* escape the lethal consequences of telomerase loss (Lundblad and Blackburn, 1993). The survivors recovered from telomerase deleted strains display global amplification and rearrangements of both telomeric and sub-telomeric repeat sequences. These extensive rearrangements are a result of recombination in sub-telomeric or telomeric repeats, as the appearance of survivors was blocked in cells lacking Rad52, which is responsible for the majority of homologous recombination events in *S. cerevisiae* (Teng and Zakian, 1999).

Recombination-based telomere maintenance in the absence of telomerase is also characteristic of *S. pombe* linear chromosome-containing survivors. The frequency of linear survivors is enhanced upon deletion of Taz1, suggesting that Taz1 suppresses telomeric recombination (Nakamura et al., 1998; Subramanian et al., 2008). Likewise, the appearance of recombination-based survivors suggests that critically short telomeres are highly

recombinogenic (Teng et al., 2000), perhaps due to the loss of binding sites for proteins like Taz1.

In immortalized mammalian cell lines, telomere maintenance in the absence of telomerase occurs by a mechanism known as ALT (Alternative Lengthening of telomeres) (Bryan et al., 1995). The mechanism of ALT is not well understood, but indirect evidence suggests it is also based on recombination mechanisms. The ALT telomeres are very heterogeneous, display a high rate of post-replicative sister chromatid exchange and show higher levels of association with proteins involved in homologous recombination, consistent with a recombinational mode of telomere maintenance (Bailey et al., 2004; Baird et al., 2000; Dunham et al., 2000).

1.3 Pot1 is an important telomere end-binding protein that controls genome stability

1.3.1 Pot1 is a highly conserved telomere binding protein

Pot1 (protection of telomeres) was first discovered in *Schizosaccharomyces pombe* using bioinformatics tools as a homolog of the ciliate *Oxytricha nova* telomere ssDNA binding protein TEBPα (Baumann and Cech, 2001). In *S. pombe*, Pot1 has a critical role in telomere capping, and Pot1 deletion results in rapid and complete telomere loss and cell death, with survival occurring only *via* chromosome circularization (Baumann and Cech, 2001), suggesting that Pot1 has an essential function in the maintenance of

linear chromosomes structure. Pot1 was also characterised in mammals (Baumann and Cech, 2001; Baumann et al., 2002), chicken (Wei and Price, 2004), *Aspergillus* (Pitt et al., 2004), *Arabidopsis* (Shakirov et al., 2005; Tani and Murata, 2005) and *C. elegans* (Raices et al., 2008) and appears to be important telomere component in eukaryotes.

Characterisation of the phenotypes generated by Pot1 disruption in a variety of organisms suggests that the function of Pot1 is highly conserved. Pot1 deletions cause defects in chromosome end structure (Hockemeyer et al., 2005), accumulation of G-rich overhang signal (Churikov et al., 2006) and massive C-rich strand resection followed by complete loss of telomeres in *S. pombe* (Chris Pitt, unpublished data). Pot1 has also been shown to play roles in telomerase recruitment, protecting telomeres from the DNA damage response (He et al., 2006; Hockemeyer et al., 2005; Wu et al., 2006) and checkpoint activation (Churikov et al., 2006; Jacob et al., 2007).

Pot1 interacts with G-rich ssDNA telomeric DNA through its N-terminal oligonucleotide/oligosaccharide-binding folds (OB folds). The OB fold comprises a five-stranded β sheet coiled to form a closed β barrel and capped by an α helix located between the third and fourth β strands (Theobald et al., 2003). Like TEBPα, Pot1 contains two OB folds in its N terminus (Baumann and Cech, 2001; Lei et al., 2004) and probably one OB fold at its C-terminus (Theobald and Wuttke, 2004). This differs from the domain arrangement in from the budding yeast ss telomere binding protein Cdc13, which contains two C-terminal OB folds.

The OB fold is a highly conserved ssDNA-binding motif. Other OB-fold domain-containing proteins include the three subunits of replication protein A (RPA) (Bochkarev et al., 1999), several types of DNA helicases and ligases, and the breast cancer susceptibility gene 2 (BRCA2) protein (Bochkarev and Bochkareva, 2004; Theobald et al., 2003; Yang et al., 2002). Among other telomere proteins, the OB fold is found in the telomerase regulation subunit Est3 (Lee et al., 2008; Young Yu et al., 2008), the Pot1 binding partner Tpp1 (a homolog of ciliate TEBPβ) (Miyoshi et al., 2008; Wang et al., 2007; Xin et al., 2007) and the Stn1 and Ten1 like proteins that are important for telomere protection in yeast (Martin et al., 2007).

1.3.2 Pot1 suppresses DNA damage response at telomeres

Pot1 plays an important role in suppressing DNA damage responses at telomeres. RNAi-mediated reduction of POT1 levels in human tumor cell lines induced a DDR at chromosome ends as evidenced by the appearance of telomeric DNA damage foci, yet remain protected from nonhomologous end-joining (Hockemeyer et al., 2005).

Disruption of the POT1 gene in chicken DT40 cells leads to similar defects (Churikov et al., 2006): a DNA damage response at telomeres as shown by telomeric H2AX accumulation, and rapid cell cycle arrest due to an ATM- and/or ATR-mediated checkpoint activation.

Conditional deletion of two mouse Pot1 orthologs POT1a and POT1b also results in a DNA damage signal at chromosome ends, endoreduplication,

aberrant homologous recombination at telomeres, and p53-dependent replicative senescence, although POT1a and POT1b have distinct functions (Hockemeyer et al., 2006; Wu et al., 2006). POT1a appears to repress a DNA damage signal at telomeres, whereas POT1b regulates the amount of single-stranded DNA at the telomere terminus. DNA damage response in the absence of Pot1 is dependent on ATR and phosphorylation of its downstream kinase targets Chk1 and Chk2 (Denchi and de Lange, 2007).

The ability of POT1 to repress the ATR kinase depends on its association with TPP1 (Hockemeyer et al., 2007). Inhibition of TPP1 gives rise to a DNA damage response at telomeres that is indistinguishable from the response to POT1 deletion (Denchi and de Lange, 2007; Xin et al., 2007). In addition, interaction between *S. pombe* Pot1 and Tpz1/TPP1 is required for telomere protection and it destruction yields a *pot1Δ* phenotype (Miyoshi et al., 2008).

The mechanism of DNA response inhibition by Pot1 is not understood. Pot1 and Pot1/TPP1 complex could block the binding of RPA to single stranded telomeric DNA. Alternatively, Pot1/TPP1 may recruit additional factors that suppress DNA damage response at telomeres. It was shown that in *S. pombe*, the Pot1-Tpz1/TPP1 complex binds to and recruits Ccq1, a protein that suppresses checkpoint activation and homologous recombination at telomeres (Miyoshi et al., 2008) and (Tomita, K et al., Genes and Dev., in press).

1.3.3 Role of Pot1 in telomerase recruitment and processivity

The role of Pot1 in telomere length regulation is not clear. Overexpression of full-length Pot1 resulted in lengthened telomeres in some mammalian cell types, suggesting that Pot1 is a positive regulator of telomere length (Colgin et al., 2003; Liu et al., 2004). However, overexpression of a dominant-negative C-terminal fragment of Pot1, which lacks the ability to bind to single-stranded DNA but is still recruited to telomeres through interaction with TPP1, leads to extensive telomerase-dependent telomere lengthening, implying that Pot1 serves as a negative regulator of telomere length (Loayza and De Lange, 2003). This result suggests that Pot1 transduces the negative telomere regulatory signal from the TRF1 complex to telomerase. In support of this idea, RNAi -mediated knock down of hPot1 resulted in longer telomeres (Ye et al., 2004).

How could Pot1 be both a negative and a positive regulator of telomere length? One possible explanation came from *in vitro* studies. There are two types of Pot1-telomere ssDNA interaction, one that sequesters the ssDNA from telomerase interaction and another that presents the ssDNA tail to telomerase in an accessible form, promoting telomere elongation (Kelleher et al., 2005; Lei et al., 2005). It was proposed that other factors could promote the switch between different stages *in vivo*.

The positive effect on telomerase is enhanced when a POT1-TPP1 complex is bound to DNA substrates, both when binding occurs at internal and 3′-terminal positions (Wang et al., 2007). In agreement with a role of TPP1-POT1 in the modulation of telomerase, an interaction of TPP1 with telomerase

through its OB fold domain has been reported (Xin et al., 2007). Thus, *in vitro* results indicate that POT1-TPP1 complex has a stimulatory action on telomerase. Because TPP1-POT1 binds terminal overhangs with high affinity and stimulates telomerase activity *in vitro*, it is possible that this dimeric complex can act as an on/off switch for telomerase action. In this scenario, an inhibitory function of TRF1/2-bound TPP1-POT1 would be reversed by an as yet unidentified mechanism, that could involve modification (for example, phosphorylation). Consistent with this possibility, the ciliate orthologs of TPP1-POT1 are phosphorylated *in vivo* (Paeschke et al., 2005).

Another explanation for how Pot1-TPP1 could promote telomerase recruitment came from the work on fission yeast (Miyoshi et al., 2008). In *S. pombe*, the Pot1-TPP1 interacting factor Ccq1 is implicated in the direct regulation of telomerase activity: whereas TPP1/Tpz1 immunoprecipitates telomerase activity, it fails to do so in the absence of Ccq1. Cells devoid of Ccq1 have short telomeres that are maintained by recombination, suggesting that Ccq1 might be necessary for telomerase activity (Miyoshi et al., 2008) and (Tomita, K et al., Genes and Dev., in press)**.**

S. pombe Poz1, on the other hand, inhibits telomere elongation. Taken together, these findings point to a model for telomerase regulation in fission yeast similar to the one suggested for the human system, with an overhang-binding Pot1-Tpz1-Ccq1 complex capable of recruiting telomerase and a double-stranded binding complex made of Taz1-Rif1-Rap1 playing an inhibitory role. Poz1-Pot1-Tpz1-Ccq1 might switch from an overhang-bound and telomerase permissive state to a Rap1-anchored state that would be

nonconducive to telomerase recruitment/action. Different conformations could be regulated by modification of Pot1 or other components of the Pot1 complex.

1.3.4 Regulation of Pot1 functions

The foregoing observations suggest that Pot1 has multiple functions at telomeres. Some of these functions are completely contradictory to others. On one hand, Pot1 stimulates the formation of a closed chromatin structure that protects telomeres from the DDR and inhibits telomerase, while on the other hand, Pot1 stimulates telomerase activity *in vitro* and *in vivo*. These completely opposite Pot1 functions can be rationalized by proposing that Pot1 can regulate different telomeric states: the close telomere conformation protects telomere from DNA damage response and is suppressive for telomerase, whereas open state favours telomerase action and telomere repeats addition (Kelleher et al., 2005; Lei et al., 2005; Miyoshi et al., 2008).

All these models predict that alternative telomere configuration states could be regulated by Pot1 cell cycle regulated modifications, although no Pot1 modifications have been detected so far. In our study we report the phosphorylation of Pot1 by the conserved DDK kinase. This is the first observation that could help to solve the mechanism of Pot1 regulation.

1.4 The role of DDK in maintaining genome integrity

1.4.1 Introduction: DDKs

DDKs or **D**bf4-**d**ependent **k**inases are cell cycle regulated enzymes that share several common features with cyclin-dependent protein kinases (CDKs) (Johnston et al., 1999). DDK and CDK regulation is accomplished by the controlled expression of their regulatory subunits (cyclins for CDK and Dbf4 related proteins for DDK), while their catalytic subunits are expressed throughout the cell cycle. Both CDKs and DDKs are crucial regulators of various processes during cell cycle progression, sometimes regulating the same mechanisms (like replication initiation and meiosis) and thereby building a highly coordinated architecture of cell cycle regulation control. Both DDK and CDK are regulated independently with interplays occurring at different levels, including mutual activity regulation and substrate specificity control.

Cdc7 (Hsk1 is the *S. pombe* ortholog), the catalytic subunit of DDK (Dbf4 dependent kinase) was originally isolated in the Hartwell cell division cycle genetic screen in budding yeast (Culotti and Hartwell, 1971). Cdc7 was shown to be a serine–threonine kinase that is activated very late in G1 phase (Yoon and Campbell, 1991; Yoon et al., 1993) and appears to serve as a final trigger for the synthesis of new DNA (Pasero et al., 1999). It was found that cell cycle dependent regulation of Cdc7/Hsk1 activity is accomplished by its regulatory subunit Dbf4 (Dfp1 is the *S. pombe* ortholog) (Jackson et al., 1993; Kitada et al., 1992). In contrast to the relatively constant levels of Cdc7 throughout the cell cycle, the amount of Dbf4 fluctuates, with up-regulation in

late G1 phase and high levels through S phase that persist until M phase when it is degraded by the APC (Weinreich and Stillman, 1999). Numerous Cdc7 and Dbf4 orthologs have been identified in other eukaryotic organisms: Hsk1 (Masai et al., 1995) and Dfp1 (Brown and Kelly, 1998) in fission yeast *Schizosaccharomyces pombe*; huCdc7 (Jiang and Hunter, 1997) and huDbf4 (Jiang et al., 1999; Kumagai et al., 1999) from human; muCdc7 and muDbf4 from mouse (Kim et al., 1998; Lepke et al., 1999); xeCdc7 and xeDrf1/xeDbf4 from *Xenopus* (Sato et al., 1997; Takahashi and Walter, 2005), the *Drosophila* Dfb4 ortholog Chiffon (Landis and Tower, 1999) and others. It appears that DDK kinase is universally involved in DNA replication control.

In addition to its replication initiation role, DDK has been implicated in several important processes: intra-S phase checkpoint response, recovery from replication fork arrest, S phase assembly of centromeric heterochromatin and cohesion, and regulation of Spo11 dependent DSB generation in meiosis. In our work, we uncovered a new role of DDK in controlling telomere functions thought phosphorylation of Pot1.

1.4.2 Hsk1 and Dfp1 form DDK in fission yeast

As in *S. cerevisiae* Cdc7*,* the abundance of the *S. pombe* catalytic DDK subunit, Hsk1, remains constant through the cell cycle whereas levels of the regulatory subunit Dfp1 are high in S phase, decrease greatly in G1, and suddenly return to maximal levels just prior to the initiation of DNA replication (Brown and Kelly, 1999; Takeda et al., 1999). The amount of Dfp1 is tightly

controlled on both transcriptional and translational levels. Interestingly, despite ongoing transcription, Dfp1 is not detected during G1, because during this period the Dfp1 protein is quickly degraded (Brown and Kelly, 1999; Takeda et al., 1999).

1.4.3 Mechanism of DDK action and substrate specificity

The consensus phosphorylation site for DDK is not known. This fact makes it much harder to determine phosphorylation sites in DDK substrates. Only little information about DDK specificity and mechanism of action is available.

S. cerevisiae Mcm4 is the best studied DDK substrate (Sheu and Stillman, 2006). It was proposed that two regions in Mcm4 - NSD (N-terminal serine/threonine-rich domain) and DDD (DDK-docking domain) are needed for Mcm4 phosphorylation. The kinase-recruitment DDD is the main determinant of DDK substrate specificity, while NSD harbors phosphoacceptor sites that are phosphorylated by DDK. The sequence of NSD is not conserved and can be replaced with any serine- and threonine-containing sequence surrounded by negatively charged amino acids (Sheu and Stillman, 2006).

How DDK substrate specificity is determined remains to be addressed. It seems that Dfp1/Dbf4 regulation subunit is responsible for DDK recruitment to its substrates. It was shown that Dfp1 interacts with the DDK substrate Swi6 in *S. pombe (Bailis et al., 2003)* and that Dbf4 is needed for DDK recruitment to origins in *S. cerevisiae* (Dowell et al., 1994).

1.4.4 Function of DDK in replication initiation

The initiation of DNA replication, or 'origin firing', is a highly controlled mechanism that involves a number of different structural and regulatory molecules. In G1, origins of replication are already occupied by pre-replicative complexes (pre-RC) (Diffley et al., 1994) comprising Cdc6, six highly conserved subunits of the origin recognition complex (ORC1-6), and six components of minichromosome maintenance (MCM) complex (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7) (Costa and Onesti, 2008). In order to activate replication, the pre-replicative complex (pre-RC) is later converted to the preinitiation complex (pre-IC) by further recruiting replication factors such as Cdc45, Sld2, Sld3, and GINS (Kamimura et al., 2001; Masumoto et al., 2002; Zou and Stillman, 2000) Cdc45 is often referred as a marker for origin activation, because it is important for recruitment of the polymerase α/ primase complex to the origin (Zou and Stillman, 2000).

DDK, together with the S phase CDKs, is required for the transition from pre-RC to pre-IC and subsequent activation of DNA replication. Several lines of evidence suggest that MCM proteins are prime targets for the DDK. Genetic evidence showed that a mutation in the MCM complex, *mcm5-bob1*, partially bypasses the essential role of the DDK (Hardy et al., 1997). Furthermore, an allele of Dbf4 has been isolated as an allele-specific suppressor of *mcm2-1* (Lei et al., 1997). *In vitro* kinase assays demonstrated that several MCM subunits are substrates of the DDK (Lei et al., 1997; Weinreich and Stillman, 1999). It

was proposed that DDK is recruited to origins through a DDK-docking domain on Mcm4 to facilitate its hyperphosphorylation, which is important for stable Cdc45-MCM complex formation on S phase chromatin (Sheu and Stillman, 2006) and activation of the MCM replicative helicase complex at origins.

Interestingly, cells harboring a phosphodeficient Mcm4 mutant are viable, but have a very long S-phase (Sheu and Stillman, 2006), suggesting that instead of functioning as a global switch, DDK-mediated phosphorylation of Mcm4 is required for activation of individual origins throughout S phase to promote timely progression. Thus, DDK recruitment might be a key determinant of both the temporal and spatial control of origin firing.

1.4.5 Intra S-phase checkpoint and recovery from replication fork arrest

There is accumulating evidence that DDK also plays an important role during the S-phase checkpoint response. First, DDK mutants display hypersensitivity to genotoxic agents like HU, UV light and MMS (Fung et al., 2002; Takeda et al., 1999). Dfp1 N-terminal truncations (amino acids 154–193) are viable but result in HU sensitivity and high frequency of the 'cut' phenotype, which is one of the hallmarks of checkpoint failure (Fung et al., 2002; Takeda et al., 1999). Unlike other Dfp1/Hsk1 mutants, those with alterations in the Dfp1 C-terminus (C-terminal truncation of amino acids 377–545, called *dfp1-376*) were found to have an intact intra-S phase checkpoint,

and yet were sensitive to MMS. This suggested an additional role for Dfp1, in the recovery from the stalled replication forks induced by MMS. Such a role is consistent with the high rate of recombination and chromosome loss, and the persistent checkpoint activation in MMS-treated *dfp1-376* cells (Fung et al., 2002). Furthermore, an *hsk1*ts allele is synthetically lethal with a null mutation of *rqh1*, which encodes a RecQ-type helicase implicated in recovery from HU arrest (Snaith et al., 2000), again consistent with a role for DDKs in recovery from replication fork stalling.

Another key finding pointing to an S phase checkpoint role for DDKs is the hyperphosphorylation of Dfp1 upon treatment with HU, which results in replication fork arrest and activation of the checkpoint kinase Cds1. When a *cds1Δ* strain is exposed to HU, there is no longer hyperphosphorylation of Dfp1 (Brown and Kelly, 1999). Hsk1 is similarly phosphorylated in a Cds1-dependent fashion following HU treatment, and *in vitro* assays using purified proteins have shown that it is a direct substrate for Cds1 (Snaith et al., 2000). The HU response is probably more complicated, because full activation of Cds1 upon HU treatment was found to depend on Hsk1 activity (Takeda et al., 2001), suggesting Cds1 and Hsk1 may be part of a regulatory loop.

What is the role of DDK in the intra-S phase checkpoint response? It was shown that Rad53-dependent phosphorylation of Dbf4 attenuates DDK activity in *S. cerevisiae* (Kihara et al., 2000; Weinreich and Stillman, 1999). Moreover, late origins normally prevented from firing following exposure of cells to either HU or MMS are released from this control in a mutant *rad53* background (Santocanale and Diffley, 1998). It was shown that Dbf4 interacts

with Rad53 and ORC through the same N-terminal domain (Duncker et al., 2002), making it possible to suggest that during checkpoint response, Rad53 prevents Dbf4 from associating with replication origins by directly targeting Dbf4 ORC-association domain, resulting in Rad53-dependent dissociation of Dbf4 from the chromatin and perhaps from Cdc7, which renders DDK unable to phosphorylate its critical targets at late replication origins, thereby preventing origin firing.

1.4.6 DDK role in centromeric heterochromatin assembly and cohesin loading

Replication folk passage is not the only factor important for proper chromatin structure formation during S-phase. It was shown that DDK is also required for Swi6 dependent centromeric heterochromatin formation (Bailis et al., 2003) and hence plays a central role in accurate chromosome segregation promoting centromeric sister chromatin cohesion (Bernard et al., 2001; Nonaka et al., 2002). Both *hsk1-1312* and *dfp1-376* mutants show loss of silencing and cohesion at the centromeres. It was shown that binding of Swi6 to methylated H3 K9 is not sufficient for heterochromatin function; instead, DDK phosphorylation of Swi6 is needed to form silent heterochromatin and establish cohesion at centromeres. It was proposed that DDK is targeted to centromeres during S phase, where it phosphorylates Swi6 and promotes the binding of additional proteins required for silencing, cohesion and assembly of the kinetochore (Bailis et al., 2003).

DDK has a more general role in cohesin recruitment. As was shown in *Xenopus* egg extracts, DDK is important for pre-RC (pre-replication complex) dependent loading of the cohesin loading factors Scc2-Scc4. Interestingly, Scc2–Scc4 binding to chromatin requires DDK kinase activity (Takahashi et al., 2008). It was demonstrated that egg extracts contain a large complex consisting of Scc2–Scc4, cohesin, and DDK, and that Scc2–Scc4 forms a bridge between DDK and cohesin (Takahashi et al., 2008). Because DDK interacts stably with pre-RCs (Takahashi and Walter, 2005) and with Scc2–Scc4, DDK could play a structural role in Scc2–Scc4 recruitment.

1.4.7 DDK function during meiotic double-stranded breaks formation

A role for DDK in meiotic progression has been reported in several organisms. In mice, reduction of Cdc7 kinase activity results in sterility with testicular and ovary atrophy, indicating an essential role for Cdc7 in gamete formation (Kim et al., 2003). In fission yeast, Cdc7 kinase activity has an essential role in meiotic progression and DSB formation (Ogino et al., 2006). In budding yeast, a *cdc7ts* mutant was shown to undergo premeiotic DNA replication but to arrest before meiosis I (Hollingsworth and Sclafani, 1993). Moreover, analysis using a *cdc7-as* (ATP analog sensitive) mutant suggested the possibility that Cdc7 is required for meiotic DSB formation in budding yeast (Wan et al., 2006).

Meiotic recombination involves the formation and repair of DSBs generated by the evolutionarily conserved Spo11 protein during meiosis prophase. DSB formation requires progression through S-phase, but is not depend strictly on replication (Hochwagen et al., 2005). Spo11 is the catalytic subunit of the meiotic DSB-forming activity, but other additional factors are required for Spo11 activity. It was shown that DDK regulates DSB formation by phosphorylation of Mer2 (Sasanuma et al., 2008; Wan et al., 2008), one of the proteins that regulates Spo11 activity. Cdc7 influences Mer2 activity by modulating Mer2 interactions with other components of the Spo11 complex, and thereby controlling the loading of Spo11 on chromatin (Sasanuma et al., 2008).

1.5 The aim of this study

In my thesis work, I found that Pot1 interacts and is phosphorylated by DDK in a cell cycle dependent manner. This finding provided an opportunity to ask whether DDK dependent Pot1 phosphorylation has a role in regulating telomere function. The aim of this work was to understand the mechanisms of Pot1 regulation and its effect on telomeres. The long-standing goal is to understand the dynamic structure of telomeres in different phases of cell cycle, and how the dynamism of telomeres regulates their functions and maintenance.

2 Materials and methods

2.1 Yeast growth and manipulation

2.1.1 Yeast strains and media

All fission yeast strains used in this study are listed in Table 1. All media and growth conditions were as previously described (Moreno et al., 1991). Cultures were usually grown at 32°C in rich medium (YES) except where noted. Strains were constructed either by mating with another mutant and selecting on appropriate selective media or by transformation with appropriate DNA integration fragment designed for gene knockout or tagging. Gene knockouts were created by one-step gene replacement of the entire ORF with a kanMX6, hphMX6 or natMX6 cassette. (Sato et al., 2005). Gene tagging with V5, myc and HA epitopes was performed as described in (Sato et al., 2005).

2.1.2 Yeast mating and tetrad dissection

Mating was induced by incubating opposite mating type yeast strains or diploids on malt-extract (ME) plates at 25˚C for 48 hours. The spore ascis were placed on a YES plate and incubated for 5 hours to breakdown the asci walls. Four released spores from each asci were placed in a line using a Singer-MSM micromanipulator. The spores were incubated at 32˚C (except where noted) until colonies formation.

Table 1 Fission yeast strains used in this study

Strain number	Genotype	Mating type	Source
JCF 6003	ade6-M210 leu1-32 ura4-D18	h+	Cooper Lab
JCF 6004	ade6-M210 leu1-32 ura4-D18	h-	Cooper Lab
JCF 6042	pot1-13xmyc:kanMX6 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6194	dfp1-3xHA:kanMX6 ade6-M210 leu1-32 ura4-D18	h-	This study
JCF 6319	dfp1-3xHA:kanMX6 pot1-13xmyc:kanMX6 ade6-M210 leu1-32 ura4-D18	h-	This study
JCF 6369	pot1-6xV5:kanMX6 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6383	pot1-6xV5:kanMX6 leu1::(dfp11-376-6his3HA leu1+) dfp1-D1 ura4-D18 ade6-M216	h+	Fung et al. 2002
JCF 6392	pot1-6xV5:kanMX6 ade6-M216 leu1-32 ura4-D18 hsk1-89:ura4+	h-	Takeda et al. 2001
JCF 6534	aur1-r::Pot1-(T58A-S89A)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6536	aur1-r::Pot1-(T111A-T154)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6539	aur1-r::Pot1-(T58A-T154A)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6541	aur1-r::Pot1-(T58A-S89A, A68T)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6542	aur1-r::Pot1-(T58A-S89A, A68T, A75T)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6545	aur1-r::Pot1-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study
JCF 6550	aur1-r::Pot1-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6551	aur1-r::Pot1-(T173A-S252A)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6553	aur1-r::Pot1-(S420A-T517A)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6555	aur1-r::Pot1-T75A-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6561	aur1-r::Pot1-(T68A, T75A)-6xV5/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study

JCF 6563	aur1-r::Pot1-(T68A, T75A)-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6581	aur1-r::Pot1-6xV5-wt pot1::kanMX6 ade6-M210	h-	This study
JCF 6598	aur1-r::Pot1-(T58A-S89A, A58S)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6601	aur1-r::Pot1-T68A-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6605	aur1-r::Pot1-T68D-6xV5/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study
JCF 6609	aur1-r::Pot1-(T68D, T75D)-6xV5/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study
JCF 6614	aur1-r::Pot1-(T68A, T75A)-6xV5 pot1::kanMX6 ade6-M216	h-	This study
JCF 6621	aur1-r::Pot1-(T58A-S89A, A89S)-6xV5 ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6622	aur1-r::Pot1-6xV5-wt ade6-M210 his3-D1 leu1-32 ura4-D18 trt1::his3+	h-	This study
JCF 6624	aur1-r::Pot1-6xV5-wt ade6-M210 leu1-32 ura4-D18 rad3::ura4 tel1::leu2	h90	This study
JCF 6625	aur1-r::Pot1-(T68A, T75A, T78A)-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6627	aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study
JCF 6635	aur1-r::Pot1-(T68D, T75D)-6xV5 pot1::kanMX6 ade6-M216	h-	This study
JCF 6645	aur1-r::Pot1-(T68A, T75A, T78A, T79A)-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 6647	aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5 pot1::kanMX6 ade6-M216	h+	This study
JCF 6657	aur1-r::Pot1-T68A-6xV5/+ pot1::kanMX6/+ ade6-M210/ade6-M216	h+/h-	This study
JCF 6673	aur1-r::Pot1-(T68A, T75A, T78A, T79A, S80A)-6xV5-wt ade6-M210 leu1-32 ura4-D18	h+	This study
JCF 7022	tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	Miyoshi et al. 2008
JCF 7023	rhp51::hygr leu1-32 ura4-D18	h-	Miyoshi et al. 2008
JCF 7024	trt1::hphMX6/+ aur1-r::Pot1-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7026	trt1::hphMX6/+ aur1-r::Pot1-(T68A, T75A)-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7029	trt1::hphMX6/+ aur1-r::Pot1-(T68D, T75D)-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study

JCF 7030	rhp51::hphMX6/+ aur1-r::Pot1-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7032	rhp51::hphMX6/+ aur1-r::Pot1-(T68A, T75A)-6xV5/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7034	rhp51::hphMX6/+ aur1-r::Pot1-(T68D, T75D)-6xV5/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7036	rhp51::hphMX6/+ aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7038	trt1::hphMX6/+ aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5-wt/+ pot1::kanMX6/+ ade6-M210/M216	h+/h-	This study
JCF 7041	aur1-r::Pot1-6xV5-wt tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7044	aur1-r::Pot1-(T68A, T75A)-6xV5 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7046	aur1-r::Pot1-(T68D, T75D)-6xV5 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7049	aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study

JCF 7078	aur1-r::Pot1-6xV5-wt pot1::kanMX6 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7080	aur1-r::Pot1-(T68A, T75A)-6xV5 pot1::kanMX6 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7082	aur1-r::Pot1-(T68D, T75D)-6xV5 pot1::kanMX6 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study
JCF 7084	aur1-r::Pot1-(T68A, T75A, T78A, S79A, S80A)-6xV5 pot1::kanMX6 tpz1:3Flag:ura4+ leu1-32 ura4-D18	h-	This study

2.1.3 Yeast transformation

All yeast transformations were performed as previously described (Sato et al., 2005) with minor modifications. Cells were grown in YES media until the cultures reach log phase; 10ml of cell culture were used per one transformation. Cells were collected by centrifugation and washed once in autoclaved, deionised water and once in LiOAc solution (0.1 M LiOAc, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cells were pelleted and resuspended in 50 μl of LiOAc solution. 50 μl of the cells were added to a tube containing the mix of transformation DNA and 3 μl of previously boiled and iced salmon sperm DNA (10 mg/ml from Stratagene). For the transformation DNA, several μg of PCR product were used or ~1 μg of supercoiled plasmid. 280 μl of PEG solution (40% polyethylene glycol 3.350 (PEG) in 0.1 M LiOAc, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) were added, mixed by inversion and incubated at the optimal growing temperature for the strain used for 30 minutes. 35μl of DMSO was added and the tube was incubated at 42°C for 5 minutes. Cells were pelleted, washed in 1 ml ddH_2O and resuspended in 100 μl of ddH_2O. Cells were plated on the appropriate media and incubated at the appropriate temperature for selection.

2.1.4 Cytological analysis

For cellular morphology analysis we collected cells from log phase cultures grown at the indicated temperatures and visualized the cells by light microscopy or by differential interference microscopy (DIC) using ZEIZZ Axioplan2 fluorescence microscope. All images were captured on a HAMAMATSU ORCA-ER digital camera. The images were analysed using Volocity 4.3.1 software (Improvision Company Ltd).

2.1.5 Cell synchronization using *cdc25-22* temperature sensitive mutant

Asynchronous cultures were grown at the permissive temperature (25°C) until mid-log phase. To block cells in G_2 the cell cultures were shifted to 36.5°C for 3 hours. For the block release, cells were shifted back to 25°C. Samples were then taken at the indicated time intervals. Mitotic index (as a measurement of cell synchronisation and cell cycle progression) was calculated as a percentage of the cells with formed septum.

2.2 General molecular biology techniques

All standard molecular biology techniques, like DNA purification, PCR, restriction endonuclease digestion, bacterial plasmid purification were carried out as described in (Sambrook and Russell, 2001) or in corresponding kit manufacturer protocol.

2.3 Protein analysis techniques

2.3.1 Protein extract preparation with trichloroacetic acid (TCA)

Yeast cells were grown in 15 ml of appropriate media until mid-log phase. Cells were than collected by centrifugation, resuspended in 1 ml of 20% trichloroacetic acid (TCA) and kept on ice for 15 minutes. We washed the cells with 1 ml of 1M Tris-Base and finally cell pellets were resuspended in 100 µl of 2X SDS-PAGE loading buffer in the tubes containing 100 µl of 0.5mm glass beads (BioSpec Products, Inc). The cells were lyzed using FastPrep FP120 cell breaker (Bio101). Protein extract was moved to another tube boiled for 5 minutes and cleared by centrifugation at 16.000g for 5 minutes.

2.3.2 Analysis of protein-protein interaction

Protein-protein interaction were analysed using yeast two-hybrid system and co-immunoprecipitation technique.

2.3.2.1 Yeast two-hybrid screen

The yeast two-hybrid screen analysis was carried out according to the manufacturer's protocol (Matchmaker Two-Hybrid System, Clontech). The bait construct was constructed by fusing full-length Pot1 to the GAL4 DNA-binding

domain (DBD) into a pBGKT7 vector (Clontech). The yeast AH109 transformant expressing the Pot1-GAL4 DBD fusion protein was transformed with *Schizosaccharomyces pombe* cDNA library expressing the GAL4 activation domain (AD) fusion proteins (America Type Culture Collection, Manassas, VA, USA). The positive clones were selected as a clones that were able to grow on minimal media lacking tryptophan, leucine, histidine and containing 5 mM of 3-amino-1,2,4-triazole (Sigma). The positives were then screened for β-galactosidase activity using X-Gal as a substrate (Roche). The cDNA clones that represented potential Pot1 binding proteins were sequenced and compared with the GenBank database using a Blast Search.

2.3.2.2 Protein immunoprecipitation

For immunoprecipitation of the Pot1-13xmyc, Pot1-6xV5 or Dfp1-3xHA 100 ml of exponentially growing cells was harvested. Cell lysates were extracted with 0.5mm glass beads (BioSpec Products, Inc) and lysis buffer (50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 0.1% NP-40, 250 mM NaCl, 1 mM DTT, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1x complete inhibitor (Roche)) using FastPrep FP120 cell breaker (Bio101) at 4°C. Soluble fractions were recovered by centrifugation for 10 min at 16.000 g and pre-cleared with 100 μl of protein-A agarose (Sigma) for 1 h at 4°C. Then, the supernatants were incubated for 1 hour with 70 μl of protein-A Dynabeads (Invitrogen) preincubated with anti-V5 (Serotec), anti-HA.11 (Covance) or anti-myc 9E10 (Covance) antibodies. The resin was washed six

times with lysis buffer. The proteins were eluted with SDS-PAGE loading buffer.

2.3.3 Two-dimensional protein gel electrophoresis

Two-dimensional protein gel electrophoresis (2D-PGE) was perform as described previously (Raggiaschi et al., 2006; Yamagata et al., 2002) and in 2-D Electroohiresis handbook, Principles and Methods (GE Healthcare) with some modifications. For separation by 2D-PGE, proteins are subjected to isoelectric focusing and then separated by size. The phosphorylation of a protein leads to a decrease in its pI and consequently changes its coordinates in a 2-D gel. To map Pot1 phosphoisoforms the samples were treated with λ Protein Phosphatase (λPpase). Phosphatase treated and untreated samples were analyzed by 2-DGE and the resulting 2-D maps compared in order to detect differences in migration corresponding to presence of Pot1 phosphorylated forms. Selectivity and sensitivity of 2-DGE were improved by combining 2-DGE with western blot protein detection.

2.3.3.1 Protein extract preparation for 2D-PGE

Cells were grown in YES media; 10ml of the mid-log phase culture were collected by centrifugation and washed twice with ultrapure deionized water. Cells were resuspended in equal volume of the lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% of appropriate IPG buffer (GE Healthcare), 40mM DTT, 1X Protease Inhibitor Mix (GE Healthcare), 1X Nuclease Mix (GE

Healthcare). The 0.5mm glass beads (BioSpec Products, Inc) were added and the cells were lyzed using FastPrep FP120 cell breaker (Bio101). Protein extract were moved to another tube and cleared by centrifugation at 16.000g for 10 minutes at +4˚C. Protein concentration was measured using the 2-D Quant kit (GE Healthcare). On average, protein concentration was about 20 μg/μl.

2.3.3.2 Phosphatase treatment

Phosphatase treatment with λ Protein Phosphatase (λ Ppase) was performed with slight modifications as described previously (Yamagata et al., 2002). In brief, two aliquots of 20 μl were mixed with 5 μl of 10% SDS and vortexed vigorously for 10 seconds. To each sample, 373 μl of deionised water, 50 μL of 20 mM MnCl2 and 50 μl of λ Ppase buffer (New England Biolabs) were added sequentially. One aliquot was incubated with 800 units of λ Ppase enzyme (New England Biolabs) and both samples were left for 2 hours at 30˚C. The protein were cleared from interfering material by precipitation using the 2-D Clean-Up Kit (GE Healthcare) and then resuspended in 100 ml of DeStreak Rehydradion Solution (GE Healthcare).

2.3.3.3 First-dimension isoelectric focusing (IEF)

Protein samples were prepared by mixing 200 μg of protein extract with 100 μl of DeStreak Rehydradion Solution (GE Healthcare). Mixed protein samples were separated on 24-cm-long Immobiline DryStrip gel strips (GE

Healthcare) with an indicated immobilized pH gradient (IPG), which were dehydrated for 12 hours with 450 µl of DeStreak Rehydradion Solution (GE Healthcare) containing 0.5% of appropriate IPG buffer (GE Healthcare). Samples were cup-loaded at the middle of the strips. The first dimension (IEF) of the 2-DGE separation was carried out on Ettan IPGphor II Manifold instrument (GE Healthcare) using the following running protocol for the strips with a liner range of pH 4-7: 0.5 kVh at 500 V, 5.2 kVh gradient up to 1000 V, 13.5 kVh up to 8000 V, and a final step for 45 kVh at 8000 V. For the strips with a liner range of pH 6-9 we used the following settings: 0.5 kVh at 500 V, 3.8 kVh gradient up to 1000 V, 13.5 kVh up to 8000 V, and 70 kVh at 8000 V.

2.3.3.4 Second-dimension SDS-PAGE

Once the IEF was finished the IPG strips were equilibrated in SDS equilibration buffer solution (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris-HCl pH 8.8, 0.002% w/v bromophenol blue) containing 65 mM DTT for 15 min, followed by a second equilibration step of 15 min with the same solution containing 135 mM iodoacetamide instead of DTT. IPG strip regions corresponding to pH range of 5.5-6.5 (for the 24-cm long IPG strips with the liner range of pH 4-7) or 6-7 (for the strips with liner range of pH 6-9) were cut out and subjected to the second dimension (SDS-PAGE) on NuPAGE 4-12% Bis-Tris Zoom IPG well polyacrylamide gradient gels with MOPS SDS running buffer (Invitrogen) using XCell SureLock electrophoresis system (Invitrogen).

Separation was carried out at 200 V, until the bromophenol blue reached the bottom of the gel.

2.3.3.5 Western Blotting

Proteins were transferred to Polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in a Mini Trans-Blot Cell (Bio-Rad) at 25mA for 1 h. The membrane was blocked in PBST buffer (10 mM Na_2HPO_4, 2mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween) with 5% MARVEL skimmed milk for 1hour. The antibodies were added and the incubation was continued for 2 hour at room temperature of at +4˚C over night. We used the following dilutions of monoclonal antibodies: anti-V5 (Serotec) at 1:4,000 dilution, anti-HA.11 (Covance) at 1:1,000, anti-myc 9E10 (Covance) at 1:2,000 and anti-FLAG M2 (Sigma) at 1:1,000. The membranes were incubated with sheep anti-mouse IgG Horseradish peroxidase conjugates in PBST containing 5% milk for 40 minutes. Proteins were detected using ECL Plus™ Western Blotting Detection Systems (GE Healthcare).

2.3.4 Expression and purification of Pot1 OB fold

The procedure of purification of *S. pombe* Pot1 OB fold expressed in Escherichia coli was described previously (Lei et al., 2002). We used this protocol with some modifications. Briefly, Pot1 OB fold mutants were expressed in Rosetta pLysS E. coli cells (Novagen) harboring plasmid pET30-Pot1-wt OB fold, pET30-Pot1-(T68A, T75A) OB fold or pET30-Pot1-(T68D,

T75D) OB fold. Cells were grown in 1 L of LB media containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C. Cells were grown in a shaker incubator to an optical density of OD600 ~0.6 and were then cooled to room temperature and grown to an OD600 of ~1.0. Production of the protein was induced by addition of isopropyl thiogalactoside (IPTG) to 1 mM, and the cells were grown for an additional 6 h at 25 °C. The cells were harvested by centrifugation and stored at -20 °C. Approximately 20 g of cells were resuspended in 50 mL of lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM 2-mercaptoethanol, 5 mM benzamidine, and 1 mM PMSF) and incubated on ice for 40 min. Then DNase I and 15 mM MgCl2 were added to the cells, which sat on ice for another 20 min. The cells were lysed by sonication, and cell debris was removed by centrifugation. The supernatant was incubated with 5 mL of Ni-NTA agarose beads (Qiagen) at 4 °C for 2 h. The beads were loaded on a column and wash with the lysis buffer. Pot1 OB fold was eluted with lysis buffer containing 50 mM imidazole. Protein was concentrated to 20 mg/mL by Centricon 10 (Millipore), dialyzed against appropriate buffer and stored at -80 °C after addition of 15% glycerol.

2.3.5 Gel mobility shift assay

Gel mobility shift assay with Pot1 OB fold was previously described in (Lei et al., 2002). Pot1 OB fold wild type or mutant dialyzed against binding buffer (25 mM HEPES/NaOH, pH 7.5, 50 mM NaCl, 40 mM KCl, 7% glycerol, 1 mM EDTA, and 0.1 mM DTT) was mixed with 0.5 μM ^{32}P-labeled telomeric

single-stranded oligonucleotide 5′-GGTTACACGGTTACAGGTTACAGGTTACAGGGTTACGGTTACGSS-3′ in a total volume of 20 μL. The reaction mixtures were incubated at room temperature for 10 min. Then the mixtures were directly loaded onto a 4-20% nondenaturing polyacrylamide gel. Electrophoresis was carried out in TBE buffer at 150 V for 85 min at 4 °C. The gels were dried, and radiolabeled telomeric ssDNA was visualized using a PhosphorImager (GE Healthcare).

2.3.6 Protein sequence alignment

Multiple protein sequences were aligned using ClustalW software.

2.3.7 *In vitro* kinase assay

For the *in vitro* kinase assay, Dfp1-3HA was immunoprecipitated as described earlier, the beads were washed twice more with kinase buffer (50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 mM β-glycero-phosphate). Beads were incubated with 20 μl reaction mix (kinase buffer supplemented with 10 μM ATP and 5 μg of purified recombinant Pot1-OB-fold (amino acids 1-185), including 0.25 μCi/μl γ-^{32}P-ATP) for 20 min at 32°C. The reaction was stopped by adding SDS-PAGE loading buffer. The proteins were separated by NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel with MOPS SDS running buffer (Invitrogen). Gel was dried out and exposed to PhosphorImaging screen

(GE Healthcare); the screen reading was carried out using STORM 840 PhosphorImager scanner (GE Healthcare).

2.4 DNA analysis and manipulation techniques

All cloning and standard DNA manipulation procedures were carried out as described in (Sambrook and Russell, 2001).

2.4.1 Oligonucleotides and vector

All plasmids that were created in this thesis are listed in Table 2.

2.4.1.1 Cloning Pot1 bait construct for yeast two-hybrid screen

For Pot1 bait construct (pGBKT7-Pot1) construction full-length Pot1 cDNA was amplified by PCR from *S. pombe* cDNA library (America Type Culture Collection, Manassas, VA, USA) using primers: forward 5′-ATCGGTCGACTCATGGGAGAGGACGTTATTGACAGTCTTCAG-3′ and reverse 5′-ACGCTGCAGTCAAACAATTTTCGTGCCAAATCCTCGC-3′. The PCR fragment was cloned into SalI and PstI digested bait expression vector pGBKT7 (Clontech).

Table 2 Plasmids that were created in this study

Plasmid number	Plasmid construct
VKP 137	pGBKT7-Pot1
VKP 203	pCST159-Pot1-(T58A-S89A)-6xV5
VKP 205	pCST159-Pot1-(T111A-T154)-6xV5
VKP 209	pCST159-Pot1-(T58A-T154A)-6xV5
VKP 221	pCST159-Pot1-(T58A-S89A, A68T)-6xV5
VKP 229	pCST159-Pot1-(T58A-S89A, A68T, A75T)-6xV5
VKP 233	pCST159-Pot1-6xV5-wt
VKP 235	pCST159-Pot1-(T173A-S252A)-6xV5
VKP 237	pCST159-Pot1-(S420A-T517A)-6xV5
VKP 246	pCST159-Pot1-T75A-6xV5
VKP 249	pCST159-Pot1-(T68A, T75A)-6xV5
VKP 250	pCST159-Pot1-T68A-6xV5
VKP 253	pCST159-Pot1-(T58A-S89A, A58S)-6xV5
VKP 257	pCST159-Pot1-(T58A-S89A, A89S)-6xV5
VKP 258	pCST159-Pot1-T68D-6xV5
VKP 260	pCST159-Pot1-(T68D, T75D)-6xV5
VKP 262	pCST159-Pot1-(T68A, T75A, T78A, T79A, S80A)-6xV5
VKP 268	pCST159-Pot1-(T68A, T75A, T78A)-6xV5
VKP 273	pCST159-Pot1-(T68A, T75A, T78A, T79A)-6xV5
VKP 305	pET30-Pot1-wt OB fold
VKP 308	pET30-Pot1-(T68A, T75A) OB fold
VKP 311	pET30-Pot1-(T68D, T75D) OB fold
VKP 314	pCST159-Pot1-(T68A, T75A)-natMX6

2.4.1.2 Creation of Pot1 serine and threonine replacement mutants

Pot1 integration expression construct (pCST159-Pot1-6xV5-wt) was created as follows. Pot1 genomic fragment containing ~800bp of Pot1 promoter region, 6xV5 C-terminal tag followed by TEF terminator was amplified by PCR from genomic DNA obtained from JCF 6369 strain using primer set: forward 5′-AATTCCTGCAGGAGCAAACTACTGTCAAAACTTAG-3′ and reverse 5′- GATCCGTCGACAGTTTCATTTGATGCTCGATGAG-3′. The amplified fragment was inserted into pCST159 vector (Chikashige et al., 2006) using PstI and SalI restriction sites.

Mutants with serine and threonine mutations in different regions of Pot1 was constructed by replacing BstEII/SapI, SapI/ApaI and MluI/NcoI Pot1 fragments in pCST159-Pot1-6xV5-wt with chemically synthesised oligonucleotides (IDT, Integrated DNA technologies, Belgium) where all serines and threonines in corresponding Pot1 sequences were replaced with alanines. We used the following synthetic oligonucleotides: for pCST159-Pot1-(T58A-T154A)-6xV5 construct: 5′-AGATTGGGTAACCGCTGTATATTTGTGGGATCCAGCTTGTGATGCTGCTGCTATCGGACTACAGATACACTTGTTCGCTAAACAGGGAAATGATTTGCCTGTAATCAAGCAGGTGGGGCAACCGCTTTTGCTTCATCAAATCGCTTTAAGAGCTTATAGAGACAGGGCTCAAGGTTTGGCTAAGGATCAATTTCGATATGCACTTTGGCCAGACTTTGCTGCTAATGCTAAAGATGCTCTCTGTCCTCAACCAATGCCTCGTTTAATGAAAGCTGGAGACAAGGAAGAGCAATTCG-3′; for

pCST159-Pot1-(T58A-S89A)-6xV5: 5′-

AGATTGGGTAACCGCTGTATATTTGTGGGATCCAGCTTGTGATGCTGCTGC

TATCGGACTACAGATACACTTGTTCGCTAAACAGGGAAATGATTTGCCTGT

AATCAAGCAGGTGGGGCAACCGCTTTTGCTTCATCAAATCACATTAAGAAG

TTATAGAGACAGGACTCAAGGTTTGTCTAAGGATCAATTTCGATATGCACTT

TGGCCAGACTTTTCTTCTAATTCCAAAGATACTCTCTGTCCTCAACCAATGCC

TCGTTTAATGAAAACGGGAGACAAGGAAGAGCAATTCG-3′; for pCST159-

Pot1-(T111A-T154)-6xV5: 5′-

AGATTGGGTAACCACCGTATATTTGTGGGATCCAACATGTGATACATCAAG

CATCGGACTACAGATACACTTGTTCAGCAAACAGGGAAATGATTTGCCTGT

AATCAAGCAGGTGGGGCAACCGCTTTTGCTTCATCAAATCGCTTTAAGAGC

TTATAGAGACAGGGCTCAAGGTTTGGCTAAGGATCAATTTCGATATGCACT

TTGGCCAGACTTTGCTGCTAATGCTAAAGATGCTCTCTGTCCTCAACCAATG

CCTCGTTTAATGAAAGCTGGAGACAAGGAAGAGCAATTCG-3′; for

pCST159-Pot1-(T173A-S252A)-6xV5: 5′-

GCTGGAGACAAGGAAGAGCAATTCGCCTTGTTGTTAAATAAAATTTGGGAT

GAGCAAGCTAATAAACATAAAAATGGCGAATTATTGGCTGCTGCTGCTGCT

CGTCAAAATCAAGCTGGATTGGCTTACCCTGCTGTCGCTTTTGCTCTGCTAG

CTCAAATAGCTCCACATCAACGTTGTGCTTTTTACGCTCAGGTAATTAAAGC

TTGGTACGCTGATAAAAACTTTGCTCTTTATGTCGCTGATTATGCTGAAAAT

GAGCTTTTTTTTCCAATGGCTCCGTATGCTGCTGCTGCTAGATGGAGGGGC

CCTTTTGGT-3′; for pCST159-Pot1-(S420A-T517A)-6xV5: 5′-

AGGCTACGCGTTCAGGTGGTAGATTTTTGGCCAAAGGCTTTGGCTCAGTTT

GCTGTGCTAGCTCAACCACCAGCTGCTTATGTTTGGATGTTTGCCTTGCTCG

TAAGGGATGTAGCTAATGTGGCTTTACCGGTCATATTTTTTGATGCTGA
CGCTGCGGAACTTATTAACGCTGCTAAAATCCAACCTTGCAATTTAGCTGAT
CACCCGCAGATGGCTCTTCAGCTTAAAGAAAGATTATTTCTGATTTGGGGG
AACTTGGAAGAACGCATTCAGCATCACATAGCTAAGGGTGAAGCTCCAGC
TCTGGCTGCTGAAGATGTTGAAGCTCCATGGTTTGAT-3′.

Integration plasmid with natMX6 selection marker, pCST159-Pot1-(T68A, T75A)-natMX6, was contracted by subcloning BglII/EcoRV natMX6 fragment from pFA6a-3xHA-natMX6 plasmid (Sato et al., 2005) into BglII/PmlI digested pCST159-Pot1-(T68A, T75A)-6xV5 vector.

2.4.1.3 Cloning of *E. coli* Pot1 OB fold expression constructs

Pot1 expression construct (pET30-Pot1-wt OB fold) was created by inserting PCR amplified Pot1 OB fold (corresponding to Pot1 amino acids 1-185) into E. coli expression vector pET30 (Novogen) digested with SalI and NotI. Pot1 OB fold was amplified from *S. pombe* cDNA library (America Type Culture Collection, Manassas, VA, USA) using forward primer 5′-CTCCGTCGACAAATGGGAGAGGACGTTATTGACAG-3′ and reverse primer 5′- GAGTGCGGCCGCTCAAGAGGTACTCAATAATTCGCCATTTTTATG-3′. Pot1 OB fold mutants for pET30-Pot1-(T68A, T75A) OB fold and pET30-Pot1-(T68D, T75D) OB fold expression constructs were amplified by RT-PCR. RNA was obtained from JCF 6614 and JCF 6635 strains respectively using AccuScript™ High Fidelity RT-PCR Kit (Stratagene). All procedures were carried out according to manufacturer protocol.

2.4.2 Site-direct DNA mutagenesis

Site-specific mutagenesis was carried out using QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer protocol. All oligonucleotides were synthesized by Sigma and were purified by HPLC. Pot1-(T58A-S89A, A58S)-6xV5 was created from pCST159-Pot1-(T58A-S89A)-6xV5 using following primer sets: 5′-GATTTTACCCCTAGTCGCCAAAGTCTACATGGAACTAAGGGTATG-3′ and 5′-CATACCCTTAGTTCCATGTAGACTTTGGCGACTAGGGGTAAAATC-3′; pCST159-Pot1-(T58A-S89A, A89S)-6xV5 was created from pCST159-Pot1-(T58A-S89A)-6xV5 using primers: 5′-CGGACTACAGATACACTTGTTCAGCAAACAGGGAAATGATTTGCCTG-3′ and 5′-CAGGCAAATCATTTCCCTGTTTGCTGAACAAGTGTATCTGTAGTCCG-3′; pCST159-Pot1-(T58A-S89A, A68T)-6xV5 was created from pCST159-Pot1-(T58A-S89A)-6xV5 using primer set: 5′-CATGTTTTTAGATTGGGTAACCACCGTATATTTGTGGGATCCAGCTT-3′ and 5′-AAGCTGGATCCCACAAATATACGGTGGTTACCCAATCTAAAAACATG-3′; pCST159-Pot1-(T58A-S89A, A68T, A75T)-6xV5 was created from pCST159-Pot1-(T58A-S89A, A68T)-6xV5 using primer set: 5′-CCGTATATTTGTGGGATCCAACATGTGATGCTGCTGCTATC-3′ and 5′-GATAGCAGCAGCATCACATGTTGGATCCCACAAATATACGG-3′; pCST159-Pot1-T68A-6xV5-wt was created from pCST159-Pot1-6xV5-wt using following

primer set: 5′-CATGTTTTTAGATTGGGTAACCGCTGTATATTTGTGGGATCCAACATG-3′ and 5′-CATGTTGGATCCCACAAATATACAGCGGTTACCCAATCTAAAAACATG-3′; pCST159-Pot1-T75A-6xV5 was created from pCST159-Pot1-6xV5-wt using following primer set: 5′-GTATATTTGTGGGATCCAGCATGTGATACATCAAGCATCGG-3′ and 5′-CCGATGCTTGATGTATCACATGCTGGATCCCACAAATATAC-3′; pCST159-Pot1-(T68A, T75A)-6xV5 was created from pCST159-Pot1-T75A-6xV5 using primer set: 5′-CATGTTTTTAGATTGGGTAACCGCTGTATATTTGTGGGATCCAGCAT-3′ and 5′-ATGCTGGATCCCACAAATATACAGCGGTTACCCAATCTAAAAACATG-3′; pCST159-Pot1-(T68A, T75A, T78A)-6xV5 was created from pCST159-Pot1-(T68A, T75A)-6xV5 using primer set: 5′-GGGATCCAGCATGTGATGCTTCAAGCATCGGACTAC-3′ and 5′-GTAGTCCGATGCTTGAAGCATCACATGCTGGATCCC -3′; pCST159-Pot1-(T68A, T75A, T78A, T79A)-6xV5 was created from pCST159-Pot1-(T68A, T75A, T78A)-6xV5 using primer set: 5′-CCAGCATGTGATGCTGCTAGCATCGGACTACAGATAC-3′ and 5‘-GTATCTGTAGTCCGATGCTAGCAGCATCACATGCTGG-3′; pCST159-Pot1-(T68A, T75A, T78A, T79A, S80A)-6xV5 was created from pCST159-Pot1-(T68A, T75A, T78A, T79A)-6xV5 using primer set: 5′-CCAGCATGTGATGCTGCTGCTATCGGACTACAGATAC-3′ and 5′-GTATCTGTAGTCCGATAGCAGCAGCATCACATGCTGG-3′; pCST159-Pot1-

T68D-6xV5 was created from pCST159-Pot1-6xV5-wt using primer set: 5′-CATGTTTTTAGATTGGGTAACCGATGTATATTTGTGGGATCCAACATG-3′ and 5′-CATGTTGGATCCCACAAATATACATCGGTTACCCAATCTAAAAACATG-3′; pCST159-Pot1-(T68D, T75D)-6xV5 was created from pCST159-Pot1-T68D-6xV5 using following primer set: 5′-CATGTTTTTAGATTGGGTAACCGATGTATATTTGTGGGATCCAGATTG-3′ and 5′-CAATCTGGATCCCACAAATATACATCGGTTACCCAATCTAAAAACATG-3′.

2.4.3 DNA sequencing

For DNA sequencing 200 ng of plasmid DNA were added to 20 μl reaction mixed containing 3.2 pmol of appropriate sequencing primer (Table 3) and 8 μl of BigDye Terminator 3.1 (Applied Biosystems). We used the following thermal cycling conditions: 96 ˚C for 1 minute; 25 cycles of 96 ˚C for 10 seconds, 50 ˚C for 5 seconds and 60 ˚C for 4 minutes.

Table 3 Pot1 sequencing primers

Primer name	Primer sequence
pot1 seq-F	5'-AGGCTAAAACTCATTTGTTGTTC-3'
pot1-seq-int--F	5'-CTGGATTGAGTTACCCTTCTGTCTC-3'
pot1-seq-C-ter-F	5'-CAAACCGAGGAAACATAGGCTAC-3'
pot1-seq-int-R	5'-GTCGTGCTCATCCCATAAAATG-3'
pot1-seq-R	5'-CTTCGATATGTGATGCTGAATGC-3'

2.4.4 Telomere Southern blotting analysis

After phenol, chloroform extraction DNA was digested with EcoRI or ApaI restriction enzymes and separated in 1xTAE, 1% agarose gels containing 0.03 mg/ml ethidium bromide. The gels were first incubated in 0.25N HCl for 15 min, followed by 30 minutes incubation in Blot 1 solution (20g NaOH, 87.6g NaCl in 1L H2O), then 60 minutes in Blot 2 solution (77g NH4Ac, 0.8g NaOH in 1L H2O). During this time, the membrane (Hybond-N, GE Healthcare) was prepared for transfer by incubating in Blot 2 solution for 5 min. To set up the dry transfer, on a top of a stack of dry paper towels three pieces of 3MM Whatman paper were placed, followed by membrane and gel. The stack was then covered with saran wrap and glass plate was put on a top to ensure even distribution of weight. The gel was allowed to transfer overnight. The membrane was then crosslinked using a Stratagene crosslinker and pre-hybridized for 1 hour in Church-Gilbert buffer (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M NaHPO4 pH 7.2) at 65 ˚C following with addition of telomere probe; the incubation was continued overnight. The membrane was washed in washing solution (2X SSC, 0.1% SDS) for 40 minutes at room temperature and was exposed to PhosphorImaging screen (GE Healthcare); the screen reading was carried out using STORM 840 PhosphorImager scanner (GE Healthcare).

The telomere probe was prepared by labelling synthetic telomere fragment using a random prime labelling kit (Stratagene). Briefly, 25 ng of purified telomere fragments (per probe) were labelled with α-^{32}PdCTP and purified using G-25 spin columns (GE Healthcare).

2.4.5 In-gel hybridisation analysis for the detection of telomere 3′ overhangs

In-gel hybridization analysis was performed as previously described (Dionne and Wellinger, 1996; Tomita et al., 2003). After phenol, chloroform extraction 1 μg of genomic DNA was digested with EcoRI restriction enzyme and separated in 0.5xTAE, 0.5% agarose gels containing 0.01 mg/ml ethidium bromide. The gel was vacuum dried at 45°C (45-60 min). Single-stranded telomeric DNA probe was labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase. G-rich probe sequence: 5′-GATCGGGTTACAAGGTTACGTGGTTACACG-3′, C-rich telomere probe: 5′-CGTGTAACCACGTAACCTTGTAACCCGATC-3′. The dried gel was pre-hybridized in hybridization buffer (AlkPhos Direct™, GE Healthcare), containing 4% of blocking reagent (AlkPhos Direct™, GE Healthcare) and 0.5 M NaCl at 37°C for 15min, and then the probe was added and the incubation was continued overnight at 37°C. The gel was washed twice with primary wash buffer (0.05 M Na_2HPO_4, pH 7.0, 0.01 M $MgCl_2$, 0.1% SDS, 2M Urea, 0.2 % blocking reagent (AlkPhos Direct™, GE Healthcare), 1.5 M NaCl) at 37°C for 10 min and then washed with secondary wash buffer (50 mM Tris base, 100 mM NaCl, 2mM $MgCl_2$) at room temperature for 15 min. The gel was placed on two layers of Whatman 3mm paper and overlaid with Saran Wrap. Detection of the signal was obtained by using a phosphoimager system (GE Healthcare). To detect total telomere signal (both double-stranded and single-stranded telomeric DNA), gels were treated with denaturing solution

(0.5 M NaOH, 150 mM NaCl) for 30 min at room temperature, and then treated with neutralizing solution (0.5 M Tris-HCl pH 8.0 150 mM NaCl) for 45 min (room temp). The gel was then re-probed with both the C and G-strand probes using the same protocol as stated above

3 Pot1 interacts with Dfp1, a regulatory subunit of DDK

Telomeres are specific chromatin structures that protect chromosome ends from been recognised as DNA double stranded breaks (DSB). Telomeres accomplish their anti-DNA damage response function by recruiting specific telomeric proteins that directly or indirectly interact with repetitive telomere DNA sequences. Pot1 interacts with ssDNA telomere overhang through its N-terminal ssDNA binding domains - OB folds. Pot1 has important functions in telomere protection: it controls telomeric C-strand resection (Churikov et al., 2006), protects telomere from DNA damage response (He et al., 2006; Hockemeyer et al., 2005; Wu et al., 2006) and checkpoint activation (Churikov et al., 2006; Jacob et al., 2007). Pot1 could accomplish these functions by forming a complex with Tpz1/TPP1, a Pot1-interacting protein that is important for telomere protection and telomerase recruitment (Hockemeyer et al., 2007; Miyoshi et al., 2008; Wang et al., 2007; Xin et al., 2007). But the precise mechanisms of Pot1 function and regulation are not well understood. Our starting premise was that by identifying new proteins that interact with Pot1, we would generate tools for understanding more precisely the mechanisms of Pot1 function and regulation.

3.1 Yeast two-hybrid screen identifies Pot1 interacting partners

In order to understand more about Pot1 function and regulation we performed a yeast two-hybrid screen to identify new proteins that interact with Pot1. Among 8 million clones screened, we isolated several potential candidates that showed strong interaction with Pot1 in the two-hybrid system (Table 4). Two positive candidates were particularly interesting for us.

Our screen identified three independent clones of Dfp1, the regulatory subunit for Hsk1 kinase. A retransformation assay shows that two-hybrid reporter activity is dependent on the presence of both Dfp1 prey and Pot1 bait constructs (Figure 1); neither Pot1 nor Dfp1 alone were able to activate the yeast two-hybrid system reporter expression. A holoenzyme formed of Dfp1 and Hsk1 (homologs of mammalian and *S. cerevisiae* Dbf4 and Cdc7, respectively) is known as DDK (Dbf4-dependent protein kinase). DDK is active during the S and G_2 phases of the cell cycle. This regulation is accomplished by cell cycle dependent regulation of Dfp1 expression, which peaks during S and G_2 phases, following by APC dependent degradation in anaphase (Brown and Kelly, 1999). In contrast, the kinase catalytic subunit Hsk1 is constitutively expressed throughout the cell cycle (Brown and Kelly, 1998; Takeda et al., 1999). DDK was implicated in several important processes during S phase. Among them are: initiation of replication by phosphorylation of the Mcm2 protein (Brown and Kelly, 1998; Masai et al., 1995); intra-S phase checkpoint response (Fung et al., 2002; Takeda et al., 2001), recovery from replication fork

arrest (Takeda et al., 1999) and S phase assembly of centromeric heterochromatin and cohesion (Bailis et al., 2003; Snaith et al., 2000).

In general, the functions of DDK are consistent with a modulation of chromatin structure during S phase. This prompted us to consider whether DDK plays a role in telomere maintenance by changing telomere chromatin structure in S phase *via* Pot1 phosphorylation. Hence, further in our study we concentrated on the function of DDK in regulation of Pot1 functions and telomere maintenance.

Among the other positive hits in the two-hybrid screen, two independent clones of Teb1/SpX were identified. Interestingly, Teb1 protein contains two Myb-like DNA-binding domains (Spink et al., 2000; Vassetzky et al., 1999). The Myb-like domains of Teb1 show high homology to the human TRF1 telomere protein. The fact that Teb1 has two N-terminal Myb-like domains makes it more similar to the Rap1 telomere protein (Kanoh and Ishikawa, 2001). It was shown previously that Teb1 binds to the human telomere sequence *in vitro* in gel shift binding assays (Vassetzky et al., 1999). These sequences are absent from fission yeast telomeres and subtelomeres, but present in the promoter sequences of numerous genes, including all the fission yeast histone genes (Vassetzky et al., 1999). We found that GFP tagged Teb1 localized throughout the nucleus, without telomere specific foci. Furthermore, Teb1 is essential (unlike other telomeric proteins), suggesting that Teb1 plays another function in addition to any telomere function (as telomeres are ultimately dispensable in fission yeast, which survives the absence of telomerase by circularizing all three chromosomes.

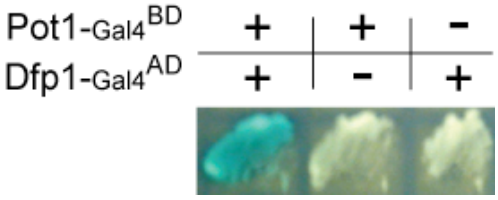


Figure 1 Pot1 interacts with Dfp1 in yeast two-hybrid system.

Pot1 bait construct Pot1-Gal4-BD interacts with prey Dfp1-Gal4-AD; β-galactosidase assay were performed on X-Gal containing media.

In our screen we also identified the product of open reading frame SPAC6F6.16c. This protein was later biochemically purified as a Pot1 interacting protein and was named Tpz1 (Miyoshi et al., 2008). Tpz1 is a structural homolog of mammalian TPP1 and telomere binding protein TEBPβ from *Oxytricha nova*. TEBPβ forms a complex with TEBPα that is important for ciliate telomere capping (Gottschling and Zakian, 1986). Importantly, the DNA binding domains of Pot1, TEBPα, TPP1 and TEBPβ have very similar crystal structures and contain multiple OB folds organized in a similar way (Lei et al., 2003; Wang et al., 2007). The fact that we identified Tpz1/TPP1 as a Pot1 interacting protein together with the later study (Miyoshi) confirm the validity of our screen and alludes to the potential for important information to be gleaned about other Pot1 interacting proteins.

Additional potential Pot1 interaction partners that we found in our two-hybrid screen are summarized in Table 4

Table 4 Results of yeast two-hybrid screen

Number of independent clones	Gene accession number and possible functions	General role	Nuclear localization, predicted	Pot1 interaction strength in two-hybrid system
6	SPBC19F5.04 Aspartate kinase (predicted)	kinase	21.7 %: cytoplasmic	**+++**
4	SPBC354.01 *S. pombe* GTP-binding protein Gtp1. Homolog of *S. cerevisiae* GIR1 which may be involved in RNA processing.	RNA processing	17.4 %: mitochondrial	**+++**
3	SPAC6F6.16c Pot1 binding partner Tpz1	telomere	17.4 %: nuclear	**+++**
3	SPCC550.13 Dfp1, regulation subunit of Hsk1 kinase. Dfp1 and Hsk1 form a kinase known as DDK	replication, cell cycle regulated kinase	82.6 %: nuclear	**+++**
2	SPAC13G7.10, Q10274 SpX/Teb1p protein containing two Myb-like DNA-binding domains	telomere, transcription factor	60.9 %: nuclear	**+++**
2	SPAC3H8.02 sec14 cytosolic factor. Has moderate similarity to *S. cerevisiae* Csr1p, which is a phosphatidylinositol transfer protein that is involved in regulation of phospholipase D	cytosolic protein	60.9 %: mitochondrial	**+++**

2	SPCC1259.12c Member of the SPRY (SPla and the RYanodine Receptor) domain containing family, has a region of moderate similarity to a region of human RANBP9, which acts in microtubule nucleation	unknown	21.7 %: nuclear	**+++**
1	SPCC285.03 ATP-dependent RNA helicase, role inferred from homology	helicase	26.1 %: mitochond-rial	**+++**
14	SPBC29A10.03c *S. pombe* conserved protein. *S. cerevisiae* homolog Rlf2p (CAC1; LRS8; PAX14) required for formation of telomeric heterochromatin-like state.	chromatin	82.6 %: nuclear	**++**
6	SPAC2F7.07c *S. pombe* hypothetical zinc finger protein. *S. cerevisiae* homolog is a catalytic component of the RPD3C(S) histone deacetylase complex. Member of the PHD-finger containing family, which may be involved in chromatin-mediated transcription regulation.	histone deacetyl-ase	43.5 %: nuclear	**++**
5	SPAC23C4.20c *S. pombe* conserved hypothetical zinc finger protein. Homolog of TRIP4 Thyroid hormone receptor interactor 4.	zinc finger transcripti on factor	30.4 %: cytoplasmic	**++**
4	SPAC12B10.13 *S. pombe* conserved hypothetical protein, homolog of GID8 (DCR1), which is involved in acceleration of the initiation of DNA replication	DNA replicati-on	65.2 %: nuclear	**++**

4	SPCC663.11 *S. pombe* hypothetical protein, sequence orphan	unknown	56.5 %: cytoplasmic	++
1	SPAC1A6.10 Moeb/ThiF domain, conserved hypothetical protein	ubiquitination	26.1 %: cytoplasmic	++
4	SPAC6B12.15 cpc2, rkp1, WD repeat protein. Protein required for normal mating, sporulation, and protein translation	mating, sporulation	69.6 %: nuclear	+
3	SPBC354.14c vac8, *S. pombe* hypothetical protein, armadillo repeat protein, role inferred from homology	unknown	52.2 %: cytoplasmic, 21.7 %: nuclear	+
3	SPCC1672.07 *S. pombe* hypothetical WD repeat protein, has low similarity to C. elegans Y45F10D.7, which is involved in positive growth regulation	unknown	73.9 %: nuclear	+
2	SPAC9.07c Putative GTP-binding protein, role inferred from homology	GTP-protein	17.4 %: mitochondrial	+
2	SPAP8A3.12c tripeptidylpeptidase (predicted), role inferred from homology	peptidase	34.8 %: cytoplasmic	+

2	SPBP23A10.11c glycoprotein, protein of unknown function, has moderate similarity to uncharacterized *S. cerevisiae* Tos1p	unknown	21.7 %: mitochondrial	+
2	SPCC320.06 *S. pombe* protein of unknown function	unknown	78.3 %: nuclear	+
2	SPCC736.16 *S. pombe* protein of unknown function, sequence orphan	unknown	73.9 %: nuclear	+
1	SPAC19A8.10 Zinc finger protein, protein ubiquitination, ubiquitin-protein ligase activity	Ubiquitination	30.4 %: endoplasmic reticulum	+
1	SPAC637.07 Moe1, Protein required for generation of a mitotic spindle and microtubule dynamics.	Microtubule dynamics	8.7 %: nuclear	+

3.2 Pot1 interacts with Dfp1 *in vivo*

DDK has an important role in cell cycle control, particularly for the regulation of replication origin firing in S-phase and replication folk progression and stability. Telomerase recruitment also occurs in S-phase and is tightly coupled with semi-conservative DNA replication. Furthermore, the maintenance of replication folk stability is an important issue for highly repetitive telomere sequences (Miller et al., 2006). Thus, we decided to concentrate on DDK and address the possible role of DDK kinase in telomere maintenance and regulation.

First, we decided to confirm the interaction between Pot1 and Dfp1 *in vivo*. We tagged Pot1 and Dfp1 endogenously with Myc and HA epitopes, respectively; both tagged Pot1 and Dfp1 were expressed from their native promoters. We immunoprecipitated Pot1-myc with anti-myc antibodies and were able to detect Dfp-HA in anti-HA Western blots (Figure 2). Dfp1-HA co-immunoprecipitates with Pot1-myc in a specific manner, as when we performed the same experiment using a strain harboring untagged Pot1, we were not able to detect Dfp1-HA in the immunoprecipitation reaction (Figure 2).

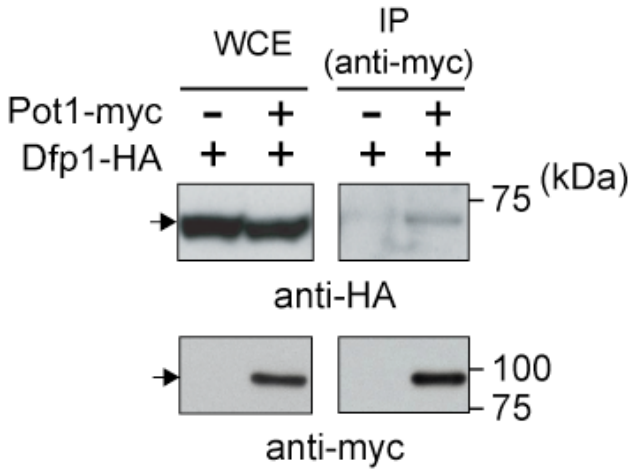


Figure 2 Pot1 interacts with Dfp1 *in vivo*.

Pot1-myc co-immunoprecipitates Dfp1-HA from the whole cell extract. Pot1-myc and Dfp1-HA were tagged endogenously and are under control of their own promoters.

4 Pot1 is phosphorylated by DDK kinase in a cell cycle dependent manner

4.1 Pot1 is a phosphoprotein

Identification of Dfp1 as a Pot1 interaction protein suggested that DDK can phosphorylate Pot1 and thus regulate Pot1 and telomere functions in a cell cycle dependent manner. Unfortunately, nothing was known about any posttranslationally modified forms of Pot1. Hence, the first challenge for us was to establish an experimental system for the detection of Pot1 modification forms, presumably phosphorylation isoforms of Pot1. We found that the most reproducible and easiest way to detect Pot1 phosphorylated forms was a two-dimensional protein gel electrophoresis (2D-PGE).

During the 2D-PGE procedure, proteins are first separated in a denaturing gel along an immobilized pH gradient; hence proteins migrate according to their isoelectric point. In the second dimension of electrophoresis, proteins are subjected to standard SDS-PAGE to separate the proteins according to their molecular mass. At the end of the 2D-PGE procedure, each protein can be identified as a spot, or a number of spots if a particular protein is represented by several posttranslational covalently modified isoforms that affect either its isoelectric point or/and molecular mass. Phosphorylation makes protein isoelectric point more acidic, which can be discerned as a shift to the more acidic region of the pH gradient in the first dimension of 2D-PGE; molecular mass of phosphorylated forms will be only

slightly different from the unphosphorylated form, or may remain virtually unchanged *vis a vis* the resolution of the gel. Sensitivity to phosphatase treatment is another criteria for identification of phosphorylated forms.

To identify Pot1 modification forms, we prepared whole cell extracts from strains carrying Pot1 endogenously tagged with a 6xV5 epitope. Protein extracts were prepared in denaturing conditions (containing 7M urea) to preserve Pot1 modification forms. The whole cell extract was then subjected to 2D-PGE. Pot1-6xV5 was subsequently detected using anti-V5 antibodies. Using 2D-PGE we were able to identify several isoforms of Pot1 (Figure 3a). One of these forms was a phosphorylated isoform, as it disappears after phosphatase treatment (Figure 3b). Using first dimension gel strips with different immobilized pH gradients, we were able to identify another phosphorylated form of Pot1 (Figure 4a). Hence, we think that Pot1 is present as two phosphorylated forms, represented by the first and the third spots if counting from the left on Figure 4a. The other three Pot1 isoforms were resistant to phosphatase treatment and could represent acetylated forms of Pot1 or some other modification.

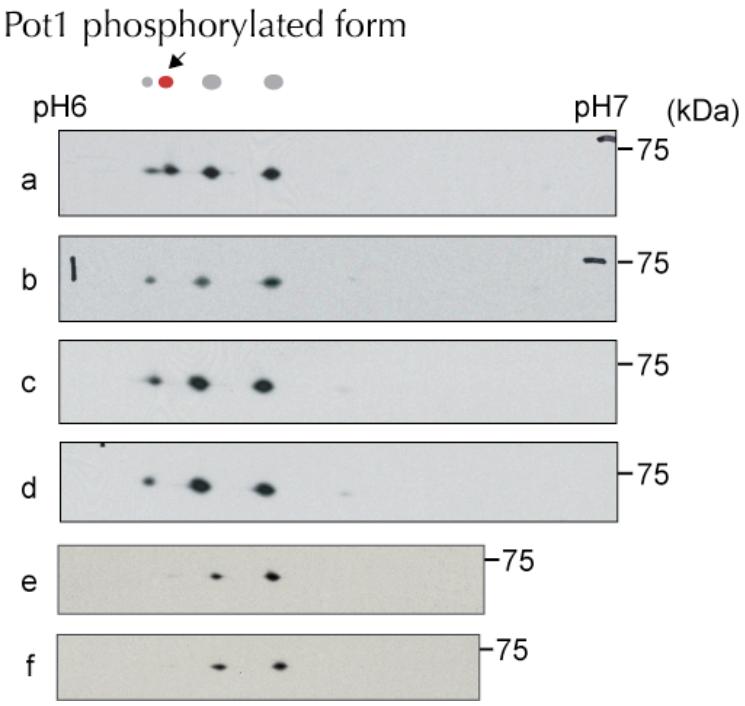


Figure 3 Pot1 phosphorylation depends on DDK activity.

Two-dimensional protein gel electrophoresis; endogenously tagged Pot1-6xV5 was detected by Western blotting. (a) wild type. (b) wild type, after λ-protein phosphatase treatment (λ-PPase). (c) *dfp1-376* (d) *dfp1-376*, λ-PPase treatment. (e) *hsk1-89* temperature sensitive mutant at 25˚C (permissive temperature). (f) *hsk1-89* ts mutant after 4 hrs incubation at 30˚C (restrictive temperature). (a-f) The first dimension was performed using 24cm Immobiline DryStrip pH 6-9.

4.2 Pot1 phosphorylation depends on DDK

Identification of Pot1 phosphoforms by 2D-PGE made it possible to check whether Pot1 phosphorylation is dependent on DDK. For this purpose, we analyzed the presence of Pot1 phosphorylated forms in mutants of both the regulatory and catalytic components of DDK. In *dfp1-376* mutants, which have very low DDK activity (Fung et al., 2002), we were not able to detect any phosphorylated forms of Pot1 (Figure 3c). We confirmed this by treating *dfp1-376* extracts with phosphatase; as expected, all Pot1 isoforms were resistant to phosphatase treatment (Figure 3d).

After determination that the phosphorylated state of Pot1 depends on Dfp1, a regulatory subunit of DDK, we sought to determine whether the catalytic subunit of DDK, Hsk1, is also essential for Pot1 phosphorylation. We were not able to detect any Pot1 phosphorylation isoforms in *hsk1-89* temperature sensitive mutant (Takeda et al., 2001) in extracts of cells grown at both permissive and restrictive temperatures (Figure 3e, f). We conclude that Pot1 is a phosphoprotein, and that its phosphorylation status is dependent on DDK activity, as mutating either the Dfp1 or Hsk1 components of DDK results in disappearance of Pot1 phosphorylation isoforms.

It was important to determine whether the effect on Pot1 phosphorylation that we saw in DDK mutants is specific only to this kinase. Pot1 contains several consensus sites (TQ, SQ) for the Rad3 (fission yeast ATR) and Tel1 (fission yeast ATM) kinases; thus, Pot1 potentially could be phosphorylated by these kinases. Moreover, it was shown that Mec1 (the

budding yeast Rad3 homolog) and budding yeast Tel1 phosphorylate Cdc13 (Tseng et al., 2006), a presumed *S. cerevisiae* functional homolog of Pot1. Deletion of both *rad3* and *tel1* simultaneously in *S. pombe* results in complete telomere loss, followed by the appearance of survivors with circular chromosomes. Interestingly, simultaneous deletion of both *rad3* and *tel1* fails to affect the presence of Pot1 phosphorylated forms (Figure 4c). Thus, the phosphorylation status of Pot1 specifically depends on DDK kinase, and not on the Rad3 and Tel1 kinases.

Pot1 phosphorylated forms were also present in strains with circular chromosomes that were created by deleting the gene encoding the catalytic protein subunit of telomerase, $trt1^{+}$ (Figure 4b), despite the fact that these strains completely lack telomere sequences. This result indicates that the phosphorylation status of Pot1 is independent of Pot1 binding to telomeric DNA.

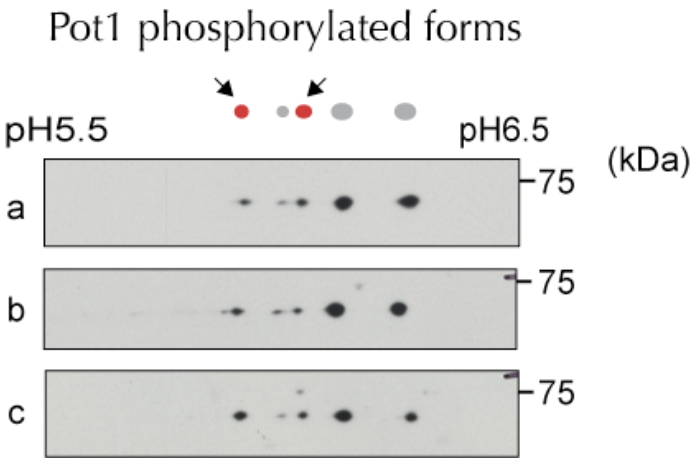


Figure 4 Pot1 phosphorylation is independent on Pot1 telomere localization and activities of Rad3 and Tel1 kinases.

(a) wild type. (b) *trtΔ* survivals with circular chromosome and without telomere sequences (c) *rad3Δ tel1Δ* circular chromosome survivors. Here for firs dimension we used 24cm Immobiline DryStrip with a pH gradient 4-7.

4.3 DDK can phosphorylate Pot1 directly *in vitro*

The fact that Pot1 phosphorylation depends on DDK may mean that DDK can phosphorylate Pot1 directly; alternatively, DDK may effect Pot1 phosphorylation indirectly by regulating the activities of other kinase(s). In order to distinguish between these two possibilities, we determined whether DDK could phosphorylate Pot1 directly *in vitro*. We did so by incubating bacterially expressed Pot1 with immunoprecipitated DDK from a *S. pombe* extract. (Unfortunately, we were not able to produce bacterially express full-length Pot1, we were able to purify only Pot1 OB fold). Indeed, the Pot1 OB fold was specifically phosphorylated *in vitro* by immunoprecipitated DDK (Figure 5). This data suggests that DDK directly phosphorylates Pot1, presumably within the OB fold.

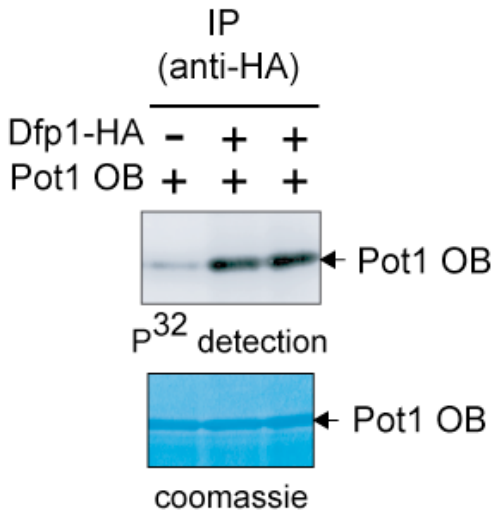


Figure 5 DDK phosphorylates Pot1 OB fold *in vitro*

Dfp1-HA was immunoprecipitated as described in Material and Methods section. Beads were incubated with 20 µl of reaction mix (kinase buffer supplemented with 10 µM ATP and 5 µg of purified recombinant Pot1-OB-fold (amino acids 1-185), including 0.25 µCi/µl γ-^{32}P-ATP) for 20 min at 32°C. The proteins were separated by NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel with MOPS SDS running buffer (Invitrogen).

4.4 Pot1 phosphorylation status is regulated through the cell cycle

Our Pot1 phosphorylation data suggest that Pot1 is phosphorylated by DDK directly. Thus, the Pot1 phosphorylation state should be regulated throughout the cell cycle in a manner that correlates with the activity of DDK. DDK is activated at the beginning of S-phase and its activity remains present for the majority of the G_2-phase of cell cycle (Brown and Kelly, 1999).

To synchronize cells, we employed the conditional mutant *cdc25-22*, whose incubation at restrictive temperature arrests cells at the G2/M transition. Pot1-6V5 *cdc25-22* cells were arrested in G_2/M by shifting the incubation temperature to 36˚C for 3.5 hrs. The block was released by shifting the temperature down to 25˚C, and samples were collected every 15 min and processed for 2D-PGE; Pot1 modification forms were then detected by Western blot. Strikingly, Pot1 phosphoforms were indeed cell cycle regulated. They appear at the beginning of S-phase and are maintained throughout G_2 (Figure 6), correlating with the Dfp1 expression profile and the activity of DDK. Base on this data, we conclude that DDK kinase directly phosphorylates Pot1 in a cell cycle dependent manner.

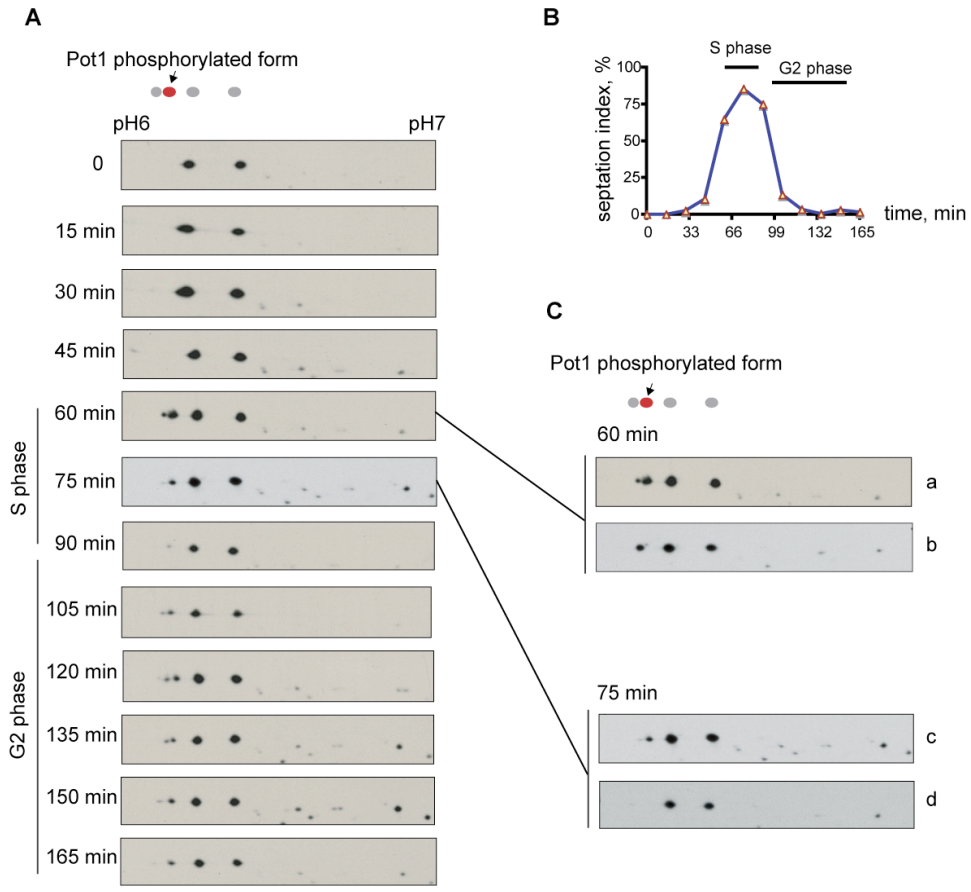


Figure 6 Phosphorylation of Pot1 is cell cycle regulated.

Pot1 is phosphorylated in the beginning of S phase and then remains phosphorylated throughout G_2. (B) Analysis of synchronicity of the cell cycle progression. The peak of septation represents the middle of S-phase. (C) Modified form of Pot1 that appears at the beginning of S-phase (60min) is a phosphoform. (a) 60 min time point (beginning of S-phase). (b) 60 min time point treated with λ-PPase . (c) 75 min time point, (d) 75 min time point after λ-PPase treatment. The first dimension IEF was performed using 24cm Immobiline DryStrip pH 6-9.

5 Pot1 is phosphorylated in a conserved region of the OB fold

The finding that Pot1 is phosphorylated by DDK in a cell cycle dependent manner was extremely exciting. This discovery opens a great opportunity for investigation of how Pot1 and telomere function are regulated through the cell cycle. In order to address this issue, the identification of Pot1 phosphorylation sites became tantamount.

5.1 Pot1 is phosphorylated in N-terminal region of Pot1

Although DDK is known to be a serine- and threonine-specific kinase, it has not been possible to discern a consensus site for DDK (Sheu and Stillman, 2006). Thus, we used a systematic mutation approach to identify serines and threonines that are phosphorylated in Pot1. We constructed Pot1 mutants where in different regions of Pot1, all serines and threonines were replaced with alanines (Figure 7). These mutant *pot1* alleles were integrated in the *aur1* locus on Chromosome I in a strain expressing endogenous Pot1; expression of the ectopically integrated *pot1* alleles was controlled by the endogenous *pot1*$^{+}$ promoter sequence. Mutation of all serines and threonines in the Pot1 C-terminus (between residues S420 and T517) and central region (between T173 and S252) failed to diminish Pot1 phosphorylation, although the amount of Pot1 phosphoisoforms in protein harboring C-terminal mutations was reduced, suggesting that this region is important for the efficient phosphorylation of

Pot1. More interestingly, mutation of all serines and threonines in the N-terminal region of Pot1 (residues between T58 and T154) completely abolished Pot1 phosphorylation. Serines and threonines in the region between T58 and T89 were essential for Pot1 phosphorylation (Figure 7); hence, we concentrated on this region of the protein (Pot1 T58A-S89A) to find the exact phosphorylation sites.

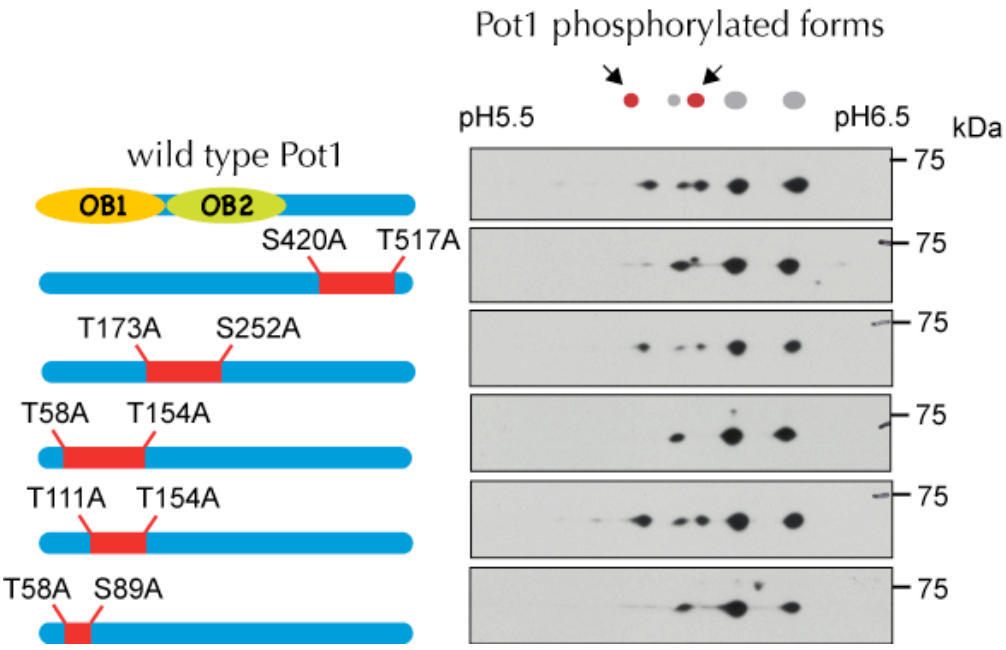


Figure 7 Pot1 is phosphorylated in N-terminal part of OB-fold between Thr58 and Ser89.

Several Pot1 mutations were constructed; red rectangles indicate the areas where all serines and threonines were mutated to alanines. Pot1 mutants were expressed from Pot1 promoter and contain C-terminally fused 6xV5 tag. 2D PAGE was followed by Western blotting with ani-V5 anybodies to detect Pot1 mutants. For first dimension IEF we used 24cm Immobiline DryStrip pH 4-7.

5.2 Identification of Pot1 phosphorylation sites

In the Pot1 T58A-S89A mutant, seven serines and threonines were mutated to alanines, resulting in the absence of Pot1 phosphorylated forms (Figure 8). To find the exact phosphorylation site, we used the following strategy. As a starting point for our mutational analyses, we chose the fully substituted Pot1 T58A-S89A mutant. We mutated individual alanines back to their wild type state (to serines or threonines as appropriate). Then we checked the phosphorylation status of the partially S/T-restored mutants to determine which serines and threonines could confer phosphorylation *in vivo*. When we mutated A58 and A89 of the Pot1 T58A-S89A mutant back to S58 and S89 respectively, the resulting Pot1 mutants were still not phosphorylated (Figure 8). In contrast, when A68 and A75 of Pot1 T58A-S89A mutant were mutated to T68 and T75, all Pot1 phosphorylated forms were restored (Figure 8), suggesting that T68 and T75 are the relevant phosphorylated residues *in vivo*.

If T68 and T75 are the only phosphorylated amino acids in Pot1, we would not expect to detect Pot1 phosphorylated forms in Pot1 harboring the T68A and T75A mutations. However, in Pot1 T68A, T75A the amount of phosphorylated forms is reduced rather than abolished (Figure 9); in order to completely remove Pot1 phosphorylated forms, mutations T68A, T75A along with T78A, T79A and S80A are required (Figure 9). These data suggest that although T68 and T75 are phosphorylated *in vivo* and are presumably the most important phosphorylation sites (see below), T78, T79 and S80 can be phosphorylated as alternative sites.

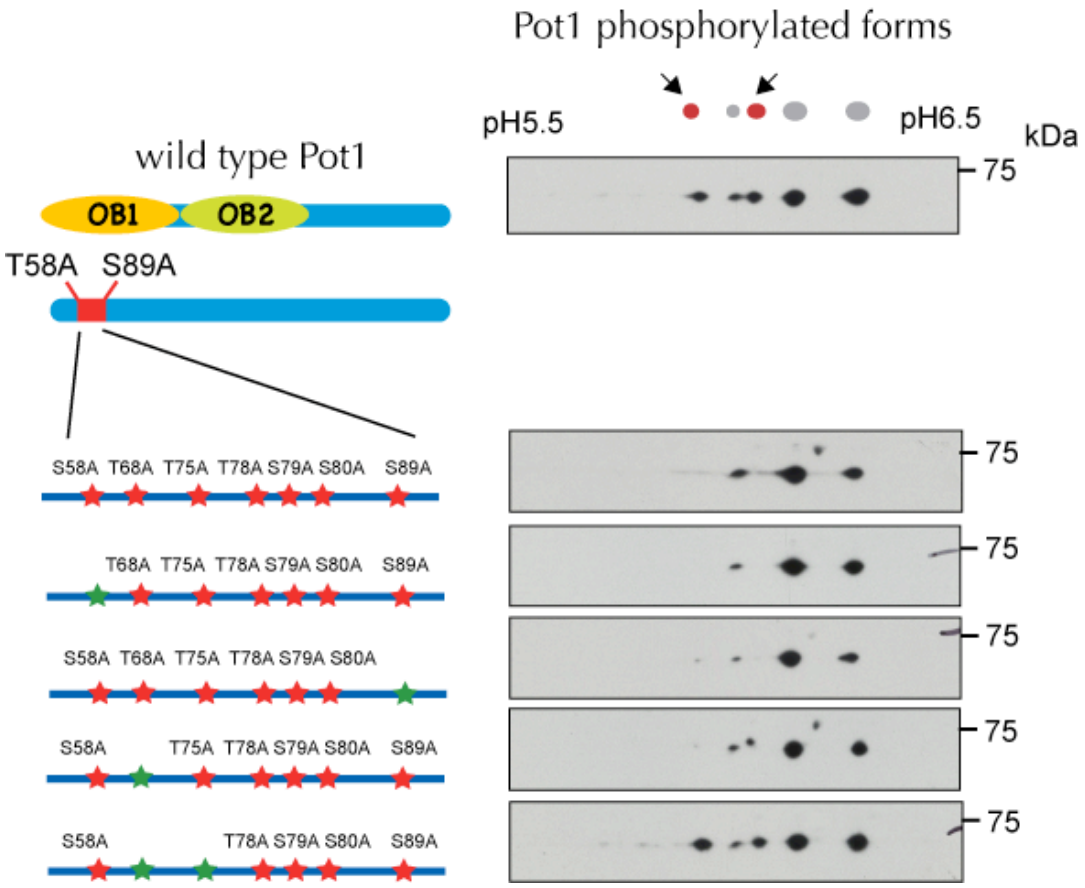


Figure 8 Pot1 Thr68 and Thr75 are phosphorylated *in vivo*.

Individual residues in Pot1 T58A-S89A mutant were mutated back to wild type, to check whether individual serines and threonines will restore wild type state of Pot1 phosphorylation. Green stars correspond to wild type amino acids, red stars corresponds to serines or threonines mutated to alanine.

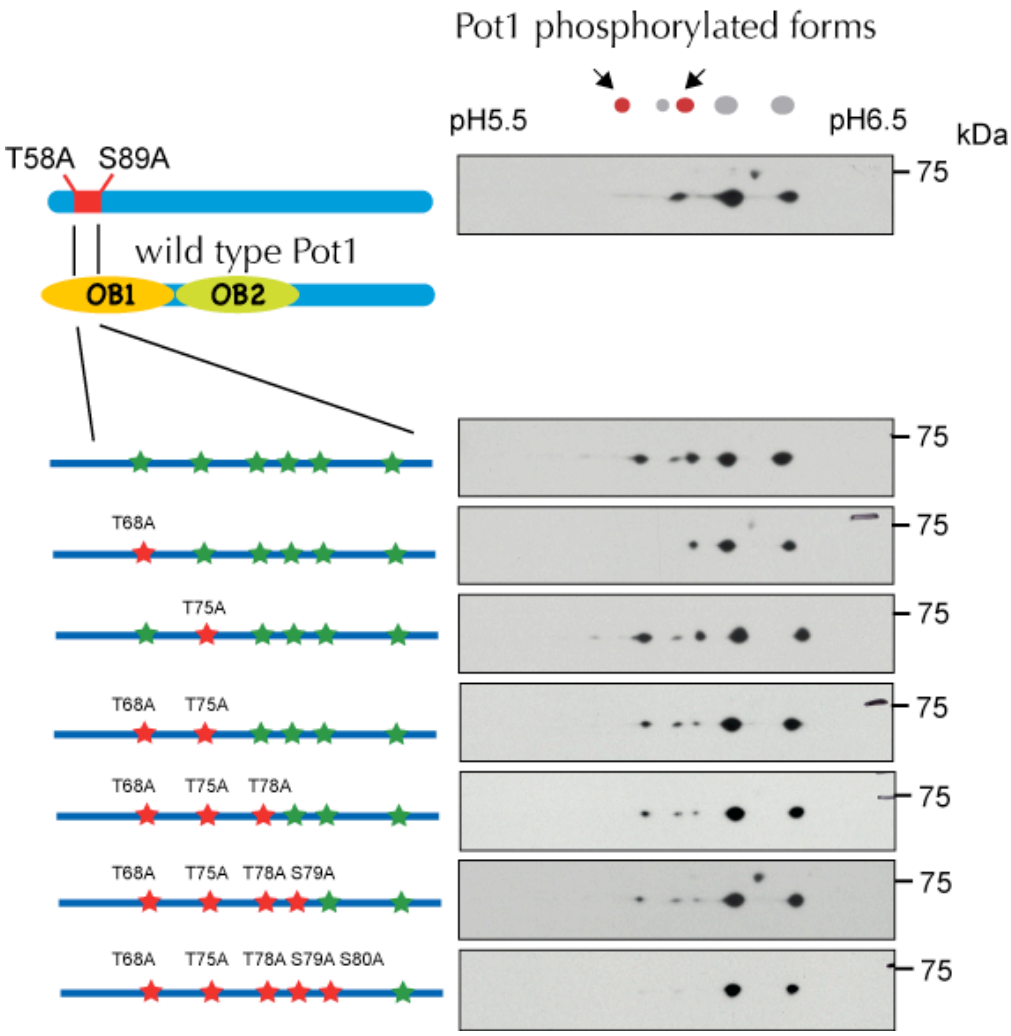


Figure 9 Pot1 $_{T68A, T75A, T78A, S79A, S80A}$ lose all phosphorylated forms.

Serines and threonines in wild type Pot1 (green stars) were replaced by alanines (red stars).

5.3 Pot1 phosphorylation sites are highly conserved

T68, T75 are located in a very conserved region of the Pot1 OB fold and these amino acids are present in Pot1 from different species (Figure 10).

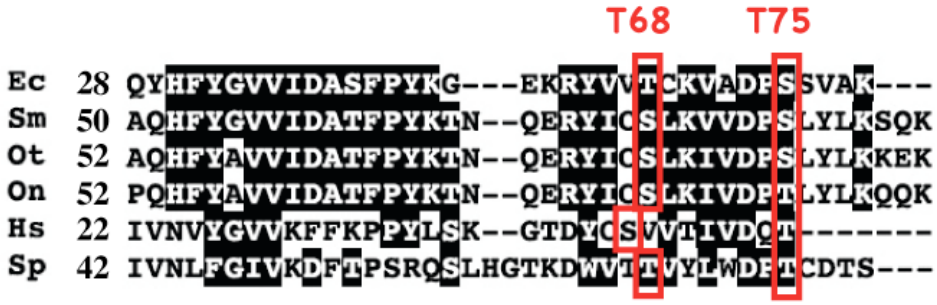


Figure 10 S. pombe Pot1 T68 and T75 are highly conserved in eukaryotes that have Pot1.

Ec, *Euplotes crassus*; Sm, *Stylonychia mytilis*; Ot, *Oxytricha trifallax*; On, *O. nova*; Hs, *Homo sapiens*; Sp, *S. pombe*.

5.4 Pot1 purification and identification of Pot1 phosphorylation sites by mass spectrometry

Mutational analysis of Pot1 phosphorylation sites suggests that residues T68A, T75A, T78A, S79A, S80A could be phosphorylated. Only by mutating all these sites to alanines can one completely abolish Pot1 phosphorylation and observe the absence of Pot1 phosphorylated forms in (2D-PGE). This genetic analysis provides us with important information about Pot1 phosphorylation sites. However, the direct identification of Pot1 phosphorylation sites by physical methods like mass spectrometry would be the ideal confirmation of our genetic data.

In order to generate enough material for mass spectrometry analysis of Pot1 modification forms, we purified endogenously tagged Pot1-6xV5 protein from 20L of yeast culture. We used a two-step purification strategy. First, Pot1 was immunoprecipitated using ani-V5 antibodies coupled with protein A-sepharose beads and eluted by V5 peptide. The eluant was then loaded on a heparin column for the second step, and the purified Pot1 complex was eluted by increasing salt concentration. The protein was then concentrated and separated by either regular SDS-PAGE or 2D-PGE (Figure 11).

Using our two-step purification strategy, we purified sufficient quantities of Pot1 complex that it was possible to identify different components of the Pot1 complex on a Coomassie stained gel. Then we used tandem mass spectrometry to identify Pot1 interacting partners and Pot1 phosphorylation sites. This was done in collaboration with two different groups, first, with Dr.

Richard Jones from FDA, Alabama and second, the group of Dr. Steven Gygi at Harvard University. We confirmed our purification results by identifying Tpz1, which we had also found in our two-hybrid screen as a Pot1 interacting partner. Two other known Pot1 interacting proteins, Poz1 and Ccq1 (Figure 11a) (Miyoshi et al., 2008), were also present in our purifications. Separating purified Pot1 by 2D-PGE followed by Coomassie staining revealed all the Pot1 modification forms we had seen previously (Figure 11a). This suggests that the modification status of Pot1 is preserved during our purification procedure.

We identified several possible phosphorylation sites by tandem mass spectrometry in collaboration with Steven Gygi's laboratory, although the amount of purified Pot1 material was insufficient for complete analysis of Pot1 phosphorylation sites. The identified possible phosphorylation sites were: S55, T78, S79, S80, S89, S183, T184, S352, S354. This is only preliminary data and further Pot1 purifications and mass spectrometry analysis are required to identify the exact Pot1 phosphorylation sites. Interestingly, three out of the five Pot1 phosphorylation sites that we identified using our genetic mutational approach, T78, S79 and S80, were also identified by mass spectrometry, suggesting that the phosphorylation sites that we found in our mutational analysis are actually phosphorylated *in vivo*.

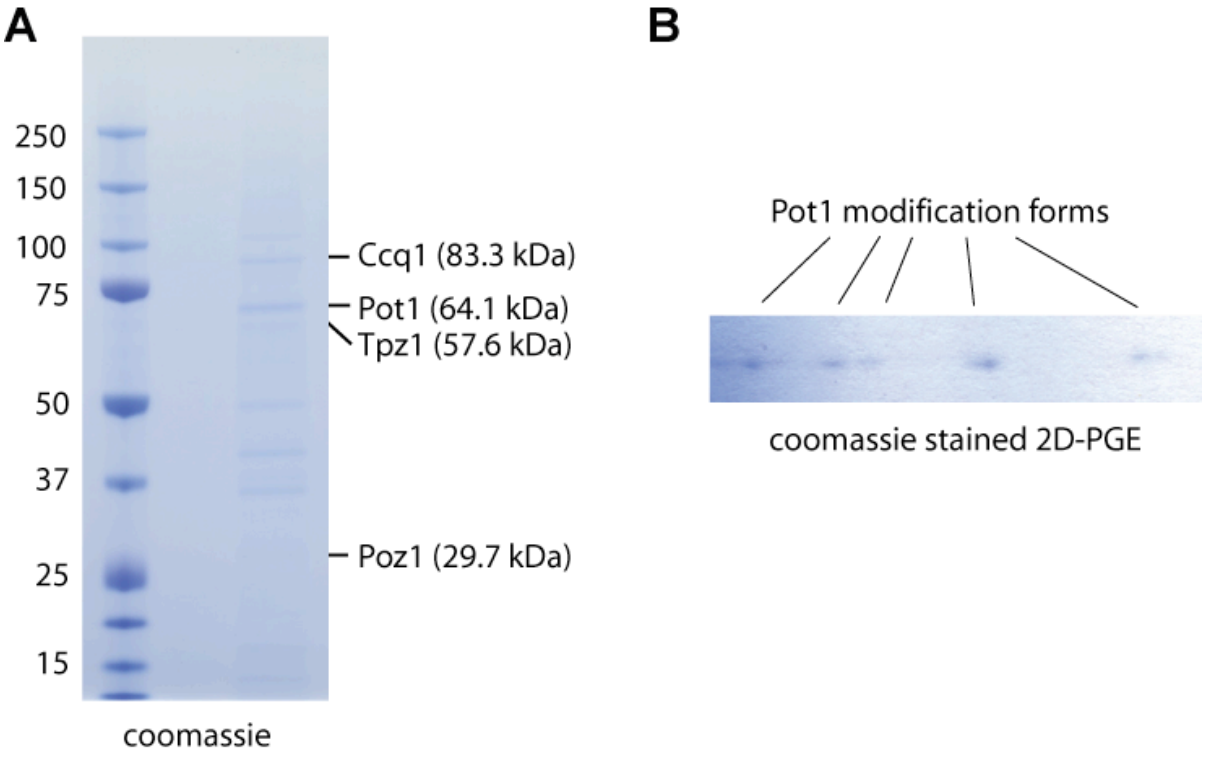


Figure 11 Purification of Pot1 complex.

(A) Pot1-6xV5 was purified from 20L of S. pombe culture in two-step purification procedure. The half of resulting purified proteins was separated on SDS-PAGE and stained with coomassie. The rest of protein was subjected to 2D-PGE (B) to confirm the presence of all Pot1 modification forms in purified Pot1.

6 The function of Pot1 phosphorylation

6.1 Pot1 phosphorylation suppresses DNA damage response at telomeres

After identification of DDK phosphorylation sites in Pot1, we focused on whether and how these phosphorylation events regulate Pot1 functions. We constructed diploid strains expressing various Pot1 phosphomutants in a heterozygous *pot1+/Δ* background. Pot1 phosphomutants were inserted in aur1 locus and were under the control of Pot1 native promoter. After these diploids were sporulated, we performed tetrad dissections and analyzed colonies that expressed only the exogenous copy of Pot1. The Pot1 phosphodeficient mutant Pot1-5A (Pot1 $_{T68A, T75A, T78A, S79A, S80A}$), which loses all phosphorylated forms, formed colonies distinct from those arising from *pot1Δ* spores (Figure 12): they were larger, but not as large as wild type. In contrast to the wild type colonies, the edges of Pot1-5A colonies were not round but instead were wrinkled and irregular, suggesting that these colonies contained dying cells. Moreover, Pot1-5A expressing cells were elongated (Figure 13) indicating that a cell cycle checkpoint arrest was activated. Mutations in the two highly conserved Pot1 phosphorylation sites identified as most important, T68A and T75A (hereafter referred to as the Pot1-2A mutant), have the same effect as the Pot1-5A mutations (Figure 12 and Figure 13).

Interestingly, the converse Pot1phosphomimetic mutations T68D and T75D (Pot1-2D) do not activate the checkpoint response (Figure 12 and Figure

13), as cell lengths were normal. This allowed us to conclude that phosphorylation of two Pot1 threonines, T68 and T75, is a minimal requirement for suppression of checkpoint activation at telomeres. The cell elongation in Pot1-2A and Pot1-5A depends on the presence of Chk1, a key component of the G2/M DNA damage checkpoint pathway and downstream target of ATR (Figure 13), suggesting that cell elongation was due to activation of the G2/M DNA damage checkpoint. We conclude that Pot1 phosphorylation by DDK prevents telomeres from activating a DNA damage checkpoint response, and this requirement could be overcome by introducing the Pot1-2D phosphomimetic mutations.

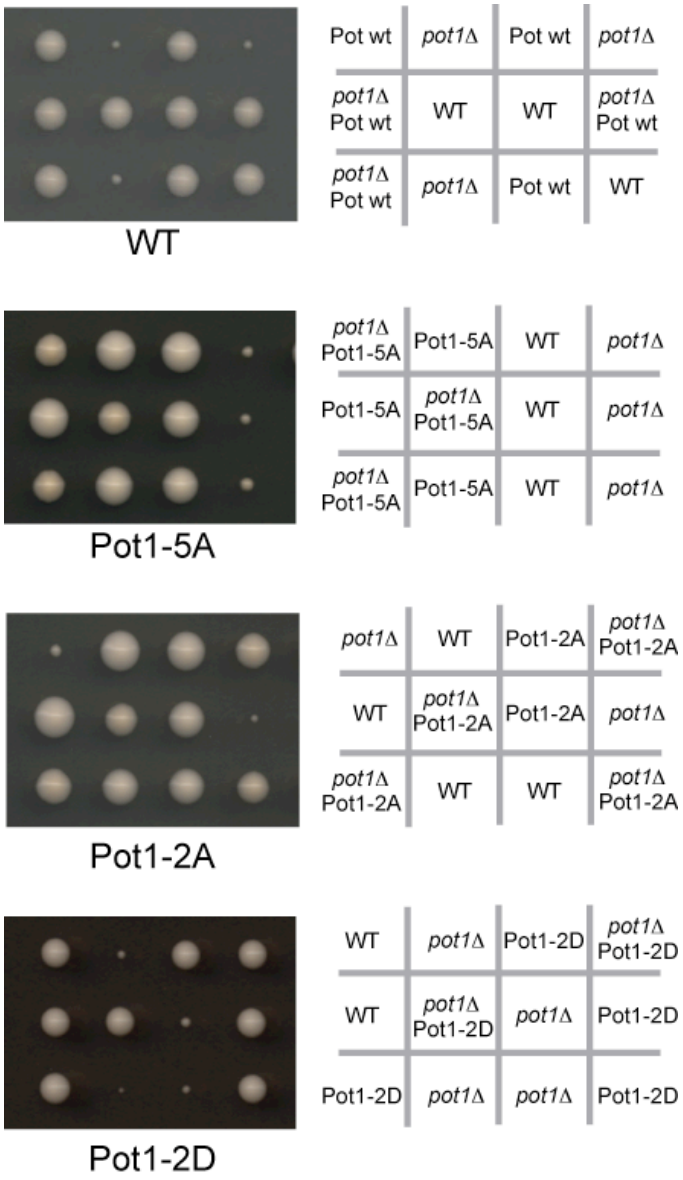


Figure 12 Tetrad analysis of Pot1 phosphomutants.

Heterozygous *pot1+/Δ* diploids containing Pot1 wt or different Pot1 phosphomutants Pot1-5A, Pot1-2A and Pot1-2D integrated in aur1 locus on chromosome I were sporulated and subjected to tetrad dissection.

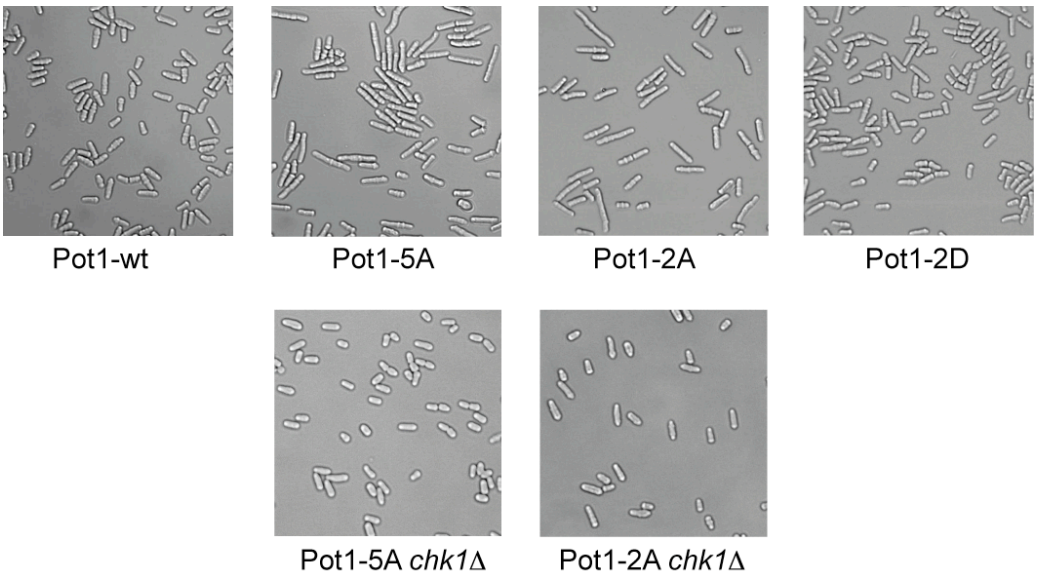


Figure 13 Cell morphology analysis of Pot1 mutants.

Cells were taken from colonies arising from sporulated and tetrad-dissected diploids on Figure 12. They were *pot1Δ* with corresponding Pot1 mutant integrated in aur1 locus. The *chk1Δ* Pot1 double mutants were obtained from *chk1+/Δ pot1+/Δ* heterozygous diploid with Pot1 mutants integrated at the *aur1* locus.

6.2 Telomere maintenance in strains harbouring Pot1 phosphomutants depends on DNA damage checkpoint

Next we checked whether DDK dependent Pot1 phosphorylation controls telomere length and telomere maintenance. The telomere length in cells harbouring Pot1-5A or Pot1-2A was longer than in wt strains, while Pot1-2D conferred the same telomere length as Pot1 wt, indicating that Pot1-2D was mimicking the wild type phosphorylation state of Pot1 in terms of telomere length homeostasis (Figure 14). Interestingly, we didn't observe any differences in G-rich overhang signal between Pot1 wt and Pot1 phospho mutants indicating that the Pot1 phospho-mutations specifically affect telomere length homeostasis and telomere checkpoint suppression, whereas Pot1 still control telomere C-strand resection Figure 16.

Surprisingly, the maintenance of Pot1-2A elongated telomeres was dependent on Chk1. Pot1-2A *chk1*Δ double mutant lose all telomeric signal immediately after sporulation (Figure 15), whereas deletion of *chk1*Δ alone had no effect on telomere maintenance. Thus, the activation of the DNA damage checkpoint is critical for telomere maintenance in Pot1-2A. Phosphomimetic Pot1-2D *chk1*Δ double mutant also lose telomeres, suggesting Chk1 function in maintenance of Pot1-2D telomeres. The simple picture of phosphorylation-deficient mutants displaying telomeric defects and phosphomimetic mutants displaying wt phenotypes turned out to be misleading (see below), suggesting an intriguing and more complex system in which cycles of phosphorylation are

important, with both phosphorylated and unphosphorylated states playing crucial roles.

The importance of Chk1 for telomere maintenance was very surprising. It was known that upstream components of DNA damage response like the Rad3 and Tel1 kinases are important for telomere maintenance. Telomeres in *rad3Δ* are very short, and telomeres completely disappear in *rad3Δ tel1Δ* double mutants resulting in survivors with circular chromosomes (Naito et al., 1998). However, a role for Chk1, a downstream component of the DNA damage response, in telomere regulation was previously unknown. As the known Chk1 functions involve its kinase activity, Chk1 could be directly involved in the maintenance of unprotected Pot1-2A telomeres *via* phosphorylating telomeric proteins or indirectly by controlling cell cycle progression.

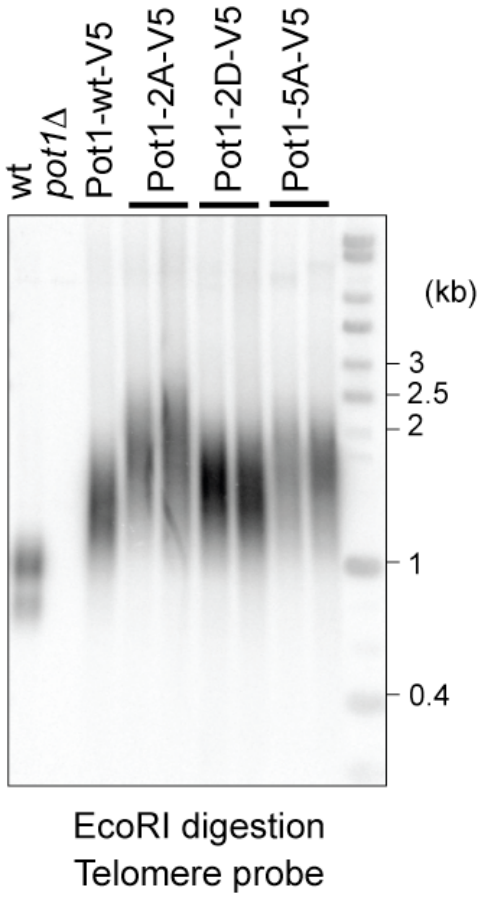


Figure 14 Telomeres in Pot1 phosphodeficient mutants are elongated compare to Pot1 wt and Pot1 phosphomimetic mutant.

Cells were taken from colonies arising from sporulated and tetrad-dissected diploids on Figure 12. They were *pot1Δ* with corresponding Pot1 mutant integrated in aur1 locus. The liquid cultures were growing overnight and cells were collected for Southern blot analysis.

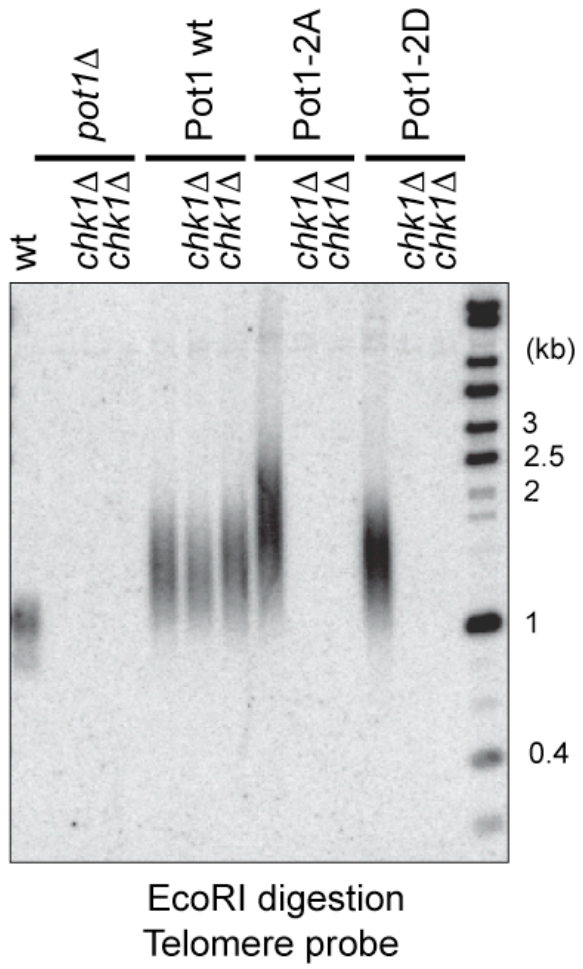


Figure 15 Telomere maintenance in Pot1 wt, Pot1-2A and Pot1-2D mutants in the presence and absence of *chk1*.

The *chk1Δ* Pot1 double mutants were obtained from *chk1+/Δ pot1+/Δ* heterozygous diploid with Pot1 mutants integrated at the *aur1* locus.

6.3 Telomere maintenance is telomerase independent in Pot1 phosphodeficient mutants

Telomeres in Pot1-5A and Pot1-2A mutants are elongated compare to Pot1-wt and Pot1-2D. Moreover, telomere maintenance in Pot1 phosphomutants depends on checkpoint activation. These data led us to suspect that telomere maintenance in the phosphodeficient mutants might be independent of telomerase and could be through recombination-based mechanisms. We checked this possibility by deleting telomerase (*trt1*) in the Pot1 phosphomutant diploid strains and monitoring telomere state over several ensuing generations. Cells harbouring endogenously tagged Pot1-6xV5 contain stably mildly elongated telomeres. This mild elongation depends on telomerase, as it is not observed in *trt1Δ* cells containing Pot1-6xV5. Gradual telomere shortening ensues upon *trt1* deletion in wild type cells (Figure 17), as is characteristic for telomerase dependent telomere maintenance.

However, we observed a very different telomere maintenance phenotype in Pot1-5A *trt1Δ* and Pot1-2A *trt1Δ* double mutants. There was no gradual telomere shortening as is usual for telomerase-deleted strains. Instead, telomere length remains constant following *trt1* deletion (Figure 17). This indicates that telomere retention in Pot1-5A and Pot1-2A mutants is telomerase independent.

Telomere maintenance in the phosphomimetic Pot1-2D *trt1Δ* double mutant depends on telomerase as in wild type cells: telomerase deletion in Pot1-2D leads to gradual telomere shortening (Figure 17).

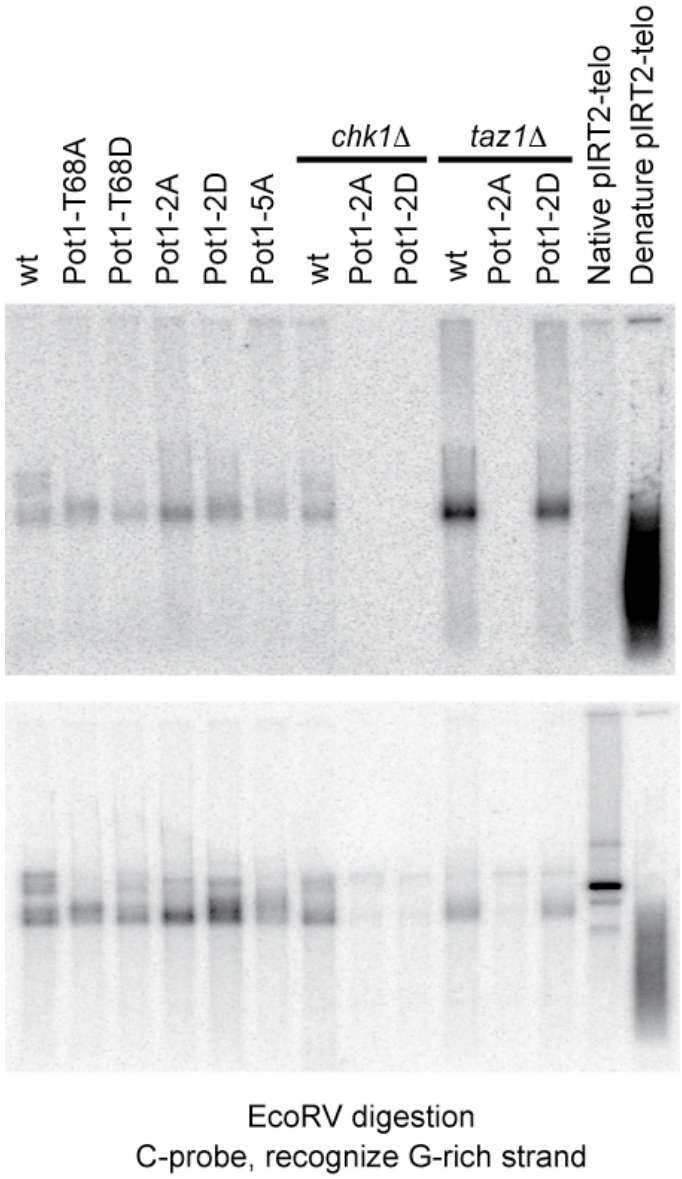


Figure 16 Detection of 3′-overhang in different Pot1 phosphomutants using in gel hybridization technique

The upper gel was running in the native conditions, the DNA was denatured and the gel was re-probed with C-probe (the bottom gel). Native and denature pIRT2-telo plasmid were used as a negative and positive control correspondently for single stranded telomere DNA

This suggests that the DDK dependent phosphorylation of Pot1, which we were able to mimic by creating Pot1-2D mutant, is crucially required for telomerase dependent telomere maintenance.

The telomere maintenance in Pot1-5A *trt1Δ* and Pot1-2A *trt1Δ* double mutants probably involved alternative recombination dependent mechanisms that are normally not active in wild type cells. The recombination dependent telomere maintenance becomes important in telomerase deleted yeast survives with linear chromosomes and ALT surviving mammalian cell lines with constant recombination at their telomeres (Bryan et al., 1997; Lundblad and Blackburn, 1993; Nakamura et al., 1998; Reddel, 2003; Teng and Zakian, 1999). In contrast Pot1-5A and Pot1-2A phosphodeficient mutants have functional telomerase, but were not able to use it for telomere maintenance, probably because of constant activation of DNA damage response at Pot1-5A and Pot1-2A telomeres. Activation of DNA damage response at telomeres could inhibit telomerase recruitment by recruiting RPA, Rhp51 and other repair factors to telomere single stranded overhangs during DDR. A similar mechanism was described for preventing *de novo* telomere addition at a double stranded break site by competition for resected single stranded DNA ends with homologues recombination factors (Cullen et al., 2007). Thus Pot1-5A and Pot1-2A telomeres behave like double stranded breaks by activating DDR and preventing telomere addition in the presence of functional telomerase.

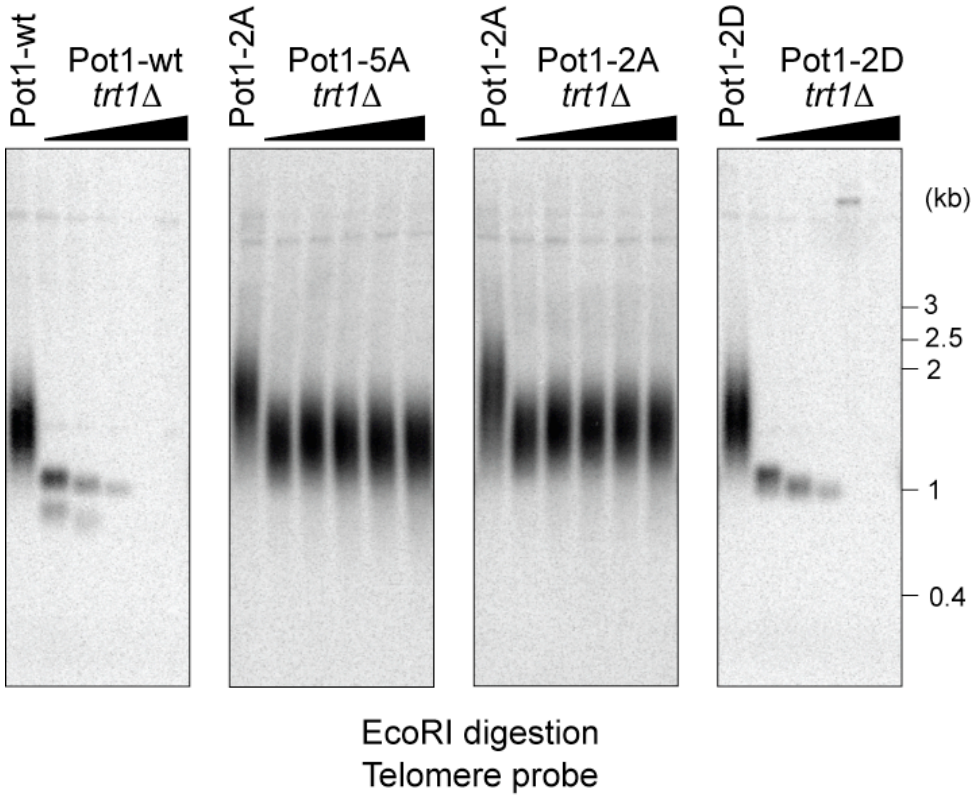


Figure 17 Telomere maintenance in Pot1 phosphomutants in the absence of telomerase.

Trt1+ and *trt1*Δ Pot1 doubles mutants were obtained from the same Pot1 phosphomutant diploids. The cell were growing for 16 days on plates, every 3 days single colonies were restreaked to the new plates and liquid cultures were inoculated, then the cultures were growing overnight and cells were collected for Southern blot analysis.

6.4 Pot1 phosphodeficient mutants maintain their telomeres by activating constant homologous recombination at telomeres

The fact that maintenance of Pot1-5A and Pot-2A telomeres is independent of telomerase led us to predict that telomere maintenance in these mutants would be due to constant homologous recombination events. In this case, we would expect to see ongoing rearrangements of the subtelomeric regions, an often-used readout for telomeric recombination. Figure 18 shows a restriction map of the fission yeast subtelomeric regions. These regions are heterogeneous, so the exact locations of subtelomeric restriction vary between different telomeres. In order to examine the stability of this region, we digested genomic DNA with NsiI restriction enzyme and performed Southern blotting using a subtelomeric probe (Figure 18). In Pot1-wt cells, the subtelomeric restriction pattern remains constant over time (Figure 19). In contrast, in Pot1-5A and Pot-2A mutants the subtelomeric regions undergo substantial rearrangement, leading to a homogenization of fragment sizes. This likely reflects progressive loss and/or rearrangement of subtelomeric restriction sites, due to hyper-recombination in the area. The Pot1-2D phosphomimetic mutant conferred a mild de-regulation of subtelomeric recombination, but this is clearly less dramatic than that conferred by Pot1 phosphodeficient mutants (Figure 19).

In order to confirm that the instability of the subtelomeric restriction pattern in Pot1-5A and Pot-2A mutants stems from elevated homologous

recombination, we compromised homologous recombination by deleting *rhp51*$^{+}$ (Figure 20). This led to the complete loss of telomeres in Pot1-2A *rhp51*Δ and Pot1-5A *rhp51*Δ double mutants. These results suggest that checkpoint activation in the absence of Pot1 phosphorylation changes the mode of telomere maintenance from telomerase dependent to recombination dependent.

Surprisingly, Pot1-2D *rhp51*Δ cells also lose telomeres at an even faster rate than the telomere loss seen in Pot1-2A *rhp51*Δ and Pot1-5A *rhp51*Δ strains (Figure 20). Hence, we suspect that the reason for Pot1-2D *rhp51*Δ telomere loss is different from that observed in the phosphodeficient mutant backgrounds, and may be connected with some *Rhp51* dependent telomere protection function that becomes important in the absence of unphosphorylated Pot1.

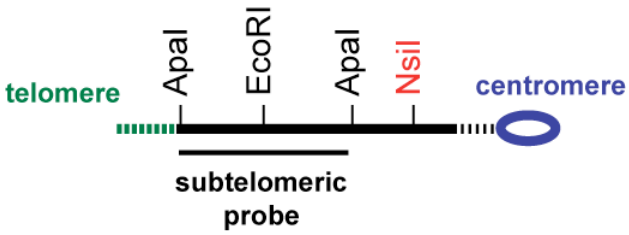


Figure 18 The restriction map of S. pombe subtelomeric region

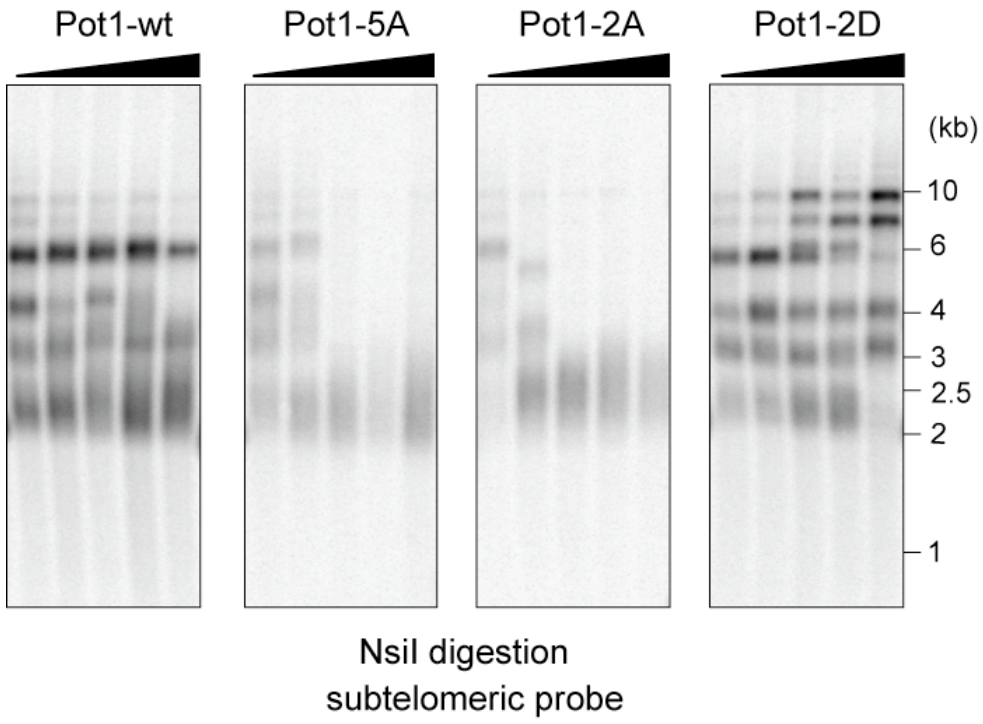


Figure 19 Massive subtelomeric rearrangements in Pot1 phosphodeficient mutant

Cells were taken from colonies arising from sporulated diploids on Figure 12. They were *pot1Δ* with corresponding Pot1 mutant integrated in aur1 locus. The cell were growing for 16 days on plates, every 3 days single colonies were restreaked to the new plates and liquid cultures were inoculated, then the cultures were growing overnight and cells were collected for Southern blot analysis.

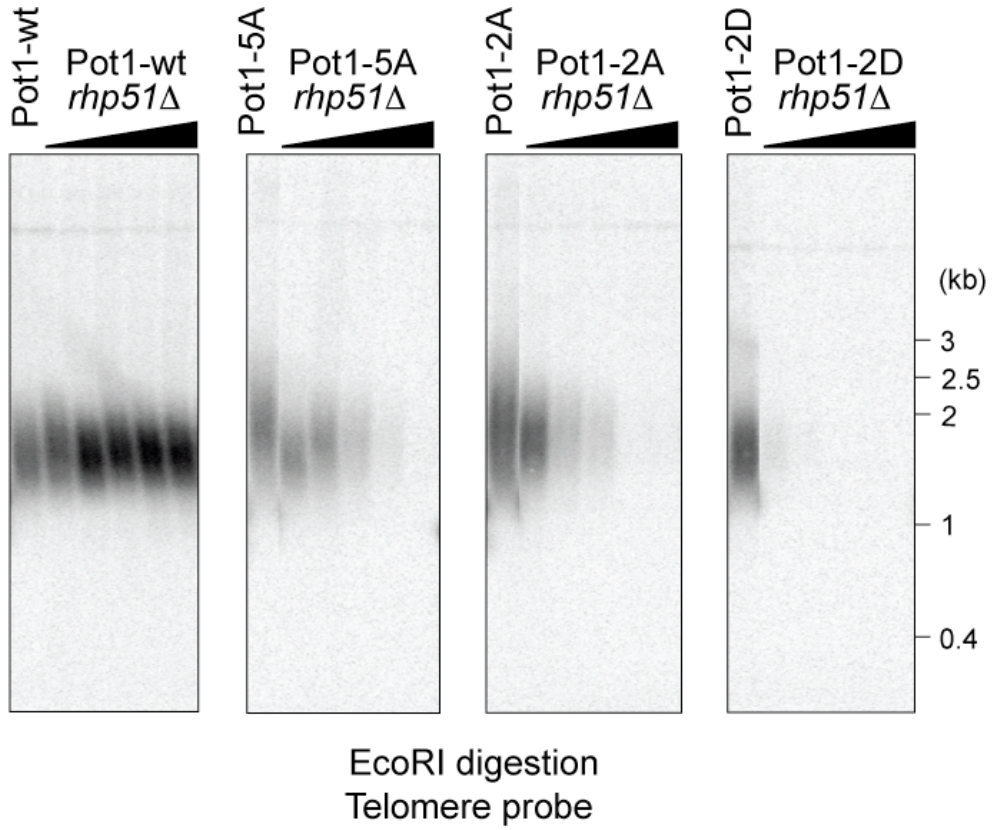


Figure 20 In Pot1 phospho mutants telomere maintenance depends on Rhp51

rhp51+ and *rhp51*Δ Pot1 doubles mutants were obtained from the same Pot1 phosphomutant diploids. The cell were growing for 16 days on plates, every 3 days colonies were restreaked to the new plates and liquid cultures were inoculated, then the cultures were growing overnight and cells were collected for Southern blot analysis.

7 How does DDK dependent Pot1 phosphorylation regulate telomere homeostasis?

7.1 Pot1 phosphorylation does not obviously alter the ssDNA binding properties of the Pot1 OB fold

What biochemical mechanisms might underlie checkpoint inactivation and HR suppression in response to Pot1 phosphorylation? As Pot1 phosphorylation sites are located in the ssDNA-binding OB-fold region, we wondered whether phosphorylation could regulate Pot1 OB binding affinity for the telomeric 3'-overhang. In order to check this hypothesis, we exploited the fact that phosphomimetic Pot1-2D mutant have very similar properties to wild type Pot1 with respect to suppression of HR, DNA damage checkpoint inactivation and ensuring telomerase dependant telomere maintenance. Hence, we used the Pot1-2D OB fold to represent constitutively phosphorylated Pot1 and the Pot1-2A OB fold as unphosphorylated Pot1. We failed to observe any significant difference in telomeric ssDNA binding between Pot1 wt, Pot1-2A and Pot1-2D mutant OB folds (Figure 21).

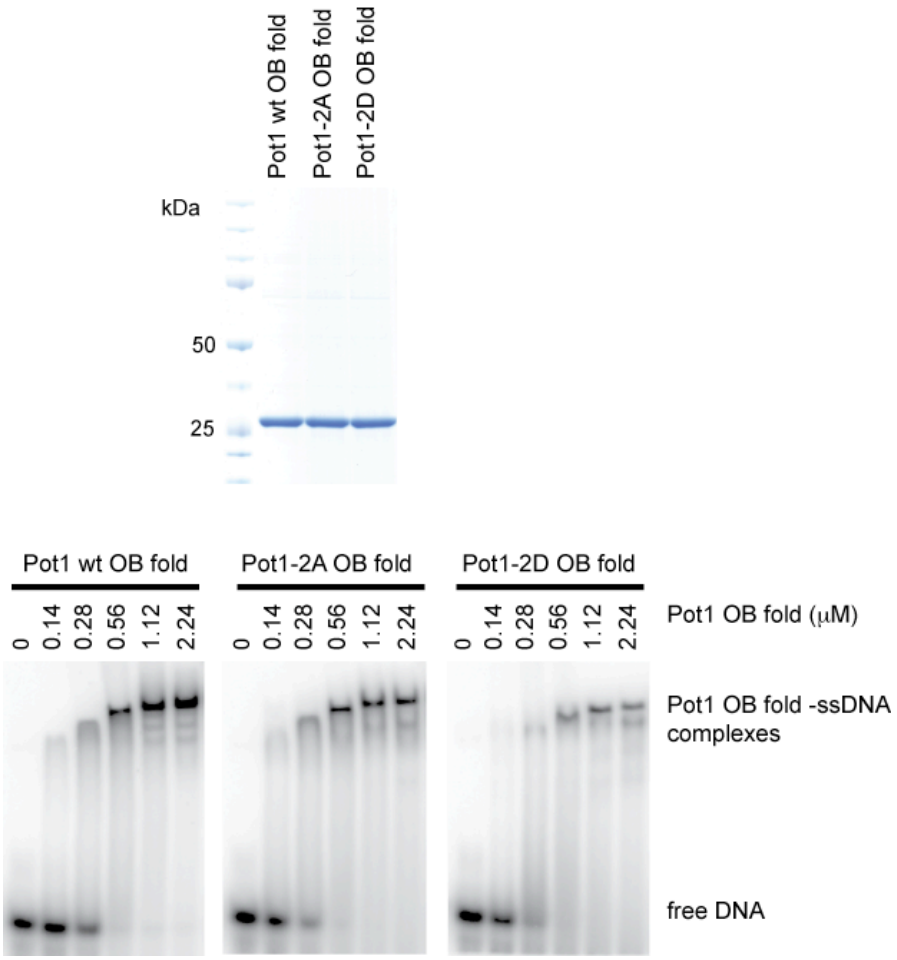


Figure 21 Pot1 phosphorylation does not drastically affect Pot1 OB fold ssDNA binding affinity.

Indicated amounts of Pot1 OB fold were incubated with 0.5 μM ^{32}P-labeled telomeric single-stranded oligonucleotide 5′-GGTTACACGGTTACAGGTTACAGGTTACAGGGTTACGGTTACGSS-3′. Pot1 OB mutants were expressed in bacteria using the pET30 expression system. The purified proteins were quantified and their concentrations were equalized as assessed by Coomassie staining.

7.2 Pot1 phosphorylation affects its interaction with Tpz1/Tpp1

In *S. cerevisiae* and *C. albicans,* the OB fold of the telomerase regulatory subunit Est3 does not mediate DNA interaction (Lee et al., 2008; Young Yu et al., 2008), but rather plays a role in interaction with other proteins of the telomerase complex. Moreover, Tpz/1TPP1, the *S. pombe* Pot1 interacting partner and Est3 structural homolog, also contains an OB fold but does not bind DNA (Miyoshi et al., 2008; Wang et al., 2007; Xin et al., 2007). These observations suggest that the OB fold is not only a single stranded DNA binding domain, but is also a complex structure that can mediate protein-protein interactions. Thus, in preliminary experiments, we checked the interaction between Pot1 phosphomutants and Tpz/1TPP1.

Interestingly, we found that the interaction between Pot1 and Tpz1/Tpp1 is less stable in Pot1-2A and Pot1-5A phosphodeficient mutants than in Pot1 wt (Figure 22). Curiously, however, we did not observe wild type levels of Tpz1/Tpp1-Pot1 interaction in the Pot1-2D phosphomimetic strain. These observations require further substantiation. However, they raise the possibility that Pot1 phosphorylation promotes its interaction with Tpz1/Tpp1 or changes the mode of Pot1-Tpz1/Tpp1 complex assembly such that checkpoint activation and HR at telomeres regulated.

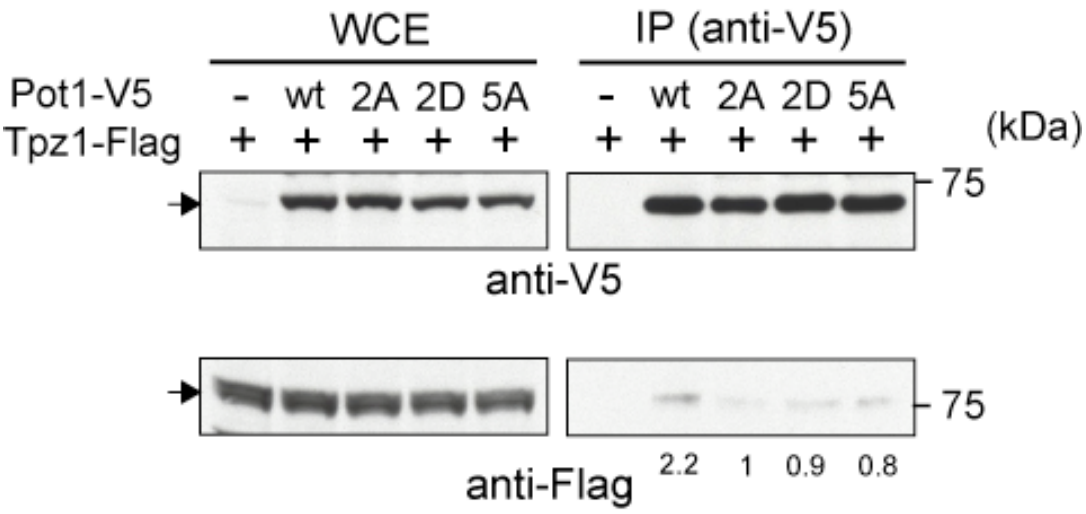


Figure 22 Pot1 phosphorylation stabilises the Pot1-Tpz1 complex.

The co-immunoprecipitated Tpz1 band intensities were quantified using ImageJ software and normalised to immunoprecipitated Pot1 band signals.

8 Pot1 phosphorylation and regulation of telomere functions

8.1 How could Pot1 phosphorylation suppress the DNA damage response at telomeres?

We showed that Pot1 phosphorylation has an important role in controlling the DNA damage response at telomeres. Mutations in Pot1 phosphorylation sites lead to activation of the DNA damage checkpoint and homologous recombination at telomeres. Hence, the chromosome ends in Pot1 phosphomutants become unprotected and appear to be treated as DNA double stranded breaks despite the presence of terminal telomere sequences. DDK phosphorylates Pot1 in its OB-fold – the domain previously characterized as a single stranded DNA binding motif. Interestingly, we found that mutations in Pot1 phosphorylation sites do not dramatically interfere with its single stranded DNA binding activity, but may affect Pot1 interaction with Tpz1/Tpp1. The Pot1-Tpz1/Tpp1 complex is widely conserved throughout evolution from ciliates to human as is its importance for telomere regulation and protection. Thus, it is perhaps not surprising that Pot1-Tpz1/Tpp1 complex stability is regulated by DDK to ensure the proper telomere function.

We do not yet know how the decrease in stability of the Pot1-Tpz1/Tpp1 complex could lead to all phenotypes that we observe in Pot1 phosphodeficient mutants. Phosphorylation of Pot1 could stabilize the Pot1-Tpz1/Tpp1 complex and/or change its 3′-overhang binding activity in a way

that allows Pot1-Tpz1/Tpp1 to win the competition with RPA for overhang binding. This could lead to suppression of the DNA damage response by preventing RPA from accumulating at the telomeres, thus suppressing the activation of the DNA damage response. These ideas should be addressed in future studies.

8.2 Why does DDK phosphorylate Pot1 in the S and G_2 phases of cell cycle?

Another question that arises from our studies centers on why such tight control of homologous recombination and DNA damage checkpoint is so important for telomeres that they employ control by the DDK. As a corollary to this question, why is this mechanism specifically activated in S and G_2? While HR is an often beneficial pathway of DNA repair, excessive HR, particularly in repeated sequences, can lead to genetic rearrangements. Thus, keeping HR inactive at telomeres may be very important for genomic stability. This task becomes more crucial during S and G_2 when HR is specifically upregulated by CDK activity and becomes the main mechanism of DSB repair, replacing nonhomologous end-joining (NHEJ) which is predominant in G_1 (Aylon et al., 2004; Ferreira and Cooper, 2001, 2004; Ira et al., 2004; Moore and Haber, 1996; Takata et al., 1998). S- and G_2- specific phosphorylation of Pot1 by DDK could be a mechanism to inactivate HR specifically at telomeres just at the time when the overall level of HR increases. By specifically inhibiting HR at telomeres, DDK could promote the advantageous use of HR as an efficient

mode of postreplicative DNA damage repair while at the same time protecting cells from dangerous and uncontrolled HR at highly repetitive telomere sequences. Such coordination between CDK dependent HR activation and DDK dependent telomeric specific HR suppression may allow the cell to use HR most efficiently to promote overall genome stability.

8.3 A new role of Rhp51 in telomere protection

In our study, we also uncovered a new role for Rhp51 in telomere protection (Figure 23), as it appears to act redundantly with the unphosphorylated form of Pot1 in allowing telomeres to be maintained. In wt cells, two forms of Pot1 are present: phosphorylated and unphosphorylated. The phosphorylated form of Pot1 plays an important role in suppressing homologous recombination and checkpoint activation at telomeres as described above. When the DNA damage response at telomeres is thus suppressed, telomere maintenance is telomerase dependent. Under these circumstances, neither Rhp51 nor homologous recombination are required for telomere maintenance.

In cells harbouring Pot1-5A and Pot1-2A phosphodeficient mutants, only the unphosphorylated form of Pot1 is present (Figure 23). Unphosphorylated Pot1 cannot suppress homologous recombination or checkpoint activation at telomeres, as evidenced by the massive subtelomeric rearrangements and cell elongation seen at Pot1-5A and Pot1-2A telomeres. These telomeres resemble those of *trt1Δ* survivors that maintain linear

chromosomes by telomeric recombination. Correlating with this observation, telomere maintenance in Pot1-5A and Pot1-2A mutants was independent of telomerase, but dependent on Rhp51. Indeed, our data also suggest that Pot1 phosphorylation is required for telomerase activity – perhaps the telomeric recruitment of telomerase is controlled by Pot1 phosphorylation. This would be an ideal mechanism for reinforcing the connection between semi-conservative DNA replication and telomerase-mediated telomere synthesis. Future experiments will explore this possibility.

The Pot1-2D phosphomimetic mutant was able to suppress both homologous recombination and checkpoint activation at telomeres. The maintenance of Pot1-2D telomeres depends on telomerase, as in wild type cells: deletion of telomerase leads to gradual telomere shortening in the Pot1-2D strain. Surprisingly, however, Pot1-2D telomeres are also dependent on the presence of Rhp51. The Pot1-2D r*hp51Δ* double mutant strain loses its telomeres immediately after germination, in contrast to r*hp51Δ* Pot1-5A and r*hp51Δ* Pot1-2A double mutants that lose telomeres after several generations. This extremely fast telomere loss resembles that seen upon Pot1 deletion, which also triggers the immediate disappearance of telomeres due to massive telomere C-strand resection; all resulting *pot1Δ* colonies contain only cells with circular chromosomes lacking telomere sequences. I think that in the Pot1-2D r*hp51Δ* double mutant, telomere protection is abolished as in *pot1Δ*. The possible explanation is that unphosphorylated Pot1 together with Rhp51 plays an important role in telomere protection. Only by deleting both factors

do telomeres become completely de-protected, leading to their disappearance in the first cell divisions (Figure 23).

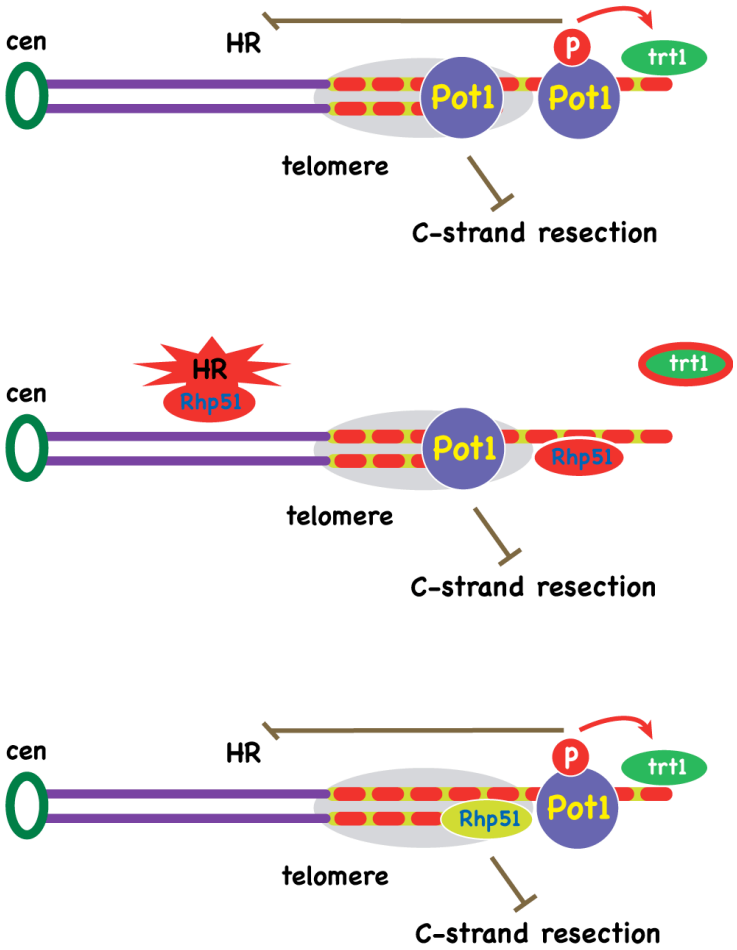
In Pot1-wt, Pot1-5A and Pot1-2A strains, deletion of Rhp51 does not have such a profound effect on telomere protection. Hence, the following model postulates the existence of an Rhp51 function in telomere protection that may be distinct from its role in homologous recombination (Figure 23).

Figure 23 Model representing the role of Pot1 phosphorylation in suppressing homologous recombination and protecting telomeres.

(See next page)

Telomere maintenance

telomerase dependent	Rhp51 dependent	
+	−	Pot1 wt
−	+	Pot1 2A
+	+	Pot1 2D



8.4 Pot1 phosphorylation ensures telomerase dependent telomere maintenance

These studies also suggest the importance of DDK phosphorylation of Pot1 to ensure telomerase dependent telomere maintenance. Why would the system evolve to favor telomerase over HR for telomere maintenance? The answer could be that telomerase dependent telomere maintenance has one important advantage over HR: the ability to confer senescence when telomerase is inactive or is not expressed. This could restrict cell proliferative potential and suppress the uncontrolled cell division that leads to cancer. Arising cancer cells deal with this problem by expressing telomerase or by choosing the recombination dependent ALT mode of telomere maintenance. In our experiments, we were able to avert cellular senescence by replacing Pot1 with a phosphodeficient form of Pot1, which makes cells unable to inactivate HR at telomeres. Constant HR events make telomerase unnecessary for telomere maintenance. Pot1 phosphorylation by DDK could play a critical role in ensuring that telomeres are subject to the highly controlled replication conferred by telomerase.

REFERENCES

Adams, A.K., and Holm, C. (1996). Specific DNA replication mutations affect telomere length in *Saccharomyces cerevisiae*. Mol Cell Biol *16*, 4614-4620.

Ancelin, K., Brunori, M., Bauwens, S., Koering, C.E., Brun, C., Ricoul, M., Pommier, J.P., Sabatier, L., and Gilson, E. (2002). Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. Mol Cell Biol *22*, 3474-3487.

Arneric, M., and Lingner, J. (2007). Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast. EMBO Rep *8*, 1080-1085.

Aylon, Y., Liefshitz, B., and Kupiec, M. (2004). The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. Embo J *23*, 4868-4875.

Bailey, S.M., Brenneman, M.A., and Goodwin, E.H. (2004). Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res *32*, 3743-3751.

Bailis, J.M., Bernard, P., Antonelli, R., Allshire, R.C., and Forsburg, S.L. (2003). Hsk1-Dfp1 is required for heterochromatin-mediated cohesion at centromeres. Nat Cell Biol *5*, 1111-1116.

Baird, D.M., Coleman, J., Rosser, Z.H., and Royle, N.J. (2000). High levels of sequence polymorphism and linkage disequilibrium at the telomere of 12q: implications for telomere biology and human evolution. Am J Hum Genet *66*, 235-250.

Baumann, P., and Cech, T.R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science *292*, 1171-1175.

Baumann, P., Podell, E., and Cech, T.R. (2002). Human Pot1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing. Mol Cell Biol *22*, 8079-8087.

Bernard, P., Maure, J.F., Partridge, J.F., Genier, S., Javerzat, J.P., and Allshire, R.C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science *294*, 2539-2542.

Blackburn, E.H., and Gall, J.G. (1978). A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. J Mol Biol *120*, 33-53.

Bochkarev, A., and Bochkareva, E. (2004). From RPA to BRCA2: lessons from single-stranded DNA binding by the OB-fold. Curr Opin Struct Biol *14*, 36-42.

Bochkarev, A., Bochkareva, E., Frappier, L., and Edwards, A.M. (1999). The crystal structure of the complex of replication protein A subunits RPA32 and RPA14 reveals a mechanism for single-stranded DNA binding. Embo J *18*, 4498-4504.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349-352.

Broccoli, D., Smogorzewska, A., Chong, L., and de Lange, T. (1997). Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat Genet *17*, 231-235.

Brown, G.W., and Kelly, T.J. (1998). Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J Biol Chem *273*, 22083-22090.

Brown, G.W., and Kelly, T.J. (1999). Cell cycle regulation of Dfp1, an activator of the Hsk1 protein kinase. Proc Natl Acad Sci U S A *96*, 8443-8448.

Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A., and Reddel, R.R. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med *3*, 1271-1274.

Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R.R. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. Embo J *14*, 4240-4248.

Celli, G.B., and de Lange, T. (2005). DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion. Nat Cell Biol *7*, 712-718.

Cesare, A.J., Groff-Vindman, C., Compton, S.A., McEachern, M.J., and Griffith, J.D. (2008). Telomere loops and homologous recombination-dependent telomeric circles in a *Kluyveromyces lactis* telomere mutant strain. Mol Cell Biol *28*, 20-29.

Cesare, A.J., Quinney, N., Willcox, S., Subramanian, D., and Griffith, J.D. (2003). Telomere looping in *P. sativum* (common garden pea). Plant J *36*, 271-279.

Chikashige, Y., and Hiraoka, Y. (2001). Telomere binding of the Rap1 protein is required for meiosis in fission yeast. Curr Biol *11*, 1618-1623.

Chikashige, Y., Tsutsumi, C., Yamane, M., Okamasa, K., Haraguchi, T., and Hiraoka, Y. (2006). Meiotic proteins bqt1 and bqt2 tether telomeres to form the bouquet arrangement of chromosomes. Cell *125*, 59-69.

Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995). A human telomeric protein. Science *270*, 1663-1667.

Churikov, D., Wei, C., and Price, C.M. (2006). Vertebrate POT1 restricts G-overhang length and prevents activation of a telomeric DNA damage checkpoint but is dispensable for overhang protection. Mol Cell Biol *26*, 6971-6982.

Colgin, L.M., Baran, K., Baumann, P., Cech, T.R., and Reddel, R.R. (2003). Human POT1 facilitates telomere elongation by telomerase. Curr Biol *13*, 942-946.

Cooper, J.P., Nimmo, E.R., Allshire, R.C., and Cech, T.R. (1997). Regulation of telomere length and function by a Myb-domain protein in fission yeast. Nature *385*, 744-747.

Costa, A., and Onesti, S. (2008). The MCM complex: (just) a replicative helicase? Biochem Soc Trans *36*, 136-140.

Cullen, J.K., Hussey, S.P., Walker, C., Prudden, J., Wee, B.Y., Dave, A., Findlay, J.S., Savory, A.P., and Humphrey, T.C. (2007). Break-induced loss of heterozygosity in fission yeast: dual roles for homologous recombination in promoting translocations and preventing de novo telomere addition. Mol Cell Biol *27*, 7745-7757.

Culotti, J., and Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division. Exp Cell Res *67*, 389-401.

Denchi, E.L., and de Lange, T. (2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature *448*, 1068-1071.

Diede, S.J., and Gottschling, D.E. (1999). Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. Cell *99*, 723-733.

Diffley, J.F., Cocker, J.H., Dowell, S.J., and Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins in vivo. Cell *78*, 303-316.

Dionne, I., and Wellinger, R.J. (1996). Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc Natl Acad Sci U S A *93*, 13902-13907.

Dowell, S.J., Romanowski, P., and Diffley, J.F. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science *265*, 1243-1246.

Duncker, B.P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S.M. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proc Natl Acad Sci U S A *99*, 16087-16092.

Dunham, M.A., Neumann, A.A., Fasching, C.L., and Reddel, R.R. (2000). Telomere maintenance by recombination in human cells. Nat Genet *26*, 447-450.

Ferreira, M.G., and Cooper, J.P. (2001). The fission yeast Taz1 protein protects chromosomes from Ku-dependent end-to-end fusions. Mol Cell *7*, 55-63.

Ferreira, M.G., and Cooper, J.P. (2004). Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. Genes Dev *18*, 2249-2254.

Fisher, T.S., Taggart, A.K., and Zakian, V.A. (2004). Cell cycle-dependent regulation of yeast telomerase by Ku. Nat Struct Mol Biol *11*, 1198-1205.

Fung, A.D., Ou, J., Bueler, S., and Brown, G.W. (2002). A conserved domain of *Schizosaccharomyces pombe* dfp1(+) is uniquely required for chromosome stability following alkylation damage during S phase. Mol Cell Biol *22*, 4477-4490.

Gottschling, D.E., and Zakian, V.A. (1986). Telomere proteins: specific recognition and protection of the natural termini of *Oxytricha macronuclear* DNA. Cell *47*, 195-205.

Greider, C.W., and Blackburn, E.H. (1985). Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell *43*, 405-413.

Greider, C.W., and Blackburn, E.H. (1987). The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell *51*, 887-898.

Greider, C.W., and Blackburn, E.H. (1989). A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature *337*, 331-337.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell *97*, 503-514.

Grossi, S., Puglisi, A., Dmitriev, P.V., Lopes, M., and Shore, D. (2004). Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation. Genes Dev *18*, 992-1006.

Hagen, K., Gilbert, D., Willard, H., Cohen, S. (1990). Replication timing of DNA sequences associated with human centromeres and telomeres. Mol Cell Biol. 10, 6348-55.

Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M., and Sclafani, R.A. (1997). mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A *94*, 3151-3155.

Harley, C.B., Futcher, A.B., and Greider, C.W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458-460.

He, H., Multani, A.S., Cosme-Blanco, W., Tahara, H., Ma, J., Pathak, S., Deng, Y., and Chang, S. (2006). POT1b protects telomeres from end-to-end chromosomal fusions and aberrant homologous recombination. Embo J *25*, 5180-5190.

Hochwagen, A., Tham, W.H., Brar, G.A., and Amon, A. (2005). The FK506 binding protein Fpr3 counteracts protein phosphatase 1 to maintain meiotic recombination checkpoint activity. Cell *122*, 861-873.

Hockemeyer, D., Daniels, J.P., Takai, H., and de Lange, T. (2006). Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. Cell *126*, 63-77.

Hockemeyer, D., Palm, W., Else, T., Daniels, J.P., Takai, K.K., Ye, J.Z., Keegan, C.E., de Lange, T., and Hammer, G.D. (2007). Telomere protection by mammalian Pot1 requires interaction with Tpp1. Nat Struct Mol Biol *14*, 754-761.

Hockemeyer, D., Sfeir, A.J., Shay, J.W., Wright, W.E., and de Lange, T. (2005). POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end. Embo J *24*, 2667-2678.

Hollingsworth, R.E., Jr., and Sclafani, R.A. (1993). Yeast pre-meiotic DNA replication utilizes mitotic origin ARS1 independently of CDC7 function. Chromosoma *102*, 415-420.

Houghtaling, B.R., Cuttonaro, L., Chang, W., and Smith, S. (2004). A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. Curr Biol *14*, 1621-1631.

Ira, G., Pellicioli, A., Balijja, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N.M.*, et al.* (2004). DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature *431*, 1011-1017.

Jackson, A.L., Pahl, P.M., Harrison, K., Rosamond, J., and Sclafani, R.A. (1993). Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol *13*, 2899-2908.

Jacob, N.K., Lescasse, R., Linger, B.R., and Price, C.M. (2007). Tetrahymena POT1a regulates telomere length and prevents activation of a cell cycle checkpoint. Mol Cell Biol *27*, 1592-1601.

Jady, B.E., Richard, P., Bertrand, E., and Kiss, T. (2006). Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol Biol Cell *17*, 944-954.

Jiang, W., and Hunter, T. (1997). Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc Natl Acad Sci U S A *94*, 14320-14325.

Jiang, W., McDonald, D., Hope, T.J., and Hunter, T. (1999). Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. Embo J *18*, 5703-5713.

Johnston, L.H., Masai, H., and Sugino, A. (1999). First the CDKs, now the DDKs. Trends Cell Biol *9*, 249-252.

Kamimura, Y., Tak, Y.S., Sugino, A., and Araki, H. (2001). Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. Embo J *20*, 2097-2107.

Kanoh, J., and Ishikawa, F. (2001). spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast. Curr Biol *11*, 1624-1630.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science *283*, 1321-1325.

Karlseder, J., Hoke, K., Mirzoeva, O.K., Bakkenist, C., Kastan, M.B., Petrini, J.H., and de Lange, T. (2004). The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol *2*, E240.

Kelleher, C., Kurth, I., and Lingner, J. (2005). Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. Mol Cell Biol *25*, 808-818.

Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Johnston, L.H., and Sugino, A. (2000). Characterization of the yeast Cdc7p/Dbf4p complex purified from insect cells. Its protein kinase activity is regulated by Rad53p. J Biol Chem *275*, 35051-35062.

Kim, J.M., Sato, N., Yamada, M., Arai, K., and Masai, H. (1998). Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae* CDC7 essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for S. cerevisiae Cdc7-related kinase. J Biol Chem *273*, 23248-23257.

Kim, J.M., Takemoto, N., Arai, K., and Masai, H. (2003). Hypomorphic mutation in an essential cell-cycle kinase causes growth retardation and impaired spermatogenesis. Embo J *22*, 5260-5272.

Kim, S.H., Beausejour, C., Davalos, A.R., Kaminker, P., Heo, S.J., and Campisi, J. (2004). TIN2 mediates functions of TRF2 at human telomeres. J Biol Chem *279*, 43799-43804.

Kitada, K., Johnston, L.H., Sugino, T., and Sugino, A. (1992). Temperature-sensitive cdc7 mutations of *Saccharomyces cerevisiae* are suppressed by the DBF4 gene, which is required for the G1/S cell cycle transition. Genetics *131*, 21-29.

Kumagai, H., Sato, N., Yamada, M., Mahony, D., Seghezzi, W., Lees, E., Arai, K., and Masai, H. (1999). A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol Cell Biol *19*, 5083-5095.

Landis, G., and Tower, J. (1999). The *Drosophila* chiffon gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. Development *126*, 4281-4293.

Lee, J., Mandell, E.K., Tucey, T.M., Morris, D.K., and Lundblad, V. (2008). The Est3 protein associates with yeast telomerase through an OB-fold domain. Nat Struct Mol Biol.

Lei, M., Baumann, P., and Cech, T.R. (2002). Cooperative binding of single-stranded telomeric DNA by the Pot1 protein of *Schizosaccharomyces pombe*. Biochemistry *41*, 14560-14568.

Lei, M., Kawasaki, Y., Young, M.R., Kihara, M., Sugino, A., and Tye, B.K. (1997). Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev *11*, 3365-3374.

Lei, M., Podell, E.R., Baumann, P., and Cech, T.R. (2003). DNA self-recognition in the structure of Pot1 bound to telomeric single-stranded DNA. Nature *426*, 198-203.

Lei, M., Podell, E.R., and Cech, T.R. (2004). Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. Nat Struct Mol Biol *11*, 1223-1229.

Lei, M., Zaug, A.J., Podell, E.R., and Cech, T.R. (2005). Switching human telomerase on and off with hPOT1 protein in vitro. J Biol Chem *280*, 20449-20456.

Lepke, M., Putter, V., Staib, C., Kneissl, M., Berger, C., Hoehn, K., Nanda, I., Schmid, M., and Grummt, F. (1999). Identification, characterization and chromosomal localization of the cognate human and murine DBF4 genes. Mol Gen Genet *262*, 220-229.

Li, B., Oestreich, S., and de Lange, T. (2000). Identification of human Rap1: implications for telomere evolution. Cell *101*, 471-483.

Lingner, J., and Cech, T.R. (1996). Purification of telomerase from *Euplotes aediculatus*: requirement of a primer 3' overhang. Proc Natl Acad Sci U S A *93*, 10712-10717.

Liu, D., Safari, A., O'Connor, M.S., Chan, D.W., Laegeler, A., Qin, J., and Songyang, Z. (2004). PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol *6*, 673-680.

Loayza, D., and De Lange, T. (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature *423*, 1013-1018.

Lundblad, V., and Blackburn, E.H. (1993). An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell *73*, 347-360.

Lundblad, V., and Szostak, J.W. (1989). A mutant with a defect in telomere elongation leads to senescence in yeast. Cell *57*, 633-643.

Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000). Cell cycle restriction of telomere elongation. Curr Biol *10*, 487-490.

Marcand, S., Gilson, E., and Shore, D. (1997). A protein-counting mechanism for telomere length regulation in yeast. Science *275*, 986-990.

Martin, V., Du, L.L., Rozenzhak, S., and Russell, P. (2007). Protection of telomeres by a conserved Stn1-Ten1 complex. Proc Natl Acad Sci U S A *104*, 14038-14043.

Masai, H., Miyake, T., and Arai, K. (1995). hsk1+, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae* CDC7, is required for chromosomal replication. Embo J *14*, 3094-3104.

Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature *415*, 651-655.

McClintock, B. (1939). The Behavior in Successive Nuclear Divisions of a Chromosome Broken at Meiosis. Proc Natl Acad Sci U S A *25*, 405-416.

McClintock, B., and Hill, H.E. (1931). The Cytological Identification of the Chromosome Associated with the R-G Linkage Group in *ZEA MAYS*. Genetics *16*, 175-190.

Miller, K.M., Rog, O., and Cooper, J.P. (2006). Semi-conservative DNA replication through telomeres requires Taz1. Nature *440*, 824-828.

Miyoshi, T., Kanoh, J., Saito, M., and Ishikawa, F. (2008). Fission yeast Pot1-Tpp1 protects telomeres and regulates telomere length. Science *320*, 1341-1344.

Moore, J.K., and Haber, J.E. (1996). Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. Mol Cell Biol *16*, 2164-2173.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol *194*, 795-823.

Muller, H.J. (1938). The remaking of chromosomes. The Collecting Net *8*, 182-195.

Munoz-Jordan, J.L., Cross, G.A., de Lange, T., and Griffith, J.D. (2001). t-loops at *Trypanosome* telomeres. Embo J *20*, 579-588.

Murti, K.G., and Prescott, D.M. (1999). Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops. Proc Natl Acad Sci U S A *96*, 14436-14439.

Naito, T., Matsuura, A., and Ishikawa, F. (1998). Circular chromosome formation in a fission yeast mutant defective in two ATM homologues. Nat Genet *20*, 203-206.

Nakamura, M., Nabetani, A., Mizuno, T., Hanaoka, F., and Ishikawa, F. (2005). Alterations of DNA and chromatin structures at telomeres and genetic instability in mouse cells defective in DNA polymerase alpha. Mol Cell Biol *25*, 11073-11088.

Nakamura, T.M., Cooper, J.P., and Cech, T.R. (1998). Two modes of survival of fission yeast without telomerase. Science *282*, 493-496.

Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S.I., and Watanabe, Y. (2002). Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat Cell Biol *4*, 89-93.

O'Connor, M.S., Safari, A., Xin, H., Liu, D., and Songyang, Z. (2006). A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. Proc Natl Acad Sci U S A *103*, 11874-11879.

Ogino, K., Hirota, K., Matsumoto, S., Takeda, T., Ohta, K., Arai, K., and Masai, H. (2006). Hsk1 kinase is required for induction of meiotic dsDNA breaks without involving checkpoint kinases in fission yeast. Proc Natl Acad Sci U S A *103*, 8131-8136.

Olovnikov, A.M. (1972). [The immune response and the process of marginotomy in lymphoid cells]. Vestn Akad Med Nauk SSSR *27*, 85-87.

Olovnikov, A.M. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol *41*, 181-190.

Paeschke, K., Simonsson, T., Postberg, J., Rhodes, D., and Lipps, H.J. (2005). Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. Nat Struct Mol Biol *12*, 847-854.

Pasero, P., Duncker, B.P., Schwob, E., and Gasser, S.M. (1999). A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. Genes Dev *13*, 2159-2176.

Pennock, E., Buckley, K., and Lundblad, V. (2001). Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell *104*, 387-396.

Pitt, C.W., Moreau, E., Lunness, P.A., and Doonan, J.H. (2004). The pot1+ homologue in *Aspergillus nidulans* is required for ordering mitotic events. J Cell Sci *117*, 199-209.

Qi, H., and Zakian, V.A. (2000). The *Saccharomyces* telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. Genes Dev *14*, 1777-1788.

Raggiaschi, R., Lorenzetto, C., Diodato, E., Caricasole, A., Gotta, S., and Terstappen, G.C. (2006). Detection of phosphorylation patterns in rat cortical neurons by combining phosphatase treatment and DIGE technology. Proteomics *6*, 748-756.

Raices, M., Verdun, R.E., Compton, S.A., Haggblom, C.I., Griffith, J.D., Dillin, A., and Karlseder, J. (2008). C. elegans telomeres contain G-strand and C-strand overhangs that are bound by distinct proteins. Cell *132*, 745-757.

Reddel, R.R. (2003). Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett *194*, 155-162.

Ritchie, K.B., Mallory, J.C., and Petes, T.D. (1999). Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol *19*, 6065-6075.

Sabourin, M., Tuzon, C.T., and Zakian, V.A. (2007). Telomerase and Tel1p preferentially associate with short telomeres in *S. cerevisiae*. Mol Cell *27*, 550-561.

Sambrook and Russell, Molecular Cloning, third edition, CSHL press, 2001

Santocanale, C., and Diffley, J.F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature *395*, 615-618.

Sasanuma, H., Hirota, K., Fukuda, T., Kakusho, N., Kugou, K., Kawasaki, Y., Shibata, T., Masai, H., and Ohta, K. (2008). Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination. Genes Dev *22*, 398-410.

Sato, M., Dhut, S., and Toda, T. (2005). New drug-resistant cassettes for gene disruption and epitope tagging in *Schizosaccharomyces pombe*. Yeast *22*, 583-591.

Sato, N., Arai, K., and Masai, H. (1997). Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. Embo J *16*, 4340-4351.

Schramke, V., Luciano, P., Brevet, V., Guillot, S., Corda, Y., Longhese, M.P., Gilson, E., and Geli, V. (2004). RPA regulates telomerase action by providing Est1p access to chromosome ends. Nat Genet *36*, 46-54.

Shakirov, E.V., Surovtseva, Y.V., Osbun, N., and Shippen, D.E. (2005). The *Arabidopsis* Pot1 and Pot2 proteins function in telomere length homeostasis and chromosome end protection. Mol Cell Biol *25*, 7725-7733.

Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M.R., Schnapp, G., and de Lange, T. (2000). Control of human telomere length by TRF1 and TRF2. Mol Cell Biol *20*, 1659-1668.

Snaith, H.A., Brown, G.W., and Forsburg, S.L. (2000). *Schizosaccharomyces pombe* Hsk1p is a potential cds1p target required for genome integrity. Mol Cell Biol *20*, 7922-7932.

Spink, K.G., Evans, R.J., and Chambers, A. (2000). Sequence-specific binding of Taz1p dimers to fission yeast telomeric DNA. Nucleic Acids Res *28*, 527-533.

Stansel, R.M., de Lange, T., and Griffith, J.D. (2001). T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. Embo J *20*, 5532-5540.

Stellwagen, A.E., Haimberger, Z.W., Veatch, J.R., and Gottschling, D.E. (2003). Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. Genes Dev *17*, 2384-2395.

Subramanian, L., Moser, B.A., and Nakamura, T.M. (2008). Recombination-based telomere maintenance is dependent on Tel1-MRN and Rap1 and inhibited by telomerase, Taz1, and Ku in fission yeast. Mol Cell Biol *28*, 1443-1455.

Szostak, J.W., and Blackburn, E.H. (1982). Cloning yeast telomeres on linear plasmid vectors. Cell *29*, 245-255.

Taggart, A.K., Teng, S.C., and Zakian, V.A. (2002). Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science *297*, 1023-1026.

Takahashi, T.S., Basu, A., Bermudez, V., Hurwitz, J., and Walter, J.C. (2008). Cdc7-Drf1 kinase links chromosome cohesion to the initiation of DNA replication in *Xenopus* egg extracts. Genes Dev *22*, 1894-1905.

Takahashi, T.S., and Walter, J.C. (2005). Cdc7-Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication. Genes Dev *19*, 2295-2300.

Takata, H., Kanoh, Y., Gunge, N., Shirahige, K., and Matsuura, A. (2004). Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres in vivo. Mol Cell *14*, 515-522.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. (1998). Homologous

recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. Embo J *17*, 5497-5508.

Takeda, T., Ogino, K., Matsui, E., Cho, M.K., Kumagai, H., Miyake, T., Arai, K., and Masai, H. (1999). A fission yeast gene, him1(+)/dfp1(+), encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. Mol Cell Biol *19*, 5535-5547.

Takeda, T., Ogino, K., Tatebayashi, K., Ikeda, H., Arai, K., and Masai, H. (2001). Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. Mol Biol Cell *12*, 1257-1274.

Tani, A., and Murata, M. (2005). Alternative splicing of Pot1 (Protection of telomere)-like genes in *Arabidopsis thaliana*. Genes Genet Syst *80*, 41-48.

Teixeira, M.T., Arneric, M., Sperisen, P., and Lingner, J. (2004). Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell *117*, 323-335.

Teng, S.C., Chang, J., McCowan, B., and Zakian, V.A. (2000). Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. Mol Cell *6*, 947-952.

Teng, S.C., and Zakian, V.A. (1999). Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in *Saccharomyces cerevisiae*. Mol Cell Biol *19*, 8083-8093.

Theobald, D.L., Mitton-Fry, R.M., and Wuttke, D.S. (2003). Nucleic acid recognition by OB-fold proteins. Annu Rev Biophys Biomol Struct *32*, 115-133.

Theobald, D.L., and Wuttke, D.S. (2004). Prediction of multiple tandem OB-fold domains in telomere end-binding proteins Pot1 and Cdc13. Structure *12*, 1877-1879.

Tomita, K., Matsuura, A., Caspari, T., Carr, A.M., Akamatsu, Y., Iwasaki, H., Mizuno, K., Ohta, K., Uritani, M., Ushimaru, T.*, et al.* (2003). Competition between the Rad50 complex and the Ku heterodimer reveals a role for Exo1 in

processing double-strand breaks but not telomeres. Mol Cell Biol *23*, 5186-5197.

Tomlinson, R.L., Ziegler, T.D., Supakorndej, T., Terns, R.M., and Terns, M.P. (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell *17*, 955-965.

Tseng, S.F., Lin, J.J., and Teng, S.C. (2006). The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation. Nucleic Acids Res *34*, 6327-6336.

van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1. Nature *385*, 740-743.

Vassetzky, N.S., Gaden, F., Brun, C., Gasser, S.M., and Gilson, E. (1999). Taz1p and Teb1p, two telobox proteins in *Schizosaccharomyces pombe*, recognize different telomere-related DNA sequences. Nucleic Acids Res *27*, 4687-4694.

Verdun, R.E., Crabbe, L., Haggblom, C., and Karlseder, J. (2005). Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. Mol Cell *20*, 551-561.

Wan, L., Niu, H., Futcher, B., Zhang, C., Shokat, K.M., Boulton, S.J., and Hollingsworth, N.M. (2008). Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. Genes Dev *22*, 386-397.

Wan, L., Zhang, C., Shokat, K.M., and Hollingsworth, N.M. (2006). Chemical inactivation of cdc7 kinase in budding yeast results in a reversible arrest that allows efficient cell synchronization prior to meiotic recombination. Genetics *174*, 1767-1774.

Wang, F., Podell, E.R., Zaug, A.J., Yang, Y., Baciu, P., Cech, T.R., and Lei, M. (2007). The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature *445*, 506-510.

Watson, J.D. (1972). Origin of concatemeric T7 DNA. Nat New Biol *239*, 197-201.

Wei, C., and Price, C.M. (2004). Cell cycle localization, dimerization, and binding domain architecture of the telomere protein cPot1. Mol Cell Biol *24*, 2091-2102.

Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. Embo J *18*, 5334-5346.

Wright, W., Tesmer, V., Liao, M., Shay J. (1999). Normal human telomeres are not late replicating. Exp Cell Res. 251, 492-9.

Wu, L., Multani, A.S., He, H., Cosme-Blanco, W., Deng, Y., Deng, J.M., Bachilo, O., Pathak, S., Tahara, H., Bailey, S.M*., et al*. (2006). Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell *126*, 49-62.

Xin, H., Liu, D., Wan, M., Safari, A., Kim, H., Sun, W., O'Connor, M.S., and Songyang, Z. (2007). TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature *445*, 559-562.

Yamagata, A., Kristensen, D.B., Takeda, Y., Miyamoto, Y., Okada, K., Inamatsu, M., and Yoshizato, K. (2002). Mapping of phosphorylated proteins on two-dimensional polyacrylamide gels using protein phosphatase. Proteomics *2*, 1267-1276.

Yang, H., Jeffrey, P.D., Miller, J., Kinnucan, E., Sun, Y., Thoma, N.H., Zheng, N., Chen, P.L., Lee, W.H., and Pavletich, N.P. (2002). BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. Science *297*, 1837-1848.

Ye, J.Z., Hockemeyer, D., Krutchinsky, A.N., Loayza, D., Hooper, S.M., Chait, B.T., and de Lange, T. (2004). POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev *18*, 1649-1654.

Yoon, H.J., and Campbell, J.L. (1991). The CDC7 protein of *Saccharomyces cerevisiae* is a phosphoprotein that contains protein kinase activity. Proc Natl Acad Sci U S A *88*, 3574-3578.

Yoon, H.J., Loo, S., and Campbell, J.L. (1993). Regulation of *Saccharomyces cerevisiae* CDC7 function during the cell cycle. Mol Biol Cell *4*, 195-208.

Young Yu, E., Wang, F., Lei, M., and Lue, N.F. (2008). A proposed OB-fold with a protein-interaction surface in *Candida albicans* telomerase protein Est3. Nat Struct Mol Biol.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol *20*, 3086-3096.