IDENTIFICATION OF >150 NOVEL GENES THAT AFFECT TELOMERE LENGTH AND DISCOVERY OF A LINK BETWEEN VITAMIN B6 SALVAGE AND TELOMERE METABOLISM

by

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(Under the Direction of Michael J. McEachern)

ABSTRACT

The nucleoprotein structures at the end of chromosomes, called telomeres, have many functions important for maintaining genomic integrity. They provide end-protection, limit cellular proliferation, regulate sub-telomeric gene expression, help in chromosome positioning, and aid homolog pairing in meiosis. Generally, telomeric DNA is composed of tandem arrays of a simple repeat. A reverse transcriptase enzyme complex, telomerase, can add telomeric repeats to the ends. In the absence of telomerase expression, as in the case of most human somatic cells, telomeres shorten with each cell division, and this sequence loss eventually triggers growth arrest. Proper length maintenance of telomeres is central to all their functions. This requires coordinated action of many proteins. In order to broaden our knowledge about the mechanisms that control telomere length, we systematically examined ~4800 haploid deletion mutants of *Saccharomyces cerevisiae* for telomere length defects. We identified more than 150 candidate genes not previously known to affect telomere length. Many of these genes have known functions in diverse cellular processes ranging from chromatin remodeling and DNA metabolism to vesicular trafficking and mitochondrial and ribosomal structure and organization. Among the

109 that showed consistently shorter telomere lengths upon growth on rich medium were deletions of a putative pyridoxal (vitamin B6) kinase, *bud16*Δ, and pyridoxine/pyridoxamine phosphate oxidase, *pdx3*Δ. This finding suggested a possible connection between vitamin B6 metabolism and telomere length maintenance. Yeast can generate the active form of vitamin B6 by *de novo* synthesis or by salvaging precursor vitamers. We demonstrate that *BUD16* and *PDX3* encode the *bona fide* kinase and oxidase of the vitamin B6 salvage pathway and are not required for biosynthesis. Our experiments have helped characterize the salvage pathway in yeast and show that the short telomere phenotypes in *bud16*Δ and *pdx3*Δ are due to B6 deficiency. The mechanism by which B6 deficiency affects telomere length remains unclear.

INDEX WORDS: yeast, *Saccharomyces cerevisiae*, telomere, screen, deletions, knockout collection, vitamin B6 salvage, pyridoxal kinase, pyridoxal oxidase, *BUD16*, *PDX3*, *YPR027W*, *BUD17*.

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DEDICATION

This dissertation is dedicated to my mother, her fierce passion for logic, and her distaste for nonsense.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Importance of being an end

The distinction between authentic chromosome ends and broken DNA ends was first recognized in the early twentieth century. Progress in cytology lead Herman Muller to identify chromosomal rearrangements in X-ray-induced mutant flies and observe that not a single one involved loss of the chromosomal ends (Muller, 1938). With some elegant cytogenetics in maize, Barbara McClintock demonstrated breakage-fusion-bridge cycles, involving loss of the terminal region, that halted only with a "chromosomal healing" at the ends (McClintock, 1941). These early findings demonstrated that ends of chromosomes, for which Muller coined the term "telomere", provide a protective cap essential to maintain genomic integrity.

In 1961, Leonard Hayflick showed that human fibroblasts grown in culture had a limited capacity to replicate (Hayflick and Moorhead, 1961). A decade later, when semiconservative DNA replication was discovered, Alexey Olovnikov and James Watson independently described a mechanism by which the ends of chromosomes are prone to shortening during each cell division (Olovnikov, 1973; Watson, 1972). Initiation of DNA replication requires an RNA primer, one for synthesis of the leading strand and one for each Okazaki fragment on the lagging strand. These RNA molecules are removed and replaced with DNA, primed by the newly synthesized DNA itself. After the removal of the most distal primer on the lagging strand, it is not possible to initiate synthesis of an Okazaki fragment ahead of the end. Consequently, there is sequence loss at one newly synthesized telomere at each end of a chromosome (see Figure 1-1).

Recognition of this problem that is inherent to conventional replication machinery, generated the idea that the shortening of telomeres may act like timepieces programmed to keep a count of cell divisions and eventually limit cell divisions. In essence, this limit applied to replication would prevent the loss of sequence with important coding information internal to the telomeric DNA. Indeed, telomeres have multiple functions in the cells that include end protection (Cervantes and Lundblad, 2002), limiting cellular proliferation (Shay and Wright, 2005), chromosome positioning during cellular division (Scherthan, 2001), help in homolog pairing in meiosis (Burgess, 2004), and regulation of sub-telomeric gene expression (Perrod and Gasser, 2003).

Telomere structure

While sequencing rRNA genes from the macronucleus of *Tetrahymena*, Elizabeth Blackburn found that the linear DNA molecules ended in tandem copies of a 6-nucleotide repeat TTGGGG (Blackburn and Gall, 1978). Within a few years telomeres in a number of organisms were found to be composed of tandem arrays of a simple repeat that was G-rich on the strand with its 3' hydroxyl oriented towards the end; e.g., TTGGGG in hypotrichous ciliates (Klobutcher et al., 1981), $T(G)_{2-3}(TG)_{1-6}$ in the yeast *Saccharomyces cerevisiae* (Shampay et al., 1984), TTTAGGG in *Arabidopsis* (Richards and Ausubel, 1988), and TTAGGG in humans (Moyzis et al., 1988). Not only does the sequence vary among species, but there is sometimes considerable variation in the size of the repeat. For example, vertebrates, trypanosomes, and *Neurospora* all encode for a 6 bp repeat, while a 25 bp repeat is found in the yeast *Kluyveromyces lactis* (McEachern and Blackburn, 1995; Zakian, 1989). Similar variation is present in the total length of the telomeric track. While telomeres of the *Oxytricha* macronucleus are only 20 bps, vertebrates such as mice have telomeres of tens of kilobases (Zakian, 1989). In

humans the total length can vary between 5-15 kb depending upon the tissue and age of the individual. For example, the average telomere length can be 5 kb more in sperm DNA than from blood cells of the same person (Cooke and Smith, 1986). Some organisms display variation in the regularity in which each repeat is present in an array. In humans the identical TTAGGG repeat is added in tandem, while *Saccharomyces cerevisiae* telomeres are composed of a more heterogeneous array that can be written as $T(G)_{2-3}(TG)_{1-6}$. One unifying characteristic of telomeres is the presence of a 3' overhang of the G-rich strand (Henderson and Blackburn, 1989; Klobutcher et al., 1981). In mammalian and plant telomeres this overhang has been shown to loop back on to the telomere and invade the double-stranded portion proximally (Cesare et al., 2003; Griffith et al., 1999). This yields a secondary lariat-like structure, termed a t-loop, which may play an important role in protecting the ends from DNA damage response.

Telomere elongation

Telomerase reaction

A revolutionary discovery in telomere biology was uncovering the telomere-specific reverse transcriptase, telomerase. This specialized enzyme extends telomere length in most eukaryotes, compensating for loss from conventional replication. In the 1980s, a graduate student in the Blackburn laboratory, Carol Greider, showed synthesis of telomere repeat addition activity in *Tetrahymena* was independent of standard DNA polymerases (Greider and Blackburn, 1985). She found the activity to be sensitive to RNase, implying the enzyme responsible for telomere repeat addition was a ribonucleoprotein (Greider and Blackburn, 1987). Greider was the first to clone and sequence the RNA subunit of this enzyme from *Tetrahymena* and demonstrated that a region on the RNA carried sequence complementary to the TTGGGG repeat of the telomeres

(Greider and Blackburn, 1989). Greider inferred that this region was being utilized as a template to add telomeric repeats onto the ends. These findings led to the discovery of essential telomerase components in many organisms (Cech et al., 1997; Lingner et al., 1997b; Nakamura et al., 1997; Shippen-Lentz and Blackburn, 1990). Indeed, telomerase is a well conserved ribonucleoprotein consisting of a catalytic reverse transcriptase protein and an integral RNA moiety that carries a species-specific template complementary to the 3' G-rich strand (Blackburn, 1997). Figure 1-2 illustrates how the telomerase template aligns at the 3' end with partial base pairing. Next, nucleotides are added onto the 3' end by the reverse transcriptase according to the remaining unpaired template. Telomerase then relocates distally to add more repeats. After the 3' strand is extended, the lagging strand can be extended, presumably by conventional replication machinery.

Telomerase-independent mechanisms

Not all organisms encode telomerase. A few organisms with linear DNA have evolved different mechanisms to deal with the end replication problem. Bacteriophages arrange many copies of DNA in one linear molecule so the sequence loss occurs only at the very end of the concatemeric DNA (Catalano et al., 1995). Repeated retrotransposition at the telomere is a mechanism described in *Drosophila* that extends the ends maintaining the integrity of the chromosomes (Pardue and DeBaryshe, 2003). Recombination-based extension of telomeres has been shown to be the route for maintaining DNA ends in *Anopheles*, *Chironomus,* and mitochondrial DNA of certain ciliates and yeasts (Biessmann et al., 1997; Morin and Cech, 1988; Nielsen and Edstrom, 1993; Nosek et al., 1995). However, the most conserved mechanism of maintaining telomere length in eukaryotes is via the expression of telomerase.

End protection and telomere length regulation

Telomerase-associated proteins

Telomerase typically maintains telomere length within a fairly tight range of length (Blackburn et al., 2000). Regulation of telomerase activity at the telomere involves the coordinated function of many proteins. The template encoding the RNA moiety (Tlc1 in yeast and Tr in humans) and reverse transcriptase protein (Est2 in yeast, Tert in humans) constitute the essential catalytic core of the enzyme (Blackburn, 1992; Mitchell and Collins, 2000). In addition, the *in vivo* activity of the enzyme requires auxiliary proteins that are part of the ribonucleoprotein complex assisting in the recruitment at the telomere (Evans and Lundblad, 2000). In yeast two proteins, Est1 and Est3, form a complex with Est2 and Tlc1 (Lin and Zakian, 1995). Est1 binds to single-stranded telomeric sequence and aids in recruitment of telomerase at the telomere in a S phase-specific way (Lingner et al., 1997a; Taggart et al., 2002). Several proteins have been found to associate with human telomerase RNA, including Tep1, hStau, L22, and some heterogenous nuclear ribonucleoproteins (C1, C2, A1 and UP1) (Dallaire et al., 2000; Fiset and Chabot, 2001; Ford et al., 2001; Ford et al., 2000). A number of snoRNA-associated proteins, Nop10, Nhp2, hGar1, and Dkc1/dyskerin have been found to bind TR and assist in its nuclear trafficking (Lukowiak et al., 2000; Mitchell et al., 1999; Pogacic et al., 2000; Vulliamy et al., 2001). These snoRNA proteins are known to bind to small nucleolar RNA and function in processing of pre-rRNA molecules. Some heat shock proteins in the Hsp90 family have been found to aid in assembly of both yeast and mammalian telomerase complex while proteins involved in nonsense-mediated decay pathway in yeast are responsible for degradation of Est1 and Est3 (Forsythe et al., 2001; Grandin and Charbonneau, 2001; Lew et al., 1998).

Telomeric proteins

Apart from the proteins that bind to components of telomerase and help in its processing, maturation, assembly, and degradation, there are a few dozen proteins that bind to telomeres either directly or indirectly and influence their function. Some of these proteins form a heterochromatin that not only coordinates repeat addition to regulate length, but also functions as a protective cap at the ends to maintain genomic integrity (Blackburn et al., 2000). Rap1 binds to double-stranded telomeric DNA in yeast and aids in regulating telomere length (Konig et al., 1996). A counting mechanism has been proposed in which the number of Rap1 molecules may be an estimate of telomere length and negatively correlate with repeat addition by telomerase (Marcand et al., 1997). Other proteins like Sir proteins bind to Rap1 and add to the telomeric heterochromatin leading to silencing of nearby genes (Bourns et al., 1998; Wotton and Shore, 1997). Telomere length regulation by Rap1p requires association with the Rap1p-interacting factors Rif1p and Rif2p (Levy and Blackburn, 2004). Likewise, the mammalian telomere binding proteins Trf1 and Trf2 bind to telomeric DNA, and other proteins in turn bind to them; namely, hRap1, Tin2, PinX1, and Tankyrase 1 and 2. These proteins protect the ends and regulate telomere length (Cook et al., 2002; Kim et al., 1999; Li et al., 2000; Lin and Blackburn, 2004). There are also specialized single strand binding proteins that bind directly to the G-rich 3' overhang and are very important for end protection. Cdc13 is the primary single-stranded protein in yeast that can alternatively bind to either components of telomerase (Est1) or other proteins (Stn1 and Ten1) to recruit or block access of telomerase at the telomere (Chandra et al., 2001; Grandin et al., 2000; Nugent et al., 1996). Pot1 binds the single-stranded end of mammalian telomeres and together with Trf1 and Trf2 assists in the formation of the secondary structure t-loop that is thought to be important for end protection (Colgin et al., 2003; Yang et al., 2005). In

addition, there are DNA replication machinery proteins known to alter telomere length (Dahlen et al., 2003). DNA repair proteins, Ku80p/Ku70p heterodimer and Mre11p/Rad50/Xrs2 complex, have functions in binding telomeric DNA, recruiting telomerase, and also contributing to the 3' overhang maintenance in yeast (Boulton and Jackson, 1996; Fisher et al., 2004; Fisher and Zakian, 2005). Human Ku proteins also associate with telomeres and bind telomerase and Trf1 and Trf2 (Hsu et al., 1999; Hsu et al., 2000; Song et al., 2000; Ting et al., 2005). Human Mre11p/Rad50p/Nbs1p complex shows S phase-specific association at the telomeres (Zhu et al., 2000). Certain DNA checkpoint proteins have also been implicated in telomere length maintenance in both yeast and humans (Ritchie et al., 1999; Ritchie and Petes, 2000). Table 1-1 lists these and some of the other proteins known to function in telomere length maintenance in yeast and humans. A balance between the positive and negative length regulation through all these proteins, functioning through telomerase, keeps telomeres within a normal range in length.

Expression of telomerase in human cells

While telomerase expression is constitutive in many organisms, not all human cells express telomerase. In an intensive survey for which tissues express telomerase activity in humans, Kim *et al.* tested cell lines derived from various human tissues as well as actual tissue samples (Kim et al., 1994). Amongst 22 normal somatic cell cultures derived from skin, joint, breast, lung, cervix, uterus, bladder, or prostate, not a single one had detectable telomerase activity as measured by PCR based TRAP assay (Kim et al., 1994). Amongst tissue samples tested, fetal and adult testes and ovarian follicles were the only normal (non-carcinogenic) tissues expressing detectable telomerase activity. Since this report, closer examination of some normal tissues has reported detectable telomerase activity either at lower levels or specifically during

high proliferation phases. For example, telomerase activity is detectable in normal T and B cells and other human cell lines (WI-38, BJ fibroblasts, and TIG-3) during S phase and in uterine endometrium during the proliferative phase of the menstrual cycle (Buchkovich and Greider, 1996; Hiyama et al., 1995; Kyo et al., 1997; Masutomi et al., 2003). In addition, cellular layers within hair follicles, intestinal mucosa, and epidermis that are enriched in stem cells have detectable telomerase activity (Harle-Bachor and Boukamp, 1996; Ramirez et al., 1997; Rubin, 2002; Yasumoto et al., 1996). These results show that telomerase activity is generally restricted to germ cells and stem cells in an adult human body.

Consequences of shortening telomeres: senescence path

Most human somatic cells do not express telomerase, and their telomeres shorten with each cell division (Harley et al., 1990). The rate of telomere sequence loss in human cells is between 50-200 bp per cell division (Vaziri et al., 1993). In fact, the length of telomeres is a fair predictor for the capacity of replication that cultured mammalian cells display (Allsopp et al., 1992). In cell culture, this telomeric shortening eventually triggers a replication arrest by signaling growth inhibition pathways dependent on pRb and p53, referred to as mortality stage 1 (M1) (Artandi et al., 2000). Expression of certain viral oncogenes, such as the large T antigen of SV40, can bypass this growth inhibition in cell cultures. As a consequence, the telomeres continue to get shorter in successive generations (Zhu et al., 1999). Eventually, the unprotected ends elicit repair mechanisms leading to chromosome end-to-end fusions, and breakage-fusion-bridge cycles ensue. This stage is characterized by widespread cell death and is termed crisis or mortality stage 2 (M2) (Romanov et al., 2001). Human cells in culture have been shown to

bypass senescence and crisis (M1 and M2) with exogenous expression of the catalytic subunit of telomerase (Counter et al., 1998; Zhu et al., 1999).

Undoubtedly, telomere shortening can be central to cessation of replication in cells. However, the extent to which this process contributes to the aging of a tissue or an individual is not entirely clear. There have been many studies in which telomere shortening in several tissues has been measured over time and related to age (Furugori et al., 2000; Hastie et al., 1990; Lindsey et al., 1991; Nakamura et al., 2000). In general, telomeres are shorter in cells derived from older individuals than younger individuals (Lindsey et al., 1991; Vaziri et al., 1993). There is a lot of variation in telomere lengths in cells of different tissues from the same individual and these differences have been suggested to be due to renewal times of the particular tissue (Takubo et al., 2002). Correlation of telomere shortening with pathologies and decreased functional capacity of hematopoietic tissue is well documented (Greenwood and Lansdorp, 2003). For example, the autosomal dominant and X-linked types of a rare genetic disorder dyskeratosis congenita (DKC) have been mapped to telomerase RNA (*TR*) and *DKC* genes, respectively (Table 1-1). *DKC* encodes dyskerin that interacts with hTR (Table 1-1). The disease progresses with premature telomere attrition. Patients present with skin pigmentation abnormalities, leukoplakia, and pancytopenia (decreased blood cell counts) due to progressive hemopoiesis inadequacy (Knight et al., 1999a; Knight et al., 1999b; Vulliamy et al., 2002; Vulliamy et al., 2001). Aplastic anemia is another serious disorder in which bone marrow stops producing blood cells of all lineages. Genetic mapping in multiple studies has led to the identification of mutations in genes that function in telomere metabolism (Table 1-2) (Knight et al., 1999a; Knight et al., 1999b).

The most compelling evidence of a role of telomere maintenance in aging comes from syndromes of premature aging. Patients of Werner's syndrome present with several typical characteristics of aging during adolescence. These include premature cataracts, skin wrinkling, premature menopause, atherosclerosis, and osteoporosis. These individuals have an average life expectancy of 45 years. The gene responsible for this disease is *WRN,* which encodes a helicase of the RecQ family with roles in DNA repair and telomere length maintenance (Bai and Murnane, 2003; Opresko et al., 2002). Cells cultured from these patients show accelerated telomere shortening and undergo fewer divisions before senescence than normal cells (Chang et al., 2004). Exogenous expression of telomerase is able to immortalize these cells (Wyllie et al., 2000). Ataxia telangiectasia is another disorder of premature aging and has been mapped to a gene with telomeric functions (Ball and Xiao, 2005). Hutchinson-Gilford progeria and Down's syndrome are other diseases where telomeres from patients are shorter than those from age-matched controls (Allsopp et al., 1992; Vaziri et al., 1993). Patients with either of these diseases age prematurely. Together all these findings point to a strong correlation between shortening of telomeres and aging of human somatic tissues.

Oxidative stress has been investigated as a major contributor to cellular damage associated with aging (Costa and Moradas-Ferreira, 2001; Saretzki and Von Zglinicki, 2002). There is accumulating evidence connecting accelerated telomere shortening and oxidative stress (Tchirkov and Lansdorp, 2003; von Zglinicki, 2002). Most studies in which chronic mild oxidative stresses were induced in cell cultures displayed increased telomere attrition (Chen et al., 2001; von Zglinicki et al., 1995). In addition, stress-induced premature senescence in human cell cultures may possibly take place via the same p53-dependent growth inhibitory pathway as induced by shortened telomeres (Toussaint et al., 2000; Vaziri et al., 1997). Like telomere length,

oxidative stress has been implicated in many aging disorders (Costa and Moradas-Ferreira, 2001; Tchirkov and Lansdorp, 2003). In fibroblast cell cultures from patients of Werner syndrome, exogenous expression of telomerase prevented telomere shortening, improved the reduction in their proliferative capacity and also decreased the hypersensitivity to an oxidant (Hisama et al., 2000; Schulz et al., 1996; Wyllie et al., 2000). There is some evidence suggesting that antioxidants protecting cells from free radical-induced damage can decelerate telomere shortening (Furumoto et al., 1998; Hisama et al., 2000). Convincing evidence of telomere attrition caused directly by an oxidative agent was provided by the demonstration of un-repaired DNA nicks as a result of hydrogen peroxide in cultured human fibroblasts (von Zglinicki et al., 2000). These single-stranded breaks accumulated preferentially at the telomeres.

Downside to telomerase up-regulation: cancerous path

As mentioned earlier, expression of viral oncogenes in pre-senescent cells allows bypass of mortality stage 1. After a crisis period, where most cells die, a few immortalized cell lines arise (Romanov et al., 2001). Such cell lines commonly have telomerase activity (Counter et al., 1992). As stated before, immortal cell lines can also be generated by exogenous expression of the catalytic subunit of telomerase in pre-senescent cells. Hence, telomerase allows immortalization of cells in culture.

When Kim *et al.* reported that telomerase activity is not detectable in somatic cells, they also tested several tissues and immortal cell lines derived from human tumors (Kim et al., 1994). They found detectable telomerase activity in 90 of 101 biopsies and 98 of 100 immortal lines. There was a clear correlation between malignancy and expression of telomerase. For example, they reported telomerase activity in none of 8 biopsies of normal prostates, 1 of 10 benign

prostatic hyperplasias, 3 of 5 prostatic intraepithelial neoplasias, and in both prostate cancer biopsies tested (Kim et al., 1994). Indeed, telomerase is activated in approximately 90% of cancers and the remainder develop an alternative mechanism (ALT) to lengthen their telomeres via recombination at the ends (Shay and Wright, 2005).

A point to be noted here is that rare syndromes with documented chromosome instability display predisposition to cancers and have been associated with accelerated telomere shortening (Callen and Surralles, 2004). A few amongst these have been mapped to genes that have functional connections to telomere length maintenance (Table 1-2). Patients of ataxia telengiectasia, Nijmegen breakage, Werner, and Bloom syndromes have a very high predisposition to several malignancies especially of soft tissue or hematopoietic origins (Franchitto and Pichierri, 2002; Wu et al., 2000a; Wu et al., 2000b).

In the past decade, telomerase has been under investigation as a major anticancer target (Shay and Wright, 2002). Advances have been made in developing ways to target human telomerase RNA using antisense oligonucleotides or hammerhead ribozymes, inhibitors directed towards the reverse transcriptase subunit, and agents capable of preventing the access of telomerase at the telomere (Kelland, 2005). Another promising area to investigate would be in mechanisms that lead to the activation of telomerase in cancer cells (Greider, 1999).

Genesis of tumors requires several cellular changes, and immortalization is one of the essential steps. Telomeres have an established function in signaling cellular senescence in mammalian cells with an obvious role in carcinogenesis. Furthermore, telomere length shortening correlates with age-related illnesses and premature aging disorders. Hence, understanding telomere biology has implications both for cancer and aging research.

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TABLES

Table 1-1

Yeast and human genes with telomere-related functions.

Gene	Function of encoded protein	References
Telomerase Catalytic proteins		
EST2 (yeast) *TERT* (human)	Reverse transcriptase adds telomeric repeats.	(Lingner et al., 1997b) (Bachand and Autexier, 2001)
TLC1 (yeast) *TR* (human)	Functional RNA that carries a template for adding telomeric repeats.	(Singer and Gottschling, 1994) (Mitchell and Collins, 2000)
Telomerase auxiliary proteins		
EST1 and *EST3* (yeast) *EST1A* (human)	Part of telomerase holoenzyme complex. Est1p recruit telomerase to telomeres.	(Hughes et al., 2000; Snow et al., 2003)
TP1/TEP1 (human)	Associate with TR.	(Harrington et al., 1997; Nakayama et al., 1997)
Telomeric double-stranded binding proteins and their associated proteins		
RAP1 (yeast)	Binds double stranded telomeric DNA in yeast and negatively regulates telomeric length.	(Barinaga, 1997)
RIF1 and *RIF2* (yeast)	Negative regulation of telomeric length via Rap1p binding.	(Bourns et al., 1998)
TRF1 and TRF2 (human)	Bind to double stranded telomeric DNA and regulate telomere length (Trf1p negative regulator) and aid in t-loop formation (Trf2p).	(Fairall et al., 2001)
TIN2 (human)	Interact with Trf1p to negatively regulate telomeric length. Connects Trf1p and Trf2p, resulting in stabilization of Trf2p at the telomere.	(Kim et al., 1999)
PINX1 (human	Interact with Trf1p to regulate telomeric length. Pinx1p also binds to telomerase components and is a negative regulator of telomere length.	(Banik and Counter, 2004; Lin and Blackburn, 2004)
TANK1 and *TANK2* (human)	Interact with Trf1p and cause ADP-ribosylation of Trf1p diminishing the ability to bind to telomeres. Positive regulator of telomere length.	(Cook et al., 2002)
RAP1 (human)	Interacts with Trf2p. Negative regulator of telomeric length.	(Li et al., 2000)
Telomere-specific single-stranded binding proteins and their associated proteins		
CDC13 (yeast)	Binding single stranded overhang. Recruiting telomerase via Est1p binding. Negative regulator of telomerase recruitment via Stn1p and Ten1p.	(Nugent et al., 1996)
STN1 and *TEN1* (yeast)	Associate with Cdc13p and negatively regulate telomerase recruitment.	(Grandin et al., 2000)

POT1 (human)	Binds single-strand telomeric DNA and interacts with Trf1p complex. Negative regulator of length.	(Colgin et al., 2003)
UP1 (human)	Binds single stranded telomeric sequence as well as TR.	(Dallaire et al., 2000)
TPP1 (human)	Interacts with Tin2p and Pot1p, connecting single stranded and double stranded binding proteins.	(Houghtaling et al., 2004)
Proteins involved in non-homologous end joining pathway		
KU70 and KU80 (yeast)	Binds telomeric DNA and is important for end-protection. Telomerase binding and recruitment.	(Fisher et al., 2004)
KU (human)		(Hsu et al., 1999)
RAD50, MRE11 and *XRS2* (yeast)	Negative regulation of telomere length. Contribution to 3' overhang.	(Ritchie and Petes, 2000)
RAD51, MRE11 and *NBS1* (human)	Interacts with Trf2 and aids in t-loop formation.	(Zhu et al., 2000)
DNA checkpoint proteins		
MEC1, TEL1 (yeast) ATM (human)	Positive regulation of telomere length. *mec1 tel1* double mutant exhibit chromosome instabililty. ATM is important for genomic stability and is mutated in Ataxia telangiectasia.	(Ritchie et al., 1999) (Celli and de Lange, 2005)
Heat shock proteins		
HSP82, HSC82 (yeast) *HSP90,* p23 (human)	Molecular chaperones for telomerase assembly.	(Grandin and Charbonneau, 2001) (Forsythe et al., 2001)
DNA replication machinery proteins		
POL1, RFC1, PIF1, TOP3 (yeast)	Telomere length regulation. Possibly lagging strand synthesis.	(Dahlen et al., 2003)
SGS1 (yeast) *RECQ2 & 3* (human)	Helicases. Regulation of recombinational elongation at telomeres.	(Watt et al., 1996) (Opresko et al., 2005)
Nonsense-mediated RNA decay pathway proteins		
UPF1-3 (yeast)	Degradation of telomerase RNA component.	(Lew et al., 1998)
snoRNA proteins		
NHP2, NOP10, GAR1 and *DKC1* (human)	Binding to telomerase RNA. X-linked disease, Dyskeratosis congenita is mapped to *DKC1* and is associated with shorter telomere length.	(Dez et al., 2001; Mitchell et al., 1999; Pogacic et al., 2000)

Table 1-2

Human diseases that have been mapped to mutations in genes with telomere-related functions.

Disease	Clinical manifestation	Associated genes	References
Aplastic anemia	Pancytopenia Acute bone marrow shut down	*TR,* *TERT,* *NBS1*	(Shimada et al., 2004; Vulliamy et al., 2002; Yamaguchi et al., 2005)
Paroxysmal nocturnal hemoglobinuria	hematuria, associated with aplastic anemia	*TR*	(Keith et al., 2004)
Autosomal dominant Dyskeratosis congenita	hyperpigmentation, dystrophic nails, leukoplakia oris, pancytopenia	*TR*	(Vulliamy et al., 2001; Vulliamy et al., 2004)
X- linked Dyskeratosis congenita	hyperpigmentation, dystrophic nails, leukoplakia, pancytopenia	*DKC1*	(Heiss et al., 1998; Knight et al., 1999b)
Hoyeraal-Hreidarsson syndrome	aplastic anemia, immunodeficiency, cerebellar hypoplasia, growth retardation.	*DKC1*	(Knight et al., 1999a)
Ataxia telangiectasia	Premature aging, progressive neuronal degeneration, immunodeficiency, cancer predisposition especially leukemias and lymphomas.	*ATM*	(Gilad et al., 1996; Pagani et al., 2002; Sandoval et al., 1999; Telatar et al., 1998)
Sporadic malignancies	T-cell Prolymphocytic leukemia/ B-cell non-hodgkin lymphoma/ Mantle cell lymphoma/ Breast cancer	*ATM*	(Bay et al., 1999; Schaffner et al., 2000; Vorechovsky et al., 1997)
Ataxia telangiectasia-like disorder	Cerebeller degeneration	*MRE11*	(Hernandez et al., 1993; Klein et al., 1996; Pitts et al., 2001; Stewart et al., 1999)
Nijmegen breakage syndrome	Microcephaly, progressive mental retardation, immunodeficiency, predisposition to lymphoid malignancy.	*NBS1*	(Tauchi et al., 1999; Varon et al., 2001)
Werner Syndrome	Premature aging, atherosclerosis, osteoporosis, diabetes mellitus, malignancies especially sarcomas.	*RECQ3*	(Yu et al., 1997)
Bloom syndrome	Growth retardation, immunodeficiency, childhood malignancies.	*RECQ2*	(Ellis et al., 1994; Foucault et al., 1997)

FIGURES

Figure 1-1

End replication problem.

The figure illustrates how DNA replication on the lagging strand remains incomplete after the distal-most RNA primer is removed. At the top, double-stranded DNA is shown with its telomeric end on the right. In the middle, replication on both strands is shown. RNA primers are shown in green stripes. At the bottom, replicated DNA is shown with the newly synthesized strands in black.

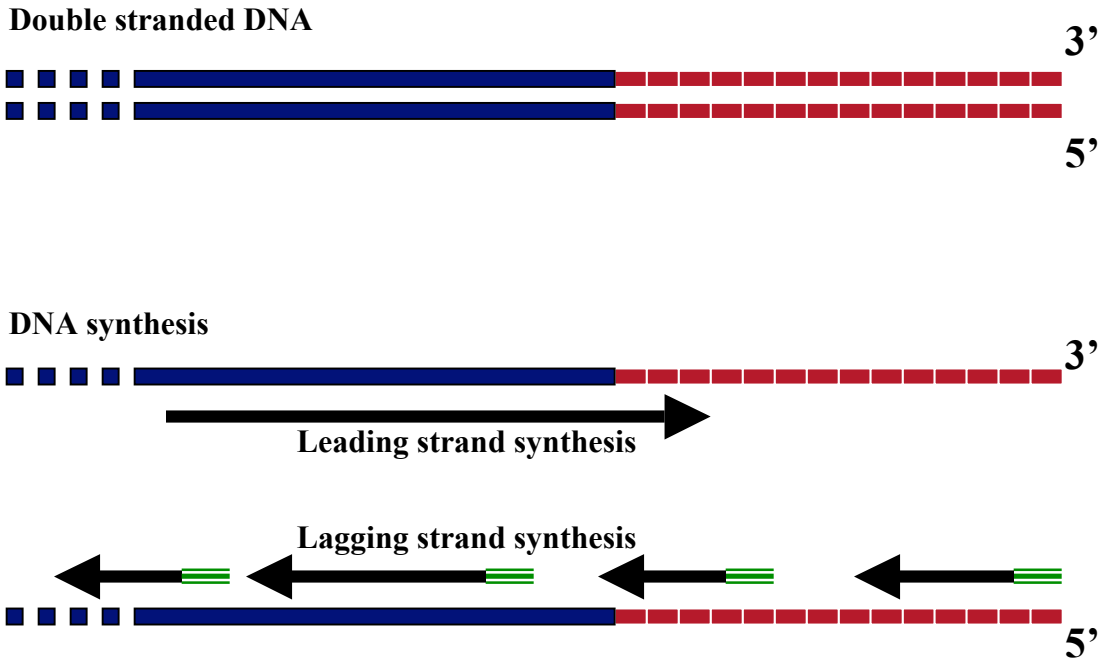
Double stranded DNA
3’
5’
DNA synthesis
3’
Leading strand synthesis
Lagging strand synthesis
5’
Replicated DNA

Figure 1-1

Figure 1-2

Telomere repeat addition by telomerase.

Diagram illustrates telomere repeat addition at the 3' overhang by telomerase. An intergral RNA moiety that carries a template complementary to the telomeric repeats is used to reverse transcribe more repeats at the end.

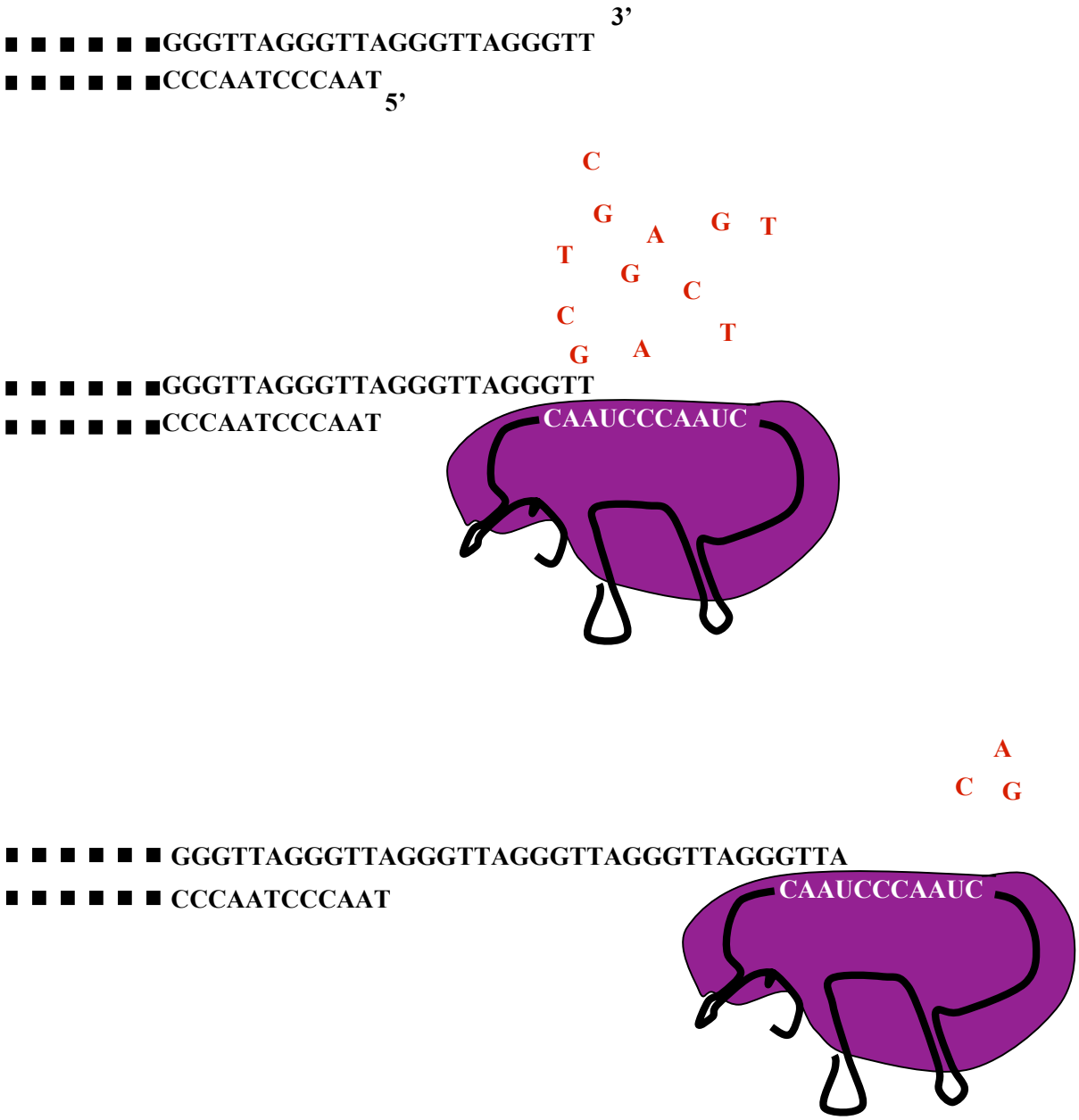
3’
GGGTTAGGGTTAGGGTTAGGGTT
CCCAATCCCAAT
5’
C
G
A
G
T
T
G
C
C
T
G
A
GGGTTAGGGTTAGGGTTAGGGTT
CCCAATCCCAAT
CAAUCCCAAUC
A
C
G
GGGTTAGGGTTAGGGTTAGGGTTAGGGTTA
CCCAATCCCAAT
CAAUCCCAAUC

Figure 1-2

Figure 1-3

Telomere length shortening in human somatic cell cultures.

The graph illustrates how telomere length decreases over successive cell divisions when human somatic cells are grown in culture. The stage of senescence is reached when shortened telomeres trigger growth arrest in normal cells. Cells that bypass this stage mostly die at the stage of crisis, but a few immortalized cell lines arise past crisis via activation of telomerase or ALT (recombination based telomere length maintenance).

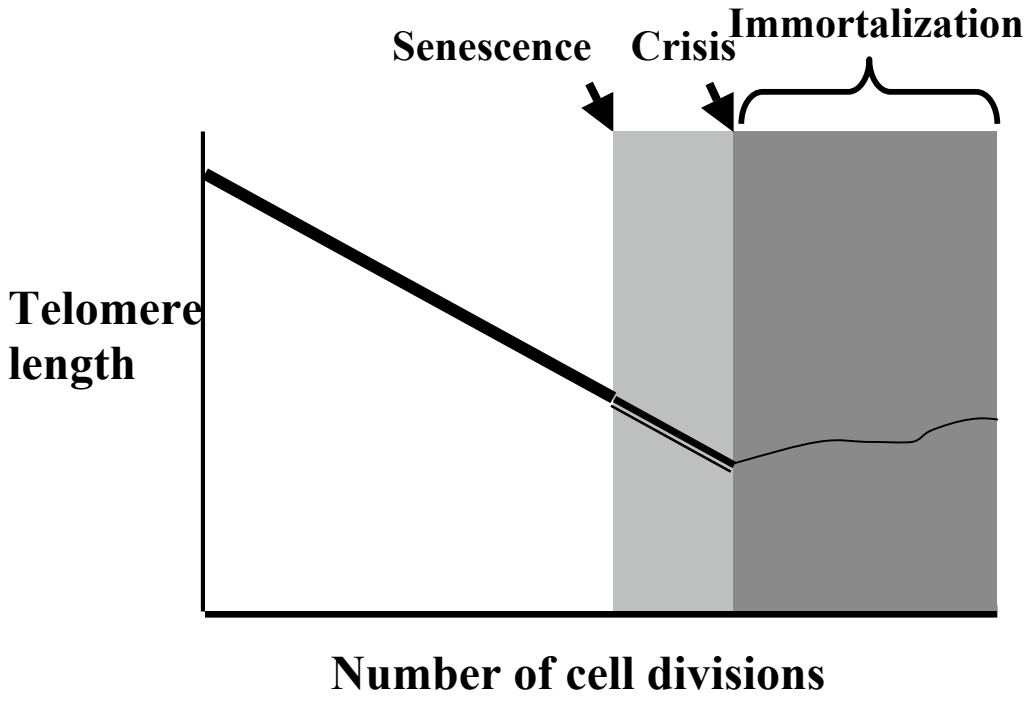
Senescence
Crisis
Immortalization
Telomere length
Number of cell divisions

Figure 1-3

CHAPTER 2

A GENOME-WIDE SCREEN FOR *SACCHAROMYCES CEREVISIAE* DELETION MUTANTS THAT AFFECT TELOMERE LENGTH[1]

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[1]Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, Coker C, Krauskopf A, Kupiec M, McEachern MJ. 2004. Proc Natl Acad Sci U S A. 2004 Jun 8;101(23):8658-63.

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ABSTRACT

Telomeres are nucleoprotein structures present at the ends of eukaryotic chromosomes. They play a central role in guarding the integrity of the genome by protecting chromosome ends from degradation and fusion. Regulation of telomere length is central to their function. In order to broaden our knowledge about the mechanisms that control telomere length, we have carried out a systematic examination of ~4800 haploid deletion mutants of *Saccharomyces cerevisiae* for telomere-length alterations. Using this screen we have identified more than 150 candidate genes not previously known to affect telomere length. In two thirds of the identified mutants short telomeres were observed, whereas in one third telomeres were lengthened. The genes identified are very diverse in their functions but certain categories, including DNA and RNA metabolism, chromatin modification and vacuolar traffic are over-represented. Our results greatly enlarge the number of known genes that affect telomere metabolism and will provide new insights into how telomere function is linked to many other cellular processes.

INTRODUCTION

In most eukaryotes, telomeres consist of tandem arrays of a short G-rich repeat which protect chromosome ends from being recognized as double strand breaks (McEachern et al., 2000). Telomeres are prone to shortening at each replication event due to an inherent inability of the replication machinery to fully replicate them (Olovnikov, 1996; Watson, 1972). This sequence loss is normally prevented by the action of the ribonucleoprotein enzyme telomerase, which reverse-transcribes telomeric repeats onto telomeric ends (Greider and Blackburn, 1985). Addition of new sequence by telomerase is typically tightly regulated resulting in the telomeres of many organisms being kept within particular size ranges.

Most human somatic cells do not express telomerase and their telomeres shorten with each cell division. This shortening can eventually trigger replicative senescence via the activation of growth inhibition pathways dependent on Rb and p53 (Artandi et al., 2000). Bypass of replicative senescence produces further telomere erosion, telomere-telomere fusions, and the eventual cell death known as crisis (Counter et al., 1998; Romanov et al., 2001; Zhu et al., 1999). Exogenous expression of the catalytic subunit of telomerase prevents both replicative senescence and crisis (Bodnar et al., 1998). >90% of human cancers display cellular immortality due to the expression of telomerase activity and the resulting stabilization of telomere lengths (Kim et al., 1994; Shay and Bacchetti, 1997). Determining the mechanisms by which telomeres and telomerase are regulated could lead to better understanding of both carcinogenesis and aging.

Much of our basic knowledge of telomere biology has come from studies of the yeast *Saccharomyces cerevisiae.* Telomeres in this organism are composed of tracts of heterogeneous $TG_{1\text{-}3}$ sequences that normally total a few hundred base pairs in length. A large number of proteins are already known to be involved in various aspects of yeast telomere function. In addition to telomerase, these include dedicated telomere binding proteins (Berman et al., 1986; Blackburn, 2000; McEachern et al., 2000), the Ku and MRX DNA repair complexes (reviewed in (Bertuch and Lundblad, 1998)), and certain checkpoint (Craven et al., 2002) and replication proteins (Adams and Holm, 1996). Genes for most of these proteins alter telomere length when mutated. Average telomere length can therefore be a very sensitive sensor of telomere function. Many genes related to telomere function in yeast have been found to have similar roles in other organisms, including humans. It is therefore reasonable to predict that identifying novel genes that alter yeast telomere will lead to useful insights into human telomere biology. In this work, we have utilized a collection of deletion mutants of all non-essential genes to systematically

search for genes affecting telomere length. We report >150 genes that were not previously known to alter telomere length. These genes affect several different cell processes, including DNA and RNA metabolism, chromatin modification and vacuolar traffic.

MATERIALS AND METHODS

Strains

A collection of 4852 haploid *Saccharomyces* strains (Giaever et al., 2002) was used, in which each strain has a single ORF replaced with the KanMX4 module, which confers G418 resistance. These strains are in the BY4741 background (MAT**a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ). The isogenic strain BY4742 (MAT**a** *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ) was used for genetic analysis.

Telomere length measurement

DNA was prepared from cells taken directly from YPD plates. In most cases, cells had been freshly stamped from thawed microtiter plate stocks. DNA from each strain was digested overnight with *Xho*I and run on agarose gels for 16-20 hrs at 1.4 volts/cm. Southern blotting was performed using Hybond N+ membrane (Amersham, Pharmacia Biotech, Piscataway, NJ) and probed with radiolabelled telomeric probe 26G (5'-TGTGGGTGTGGTGTGTGGGTGTGGTG-3') labeled with T4 Polynucleotide kinase. All hybridizations were done in 200 mM Na_2HPO_4 and 2% SDS (Church and Gilbert, 1984).

For the Southern blots shown, internal control fragments of sizes 1865 bp and 644 bp, containing *S. cerevisiae* telomeric repeats were generated by *Bsm*AI and *Taq*I digests, respectively, of the plasmid pYt103 (Shampay et al., 1984). Approximately 10 ng of each digested DNA was added to each lane together with the *Xho*I digested genomic DNA. For better resolution of size differences, 25 cm long gels were run at 1.4 volts/cm for 600 minutes and an

additional 600 minutes at 2 volts/cm. The average telomeric length for each lane was estimated by plotting the peak of signal intensity of the shortest telomere band (Y' telomeres) against the position of the added internal telomere size standards.

PCR

To confirm ORF deletion identity, a general primer from inside the KanMX cassette (GCCATCAAAATGTATGGATGC) and a specific primer from the 5' UTR of each ORF (Shoemaker et al., 1996) were used. When necessary, the reaction was repeated using the KanMX primer and a primer complementary to the UPTAG primer that was used to create the deletion strains (GATGTCCACGAGGTCTCT). This PCR product was sequenced in order to identify the strain by its unique tag.

Co-segregation Test

Telomere length phenotypes of 27 candidate strains were checked by testing co-segregation of the length phenotype with the deletion ($G418^r$). The candidate mutant strains were mated with BY4742 wild type and the resulting heterozygous diploids were sporulated by the recommended method (Giaever et al., 2002). Tetrads picked were scored for G418 resistance on YPD plates supplemented with 400 mM G418 (Amersham). Telomere length was estimated by Southern blot analysis. The presence of the KanMX casette was confirmed by re-probing the Southern blots with a labeled ~800 bp *Sca*I/ *Nco*I fragment of pFA6-KanMX4 plasmid (Wach et al., 1994) All hybridizations with 26G and G418 were done at 55º and 65º C, respectively.

RESULTS

We have carried out a genome-wide screen for mutants affecting telomere length. A collection of 4,852 haploid-viable yeast strains, each deleted for a single ORF, was used. DNA

from each strain in the collection was digested with *Xho*I, separated by gel electrophoresis under standardized conditions and subjected to Southern blot analysis using a telomere-specific probe. The telomeric probe produces a complex pattern, derived from hybridization to many telomeric fragments, and also to several subtelomeric repeats (Zakian and Blanton, 1988). The latter served as internal controls, as their electrophoretic mobility was usually not affected by the various mutations. The shortest fragments (~1.3 kb in wild type cells), resulting from Y' -containing telomeres, were the most reliable in determining telomeric length differences between strains, both because they represent multiple telomeres and because length differences are most pronounced in the shortest fragments. The next few bands, corresponding to telomeres containing an adjacent X element and no Y' element (X' telomeres), were also observed carefully in order to identify mutants with length alterations.

After the first round of screening, ~600 mutants exhibiting possible short or long telomere phenotypes were re-tested by at least two additional rounds of fresh DNA preparations and Southern blotting. DNA from wild type cells was run interspersed amongst the mutants in these gels to maximize the chances of correctly identifying mutants with modest effects on telomere length. By this very stringent criterion, 173 strains were identified that consistently exhibited either shorter or longer telomeres than wild type (Table 2-1). In addition, 26 other mutants were labeled as questionable gene candidates for telomere length phenotypes because they showed very mild telomere length phenotypes that failed to be detected in at least one repeat Southern (see Table 2-2).

One additional DNA preparation and Southern blot was then carried out to measure telomere length in each of the 173 mutants identified in the screen. The average telomeric length for the shortest telomeric fragment (Y' telomeres) was then estimated by utilizing fragments

generated by restriction digests of a plasmid containing a cloned *Saccharomyces* telomere that were added to each genomic DNA sample. These telomeric fragments ran at positions above and below the Y' telomeric bands and served as internal controls to measure size and to assure uniform migration of different samples (see Materials and Methods). Figure 2-1 shows an example of one of these Southern blots, each of which was run on long gels (25 cm) to maximize resolution of telomeric length differences. The rest of these Southern blots are shown as Figure 2-2. Average Y' telomere length for the wild type strain was estimated from multiple samples run on the same gels. The change in Y' telomere length relative to the wild type was then estimated for each of the 173 mutants. These values are presented in Table 2-3. 25 of the 173 mutants in this last Southern blot analysis showed Y' telomeres that had only slight length differences with the wild type control (less than 25 bp). We have not excluded them from our list since they had reproducibly shown slight length differences in all other Southern blots carried out. These mutants are marked with double asterisk in Table 2-1.

The identity of each of the 173 mutants shown in Table 2-1 was confirmed by PCR analysis (see Materials and Methods). The sizes of the Y' telomeres were grouped into the following categories: slightly short (<50 bp shorter than wild type), short (50-150 bp shorter than wild type), very short (>150 bp shorter than wild type), and equivalent categories for long telomere mutants (Table 2-1). While consistently showing either long or short telomere length, many of the deletion strains exhibit some variation in the degree of their length phenotype when observed over repeated Southern blots. We have considered the length phenotypes from all Southern blots before assigning a gene to one of the phenotypic groups shown in Table 2-1. Of the mutants identified, 123 exhibited shorter and 50 showed longer telomeres than wild type. None of the newly identified mutants appeared to have the ever shortening (EST) phenotype

characteristic of telomerase deletion mutants (Lendvay et al., 1996; Lundblad and Szostak, 1989). Several other mutants, listed in the Table 2-4, repeatedly had telomeres that were heterogeneous in length. Some of these appear to have Y' telomeres of normal length but have elongated X telomeres, others displayed Y' telomeres with a bimodal length distribution. Conceivably, the former might be altered in the uncharacterized mechanism that leads to size differences between X and Y' telomeres (Craven and Petes, 1999).

Twenty seven of the mutants (marked with †† in Table 2-1), were tested for co-segregation between the telomere phenotype and the G418 resistance determinant inserted in place of the deleted gene. All of these except one showed clear segregation of telomeric phenotype with G418 resistance. The single exception, *yel033w*, showed a short telomere phenotype that segregated independently from the antibiotic resistance. From our analysis we conclude that the *yel033w* deletion segregates with a very slow growth phenotype and the short telomeres segregate with a suppressor mutation responsible for restored growth. Thus, *YEL033W*, a dubious ORF, appears to genetically interact with an unidentified gene with telomere function. Our results imply that in the vast majority of our strains the telomere phenotype was caused by the relevant deletion.

DISCUSSION

Saccharomyces cerevisiae is the best understood system for studying telomere biology and many genes with roles in telomere function are already known in this organism. Nonetheless, our efforts have resulted in a wealth of additional candidate genes that alter telomere length when deleted. Our results indicate that a surprisingly large percentage of the yeast genome is in some way linked to telomere metabolism. The 173 genes listed in Table 2-1 represent ~3.2% of the

estimated 5538 genes of *S. cerevisiae*. This number is certainly an underestimate of the total as >1000 essential genes were not examined and because many genes known to mildly affect telomere length in other strains were not found in our screen.

Out of the 32 genes previously known to exhibit a telomeric phenotype when singly deleted, our screen identified 18 (bold in Table 2-1). Genes not identified in our screen include some whose reported phenotype was very mild. These include *FOB1* (Weitao et al., 2003), *SWD1* and *SWD3* (Roguev et al., 2001), *RRM3* (Ivessa et al., 2002), *EBS1* (Zhou et al., 2000), *MLP1* and *MLP2* (Hediger et al., 2002), *SIR3* (Palladino et al., 1993), *DDC1* and *RAD17* (Longhese et al., 2000). We have not determined whether these genes were missed in our screen or whether they simply behave differently in our strain background. Of the four additional genes not identified in our screen, one (*TOP3*) (Kim et al., 1995) was not present in the collection. Two of the three others (*GAL11* (Suzuki and Nishizawa, 1994) and *SIR4* (Palladino et al., 1993) but not *CTF18* (Hanna et al., 2001)) showed mild telomere length phenotypes when individually re-examined (data not shown). It is not unexpected that mutants with slight telomere length alterations could be difficult to distinguish from wild type or even overlooked by our screen. Yeast telomeres are not only heterogeneous in size, but they are subject to size fluctuations even within clonal lineages (Shampay and Blackburn, 1988). While it is clear that our screen failed to detect all mutants with slight telomere length phenotypes, we are confident that it successfully identified the great majority of the nonessential genes that appreciably affect telomere length when deleted. Although absolute confidence that individual genes identified in this study affect telomere length will require additional experimentation, it is unlikely that our screen identified many false positives. Each mutant has had its telomere length examined several times and the identity of all has been confirmed through PCR testing. Moreover, 26 of 27 randomly sampled mutants have

shown the expected meiotic co-segregation between the KanMX marker and the telomeric phenotype.

The genes identified in our screen have very diverse functions. Although some of them are probably directly involved in telomere metabolism, most are likely to affect telomere length indirectly either by altering the activity of proteins directly involved in telomere maintenance, or by eliciting cellular mechanisms that lead to changes in telomere length. The genes most likely to be directly involved in telomere size maintenance are those affecting DNA metabolism. In addition to the known DNA repair genes involved in telomere size control, such as components of the MRX (*Mre11, Rad50, Xrs2*) and Ku (*Yku70, Yku80*) complexes, we have identified *LRP1*, the yeast homolog of the human protein C1D (Erdemir et al., 2002). In mammalian cells, this is a Gamma-Irradiation-inducible nuclear matrix protein that activates DNA PK (Yavuzer et al., 1998). The yeast homologue has roles both in homologous recombination and in non-homologous end-joining (Erdemir et al., 2002).

DCC1 encodes a component of an RFC-like (RLC) clamp loader complex that works with the Trf4 DNA polymerase, to ensure proper sister chromatid cohesion during DNA replication (Hanna et al., 2001). Although both *Ddcc1* and *Dtrf4* were identified among the short telomere strains, mutations in *ELG1*, the main component of an alternative RLC, lead to elongated telomeres (Table 2-1; (Ben-Aroya et al., 2003; Smolikov and Krauskopf, 2003)), and the *Drad24* strain, defective in a third RLC (Green et al., 2000), exhibited no telomeric phenotype (data not shown). Another mutant with links to DNA replication, whose deletion causes telomere shortening, is *RNH35*. This gene encodes an RNase H required for RNA primer removal during DNA synthesis (Chen et al., 2000). Its activity may be required for the coordination between

replication of subtelomeric regions by the DNA polymerases and telomeric elongation by the telomerase. A similar role has been proposed for Elg1p (Smolikov and Krauskopf, 2003).

Genes located in the proximity of the chromosomal ends are often subjected to epigenetic silencing, also known as Telomeric Position Effect (TPE) (Gottschling, 1992). Although many mutants have been isolated that affect telomeric silencing, not all of them exhibit changes in telomere length. Our screen has identified components of several complexes previously known to affect silencing that produce short telomeres. These include: the *HST1-SUM1-RFM1* histone deacetylase (McCord et al., 2003), and the *SIN3, SAP30, OPI1* and *DEP1* genes, encoding components of the Rpd3 histone deacetylase complex (Lamping et al., 1994; Zhang et al., 1998). In addition, we have identified several components of the Paf1, Set1 and Tho complexes, which seem to interact both in chromatin remodeling and during transcription elongation (reviewed in (Krogan et al., 2003)). Moreover, mutations in certain components of the RSC, Mediator and CTD phosphorylation complexes, which are located at the interphase of chromatin remodeling and RNA polymerase activation, also caused shortening of the telomeres. Not all of the nonessential members of these complexes reduced telomere length, suggesting that the link to telomere homeostasis may be due to the individual proteins in these complexes. The isolation of so many mutants that lead to shortened telomeres by interfering with chromatin remodeling functions suggests that chromatin integrity/modification plays an important role in elongating telomeres. We have also identified *Dnup60* as a strain exhibiting short-telomeres. Nup60 is required to anchor telomeres to the nuclear periphery and a link between telomere position within the nucleus and chromatin remodeling affecting TPE has been shown (Feuerbach et al., 2002). It is possible that telomere elongation also requires anchoring of telomeres to the nuclear periphery.

A large number of genes with known vacuolar functions showed telomere length alterations when individually deleted. The yeast vacuole is the functional analogue of the mammalian lysosome, the major site of degradation of both exogenous and endogenous macromolecules (reviewed in (Teter and Klionsky, 2000)). Prominent amongst these genes are components of the ESCRT (endosomal sorting complex required for transport) complexes. Three ESCRT complexes are known to bind in succession to ubiquinated cargos in late endosomes and function in the sorting of proteins to be degraded by vacuole/lysosome in the MVB (MultiVesicular Bodies) pathway, a well conserved process in eukaryotes (reviewed in (Bache et al., 2003)). Ten genes involved in this process display a short telomere phenotype when deleted. These encode components of the ESCRT complexes (Vps23, Vps28, Vps22, Vps25, Vps36, Vps32, Yel057c), a phosphatidylinositol-3-kinase, Vps34, its associated kinase Vps15 and a downstream player in the process, Bro1 (Bilodeau et al., 2002; Nikko et al., 2003; Stack et al., 1995). Additional vacuolar genes identified include those that act in vacuolar targeting and fusion.

This connection between vacuolar targeting and telomere metabolism may be due to one or more telomeric proteins being regulated by degradation in the vacuole. Interfering with the vacuolar pathway could cause an increase in the level of these proteins, creating an imbalance in telomere size. At this moment there are no obvious telomeric proteins that are known to be degraded via this pathway.

In contrast to the >100 genes whose mutations led to short telomeres, only 50 deletion mutants exhibited a clear phenotype of elongated telomeres. The reason for this asymmetry is unclear. It likely indicates that there are more genes connected to telomerase-mediated sequence addition than there are to the negative regulation of that process. The mutants causing lengthening were more difficult to organize in clear functional categories. In addition to *ELG1*,

discussed above, *POL32*, a nonessential subunit of DNA polymerase Delta (Gerik et al., 1998), is a candidate for having direct link to telomere metabolism. Two additional genes causing telomere elongation affect chromosome segregation: *CSM1*, encoding a component of the kinetochore, and *SRC1*, which affects sister-chromatid segregation (Rodriguez-Navarro et al., 2002) and seems to be a target for Cdk phosphorylation (Ubersax et al., 2003).

Deletion of only a few genes affecting chromatin/silencing caused telomere elongation. These include two components of the Mediator complex (*NUT1* and *SSN8*) (Kuchin et al., 1995; Tabtiang and Herskowitz, 1998), two components of the RSC complex (*HTL1* and *SFH1*) (Cao et al., 1997; Romeo et al., 2002) and several less well-characterized genes. For example, *NPL6* was defined as a nuclear pore component; however it binds histone H2B and many chromatin-remodeling factors, and localizes to the nucleus (Nelson et al., 1993).

Among the genes with long telomeres identified in our screen, there is only one clear case where deletion of each subunit of a known complex caused a similar telomere phenotype. Deletion of each subunit of the NatC N-terminal acetyltransferase led to elongated telomeres. N-terminal acetylation is one of the most common cotranslational modification processes in eukaryotes (reviewed in (Polevoda et al., 2003)) and is carried out by one of three complexes in a substrate-specific fashion. In several cases, genes affecting telomere length are physically next to one another. Deletion of one potentially could alter telomere length by changing the expression of its neighbor. Examples include one neighbor of *STN1* and both neighbors of *PIF1* (including the DNA repair gene *OGG1*). In addition, there are at least six more pairs of neighboring genes in our list of candidates that show telomere length phenotype when individually deleted. These are listed in table 2-5.

Our genome-wide approach has allowed us to identify many novel genes that affect telomere length control. These reveal novel and unexpected links to various aspects of the cellular metabolism. Given the conservation of telomere maintenance mechanisms throughout evolution, analysis of the new genes will certainly be relevant to other eukaryotes, and may have important consequences for therapeutic treatment of cancer.

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TABLES

Table 2-1

List of *S. cerevisiae* genes that affect telomere length when deleted.

Single knockout mutants of the listed 173 genes screened positive for telomere length defects in our analysis. Genes are listed grouped according to broad cellular function. The second column shows estimated average Y' telomere lengths relative to wild type length: "ss", slightly short (<50 bp shorter than wild type); "S", short (50-150 bp); "VS", very short (>150 bp); "sl", slightly long (<50 bp longer than wild type); L, long (50-150 bp); and "VL", very long (>150 bp). Telomeric length was measured by plotting the peak signal of the shortest telomeric band (Y' telomeres) against the positions of the added internal controls of the gels shown in Fig. 1 and supplementary Fig. 2 using a phosphoimager and ImageQuaNT version 4.1 software. Final telomeric length classification shown here is additionally based on data from other gels. Double asterisks indicate mutants that either showed average measured Y' telomere length <25 bp different in size from those of wild type cells in the gels shown in Fig. 1 and Fig. 2 (see supplementary material) or they showed length phenotypes inconsistent with reproducible phenotypes noted in all previous Southerns. Because these mutants showed altered telomere lengths in all previous Southerns, they are included in this Table. Co-segregation test was carried out with the deletion mutants marked with "††

Gene	Telomere phenotype	Function
DNA Metabolism		
EST1	***VS***	Telomerase holoenzyme complex
EST2	***VS***	Telomerase reverse transcriptase
EST3	***VS***	Telomerase holoenzyme complex
TEL1	***VS***	DNA damage response kinase
YKU70	***VS***	DNA repair, Ku70-Ku80 complex
YKU80	***VS***	DNA repair, Ku70-Ku80 complex
MRE11	***VS***	DNA repair, MRX complex
RAD50	***VS***	DNA repair, MRX complex
XRS2	***VS***	DNA repair, MRX complex

*RNH35***	*VS*	RNaseH, DNA replication. Interacts with Rif2
DCC1	*S*	Sister chromatid cohesion
HUR1	*S*	DNA replication, interacts with Mec3
LRP1	*S*	Homologue of C1D, Double-strand break repair
YPL205C	*S*	Overlaps with *HRR25* (an essential DNA repair gene)
RIF1	***VL***	Telomere maintenance, silencing
RIF2	***VL***	Negative telomere regulator
ELG1	***VL***	Genome stability
PIF1	***VL***	Telomere maintenance, recombination
OGG1	*L*	Base excision repair, shares *PIF1* promoter
*POL32***	*sl*	DNA polymerase Delta complex
*MLH1***	*sl*	Mismatch DNA repair
CSM1	*sl*	Meiotic chromosome segregation, interacts with *zds2*
*YML035C-A***	*sl*	Antisense to *SRC1* (sister chromatid segregation gene)
Chromatin, silencing, PolII transcription		
HST1	*S*	*SIR2* homolog, histone deacetylase complex
SUM1	*S*	Supressor of *sir* mutants, increases silencing in telomere
RFM1	*S*	Silencing, part of Hst1 histone deacetylase complex
SIN3	*S*	Silencing, Part of Rpd3 histone deacetylase complex
SAP30	*S*	Silencing, Part of Rpd3 histone deacetylase complex
OPI1	*S*	Interacts with Sin3
DEP1	*S*	Part of the Rpd3 histone deacetylase complex
HDA2	*S*	Part of the HAD Histone deacetylase complex
CDC73	*S*	Part of the Paf1 complex
RTF1	*S*	Part of the Paf1 complex
BRE2	*S*	Part of the SET1 histone methylase complex
††*MFT1*	*ss*	Tho and Paf1 complexes
THP2	*S*	Tho and Paf1 complexes
††*SOH1*	*S*	Supressor of *hpr1* mutants (Tho and Paf1)
RPB9	*S*	DNA-directed RNA polymerse II subunit
RPB4, CTF15	*S*	DNA-directed RNA pol II subunit
SRB2	*S*	Pol II transcription, mediator complex
††*SRB5*	*S*	Subunit of RNA polII, mediator complex
RSC2	*S*	RSC complex- chromatin modeling
CTK1	*S*	Pol II transcription regulation, protein kinase
††*SPT21*	*S*	Pol II transcription, chromatin
CST6	*ss*	Transcriptional activator, chromosome stability
*NUP60***	*ss*	Silencing, part of the nuclear pore
††*HTL1*	*VL*	DNA replication and chromosome cycle
HPR1	*L*	Tho and Paf1complexes.
HCM1	*L*	Transcription factor (forkhead2)
††*MMS19*	*L*	Pol II transcription (TFIIH) and nucleotide excision repair
*YDJ1***	*sl*	Forms a complex with Mms19
SSN8	*L*	Part of the mediator complex
*NUT1***	*sl*	Part of the mediator complex
NFI1	*sl*	SUMO ligase, chromatin
*FMP26***	*sl*	Interacts SAGA, reported mitochondral.
*VPS65***	*sl*	Deletion affects *SFH1*, (RSC complex)
*HMO1***	*sl*	ss-DNA binding, HMG-box protein
NPL6	*sl*	Protein-nucleus import
Vesicular traffic (Vacuole, Golgi, ER, membrane biosynthesis)		
CAX4	*VS*	ER, N-glycosylation, phosphatase
VPS3	*S*	Vacuolar sorting protein
VPS9	*S*	Vacuolar sorting protein
††*VPS15*	*S*	Vacuolar sorting protein
VPS18	*S*	Vacuolar sorting protein
††*VPS23*	*S*	Vacuolar sorting protein. ESCRT-I
††*VPS28*	*S*	Vacuolar sorting protein. ESCRT-I
††*VPS22*	*S*	Vacuolar sorting protein. ESCRT-II
††*VPS25*	*ss*	Vacuolar sorting protein. ESCRT-II
††*VPS36*	*S*	Vacuolar sorting protein..ESCRT-II
VPS32	*S*	Vacuolar sorting protein. ESCRT III.
YEL057C	*S*	Vacuolar sorting protein. ESCRT-III
BRO1	*S*	Vacuolar sorting protein. After ESCRT-III

VPS34	*S*	Vacuolar sorting protein.
VPS39	*S*	Vacuolar sorting protein
VPS75	*S*	Vacuolar sorting protein
VPS43	*ss*	Vacuolar sorting protein
APE3	*S*	Aminopeptidase, vacuolar protein degradation
ATG11	*S*	Peroxisome degradation and vacuolar targeting
MOT3	*S*	Suppressor of *spt3*, increased sterol levels
ARV1	*S*	Sterol metabolism and transport
AGP2	*S*	Carnitine transporter, fatty acid metabolism
YTA7	*S*	Affects Ergosterol and dolicol synthesis
††*PDX3*	*S*	Pyridoxine phosphate oxidase
ERJ5	*S*	Golgi transport to ER
LST7	*S*	Golgi-to-surface trafic protein
††*SUR4*	*S*	fatty acid synthesis post golgi transport
RPN4	*S*	Ubiquitin degradation pathway.
*PMT3***	*ss*	Protein-O-mannosyl-transferase, ER
YSP3	*ss*	Subtilisin-like peptidase
RNA metabolism		
UPF1	***VS***	Nonsense mediated mRNA catabolism
UPF2	***VS***	Nonsense mediated mRNA catabolism
UPF3	***VS***	Nonsense mediated mRNA catabolism
KEM1	*VS*	Exonuclease, mRNA degradation
RRP8	*sl*	Methyltransferase, pre-rRNA processing
STO1	*sl*	mRNA splicing and snRNA cap binding complex.
LEA1	*sl*	mRNA splicing
*YPL105C***	*sl*	Unknown. Interacts with splicing and translation genes.
*YMR269W***	*sl*	Unknown. 2H with eIF2B
Cell polarity, cell wall and bud site selection		
††*BEM4*	*S*	Cell polarity, actin organization,
YOR322C	*S*	sl with cell polarity mutants
††*BUD16*	*S*	Unknown, putative pyridoxal kinase
YPL041C	*S*	sl with cell wall, phosphoglucomutase
YPL144W	*S*	Cell wall, phospholipids
SMI1	*S*	Cell wall synthesis, chromatin binding
CCW14	*S*	Cell wall structural protein
*CSR2***	*ss*	Cell wall organization
GPB2	*ss*	Pseudohyphal growth
SPS100	*ss*	Spore wall assembly
LDB7	*L*	Cell wall organization
BUD30	*sl*	Bud site selection
*BUD23***	*sl*	Bud site selection
*BEM2***	*sl*	Polarity, cytoskeleton, budding
Protein modification and heat shock proteins		
††*SSE1*	*S*	Anti-oxidative response, co-chaperone
††*XDJ1*	*S*	Chaperone regulator.
HSC82	*S*	Heat shock protein
HCH1	*ss*	Chaperone activator, Hsp90 supressor.
††*MAK10*	*L*	N-acetyltransferase
††*MAK31*	*L*	N-acetyltransferase
††*MAK3*	*L*	N-acetyltransferase
HSP104	*sl*	Heat shock protein.
CDH1	*sl*	degradation of cyclin: metaphase/anaphase transition
Ribosome and translation		
RPP1A	*VS*	Large ribosomal subunit
RPL12B	*S*	Large ribosomal subunit
RPL13B	*S*	Large ribosomal subunit
RPL1B	*S*	Large ribosomal subunit
MRT4	*S*	Ribosomal large subunit biogenesis
EAP1	*S*	Translation regulation
RPS17A	*VL*	Small ribosomal subunit
*RPS10A***	*L*	Small ribosomal subunit
RPS14A	*L*	Small ribosomal subunit
ASC1	*L*	Ribosomal subunit
*RPS16A***	*sl*	Small ribosomal subunit.
RPS4B	*sl*	Small ribosomal subunit

††*HIT1***	*sl*	Interacts BTT1 and RSA1 (ribosome assembly)
Mitochondria		
MRPL44	*S*	Mitochondrial ribosomal subunit.
MRPL38	*S*	Mitochondrial ribosomal protein
MMM1	*S*	Mitochondrial organization
ISA1	*S*	Iron sulfur Protein maturation, Mitochondrial
PTC1	*S*	Mitochondrial inheritance
*TOM5***	*ss*	Protein transport, mitochondrial outer mebrane
PCP1	*ss*	Mitochondrial protease
YIL042C	*ss*	Mitochondrial kinase
YDR115W	*ss*	Mitochondrial ribosomal protein
*UGO1***	*sl*	Mitochondrial fusion
Nucleotide Metabolism		
PRS3	*VS*	Ribose-phosphate pyrophosphokinase
††*MET7*	*VS*	Folylpolyglutamate synthetase.
ADO1	*S*	Adenosine kinase
ADE12 BRA9	*S*	Adenylosuccinate synthetase
GCV3	*S*	Glycine dehydrogenase, required for folate production
Phosphate Metabolism		
††*PHO85*	*S*	CDK phosphate and glycogen metabolism and cell-cycle progression
††*PHO80*	*S*	Phosphate metabolism
GTR1	*S*	Phosphate transport
††*PHO87*	*L*	inorganic phosphate uptake with pho86 and pho84.
TAT2	*sl*	Interacts with Pho23, sl with *pho85*
Nitrogen Metabolism		
URE2	*S*	Nitrogen catabolite repression regulator
ARG2	*S*	Glutamate acetyl transferase
Glycerol Uptake		
GUP1	*S*	Glycerol uptake
*GUP2***	*sl*	Glycerol uptake
Potassium Transport		
TRK1	*S*	potassium transporter
Killer toxin-related		
*FYV12***	*sl*	Killer toxin sensitive
KRE21	*sl*	K1 killer toxin resistant, sl with cell wall mutations
KRE28	*sl*	Killer toxin resistant
PP2A-related		
*TPD3***	*S*	Phosphatase type 2A subunit
SIT4	*S*	Phosphatase type 2A subunit
YOR1	*L*	Multidrug resistant transporter. Interacts with PP2A
PPE1	*L*	Protein modification. Interacts with PP2A.
Unknown		
YDL118W	*VS*	Unknown
††*YEL033W*	*VS*	Unknown- dubious ORF (Failed co-segregation test)
YGR042W	*S*	Unknown
YOL138C	*S*	Unknown
YMR031W-A	*S*	Unknown, overlaps YMR031c, also unknown
YGL039W	*sl*	NADPH-related oxidoreductase
YOR008C-A	*sl*	Diepoxybutane and mitomycin C resistance

Table 2-2

***Saccharomyces cerevisiae* genes that produce questionable telomere length alterations when deleted.**

Single knockout mutants of the listed genes showed slight telomere length defects in more than one but not all of initial Southern blots.

Questionable short			Questionable long		
ORF name	Common name	Function	ORF name	Common name	Function
YAL035W	*FUN12*	Translation initiation, interacts with CDC13	*YAL018C*		Unknown
YJL120W		Unknown	*YDL002C*	*NHP10*	Unknown, HMG-box protein
YJL121C	*RPE1*	Pentose phosphate shunt	*YDL021W*	*GPM2*	Glycolysis, gluconeogenesis
YKL003C	*MRP17*	Mitochondria small ribosome subunit	*YDR391C*		Unknown
YLR021W		Unknown	*YEL007W*		Unknown
YLR122C		Unknown	*YJR063W*	*RPA12*	POL 1 complex
YLR239C	*LIP2*	Protein-lipoylation	*YMR038C*	*LYS7*	Copper chaperone activity
YLR262C	*YPT6*	Enndosome to golgi transport	*YMR316C-B*		Unknown
YMR175W	*SIP18*	Osmotic stress response	*YNL016W*	*PUB1*	Nonsense-mediated mRNA decay
YMR315W		Unknown	*YOR324C*		Unknown
YOR327C	*SNC2*	Endocytosis	*YOR357C*	*GRD19*	Protein retention in golgi
YPR066W	*UBA3*	Ubiquitin cycle	*YPL226W*	*NEW1*	Amino terminus acts as a prion
			YPL239W	*YAR1*	Unknown
			YPR083W	*MDM36*	Mitochondrial organization

Table 2-3

Measured length differences of Y' containing telomeres of S. cerevisiae mutants.

Measured length differences of the Y' containing telomeres of S cerevisiae deletion mutants relative to the average length for wild type Y' telomeres. These measurements were made by plotting the peak signal of the shortest telomeric band against the positions of the added internal controls of the gels shown in Fig. 2-1 and Fig. 2-2 using a phosphoimager and ImageQuaNT software. The column labeled Measurement 1 is data from gels shown in Figure 2-2. Measurement 2 shows additional data for which gels are not shown. Asterisks indicate mutants that either showed average measured Y' telomere length inconsistent with the reproducible phenotype noted in all previous Southern blots or that showed <25-bp length difference from wild type. As explained in the text, this latter category displayed altered telomere lengths in all previous Southern blots. Genes previously known to alter telomere length are shown in bold. ND, no data. H indicates mutants with heterogeneous telomere length phenotypes (see table 4). VS, very short. ss, slightly short. sl, slightly long. L, long.

ORF name	Common name	Measurement 1	Measurement 2	Comments
YLR233C	***EST1***	-293		
YBL088C	***TEL1***	-240		
YNL250W	***RAD50***	-233		
YMR106C	***YKU80***	-227		
YDR369C	***XRS2***	-225		
YMR284W	***YKU70***	-222		
YMR080C	***UPF1***	-211		
YHR077C	***UPF2***	-208		
YGR072W	***UPF3***	-193		
YEL033W		-188		
YDL118W		-183	-123	
YHL011C	*PRS3*	-173		

YDL077C	*VAM6, VPS39*	-162	-73	
**YNL064C*	*YDJ1, MAS5*	-155		sl in other Southern blots
YGL173C	***KEM1***	-151		
YDL081C	*RPP1A*	-150	-112	
YDR310C	*SUM1*	-146		
YLR418C	*CDC73*	-144		
YLR240W	*VPS34*	-141		
YBR097W	*VPS15, GRD8*	-138		
YGR104C	*SRB5*	-136	-119	
YGL244W	*RTF1, CSL3*	-135	-109	
YLR357W	*RSC2*	-127		
YNL220W	*ADE12 BRA9*	-127		
YLR148W	*PEP3, VPS18*	-122		
YPL002C	*SNF8*	-121		
YCL016C	*DCC1*	-120		
YOR279C	*RFM1*	-117		
YLR372W	*SUR4*	-117		
YPL031C	*PHO85*	-117		
YPR049C	*CVT9*	-117		
YDR295C	*PLO2*	-116		
YPL065W	*VPS28/ VPT28*	-115		
YMR142C	*RPL13B*	-115		
YDL020C	*RPN4*	-115		
YJL140W	*RPB4, CTF15*	-114		
YOL004W	*SIN3*	-113		
YOR322C		-112		
YGR229C	*SMI1/ KNR4*	-111	-100	
YDR418W	*RPL12B*	-110		
YLR417W	*VPS36 GRD12*	-109		
YLR025W	*SNF7*	-108		
YHR041C	*SRB2*	-107		
YEL029C	*BUD16*	-106		
YGL127C	*SOH1*	-105		
YJR105W	*ADO1*	-105		
YGL135W	*RPL1B*	-103		
YMR225C	*MRPL44*	-103		
YPL106C	*SSE1*	-102		
YLR391W	*CCW14, SSR1*	-101		
YOL068C	*HST1*	-100		
YNL229C	*URE2*	-100		
YDL047W	*SIT4, LGN4*	-99		

YMR179W		-98		
YPL144W		-97		
YOL115W	***TRF4***	-94		
YML121W	*GTR1*	-94		
YPL084W	*BRO1 LPF2*	-94		
YGL084C	*GUP1*	-94		
YGL168W	*HUR1*	-94		
YJR102C	*VPS25*	-93		
YDR495C	*VPS3 PEP6*	-90		
YGR057C	*LTS7*	-90	-98	
YML097C	*VPS9*	-89		
YEL057C		-89		
YIL036W	*CST6/ ACA2*	-89		
YLR242C	*ARV1*	-88		
YMR031W-A		-88		
YPL041C		-87		
YDL006W	*PTC1*	-86		
YKL139W	*CTK1*	-85		
YMR263W	*SAP30*	-85		
YLL027W	*ISA1*	-84		
YBR132C	*AGP2*	-83		
YHR081W	*LRP1*	-83		
YOL001W	*PHO80*	-83		
YAL013W	*DEP1*	-82	-87	
YGL070C	*RBP9, SHI1*	-81		
YMR070W	*MOT3*	-80		
YJL071W	*ARG2*	-79		
YLR015W	*BRE2*	-79		
YKL204W	*EAP1*	-79		
YOL138C		-78		
YGR270W	*YTA7*	-78		
YCL008C	*VPS23*	-77		
YBR035C	*PDX3*	-76		
YIL042C		-76		
YDR115W		-75	-60	
YMR186W	***HSC82***	-72		
YPL161C	*BEM4*	-69		
YHR167W	*THP2*	-69		
YLR028C	*ADE16*	-65		H
YAL044C	*GCV3*	-62	-73	
YLL006W	*MMM1*	-62		

YNL246W	*VPS75*	-60		
YLR090W	*XDJ1*	-58		
YKL009W	*MRT4*	-57		
YHL020C	*OPI1*	-57		
YLR338W	*KRE21*	-57		
YLR231C	*BNA5*	-56		H
YFR041C		-56		
YKL170W	*MRPL38*	-55		
YJL129C	*TRK1*	-52		
YPL205C		-51		
YBR286W	*APE3, APY1*	-47		
YER093C-A		-47		
YNL281W	*HCH1*	-47		
YOR008C-A		-42		
YOL082W	*CVT19*	-40		H
YAL053W		-39	-42	H
**YMR167W*	*MLH1*	-39		sl in other Southern blots
YLR074C	*BUD20*	-37		H
YOR003W	*YSP3*	-37		
**YML035C-A*		-36		sl in other Southern blots
YLR318W	***EST2***	-36		
YLR057W		-34		H
YAL056W	*GPB2*	-34		
YHR139C	*SPS100*	-33		
YMR192W	*APP2*	-31		H
YMR164C	*MSS11*	-29		H
**YJR080C*		-29		sl in other Southern blots
**YDR470C*	*UGO1*	-28		sl in other Southern blots
**YPR030W*	*CSR2*	-25		
**YAR002W*	*NUP60*	-24	-11	
**YOR321W*	*PMT3*	-24		
YGR101W	*PCP1*	-19	-40	
**YAL016W*	*TPD3, FUN32*	-16	-23	
**YPL189W*	*GUP2*	-15		sl in other Southern blots
**YCR047C*	*BUD23*	-10		sl in other Southern blots
**YPL105C*		-9		sl in other Southern blots
YJL206C		-8		H
**YJR043C*	*POL32*	-6		sl in other Southern blots
**YMR143W*	*RPS16A*	-6		sl in other Southern blots
YDR083W	*RRP8*	-2	35	
**YNL072W*	*RNH35*	-2		VS in other Southern

				blots
YLR325C	*RPL38*	-1		H
**YGL151W*	*NUT1*	3		sl in other Southern blots
**YOR293W*	*RPS10A*	7		L in other Southern blots
**YPR133W-A*	*TOM5, MOM8A*	8		ss in other Southern blots
**YER155C*	*BEM2*	9		H
**YMR269W*		9		
**YLR322W*	*VPS65*	11		
YJR110W		13		H
**YJR055W*	*HIT1*	22		
**YOR183W*	*FYV12*	22		
**YDR174W*	*HMO1*	24		
YGR042W		24	-56	
YEL053C	*MAK10*	25		
YLL026W	*HSP104*	26		
YDL151C	*BUD30*	27	44	
YOL020W	*TAT2*	27		
YOR156C	*NFI1/SIZ2*	28		
YGL039W		30		
YHR203C	*RPS4B*	30		
YPL213W	*LEA1*	34		
YMR091C	*NPL6*	41		
YCR086W	*CSM1*	47		
YMR125W	*STO1*	49		
YPR051W	*MAK3*	54		
YGL003C	*CDH1/HCT1*	55		
YGR281W	*YOR1*	55		
YCR020C-A	*MAK31*	62		
YBL006C		65	49	
YIL128W	*MMS19*	70		
YCR065W	*HCM1*	77		
YHR075C	*PPE1*	80		
YCR037C	*PHO87*	82		
YMR116C		85		
YNL025C	*SSN8*	85		
YCR031C	*RPS14A*	97		
YCR020W-B	*HTL1*	157		
YLR453C	***RIF2***	295		
YDR532C	*KRE28*	ND		
YGL212W	*VAM7*	ND	-38	
YGR036C	*CAX4/CWH8*	ND		

YLR096W	*KIN2*	ND	H
YML024W	*RPS17A*	ND	
YML060W	*OGG1*	ND	
YML062C	*MFT1*	ND	
YOR144C	*ELGI*	ND	
YOR241W	*MET7*	ND	

Table 2-4

S. cerevisiae genes showing heterogeneous telomere lengths when deleted.

Single knockout mutants of the listed genes reproducibly showed heterogeneity in their observed telomere lengths. The observed phenotype of the shortest restriction fragment observed (Y' containing telomeres) are described. Most of the mutants showed a bimodal distribution for the lengths of these telomeres. Four of the mutants (see last column) had elongated X telomeres as the higher bands were longer than those in the wild type. ADE16 deletion showed short telomeres in two Southerns and long telomeres in two Southerns.

ORF name	Common name	Function	Y' telomere length phenotype	X telomeric bands
YAL053W		Unknown	Mix of wild type and long	
YER093C-A		Unknown	Mix of wild type and long	
YJL206C		Unknown	Mix of wild type and long	
YJR110W		Phophoric monoester hydrolase	Mix of wild type and long	Long
YLR028C	*ADE16*	IMP cyclohydrolase activity, aerobic respiration	Variable: short or long	
YLR057W		Unknown	Wild type	Long
YLR074C	*BUD20*	Bud site selection	Mix of wild type and long	
YLR096W	*KIN2*	Membrane associated serine/threonine protein kinase	Mix of wild type and long	
YLR231C	*BNA5*	NAD biosynthesis, kynureninase in trytophan degradation	Mix of wild type and long	Long
YLR325C	*RPL38*	Cytosolic ribosomal subunit	Mix of wild type and long	Long
YMR164C	*MSS11*	Specific RNA polymerase II transcription factor activity	Mix of wild type and long	
YMR192W	*APP2*	Unknown	Mix of wild type and long	
YOL082W	*CVT19*	Protein-vacuolar targeting	Mix of wild type and long	

Table 2-5

Neighboring ORFs that produce telomere length alterations when deleted.

Single knockout mutants of the listed neighboring genes showed telomere length defects in Southern blots. VS, very short. S, short. ss, slightly short. qs, questionably short. VL, very long. L, long. sl, slightly long. ql, questionably long. H, heterogeneous. Genes previously known to alter telomere length are shown in bold.

YMR142C	*RPL13B*	S	
YMR143W	*RPS16A*	sl	
YOR321W	*PMT3*	ss	
YOR322C	unknown	S	
YMR224C	***MRE11***	VS	
YMR225C	*MRPL44*	S	
YDR082W	***STN1***	inviable	
YDR083W	*RRP8*	sl	
YML060W	*ODD1*	L	
YML061C	***PIF1***	L	
YML062C	*MFT1*	ss	
YBR035C	*PDX3*	S	
	TLC1	Deletion not in the mutant collection	
YLR231C	*BNA5*	h	*ylr232w* has normal telomeres
YLR233C	***EST1***	VS	
YHR075C	*PPE1*	L	*yhr076w* has normal telomeres
YHR077C	***UPF2***	VS	
YLR239C	*LIP2*	qs	
YLR240W	*VPS34*	S	*ylr241w* has normal telomeres
YLR242C	*ARV1*	S	

YJL120W	unknown	qs
YJL121C	*RPE1*	qs
YDL020C	*RPN4*	S
YDL021W	*GPM2*	ql
YMR315W	unknown	qs
YMR316C-B	unknown	ql

FIGURES

Figure 2-1

Representive Southern blot of mutants that alter *S. cerevisiae* telomere length.

Southern blot of *Xho*I digested DNA of single gene knockout mutants of *S. cerevisiae* probed with telomeric sequence. Gene or ORF names are indicated as are strain BY4741 controls (WT). Samples of DNA from mutants were combined with restriction-digested plasmid DNA to provide internal size standards. The two internal control bands are fragments of the plasmid pYt103 containing telomeric sequence that were generated by mixing separate digests done with *Bsm*AI and with *Taq*I (producing the 1835 bp and 644 bp fragments, respectively). The panel on the left shows a strain BY4741 control (WT p-) as well as the size standards without added yeast DNA.

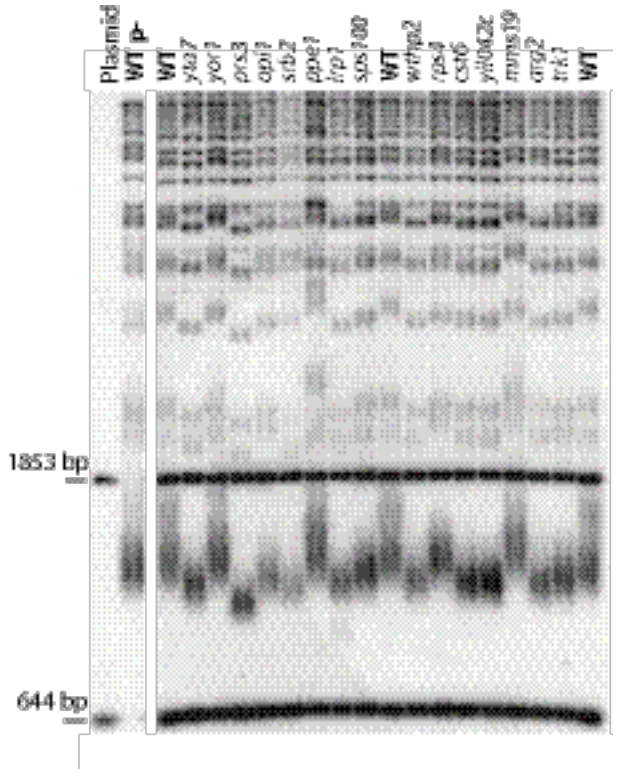
Plasmid
WT p-
WT
ykl7
yor1
prs3
epl1
srb2
ppe1
frp1
sps100
WT
wthp2
rpa1
cst6
ylr042c
mms19
drg2
trk1
WT
1853 bp
644 bp

Figure 2-1

Figure 2-2

Examples of Southern blots that were used to measure telomeric length of mutants.

Southern blots of *Xho*I-digested DNA of single gene knockout mutants of *S. cerevisiae* probed with telomeric sequence. Gene or ORF names are indicated as are strain BY4741controls (WT). Samples of DNA from mutants were combined with restriction-digested plasmid DNA to provide internal size standards. The two internal control bands are fragments of the plasmid pYt103 containing telomeric sequence that were generated by mixing separate digests done with *Bsm*AI and with *Taq*I (producing the 1835 bp and 644 bp fragments, respectively). The first panel shows a strain BY4741 control (WT p-) as well as the size standards without added yeast DNA. Asterisks indicate mutants that showed average measured Y' telomere lengths appreciably different from the observed telomere length phenotypes in all previous Southerns. Note that a few mutants shown here were judged not to have significantly altered telomere lengths and are not listed in Table 1.

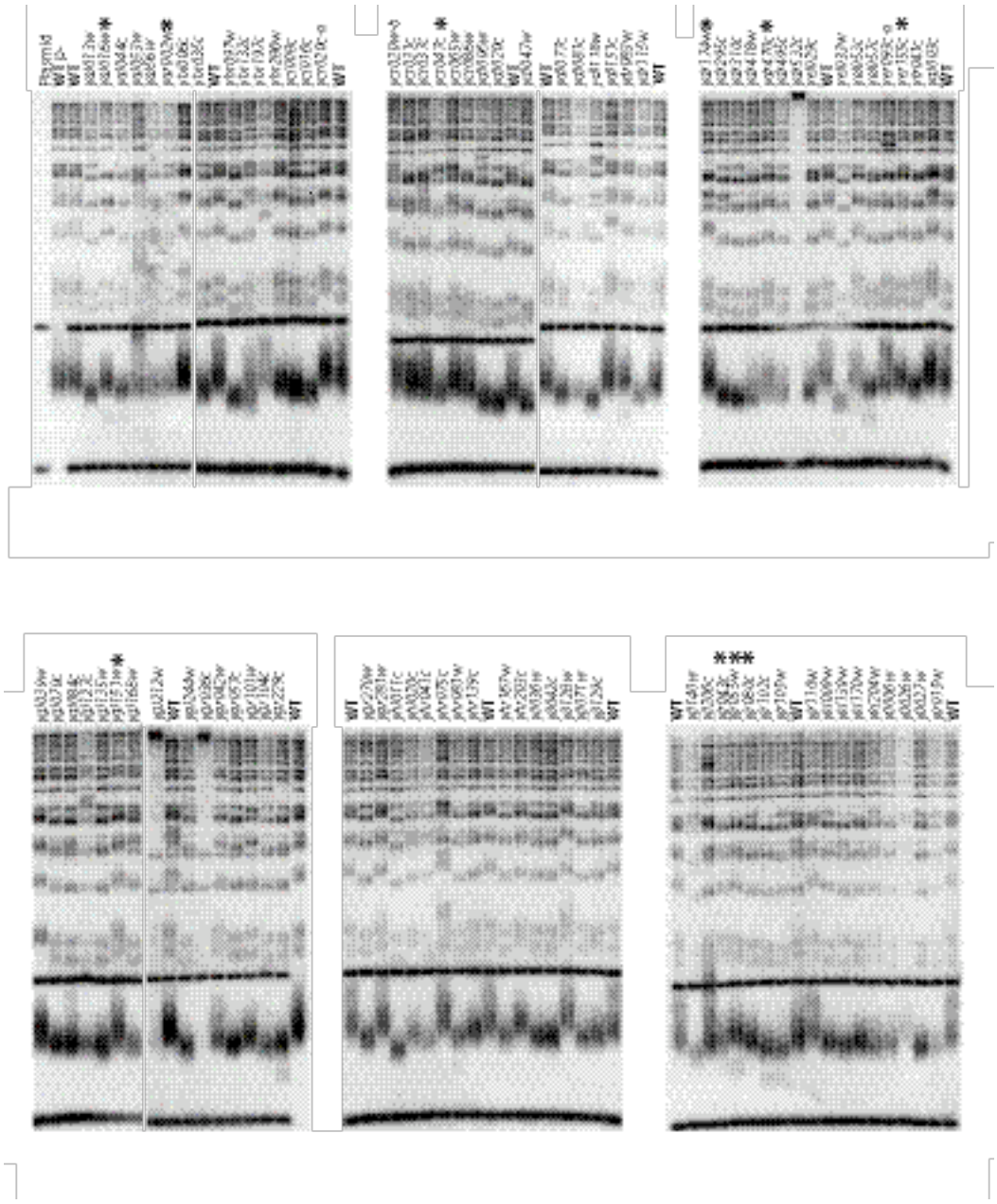

Figure 2-2A

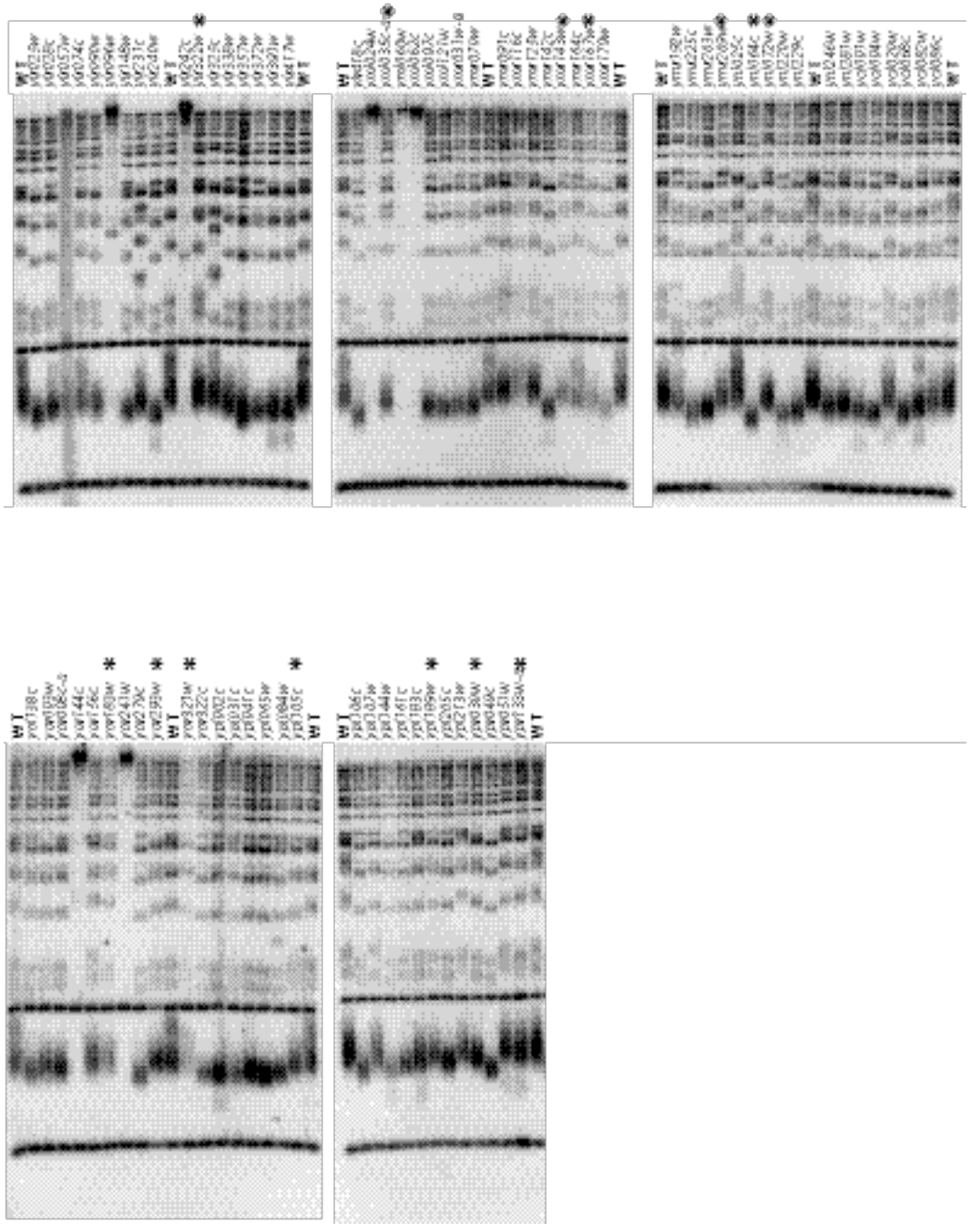

Figure 2-2

CHAPTER 3

VITAMIN B6 SALVAGE IN *SACCHAROMYCES CEREVISIAE*: CHARACTERIZATION OF COMPONENTS AND A LINK TO TELOMERE FUNCTION [1]

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[1]Askree S. H., Waters J., Hawk J. and McEachern M. J. To be submitted to *Eukaryotic cell*.

ABSRACT

Vitamin B6 is an important coenzyme in many cellular reactions involving amino acid and nucleotide metabolism and has recently been shown to be a potent antioxidant in yeast and mammalian cells. While de novo synthesis is restricted to bacteria, yeast, and plants, all organisms encode enzymes that can convert B6 vitamers taken up exogenously or salvaged within the cells to the active coenzyme form, pyridoxal phosphate or PLP. Neither biosynthetic nor salvage pathways are well understood in yeast. In a screen using single ORF deletion *Saccharomyces cerevisiae* strains, we previously identified two vitamin B6-deficient mutants that maintain telomeres at a shorter length than wild type. Here we show that deletion of *BUD16*, which encodes a putative pyridoxal kinase of the vitamin B6 salvage pathway, causes slow growth and short telomeres on rich media. Supplementation with certain B6 vitamers, pyridoxine and pyridoxal, suppresses these defects, tying the gene functionally to the B6 salvage pathway. Interestingly, deletion of pyridoxine/pyridoxamine phosphate oxidase, *pdx3*Δ, has a milder short telomere phenotype that worsens upon supplementation with high amounts of pyridoxine. Here, we demonstrate that the telomeric defects in *bud16*Δ and *pdx3*Δ, are due to vitamin B6 salvage deficiency. Our experiments show that *BUD16* encodes the major PL/PN/PM kinase of the salvage pathway in yeast. A homolog of *BUD16*, *BUD17*, also has ties to vitamin B6 metabolism but is not required for salvage or biosynthesis. *YPR127W* encodes a pyridoxal reductase activity that converts PL to PN. A major question is how depletion of vitamin B6 leads to telomere length defects. Since thymine nucleotide synthesis requires vitamin B6-dependent folate activation, we hypothesize that depletion in thymine nucleotide pools is responsible for telomere shortening in B6 deficient mutants. Our findings are inconsistent with thymine depletion being the only route to these phenotypes.

INTRODUCTION

In a recent report involving a systematic genomic survey in budding yeast, we reported more than a hundred candidate genes that affected telomere length when deleted (Askree et al., 2004). Due to their generally modest phenotypes and very diverse known functions, most genes disrupted in these strains can be predicted to affect telomere length via indirect mechanisms. Among those reported to lead to shorter telomere length than wild type were deletions of pyridoxal oxidase, *pdx3*Δ, and a putative pyridoxal (vitamin B6) kinase, *bud16*Δ (Askree et al., 2004). This finding suggested a possible connection between the yeast vitamin B6 salvage pathway and telomere length maintenance in yeast.

The active form of vitamin B6, pyridoxal-5'-phosphate or PLP, serves as a versatile coenzyme for several enzymatic activities in diverse cellular processes. These include transamination, racemization, decarboxylation, and α, β and γ-eliminations. The enormous versatility in substrate specificity and mechanism of action is attributable to electrophilic catalytic activity that allows PLP to stabilize various reaction intermediates (Eliot and Kirsch, 2004). There are 145 PLP-dependent enzyme activities identified across all organisms that span five of the six general classes specified by the Enzyme Commission (Percudani and Peracchi, 2003). Preliminary reports from sequenced genomes suggest 0.5-1.5% of genes to encode PLP-dependent enzymes across a diverse group of organisms including yeast and humans (Percudani and Peracchi, 2003). The number of genes may be an under-representation of the total PLP-dependent activites because of "catalytic promiscuity" displayed by some of PLP-dependent enzymes where one enzyme carries out more than one catalytic activity (Cooper et al., 2002; Han et al., 2001b; Percudani and Peracchi, 2003; Strisovsky et al., 2003).

Among PLP-dependent activities, there are several in amino acid metabolism. There are many important PLP-dependent transaminase reactions where amino acids interconvert with their corresponding keto acid (Christen and Mehta, 2001; Eliot and Kirsch, 2004; Percudani and Peracchi, 2003). Examples include steps in glycolysis (alanine and pyruvate) and the tricarboxylic acid cycle (glutamate and keto-glutarate). A group of PLP-dependent branched chain aminotransferases catalyze valine, leucine, and isoleucine conversions (Hutson, 2001). One of the most well conserved PLP-dependent enzymes is serine hydroxymethyltransferase (SHMT) (Percudani and Peracchi, 2003). This enzyme catalyzes transfer of a hydroxymethyl group from serine to tetrahydrofolate to form glycine and an active form of folate. The latter catalyses thymine nucleotide synthesis (Rao et al., 2000; Snell and Riches, 1989). Several neurotransmitters including serotonin, catecholamines, histamine, and GABA, are synthesized via PLP-dependent decarboxylation reactions (Bender, 1999; Pearl and Gibson, 2004; Williams et al., 2005). Generation of aminolevulinic acid is a PLP-dependent step in heme biosynthesis (Ferreira and Gong, 1995; Gong and Ferreira, 1995). Inborn errors of metabolism due to mutations in PLP-dependent enzymes include homocystinuria, histidine intolerance, and xanthurenic aciduria (Jarisch and Wantke, 1996; Kraus et al., 1999; Mudd et al., 1970; Tada et al., 1968).

Estimation of vitamin B6 nutritional status in human populations has often suggested that a substantial number of people, especially the elderly, preschool children, and adolescent girls, may have 10-25% less than the recommended B6 levels (Brown et al., 1975; Fries et al., 1981; Kirksey et al., 1978; Tolonen et al., 1988). Even higher deficiencies, 40-60% below normal, have been reported in pregnant and low income women (Heller et al., 1973; Schuster et al., 1985; Schuster et al., 1981). In all these studies, dietary intake, plasma PLP levels, or serum and/or

erythrocyte transaminase activation measurements were used as indicators for vitamin B6 status of an individual. These measurements may be misleading because plasma PLP levels may not represent the total B6 status (sum of all vitamer forms, free and albumin-bound) of an individual, and tests based on transaminase enzymatic activity may be affected by liver functions (Leklem, 1990). However, the effects of low vitamin B6 intake remain unclear but are of growing concern.

While vitamin B6 is an essential micronutrient for most animals and some obligate parasites, there are two distinct pathways for *de novo* synthesis of vitamin B6 across bacteria, fungi, and plants. Vitamin B6 biosynthetic genes *GAPB*, *SERC*, *PDXA*, *PDXB*, *PDXJ*, and *PDXH* were identified in *Escherichia coli* and this pathway has now been well characterized (Hill et al., 1996; Hill et al., 1975; Hill et al., 1977; Hill and Spenser, 1970; Hill and Spenser, 1973; Laber et al., 1999). However, homologs of *E. coli* B6 biosynthetic genes are only present in a certain fraction of eubacteria (Mittenhuber, 2001). The second unrelated pathway was first discovered in *Cercospora nicotianae* where *SOR1* gene was found to be essential for vitamin B6 biosynthesis (Ehrenshaft et al., 1999; Ehrenshaft et al., 1998). *SOR1* lead to the discovery of the second biosynthetic pathway well-conserved across archaebacteria, fungi, plants, and some eubacteria (Ehrenshaft et al., 1999). Invariably, organisms have homologs to only one of the two biosynthetic genes: *PDX* gene or *SOR1*. The yeast homolog of *SOR1* is *SNZ1*. There are three related *SNZ* genes in *Saccharomyces cerevisiae*, each with an upstream *SNO* gene that is divergently transcribed from the same promoter (Padilla et al., 1998). *SNZ1* is 80% identical to *SNZ2* and *SNZ3*, which in turn are 99% identical to each other. Similarly, *SNO2* and *SNO3* are almost identical and share 72% homology with *SNO1* (Padilla et al., 1998). While the *SNZ1/SNO1* gene pair functions in vitamin B6 biosynthesis, *SNZ2/SNO2* and *SNZ3/SNO3* are involved in thiamine (vitamin B1) biosynthesis (Rodriguez-Navarro et al., 2002). The steps in

the *SOR1* type biosynthetic pathway are currently under investigation. The few established facts about the pathway include that the ring nitrogen of the vitamin B6 originates from an amide group of glutamine and is supplied by glutaminase activity of Sno1p, complexed with Snz1p (Dong et al., 2004). This is different from *E. coli* where the α-amino group of glutamate provides the nitrogen of the pyridine ring (Hill et al., 1996; Hill et al., 1977). Where it is clear that a condensation between 1-deoxy-D-xylulose and 4-hydroxy-L-threonine yields the carbon-nitrogen structure of B6 synthesized in *E coli*, the only clue to the yeast counterpart in the process is the involvement of D-ribulose-5-phophate (Kondo et al., 2004).

In contrast to the situation with biosynthetic genes, all organisms, from *E. coli* to mammals, have a vitamin B6 salvage pathway where three vitamer forms, pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM), are taken up exogenously as nutrients or salvaged from within the cell and converted to PLP. The pathway is well understood in *E. coli* and is illustrated in Figure 3-1. Pyridoxal kinase (EC, 2.7.1.35) phosphorylates vitamer forms PL, PN, and PM in the presence of ATP and a divalent metal ion (McCormick et al., 1961). Phosphorylation of PL yields PLP directly, whereas phosphorylated PN and PM (PNP and PMP, respectively) are oxidized to PLP by a FMN-dependent pyridoxine (pyridoxamine) phosphate oxidase (PNP/PMP oxidase) (EC, 1.4.3.5) (Kazarinoff and McCormick, 1975; Turner and Happold, 1961). There are two genes encoding pyridoxal kinase in *E. coli*. PdxKp has activity for all three vitamer substrates, PL, PN, and PM, while PdxYp phosphorylates only PL and with much lower affinity than PdxKp (Yang et al., 1998; Yang et al., 1996). The PNP/PMP oxidase activity in *E. coli* is encoded by *PDXH* (Zhao and Winkler, 1995). The vitamin B6 biosynthetic pathway catalyzed in *E. coli* yields PNP, making PdxHp an essential enzyme for both biosynthesis and salvage in the final conversion of PNP to PLP (Garrido-Franco, 2003; Laber et al., 1999).

Mammalian tissue has ubiquitous presence of both enzymes for salvaging B6 vitamers (Hanna et al., 1997). Pyridoxal kinase and PNP/PMP oxidase have been purified from many different mammalian tissues (Choi et al., 1987; Churchich, 1984; di Salvo et al., 2004; Kang et al., 2004; Kazarinoff and McCormick, 1975), and the crystal structures have been determined (Li et al., 2002; Li et al., 2004; Musayev et al., 2003). However, there is limited information on genes encoding these activities in yeast. *BUD16* and *BUD17* are the only two *Saccharomyces cerevisiae* genes with sequence similarity to known pyridoxal kinases in other organisms (see Table 1). Both these genes were named after their random bud site phenotype in a homozygous diploid deletion mutant state (Ni and Snyder, 2001). *PDX3* encodes the *S. cerevisiae* PNP/PMP oxidase and was discovered as a suppressor mutation that allowed heme synthesis to continue under anaerobic conditions where it is usually switched off (Loubbardi et al., 1995).

Another enzyme with a role in vitamin B6 salvage is pyridoxal reductase (pyridoxine dehydrogenase, 1.1.1.65) which catalyzes the reversible reaction where pyridoxal (PL) is reduced to pyridoxine (PN). The activity has been purified to homogeneity from *S. cerevisiae* cytoplasm, but the gene encoding it has not been cloned (Guirard and Snell, 1988). The same activity has been described in *Schizosaccharomyces pombe* where the gene *PLR1* encodes pyridoxal reductase. Even though the reaction is reversible, under physiological pH the enzyme has a dramatically higher substrate preference for PL relative to PN in both yeasts (Chumnantana et al., 2005; Morita et al., 2004; Nakano et al., 1999).

In this paper we describe short telomeres in deletion mutations of pyridoxal kinase, *BUD16*, and pyridoxal oxidase (*PDX3*) of *Saccharomyces cerevisiae* and demonstrate that vitamin B6 deficiency is responsible for these defects. Our results argue that Bud16p is the *bona fide* pyridoxal kinase and *YPR127W* is the ORF encoding pyridoxal reductase in yeast.

MATERIALS AND METHODS

Strains

Yeast strains *BUD16*/*bud16*, *BUD17/bud17*, *PDX3/pdx3*, *YPR127W/ypr127w*, *SNZ1*/*snz1*, and *EST1/est1* were taken from a heterozygous diploid *Saccharomyces* strain collection in which each strain has one copy of an ORF replaced with the KanMX4 module that confers resistance to G418 (Giaever et al., 2002). These strains were in BY4743 (*his3/his3 leu2/leu2 ura3/ura3 MET15/met15 LYS2/lys2*) background. Heterozygous strains were sporulated by the method recommended (www-sequence.stanford.edu/group/yeast_deletion_project/spo.html). Spores were isolated by tetrad dissection and scored for G418 resistance to identify haploid strains that inherited deletions for *bud16*, *bud17*, *pdx3*, *ypr127w*, *snz1*, and *est1*. Spores were also scored for mating type and lysine and methionine auxotrophy. Spores with favorable genotype were grown and saved at -80º. To generate double mutants, two different single deletion strains (saved from the first round of tetrad dissections) that were compatible in terms of mating type and selectable markers, were mated, sporulated, and dissected. Spores were scored as described above. The identity of spores with two ORF deletions was confirmed by Southern blot analysis with a labeled ~800-bp *Sca*I/ *Nco*I fragment of pFA6KanMX4 plasmid (Wach et al., 1994). All hybridizations were done at 65º C. Double mutants with several genotypes, in terms of mating type and markers, were saved at -80º. The term "wild type" in this paper refers to strains that are either the original BY4741 (Mat A *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ), BY4742 (Mat α *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ), or BY4743 (BY4741 X BY4742 diploid strain), or the G418 sensitive spores segregated in the course of this study with any combination of selectable markers

(methionine and lysine) and either mating type. Plasmid p*TK* and the strains RWY42-22A (Mat A *ade2*Δ *leu2*Δ *lys2*Δ *trp1*Δ*63 ura3*Δ) and RWY42-22B (Mat α *ade*Δ *his3*Δ *lys2*Δ *trp1*Δ*63 ura3*Δ *cdc8-1*) were received as a gift from Raymund Wellinger's lab (Toussaint et al., 2005).

Media

Rich medium, YPD (yeast extract /peptone /dextrose) was used unless stated otherwise. Synthetic defined medium lacking vitamin B6 (SD-B6$^-$) was synthesized in the lab based upon synthetic minimal glucose medium described by Sherman (Sherman, 2002). Pyridoxal phosphate, pyridoxal hydrochloride, pyridoxine hydrochloride, pyridoxamine hydrochloride hydrate, and thymidine were purchased from Sigma-Aldrich (St. Louis, MO), dissolved in deionized water, filter-sterilized, and stored at -20º. All chemicals were added to media after autoclaving.

For *Herpes simplex* thymidine kinase complementation experiments, synthetic complete (SC) plates were used. These were made by adding several amino acids and other nutrients to SD plates as described previously (Sherman, 2002). Yeast transformants were selected on SC plates lacking histidine (in the case of 4741 wild type and *bud16*Δ) or SC lacking tryptophan (in the case of RWY42-22A and 22B).

Growth rate measurements

Yeast strains were cultured in sterile glass tubes in liquid media in a shaking water bath at 30º C. Optical density readings were recorded with a spectronic 20D spectrometer at a wavelength of 600 nm. Optical density readings were taken at 1.5 hour intervals and plotted against time. Experiment was performed in triplicate, and error bars represent the error based on standard deviation across the three independent readings.

For culturing senescing strains, wild type, *pdx3*Δ*, est1*Δ, and *pdx3*Δ *est1*Δ spores were picked off of YPD plates (where they were dissected) and moved to 3 ml liquid YPD media in sterile glass tubes. The cultures were grown at 30º C in a shaking water bath. Optical density readings were taken every 2-4 hours. When all cultures had grown to an OD_{600} of ~ 5, 250 µl were re-cultured in 3 ml YPD, and optical densities were measured every hour to bring all cultures to the exact same optical density. Next, cells were diluted 1000 times and a second round of culturing was done till OD_{600} ~5, followed by an intermediary culture to bring the slow growing cultures to the same generation and eliminate the differences in cell densities before re-culturing. This cycle of re-culturing with a short intermediate culture to keep all cell lines at the same doubling age was repeated 8 times. At each cycle, cells were harvested for DNA preparation and also streaked on plates to look for suppressors.

Telomere length analysis

DNA prepared from cells was digested with *Nci*I overnight and run on 1.5% agarose gels in 1X TBE buffer, for 960 minutes at 35 volts and an additional 420 minutes at 50 volts. *Xho*I or *Alu*I/*Hae*III/*Hin*fI/*Msp*I digested DNA was run on 1% agarose for 960 minutes at 35 volts and 240 minutes at 50 volts. Southern blotting was performed using Hybond-N+ membrane (Amersham Biosciences), and probing was done with a radiolabeled telomeric probe 26G (5'-TGTGGGTGTGGTGTGTGGGTGTGGTG-3') labeled with T4 polynucleotide kinase and gamma-^{32}P-ATP. All hybridizations were done in 200 mM Na_2HPO_4 and washed with 2% SDS (Church and Gilbert, 1984).

For the Southern blots shown, internal controls fragments of sizes 136 bp and 750 bp were generated by *Msp*I and *Pst*I/*Sal*I digests, respectively, of the plasmid pYt103 (Shampay et al., 1984). We added ~10 ng of digested plasmid DNA to each lane with *Nci*I-digested genomic

DNA. A telomere ladder was made by digesting pYt103 plasmid in 15 separate tubes (each with a separate restriction enzyme/s), followed by heat inactivation of the enzymes and pooling the 15 digests. The 15 digests were with *Msp*I, *Hin*PI/*Rsa*I, *Bsm*AI/*Taq*I, *Rsa*I/*Nae*I, *Apo*I/*Taq*I, *Taq*I, *Pst*I/*Sal*I, *Hin*PI, *Sph*I/*Ava*II, *Pst*I/*Bgl*I, *Ava*II, *Dde*I/*Rsa*I, *Dde*I/*Apo*I, *Dde*I/*Pst*I, *Bsm*AI, and *Rsa*I. These digests yield fragments containing telomere sequence of the sizes 136 bp, 254 bp, 364 bp, 476 bp, 561 bp, 644 bp, 750 bp, 850 bp, 936 bp, 1034 bp, 1169 bp, 1286 bp, 1490 bp, 1680 bp, 1835 bp, and 1988 bp, respectively.

Flow cytometry

Unsynchronized cells were grown in liquid media culture and kept in exponential growth for 5 hours or more before alcohol fixation (Lloyd, 1999). Fixed cells were stained with propidium iodide (Sigma-Aldrich) and sorted through FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) at the Center for Tropical and Energing Global Diseases Flow Cytometry Facility at the University of Georgia. FlowJo (Tree Star, Inc., Stanford University) software was used for analysis of data. Data was gated on FSC vs SSC and FL2-area vs FL2-width to exclude debris and doublets. Total cell fluorescence (FL2-A: PI-A) was plotted against number of counts (number of cells).

RESULTS

Mutations in the yeast vitamin B6 salvage pathway lead to slowed growth and shortened telomeres.

Deletions of *BUD16* and *PDX3* were recently reported to have short telomeres when grown on YPD rich medium (Askree et al., 2004). *PDX3* encodes a pyridoxine (pyridoxamine) phosphate (PNP/PMP) oxidase (Loubbardi et al., 1995), and *BUD16* is a putative pyridoxal

kinase based on sequence similarity to *E. coli* and human enzymes (see Table 3-1). Deletions of both these genes also produce a slow growth phenotype on YPD. Both the short telomere phenotype and the slow growth phenotype are more pronounced in *bud16*Δ than in *pdx3*Δ (Figure 3-2). The mutant strains were part of a collection in which each haploid *Saccharomyces* strain has a single ORF replaced with the KanMX4 module that confers G418 resistance (Giaever et al., 2002). To confirm that telomeric phenotypes of *pdx3*Δ and *bud16*Δ were due to the disruption of the specific gene, tetrad analysis was performed. As expected, both phenotypes were found to co-segregate with G418 resistance in more than a dozen dissected tetrads from sporulated diploid strains heterozygous for each deletion (data not shown). Strains deleted for *BUD17*, a homolog of *BUD16,* or for *YPR127W*, a putative pyridoxal reductase do not lead to a growth defect or an altered telomere length phenotype on YPD (Figure 3-2). As both PNP/PMP oxidase and pyridoxal kinase are involved in vitamin B6 salvage, our results suggest that the defects in *pdx3*Δ and *bud16*Δ are due to a deficiency in pyridoxal-5'-phosphate (PLP), the active form of vitamin B6.

Faster growing presumptive suppressors appear very rapidly in *bud16*Δ making it difficult to grow the strain in liquid media. The telomere length defect is less pronounced or even lost when the growth reverts to a wild type rate in these suppressors (data not shown). Suppressors also occur with the *pdx3*Δ mutant but were less of a problem with this strain, presumably due to its faster growth rate. There is a characteristic slightly whiter color to the colonies in both mutants relative to the creamy white of the wild type strains. Since the suppressors show the wild type color, it was generally easy to avoid these colonies on plates.

Biosynthesis is responsible for the residual growth of *bud16*Δ on YPD.

Salvage of all three vitamer forms, pyridoxine (PN), pyridoxal (PL), or pyridoxamine (PM) requires a kinase (Figure 3-1). *BUD16* may encode a kinase active on one or more of these vitamers. The residual growth in the absence of *BUD16* indicates that *S. cerevisiae* cells have an alternative minor pathway for generation of PLP on YPD medium. We first asked if a second pyridoxal kinase activity is responsible for allowing the residual slow growth of *bud16*Δ on YPD. *BUD17* was the only other obvious gene with homology to known pyridoxal kinases in *S. cerevisiae* genome. Using tetrad analysis, we isolated *bud16*Δ *bud17*Δ double deletion spores. We found that the growth rate of the double mutant strain was indistinguishable from a single *bud16*Δ on rich medium (Figure 3-3). This indicates that Bud17p is not required for the residual growth in *bud16*Δ mutant.

We next asked whether PLP biosynthesis was responsible for the residual growth in *bud16*Δ mutant. The *SNZ1* gene encodes a protein essential for *de novo* synthesis of vitamin B6 in yeast (Rodriguez-Navarro et al., 2002). We generated strains deleted for both *bud16* and *snz1* and found them to be inviable on rich media (Figure 3-3, bottom row). This shows that the residual growth of a *bud16*Δ strain is *SNZ1*-dependent and therefore due to biosynthesis. These results further indicate that salvage of vitamin B6 on YPD medium is completely blocked by deletion of *BUD16*.

All three vitamers can be utilized in yeast to form PLP.

Strains deleted for *snz1* failed to grow on synthetic defined (SD) media that lacks vitamin B6, SD-B6$^-$ (Figure 3-4, second row from top) (Rodriguez-Navarro et al., 2002). When SD-B6$^-$ media was supplemented with 2 μM of any one of the three vitamers, PL, PN, or PM, *snz1*Δ

strain was able to grow at a rate equal to that of a wild type strain (Figure 4). This low concentration of the vitamin corresponds to the level usually added in SD media (Sherman, 2002). This finding shows that yeast is capable of salvaging any of the three vitamers, but does not address whether one vitamer is converted to another before being phosphorylated. To test if there is a kinase present in yeast to phosphorylate PL directly to PLP, we generated a strain deleted for both *snz1* and *ypr127w*. *YPR127W* encodes a protein homologous to pyridoxal reductase in other organisms and generates PN from PL (Figure 3-1). A single *ypr127w*Δ strain was able to grow well on SD-B6$^-$ media (data not shown). Similar to a single *snz1* deletion strain, a *ypr127w*Δ *snz1*Δ double deletion strain is auxotrophic for vitamin B6 but able to grow well on media supplemented with 2 μM of any vitamer including PL (Figure 3-4, third row from top). Presuming that *YPR127W* encodes the only pyridoxal reductase in yeast, our result suggests that even if the activity of the pyridoxal reductase shunts PL into the path of PN→PNP → PLP normally, yeast is capable of phosphorylating PL directly to PLP.

Salvage of 2 μM B6 vitamers is Bud16p-dependent.

Both *pdx3*Δ and *bud16*Δ mutant strains grew at a rate comparable to wild type strain on synthetic defined minimal media that lacks B6 (SD-B6$^-$), consistent with these genes functioning only in the salvage pathway and not in the biosynthetic pathway (data not shown). The telomeres of both strains reverted to wild type length upon a single streak on a minimal plate (data not shown).

Strains deleted for both *bud16* and *snz1* were streaked on SD-B6$^-$ media with or without added vitamers. Supplementation with 2 μM PL, PN, or PM was not able to support growth of a *bud16*Δ *snz1*Δ double deletion strain (Figure 3-4, fifth row from top). This demonstrated that

BUD16 is essential for utilization of vitamers at concentrations typically present in standard yeast media. Double deletion of *snz1* and *bud17*, the homolog of *BUD16*, showed no growth difference from a single *snz1*Δ strain on SD-B6$^-$ plates supplemented with 2 μM of all vitamers (Figure 3-4, sixth row from top).

Strains were generated that were deleted for both *pdx3* and *snz1* genes and streaked on SD-B6$^-$ with or without vitamer supplementation. 2μM of PM, but not PL or PN, was able to support growth of *pdx3*Δ *snz1*Δ double deletion strain (Figure 4, fourth row from top). These results suggest that while *PDX3* is required for converting PNP to PLP, there may be an alternative route for PMP oxidase activity. The dispensability of Pdx3p in PM utilization to convert PMP to PLP has been reported earlier in *Clostridium* and suggested to be via an alternative pathway utilizing transaminases (Loubbardi et al., 1995).

Short telomeres in *bud16*Δ and *pdx3*Δ are linked to B6 deficiency.

To test how the mutant phenotypes of *bud16*Δ and *pdx3*Δ responded to higher amounts of B6 vitamers, mutant strains were grown on YPD media supplemented with 40 μM, 400 μM or 4 mM of either PN, PL, or PM. We found that 4 mM of PN or PL, but not PM, were able to suppress both the growth and the short telomere phenotypes of *bud16*Δ (Figure 3-5). This suggests that both phenotypes were due to deficiency of the active PLP form of vitamin B6. 400 μM of PN or PL were also able to improve *bud16*Δ growth and telomere length but to a lesser degree (data not shown). The suppression with high concentration of certain vitamers ties *BUD16* functionally to yeast's vitamin B6 salvage pathway and strongly supports the idea that the short telomere phenotype in *bud16*Δ is due to a deficiency of the active form of vitamin B6, PLP. However, because the suppression from high levels of PN and PL was *BUD16*-

independent, it indicates that there is likely to be another kinase able to phosphorylate PL and/or PN that can function at high levels of those vitamers. Addition of B6 vitamers did not improve the growth or telomeric defects of *pdx3*Δ. In contrast, high levels of PN, but not PL or PM, had a strong inhibitory effect on growth of *pdx3*Δ strain on YPD (Figure 3-5 A). There was also a further reduction in telomere length upon high PN levels in a *pdx3*Δ strain (Figure 3-5 B).

The effects of high levels of PN on growth of *bud16*Δ and *pdx3*Δ were further analyzed in liquid culture. Cells from three separate colonies for wild type, *bud16*Δ*, pdx3*Δ, and *bud16*Δ *pdx3*Δ double mutant strains were picked from YPD plates and transferred to liquid YPD media with or without added 4 mM PN. The growth rate was monitored with optical density measurements of cultures. Both growth inhibition of *pdx3*Δ and growth rate improvement of *bud16*Δ upon PN addition was found to occur very rapidly (Figure 3-5 C).

Suppression of *bud16*Δ by high levels of PN or PL is *PDX3*-dependent but *BUD17*-independent.

As described above, high levels of PN or PL, but not PM are able to restore growth and telomere length to normal in a *bud16*Δ strain. This suggests there is a second pyridoxal kinase capable of functioning at high levels of PL and/or PN. *BUD17,* a homolog of *BUD16*, was an obvious candidate. However, we found that a *bud16*Δ *bud17*Δ double mutant strain was restored to normal growth with added 4 mM PN or PL, but not PM, similar to a single *bud16* deletion strain (Figure 3-6). This demonstrates that *BUD17* is not required for the suppression of *bud16*Δ by high levels of PL or PN.

We next generated strains deleted for both *bud16* and the putative PL reductase Ypr127w. 4 mM PN, but not PL, was able to restore the growth defect of *bud16*Δ *ypr127w*Δ on

YPD (row 3, Figure 3-6). The inability of PL supplementation to help growth is consistent with the suppression occurring from a PL→ PN → PNP → PLP route. The kinase activity responsible for suppression of the poor growth of *bud16*Δ by high levels of PL or PN remains unclear.

PL counteracts growth inhibition on *pdx3*Δ by PN in a *BUD17*-dependent manner.

Improvement of growth of a pyridoxal kinase mutant, *bud16*Δ, with added excess PN can easily be explained with the possible presence of an unidentified kinase that has weak affinity for one or more B6 vitamers. However, the negative effect of high level PN upon growth of *pdx3*Δ does not have an obvious explanation. Since PL and PM did not have these effects on *pdx3*Δ, we tested effects of all possible combinations of vitamers upon growth of *pdx3*Δ and *bud16*Δ. Combination of any two vitamers and all three vitamers, at 4 mM concentrations each, added to YPD, had no effect on growth of wild type strain (Figure 3-7, top row), proving that none of the amounts used were toxic to wild type yeast. All combinations improved growth of *bud16*Δ strain (data not shown). An interesting finding in the experiment was that simultaneous addition of PL, but not PM, can counteract the inhibitory effects of PN on *pdx3*Δ growth (Figure 3-7, second row from top). This relief of PN inhibition via PL was found to be *BUD17*-dependent as the double mutant *pdx3*Δ *bud17*Δ was inhibited by a combination of high PL and high PN (Figure 3-7, third row from top).

High levels of PN fail to inhibit *pdx3*Δ when B6 biosynthesis is on.

As described above, high PN (4 mM) supplementation on rich YPD media led to a growth inhibition of a *pdx3*Δ strain. However, *pdx3*Δ grown on a minimal media, SD-B6$^-$, supplemented with high levels of PN (4 mM) showed no growth inhibition (Figure 3-8, third row

from top). The question arises how *pdx3*Δ strain withstands these high levels of PN on minimal media and not on rich media. We found that deleting biosynthesis gene *snz1*Δ in a *pdx3*Δ background results in a strain unable to grow on high PN on a minimal media plate (Figure 3-8, fourth row from top). Since biosynthesis is mostly off on rich media, we concluded that inhibition by high level PN in a *pdx3*Δ mutant could be suppressed by expression of the biosynthetic pathway. Another finding that added more complexity to this puzzle was that high level PL also inhibited growth of a *snz1*Δ *pdx3*Δ double mutant on minimal plate (Figure 3-8, fourth row from top). This is in contrast to what we saw on rich media where PL counteracts the PN inhibition of a single *pdx3*Δ. Although we are unable to give a clear explanation for all these findings, it seems that PN inhibition on *pdx3*Δ occurs where biosynthesis is mostly off.

Mutants of vitamin B6 deficiency have a cell cycle progression defect.

When exponential phase *bud16*Δ cells grown in YPD were visualized under the microscope, we observed a higher budded versus unbudded proportion relative to wild type control cells. 44% of *bud16*Δ cells were budded in the mutant as opposed to 23% in wild type. When scored for bud sizes (small versus large), the difference seemed to be entirely in the number of medium to large buds. To investigate these preliminary observations, we performed FACS analysis to profile cell cycle phase distribution amongst cells in culture. Asynchronous cell populations of wild type, *bud16*Δ, and *pdx3*Δ strains were cultured in exponential growth phase in liquid YPD medium alone and YPD medium supplemented with 4 mM PL, 4 mM PN, or 4 mM PL/PN. Harvested cells were fixed and stained with propidium iodide and sorted for fluorescence-labeled DNA content using flow cytometry (see Materials and Methods). Relative to wild type, *bud16*Δ exhibited a major decrease in the proportion of cells that had duplicated

their DNA (Figure 3-9). Addition of 4 mM PN or PL suppressed these defects and restored the G1/G2 proportion of unsynchronized mutant cells to wild type. In a *pdx3*Δ strain a similar decrease in the G2 population of cells was evident when growth was slowed by the presence of 4 mM PN. This defect was corrected when 4 mM PL was also present (Figure 3-9). Similarity between the cell cycle phase profiles of *bud16*Δ on YPD and *pdx3*Δ with high PN suggests a shared mechanism for slow growth in both these mutants, possibly PLP deficiency.

Are short telomeres of vitamin B6 deficiency due to nucleotide depletion?

A possible link between vitamin B6 and telomere function could be nucleotide metabolism. Through its roles in folate, aspartate, glutamate, and glycine metabolism, vitamin B6 has indirect links to purine and pyrimidine synthesis. One direct PLP-dependent step is in the synthesis of thymine nucleotide. Serinehydroxymethyl transferase (SHMT) is a PLP-dependent enzyme required for activation of folate that converts deoxyuridine monophosphate to deoxythymidine monophosphate (Figure 3-10) (Rao et al., 2000; Snell and Riches, 1989). We first tested whether loss of the PLP-dependent SHMT led to telomere shortening or growth defect. There are two SHMT-encoding genes in yeast, *SHM1* and *SHM2* (Kastanos et al., 1997). A double mutant deleted for both *shm1* and *shm2* was constructed and was found to grow at or near a wild type rate and to have wild type telomere length (data not shown). We concluded that loss of SHMT function is insufficient to explain the phenotype of *bud16*Δ and *pdx3*Δ cells. However, *SHM1*- and *SHM2*-independent alternative routes have been described previously in which glycine synthase activity is able to replace the function of SHMT activity. (McNeil et al., 1994; Monschau et al., 1997).

The notion that nucleotide pools can affect telomere length was first described in temperature-sensitive mutations of two enzymes essential for de novo dTTP synthesis, thymidylate synthetase (*cdc21-1*) and thymidylate kinase (*cdc8-1*) (Toussaint et al., 2005). Both these mutants result in shortened telomere length even at permissive temperature (Adams and Holm, 1996; Toussaint et al., 2005). Furthermore, a thymidine depletion causes an S phase arrest in both mutants (Vernis et al., 2003). We proceeded to address the problem by supplementing *bud16*Δ with thymine nucleotides in a way that bypasses all B6-dependent steps. *S. cerevisiae* lacks the ability to take up exogenous nucleotides (Ahmad et al., 1998). It also lacks thymidine kinase acitivity and is unable to utilize exogenous thymidine (Grivell and Jackson, 1968) (Figure 3-10). *Herpes simplex* thymidine kinase gene, *hsTK*, has both thymidine kinase and thymidylate kinase activity (McNeil and Friesen, 1981) (Figure 3-10). Transformation with *hsTK* has been shown to complement the telomere length defect of the thymidylate kinase temperature-sensitive mutant strain, *cdc8-1* (Toussaint et al., 2005). Along the same lines, we transformed *bud16*Δ with *hsTK*. Since YPD plates do not allow selection of transformed yeast strains and a *bud16*Δ mutant does not exhibit any growth or telomere defects on SD plates, we performed the experiment on synthetic complete (SC) plates. SC plates are SD plates with added amino acids and other nutrients (see Materials and Methods). As a control, we were able to duplicate suppression of a *cdc8-1* strain with the expression of thymidine kinase plasmid. However, our results established that the growth of *bud16*Δ does not improve after transformation with *hsTK* (Figure 3-11). The short telomere length phenotype of a *bud16*Δ strain was also unaffected with *hsTK* (data not shown). These findings argue that TTP depletion alone does not explain the slow growth and short telomere length defects of a vitamin B6-deficient *bud16*Δ mutant.

A *pdx3* mutation does not perturb senescence or survivor formation in the absence of telomerase.

In vitamin B6 salvage mutants, telomeres are maintained at a shorter length in the presence of telomerase. This excessive shortening might be attributable to either a deficiency in the ability of telomerase to lengthen the telomeres, or to an acceleration of telomere shortening that would be expected to be independent of telomerase. As a first test to try to distinguish between these possibilities, we generated double mutants of telomerase (*est1*Δ) with either *bud16*Δ or *pdx3*Δ. If the phenotype of B6-deficient mutants was dependent on telomerase, then telomeres of a single telomerase deletion strain, *est1*Δ, would shorten at the same rate as those in a *estl*Δ *bud16*Δ or *est1*Δ *pdx3*Δ double mutants. On the other hand, if the mechanism underlying short telomeres caused by deficiency of B6, is due to accelerated telomere shortening, then the telomeres of a double mutant would shorten at a faster rate than a single mutant. We found that *bud16*Δ strains generated suppressors at a rate too high to permit completion of this experiment. Apart from suppressors, a second difficulty of the experiment was differential growth of the strains. Careful re-culturing at the exact same age (doublings), calculated with optical density measurements of liquid cultures, made it possible to compare *est1*Δ with a double mutant *est1*Δ *pdx3*Δ sporulated from the same diploid heterozygote (details in Materials and Methods). Among the spores from five tetrads that were followed until generation 105, we detected no obvious difference either in the rate of telomere shortening or in the time (in generations) at which survivors appeared in culture between the two genotypically different senescent strains (Figure 3-12). In three of the five comparisons, we observed survivor formation (as indicated by elongated telomeres) one passage earlier in the double mutant than in a control single mutant.

However, the difference was very slight and not accompanied by any detectable faster telomere shortening.

Two types of survivors are known to form in telomerase deletion yeast strains: Type I survivors that arise with recombination within subtelomeric DNA, thereby extending the ends with tandem duplication of the subtelomeric Y' element, and Type II with extensions involving mostly telomeric repeats only. Distinction between the types of survivors can be made with patterns of telomeric *Xho*I restriction fragments. *Xho*I cuts in the distal portion of Y' elements, resulting in telomeric bands of specific sizes (~1.3, 5.2, and 6.7 kb). Type I survivors show stronger hybridization at specific large fragments, and Type II survivors show shifts of fragments to a larger size. A quadruple digest with *Alu*I/*Hae*III/*Hin*fI/*Msp*I leads to digestion of the Y' elements to smaller size, resulting in telomeric bands being brought to shorter sizes in DNA from Type I survivors, but the elongated telomeric fragments of Type II survivors remain unaffected. Using these digests, we tested for survivor types in *est1*Δ and *est1*Δ *pdx3*Δ strains. We found that both types of survivors appear in both *est1*Δ and *est1*Δ *pdx3*Δ strains (Figure 3-13). Since the cells were kept in liquid culture, we see both kinds of survivor types in the same strain. Clearly, deletion of *pdx3*Δ did not prevent formation of either type of survivors.

DISCUSSION

Generation of active vitamin B6, pyridoxal 5'-phosphate (PLP) requires phosphorylation of inactive precursors pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM). Vitamin B6 specific kinases (often termed pyridoxal kinase in spite of their activity on PN and/or PM) have been purified and characterized from bacteria, plants, and mammals (di Salvo et al., 2004; Hanna et al., 1997; Lum et al., 2002). While there is only one enzyme with activity for all three vitamers

in humans, there are two genes encoding for pyridoxal activity in *E. coli*: PdxKp which has PL/PN/PM kinase activity, and PdxYp, which has activity only for PL (di Salvo et al., 2004). The crystal structure has been reported for pyridoxal kinase purified from sheep brain and for PdxYp of *E. coli* (Li et al., 2002; Safo et al., 2004). In both cases the protein was purified as a homodimer, and the detailed analysis of the active sites and ligand binding sites placed pyridoxal kinases in a new class within the ribokinase superfamily. The human pyridoxal kinase has received attention because of its drug interactions that may cause clinically significant neurological side effects (Laine-Cessac et al., 1997). In addition, a rare autoimmune disease, Polyglandular Deficiency Syndrome Type I, has been mapped to the human pyridoxal kinase gene (Aaltonen et al., 1994).

In this study, we have demonstrated that *BUD16* encodes the *bona fide* pyridoxal kinase of the *S. cerevisiae* vitamin B6 salvage pathway. It is essential for the utilization of all three vitamer forms (Figure 3-3). When grown on rich YPD media, a strain deleted for *BUD16* showed complete shut down of the salvage pathway. Slow residual growth of a *bud16*Δ mutant was entirely due to incomplete repression of the B6 biosynthetic pathway (Figure 3-4). However, extremely high levels (~ 2000 times) of PN and PL were able to improve growth of a *bud16*Δ strain to wild type levels (see Figure 3-6 and 3-9). Our findings argue that a second kinase activity phosphorylates only PN when the substrate is present at high levels. This suggests that this second kinase does not normally phosphorylate B6 vitamers. Our results have ruled out the closest relative of *BUD16*, *BUD17*, as the gene encoding this activity. Another possible candidate for this activity would be hydroxymethylpyrimidine (HMP) kinase of the vitamin B1 salvage pathway. HMP kinase purified from *E coli* was previously reported to be capable of kinasing PL, PM, and PN in *E. coli* (Mizote and Nakayama, 1989).

Another enzyme activity that has been implicated in vitamin B6 salvage is pyridoxal reductase. This activity has been described in *Schizosaccharomyces pombe* where the protein, encoded by the *PLR1* gene, routes PL to PN before conversion to PLP (Nakano et al., 1999). Based on sequence similarity, *YPR127W* was reported as the closest *S. cerevisiae* homolog of *PLR1*. Our results confirm that *S. cerevisiae* gene *YPR127W* does indeed encode pyridoxal reductase activity that routes PL to PN (Figure 3-6). However, disruption of *S. cerevisiae* pyridoxal reductase led to no obvious vitamin salvage deficiency (Figure 3-4), indicating that it is not absolutely required for vitamin B6 salvage. Similar findings were reported for a *S. pombe plr1* deletion strain which showed no growth defect (Morita et al., 2004; Nakano et al., 1999). It has been argued that the main function of pyridoxal reductase may be to maintain a PL → PN → PNP → PLP route in both yeast species (Guirard and Snell, 1988; Morita et al., 2004; Nakano et al., 1999). The basis for this hypothesis was two-fold. First, at physiological pH, the *S. cerevisiae* pyridoxal reductase activity has a V/K_m for PL 600 times that for the reverse reaction substrate, PN (Guirard and Snell, 1988). Secondly, *S. pombe* excretes PN upon incubation with excess PL, and this excretion decreases in a *plr1*Δ strain (Morita et al., 2004). Our results are also consistent with this hypothesis since salvage by 2 μM PL was found to be dependent upon Pdx3p, which converts PNP to PLP (Figure 3-4 and 3-8).

Apart from a kinase activity to phosphorylate inactive vitamers, salvage of PN and PM also requires an oxidase activity to convert their phosphorylated forms, PNP and PMP, to PLP. This activity has been studied in *E. coli* (Zhao and Winkler, 1995) and in various mammalian tissues, including rabbit liver (Kazarinoff and McCormick, 1975), pig brain (Churchich, 1984), sheep brain (Choi et al., 1987), and human brain (Kang et al., 2004). The *E. coli* and human proteins have been crystalized (di Salvo et al., 2002; Musayev et al., 2003). In addition to its role

in salvage, the *E. coli* homolog, *PDXH*, is also essential for *de novo* synthesis of vitamin B6, for the final conversion of PNP to PLP (Laber et al., 1999). In yeast, *PDX3* encodes the *S. cerevisiae* pyridoxine/pyridoxamine phosphate (PNP/PMP) oxidase (Loubbardi et al., 1995). Our experiments show that *PDX3* is not required for vitamin B6 biosynthesis (Figure 3-3), which may argue against PNP being the first vitamer formed in yeast vitamin B6 biosynthesis. According to our experiments, salvage of PL and PN, but not PM, are dependent upon *PDX3* (Figure 3-4). This result argues that PL is converted to PN before being utilized and that there is a *PDX3*-independent oxidase activity that is able to convert PMP to PLP in yeast (Loubbardi et al., 1995).

An interesting observation was that *pdx3*Δ grows poorly on rich media supplemented with high amounts of PN (Figure 3-5). This inhibition by PN could be explained either by further deficiency in PLP formation or by accumulation of PNP, a possible competitive inhibitor of PLP function. We favor the second possibility, as high levels of PL were able to counteract PN inhibition of a *pdx3*Δ strain. Conceivably, simultaneous addition of PL and PN could be readjusting the PNP/PLP ratio to favor formation of more PLP. Pyridoxal kinase activity from *E. coli* and yeast crude extracts was found to be inhibited by its substrate PL, but not PN or PM, in a concentration-dependent manner (Furukawa et al., 1981; White and Dempsey, 1970). Hypothetically, such an inhibition of the kinase could help explain how PL decreases PNP production in a *pdx3* deletion strain. However, little can be said with certainty about the mechanisms by which these high vitamer levels affect a *pdx3*Δ strain. Our data did show that the ability of PL to counteract PN inhibition on *pdx3*Δ is *BUD17*-dependent. From other experiments we had established that Bud17p does not serve as an essential kinase for PL, PN, or PM in a *bud16*Δ strain (Figure 3-3). The actual role of this gene is still unclear to us, but its ability to

route PL in a compensatory mechanism over PN inhibition ties the gene to vitamin B6 metabolism.

During our experiments, fast growing suppressors appeared in vitamin B6-deficient strains. Strains deleted for *BUD16* that were growing on YPD and *snz1* deletion strains on media lacking vitamin B6 showed a high rate of suppressors. Strains deleted for *PDX3* grown on plates with 4 mM PN, had a lower occurrence of fast growing suppressors. Conceivably, salvage mutants on rich media could be generating suppressors by activating biosynthesis. But suppressors also appear in salvage and biosynthesis double mutants (*bud16*Δ *snz1*Δ). This finding has also been reported in *E. coli,* where fast growing suppressors appeared in a *pdxB pdxK pdxY* triple mutant where both salvage kinase activities as well as the biosynthetic pathway were knocked out (Yang et al., 1998). It is possible that suppressors appear from vitamin B1 metabolic machinery, since biosynthesis of vitamin B1 and B6 are closely related. Addition of thiamine (vitamin B1) has been shown to cause shut down of B6 biosynthesis and drive all tested *Saccharomyces* wild type strains to B6 auxotrophy (Kamihara and Nakamura, 1984). At least some genes involved in vitamin B6 and vitamin B1 have very high sequence similarity. The *SNZ2/SNO2* and *SNZ3/SNO3* gene pairs that are involved in thiamine (vitamin B1) biosynthesis have 70-80% identity to the *SNZ1/SNO1* gene pair that functions in vitamin B6 biosynthesis (Padilla et al., 1998; Rodriguez-Navarro et al., 2002).

Genes involved in Vitamin B6 biosynthesis in yeast, *SNZ1* and *SN01,* had been discovered as exceptionally well-conserved genes that were highly expressed during stationary phase (Braun et al., 1996; Fuge et al., 1994). Interestingly, the *SNZ1* homolog in *Cercospora nicotianae*, *SOR1*, was found to be responsible for conferring resistance to singlet oxygen radicals from photosensitizers (Ehrenshaft et al., 1999; Ehrenshaft et al., 1998). This finding led

to the recent investigation and establishment of an antioxidant function for vitamin B6 (Bilski et al., 2000; Chumnantana et al., 2005; Kannan and Jain, 2004). Deletion mutants of *snz1* and *sno1* were reported to be sensitive to methylene blue, a singlet oxygen producer and 6-azauracil, an inhibitor of purine and pyrimidine synthesis (Padilla et al., 1998). These early findings are consistent with vitamin B6 functioning as an antioxidant and in nucleotide metabolism, but the functional significance of high expression of B6 biosynthetic genes during stationary phase is not known.

We have shown that the short telomere phenotypes of *bud16*Δ and *pdx3*Δ respond to supplementation with certain vitamers (Figure 3-5). Our experiments have established that vitamin B6 deficiency is responsible for the telomere defects in these B6 salvage mutants. This finding could be of importance to humans that lack the ability for B6 biosynthesis and rely solely on salvage for PLP generation. The expression of the enzymes pyridoxal kinase and PNP/PMP oxidase were found to be ubiquitous in all human tissues tested (Hanna et al., 1997; Kang et al., 2004). One study examining the levels of activity of pyridoxal kinase in erythrocytes from Caucasians and African Americans found that individuals had either of two distinct levels, "high" or "low" (Chern and Beutler, 1976). The frequencies of these phenotypic traits were very different for the two racial groups. Whether these observations actually translate into different amounts of PLP in the cell is not known. In other studies, vitamin B6 status in individuals was estimated from dietary intake or measured from plasma PLP levels and/or PLP-dependent enzyme activation. These studies showed that vitamin B6 deficiency is common in humans especially in elderly and low income women (Brown et al., 1975; Schuster et al., 1985; Schuster et al., 1981; Tolonen et al., 1988).

We hypothesized that the shortening of telomeres from vitamin B6 deficiency in yeast was due to a depletion of nucleotides. Since at least one important step in thymidylate synthesis is catalyzed by a PLP-dependent enzyme, we transformed a *bud16*Δ strain with the *Herpes simplex* thymidine kinase and tested for its effect on growth. Expression of this enzyme in yeast has previously been shown to generate thymine nucleotides via a route expected to bypass the PLP-dependent step (Figure 3-11) (Toussaint et al., 2005). The inability of *Herpes simplex* thymidine kinase to improve growth of bud16Δ argues against the possibility that defects in *bud16*Δ are entirely due to thymine nucleotide deficiency. However, vitamin B6 also has indirect roles in purine synthesis via folate, glutamate, and glycine metabolism and we have not tested for deficiency of purine nucleotides. Since telomeric DNA is rich in Gs and Ts on the strand that is extended by telomerase, it remains possible that a combination of thymine and guanine nucleotides will suppress the short telomere defect in a *bud16* deletion strain.

Another possible mechanism that could lead to telomere shortening from vitamin B6 deficiency is from direct DNA damage. Chronic oxidative stress accelerates telomere shortening of human somatic cells in culture. There are multiple possible links by which vitamin B6 deficiency could lead to oxidative DNA damage. Vitamin B6 could potentially lead to uracil incorporation into DNA from lack of thymine. This mechanism has certainly been shown to cause DNA damage in folate deficiency in human cells (Blount et al., 1997; Mashiyama et al., 2004). Also, synthesis of heme is dependent upon a PLP-dependent production of α-aminolevulinic acid (Ferreira and Gong, 1995). Heme deficiency leads to mitochondrial decay and release of oxidants into the cell (Atamna, 2004; Atamna and Frey, 2004; Han et al., 2001a). If oxidative damage causes DNA damage in B6 deficient yeast mutants, a more heterogeneous

telomere shortening would be the expected phenotype. However, we observed telomeres in vitamin B6 deficient yeast mutants to stay consistently at a specific shorter length.

Our results suggest that vitamin B6-deficient yeast mutants accumulate in G1 and do not progress through S-phase at a wild type rate (Figure 3-9). The thymine-deficient temperature sensitive mutants, thymidylate synthetase (*cdc21-1*) and thymidylate kinase (*cdc8-1*), have previously been described with an S phase arrest (Vernis et al., 2003). We observed an increased number of budded cells in asynchronous *bud16*Δ strain growing exponentially in YPD. This would be consistent with an S phase block in these cells. However, since cells were not synchronized before analysis, we cannot conclude with certainty whether these slow growing vitamin-deficient cells have a delay in entering S phase or during the progression of DNA replication. In summary, we have demonstrated that deficiency of vitamin B6 in yeast salvage mutants causes short telomeres and a cell cycle progression defect via a mechanism remains to be defined.

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TABLES

Table 3-1

Genes of vitamin B6 Salvage pathway.

Table lists putative and known genes of *S. cerevisiae* vitamin B6 salvage pathway. The best BLASTP hits in GenBank for homologs in *E. coli* and *Homo sapiens* are written with E-value (E), percent similarity (S) and percent identity (I). Included are functional annotations from *Saccharomyces* Genome database (SGD) together with their GO (Gene Ontology) terms; ISS (inferred from sequence or structure similarity) and IDA (inferred from direct assay). Also listed are conserved protein domains based on amino acid sequences from Conserved Domain Database (CDD). PL, pyridoxal. PN, pyridoxine. PM, pyridoxamine. PNP, pyridoxine phosphate. PMP, pyridoxamine phosphate. HMPP, hydroxymethypyrimidine phosphate, pfkB, superfamily of carbohydrate kinases that includes phsophofructokinases.

S. cerevisiae ORF/gene name	***YBR035C/ PDX3***	***YEL029C/ BUD16***		***YNR027W/ BUD17***		***YPR127W***
Known annotated function from SGD.	Verified PNP/ PMP oxidase (IDA).	Putative PL/PN/PM kinase (ISS). Bud site selection.				Putative PL reductase (ISS). Uncharacterize d ORF.
Protein domain information from CDD.	PL oxidase, PMP oxidase.	PL/ PM kinase, HMPP kinase, pfkB family kinase.		PL kinase, HMPP kinase, ribokinase.		Aldo/keto reductase family
E. coli	PdxHp E, $9e^{-38}$; I, 41%; S, 59%	PdxKp E, $7e^{-10}$; I, 25%; S, 45%	PdxYp E, $5e^{-16}$; I, 28%; S, 44%	PdxKp E, $7e^{-11}$; I, 27%; S, 43%	PdxYp E, $1e^{-17}$; I, 27%; S, 47%	yghZ E, $2e^{-08}$; I, 25%; S, 45%
H. sapiens	PN(M)PO E, $4e^{-33}$; I, 37%; S, 57%	PLK E, $1e^{-19}$; I, 30%; S, 54%		PLK E, $9E^{-37}$; I, 39%; S, 59%		KCNA3B E, 0.031; I, 24%; S, 44%

FIGURES

Figure 3-1

Generalized scheme of vitamin B6 salvage pathway.

Figure illustrates the B6 salvage pathway based on information known in *E. coli*. Proteins assigned to enzyme activities are written in bold for *E. coli* and within parenthesis for *Saccharomyces cerevisiae*. Question mark denotes that assignment of protein is based on sequence similarity alone.

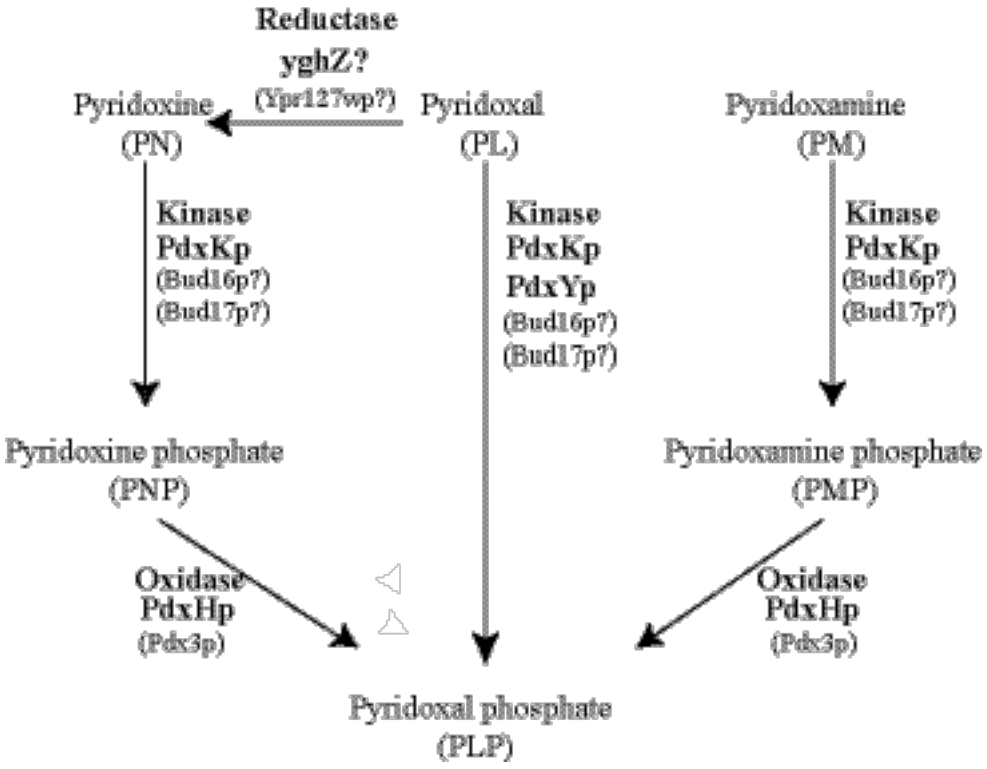
Reductase
yghZ?
(Ypr127wp?)
Pyridoxine
(PN)
Pyridoxal
(PL)
Pyridoxamine
(PM)
Kinase
PdxKp
(Bud16p?)
(Bud17p?)
Kinase
PdxKp
PdxYp
(Bud16p?)
(Bud17p?)
Kinase
PdxKp
(Bud16p?)
(Bud17p?)
Pyridoxine phosphate
(PNP)
Pyridoxamine phosphate
(PMP)
Oxidase
PdxHp
(Pdx3p)
Oxidase
PdxHp
(Pdx3p)
Pyridoxal phosphate
(PLP)

Figure 3-1

Figure 3-2

Slowed growth and shortened telomeres in yeast vitamin B6 salvage mutants.

A) Colonies of wild type and single ORF deletion mutants after three days of growth on rich, YPD media. B) Southern blot of *Nci*I-digested DNA of wild type and mutant strains probed with telomeric probe. A plasmid generated ladder containing telomere sequence (see Material and Mehods) was loaded in lane marked M. *Nci*I-digested genomic DNA from yeast strains were combined with restriction-digested plasmid DNA to provide internal size standards (arrows). The two internal control bands are fragments of the plasmid pYt103 containing telomeric sequence that were generated by mixing separate digests done with *Msp*I and *Pst*I/*Sal*I (producing 750 bp and 136 bp fragments, respectively). The last lane, marked "Int", is loaded with only the internal control plasmid DNA.

Figure 3-2 A

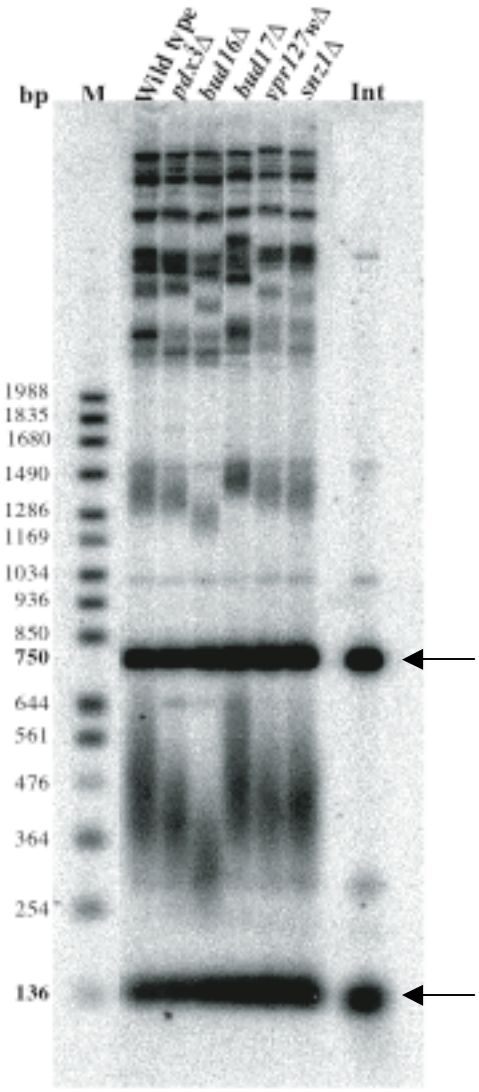

Figure 3-2 B

Figure 3-3

Residual growth of *bud16*Δ on YPD requires *SNZ1*.

Photos showing three days growth of wild type and mutant strains on rich, YPD media. Top row strains were generated by isolating spores of a single tetrad from sporulated heterozygous *bud16*Δ *bud17*Δ diploid strain. Bottom row strains were generated by isolating spores of a single tetrad from heterozygous *bud16*Δ *snz1*Δ diploid strain.

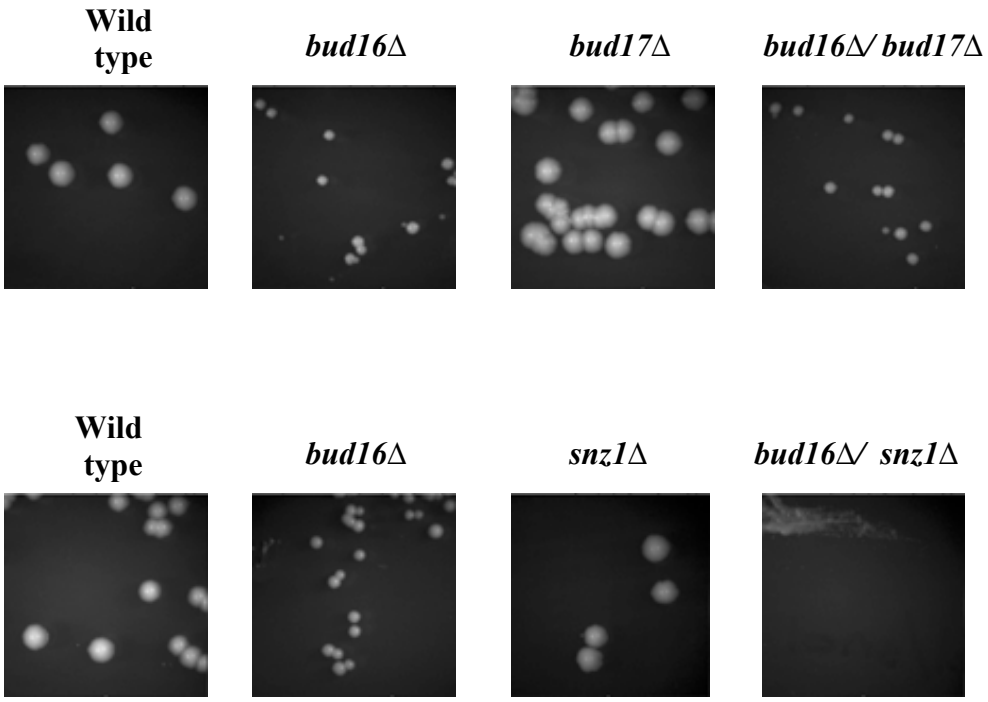
Wild type
bud16Δ
bud17Δ
bud16Δ/ bud17Δ
Wild type
bud16Δ
snz1Δ
bud16Δ/ snz1Δ

Figure 3-3

Figure 3-4

Bud16 and Pdx3 are required for salvage of 2 μM B6 vitamers.

Photos show growth of wild type and mutant strains on synthetic media lacking vitamin B6 (SD-B6$^-$) and SD-B6$^-$ supplemented with 2 μM of PL, PN, or PM. All strains were streaked from YPD onto the minimal media plates shown, and pictures were taken on the third day of growth. The little growth of *snz1Δ* on SD-B6$^-$ plate is not observed on the second streak. There is no difference in growth rates of all strains showing large colonies in the figure.

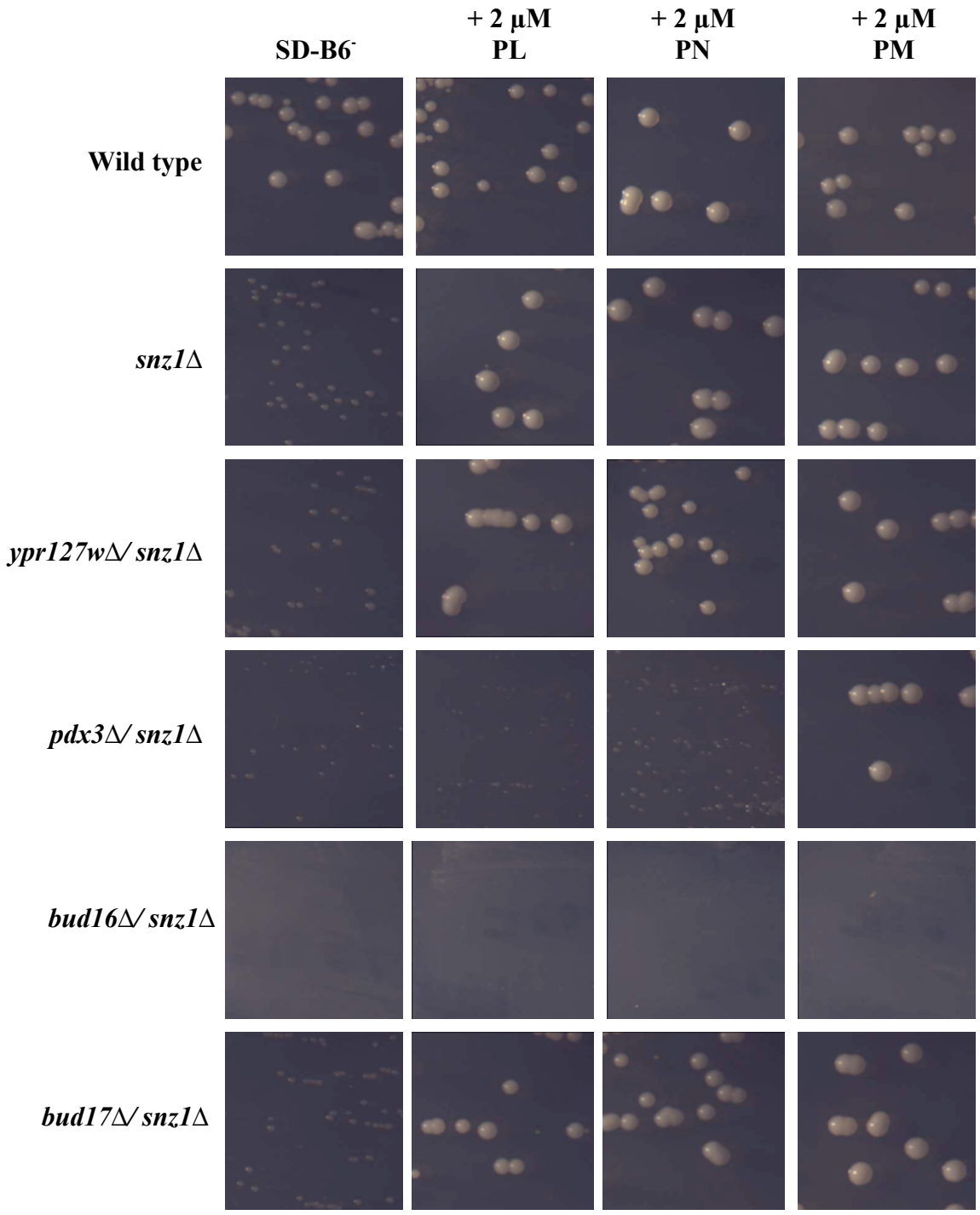
SD-B6-
+ 2 µM PL
+ 2 µM PN
+ 2 µM PM
Wild type
snz1Δ
ypr127wΔ/ snz1Δ
pdx3Δ/ snz1Δ
bud16Δ/ snz1Δ
bud17Δ/ snz1Δ

Figure 3-4

Figure 3-5

Effects of high level (4 mM) vitamer supplementations on *bud16*Δ and *pdx3*Δ.

A) Photos showing growth of wild type and mutant strains on YPD and YPD supplemented with 4 mM PL, PN, or PM. Cells from a single colony of each strain were picked from YPD plates and streaked on all four types of plates, YPD or YPD + PL, PN or PM. Colonies were re-streaked every third day, and photographs were taken on the third streak. B) Southern blot of *Nci*I-digested DNA of wild type and mutant strains from the third streak shown in A. Only the shortest Y' telomere bands are shown. The lane marked M is plasmid-generated ladder (see Material and Methods). DNA in each lane was combined with restriction-digested plasmid DNA to provide internal size standards of 750 bp and 136 bp (arrows). C) Growth curves for wild type and mutant strains grown in liquid YPD and liquid YPD + 4 mM PN. Strains were picked from YPD plates (without added PN) and grown in liquid culture for 16 hours. Optical density readings were taken at 1.5 hour intervals and plotted against time. Experiment was performed in triplicates and error bars represent the standard deviation.

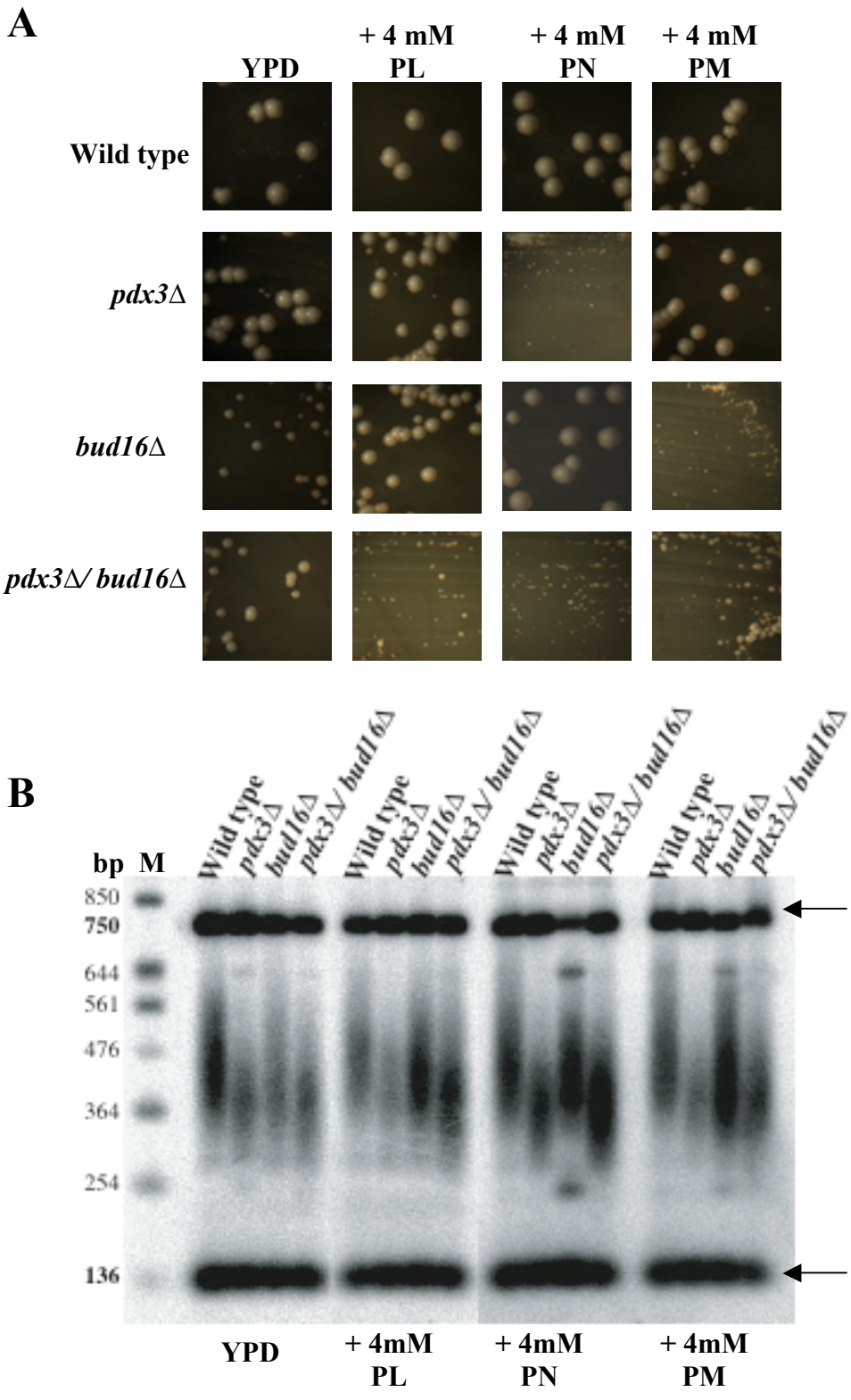

Figure 3-5 A and B

C

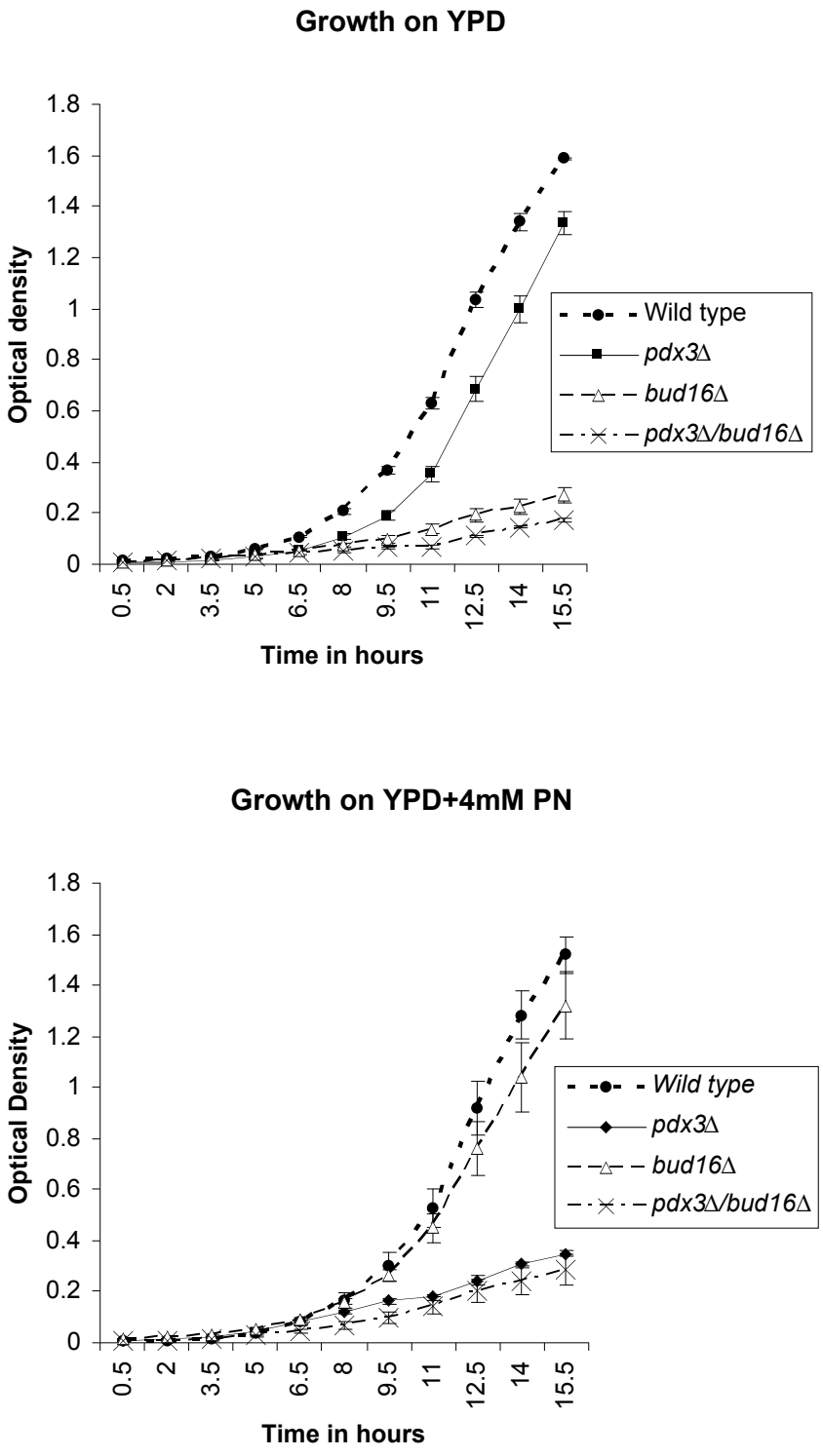
Growth on YPD
Optical density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.5
2
3.5
5
6.5
8
9.5
11
12.5
14
15.5
Time in hours
Wild type
pdx3Δ
bud16Δ
pdx3Δ/bud16Δ
Growth on YPD+4mM PN
Optical Density
Time in hours
Wild type
pdx3Δ
bud16Δ
pdx3Δ/bud16Δ

Figure 3-5C

Figure 3-6

The growth improvement of *bud16*Δ by 4 mM PL is *YPR127W*-dependent.

Photos showing growth of *bud16*Δ*, bud16*Δ *bud17*Δ*,* and *bud16*Δ *ypr127w*Δ strains on YPD and YPD with added 4 mM PL, PN, or PM. All strains were picked from YPD + 4 mM PN plates where they grow at wild type rate and streaked on the plates shown. Pictures were taken on the third day after streaking.

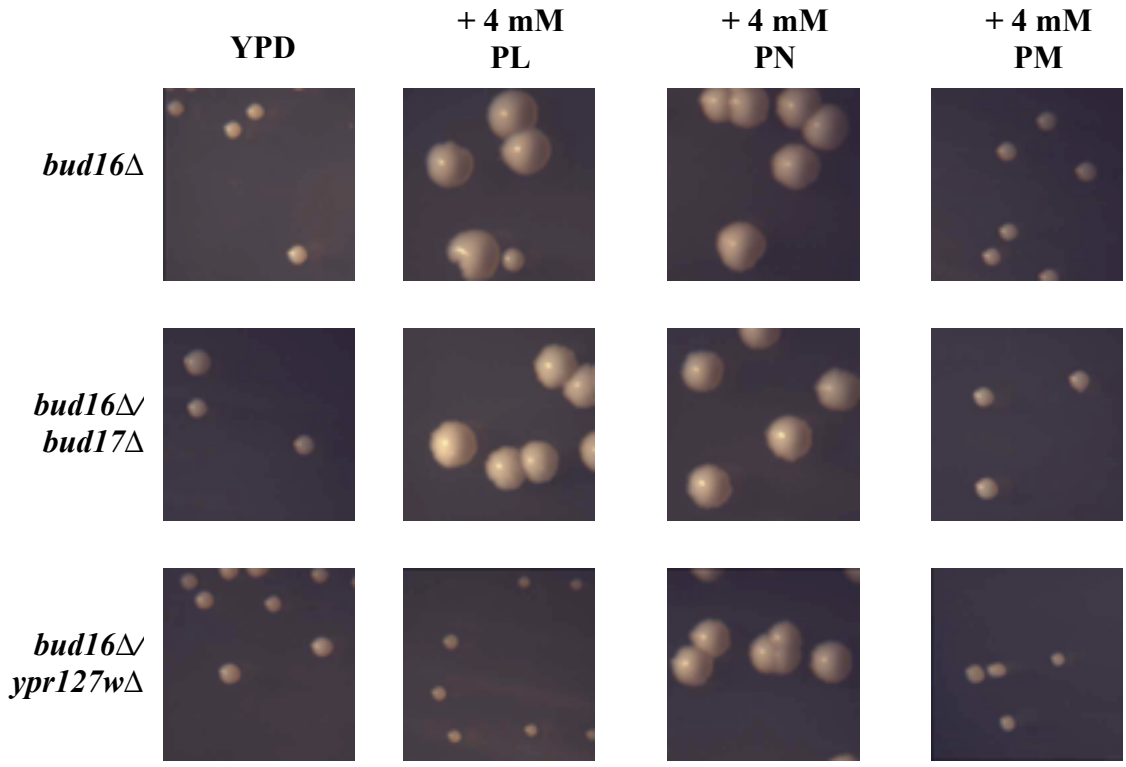
YPD
+ 4 mM PL
+ 4 mM PN
+ 4 mM PM
bud16Δ
bud16Δ/ bud17Δ
bud16Δ/ ypr127wΔ

Figure 3-6

Figure 3-7

***BUD17*-dependent improvement of PN-inhibited *pdx3*Δ growth by added PL.**

Figure shows growth of wild type, *pdx3*Δ*, pdx3*Δ *bud17*Δ, and *pdx3*Δ *snz1*Δ strains on YPD and YPD with added PL, PN, and PM alone and in all possible combinations. Each vitamer was added at 4 mM each. All strains were picked from YPD plates and streaked on the plates shown. Pictures were taken on the third day after streaking.

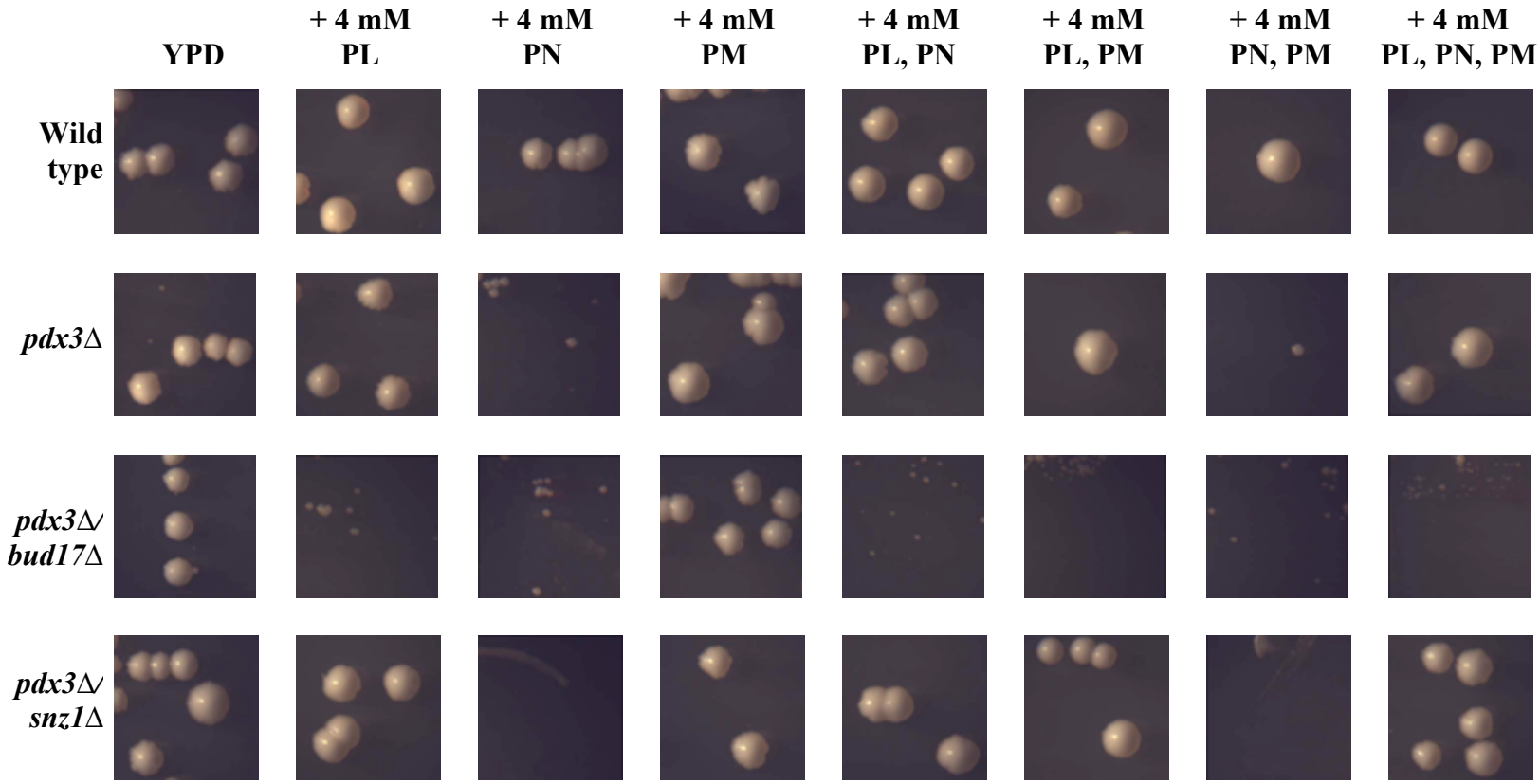
YPD
+ 4 mM PL
+ 4 mM PN
+ 4 mM PM
+ 4 mM PL, PN
+ 4 mM PL, PM
+ 4 mM PN, PM
+ 4 mM PL, PN, PM
Wild type
pdx3Δ
pdx3Δ/ bud17Δ
pdx3Δ/ snz1Δ

Figure 3-7

Figure 3-8

Growth inhibition of *pdx3*Δ *snz1*Δ, but not *pdx3*Δ, on minimal media by added PN.

Figure shows growth of wild type and mutant strains on SD-B6$^-$ and SD-B6$^-$ with 2 μM and 4 mM PL, PN, or PM. Wild type, *pdx3*Δ, *snz1*Δ, and *pdx3*Δ *snz1*Δ strains were picked off YPD plate and streaked on the plates shown. The *bud16*Δ *snz1*Δ strain was picked from a YPD + 4 mM PN plate and streaked on the plates shown. Pictures were taken on the third day from streaking.

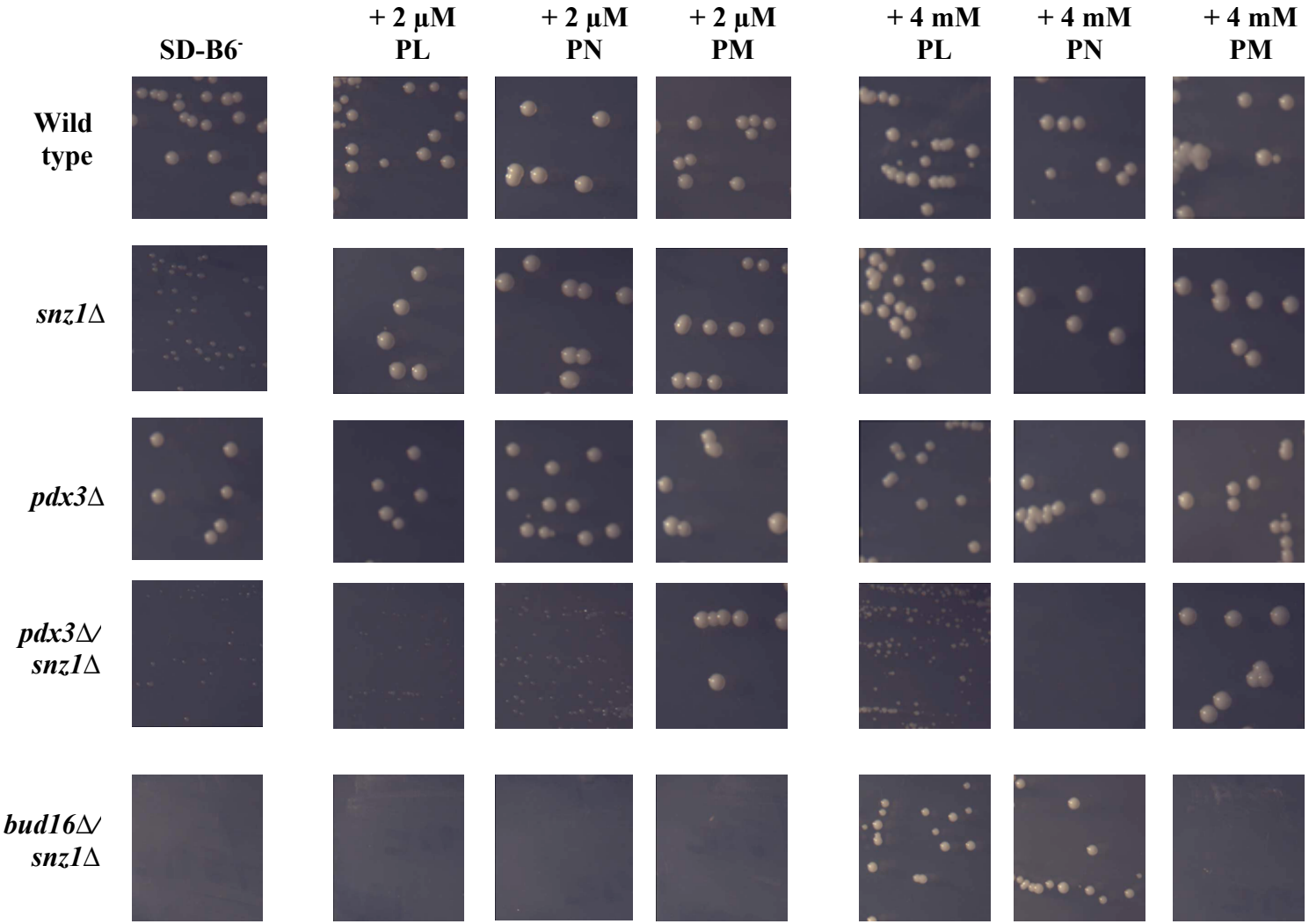
SD-B6-
+ 2 µM PL
+ 2 µM PN
+ 2 µM PM
+ 4 mM PL
+ 4 mM PN
+ 4 mM PM
Wild type
snz1Δ
pdx3Δ
pdx3Δ/ snz1Δ
bud16Δ/ snz1Δ

Figure 3-8

Figure 3-9

Flow cytometric analysis of vitamin B6-deficient mutants.

Unsynchronized, exponentially growing wild type and mutant cells from the media specified were fixed, stained with propidium iodide, and analyzed for amount of fluorescence labeling of DNA per cell. Data was gated to exclude debris and cell aggregates. In each graph shown, total cell fluorescence (FL2-A: PI-A) is plotted against number of counts (number of cells). The resulting histogram for each culture has a cell population in G0/1 that results in the peak on the left in each graph. Cells in G2/M phase that have duplicated their DNA result in the second peak with higher total cell fluorescence value. Cells in S phase have fluorescence value in between the G0/1 and G2/M peaks. The two percent age values given in each graph represent the estimated cells in the two peaks.

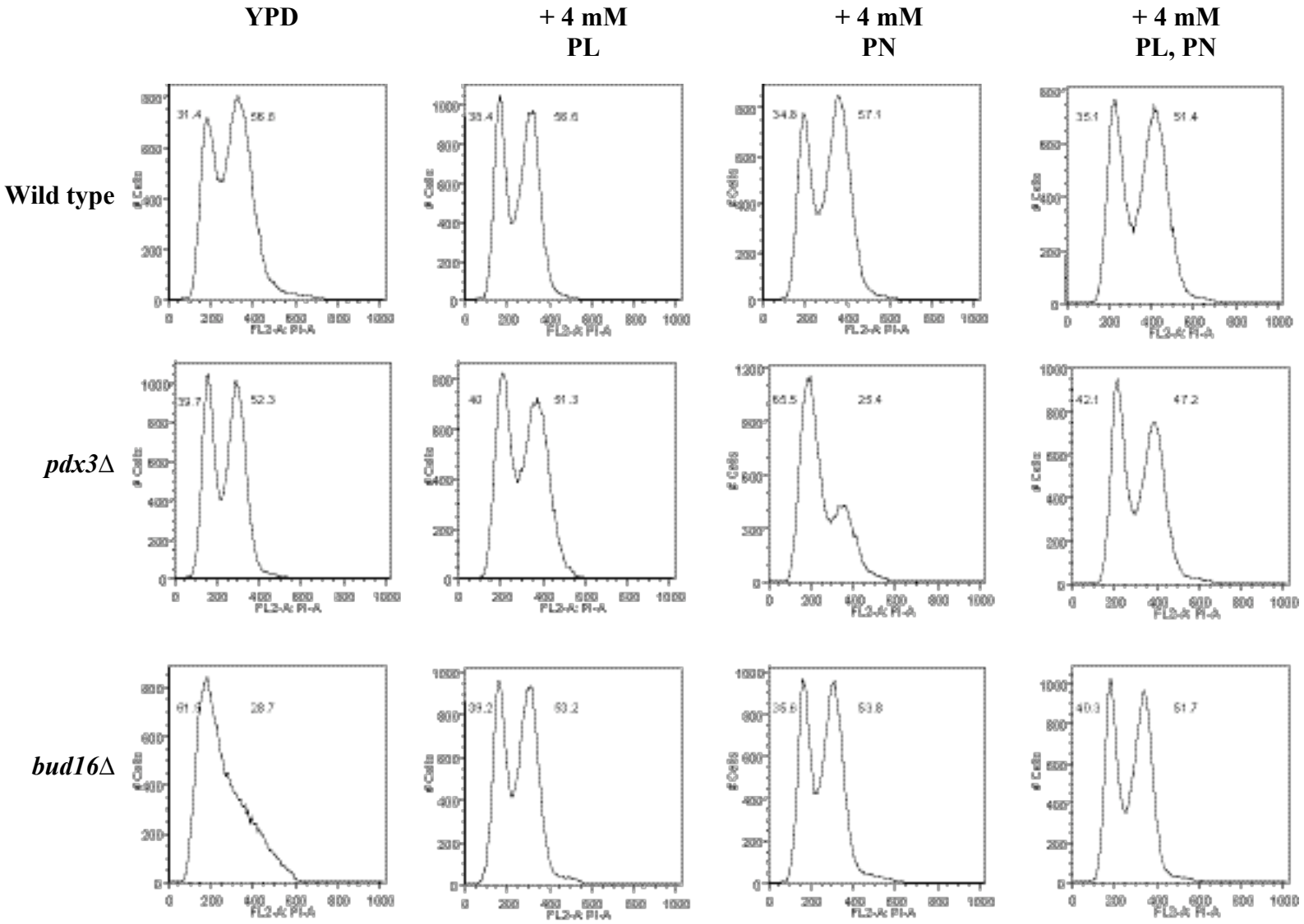
YPD
+ 4 mM PL
+ 4 mM PN
+ 4 mM PL, PN
Wild type
pdx3Δ
bud16Δ
Cells
FL2-A: PI-A

Figure 3-9

Figure 3-10

Thymine nucleotide synthesis in yeast

The PLP-dependent enzyme serine hydroxymethyltransferase activates folate in thymine nucleotide synthesis. Yeast genes encoding the activities are written under the names of enzymes. Asterisk denotes activities catalyzed by *Herpes simplex* thymidine Kinase (*hsTK*).

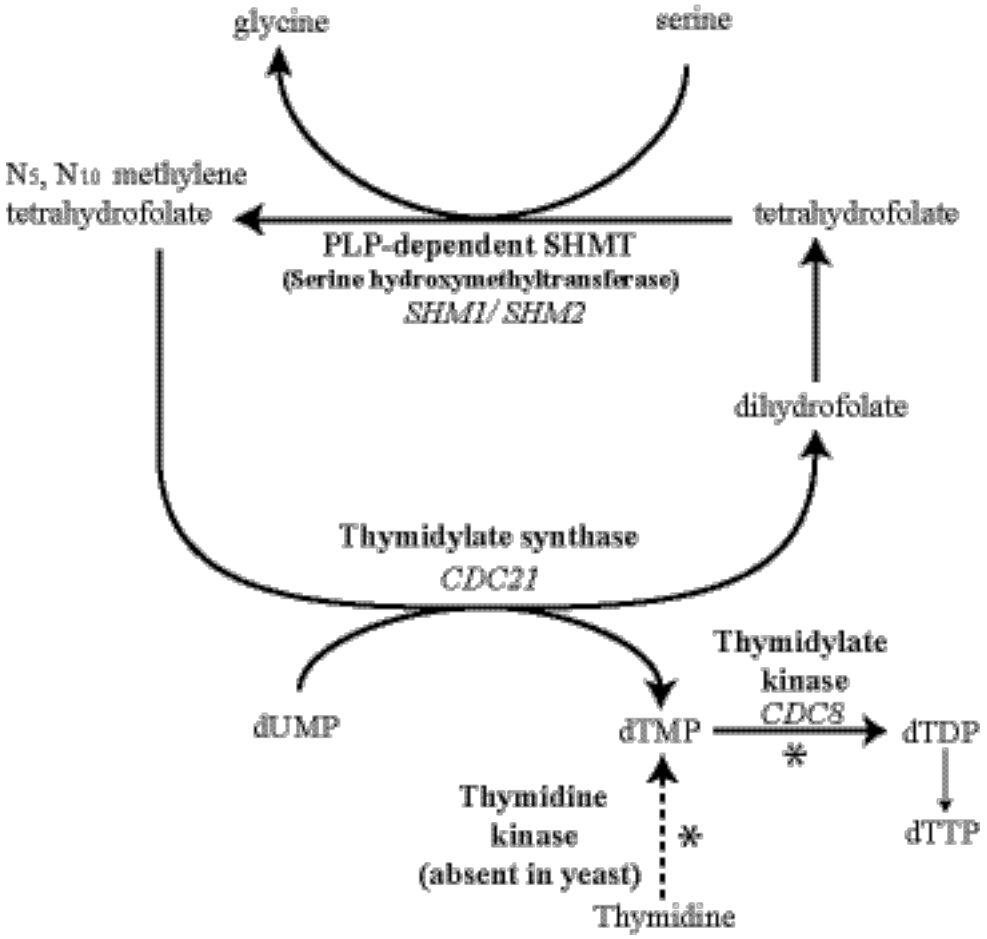
glycine
serine
N5, N10 methylene tetrahydrofolate
tetrahydrofolate
PLP-dependent SHMT
(Serine hydroxymethyltransferase)
SHM1/ SHM2
dihydrofolate
Thymidylate synthase
CDC21
Thymidylate kinase
CDC8
dUMP
dTMP
dTDP
dTTP
Thymidine kinase (absent in yeast)
Thymidine

Figure 3-10

Figure 3-11

Expression of *Herpes simplex* thymidine kinase gene in *bud16*Δ.

Photos showing growth of wild type and mutant strains with or without transformation with a plasmid carrying *Herpes simplex* thymine kinase gene *hsTK*. The experiment was performed on Synthetic Complete (SC) media and SC lacking histidine (for selection of *bud16* and 4741 wild type) or tryptophan (RWY42-22A and 22B). RWY42-22B is a strain carrying a temperature-sensitive mutation, *cdc8-1*. RWY42-22A is the isogenic wild type strain. Both strains were received from Wellinger lab (see Materials and Methods). Photos shown are from three day growth at restrictive temperature, 30º. The two small colonies of RWY42-22B without the plasmid failed to grow upon re-streaking.

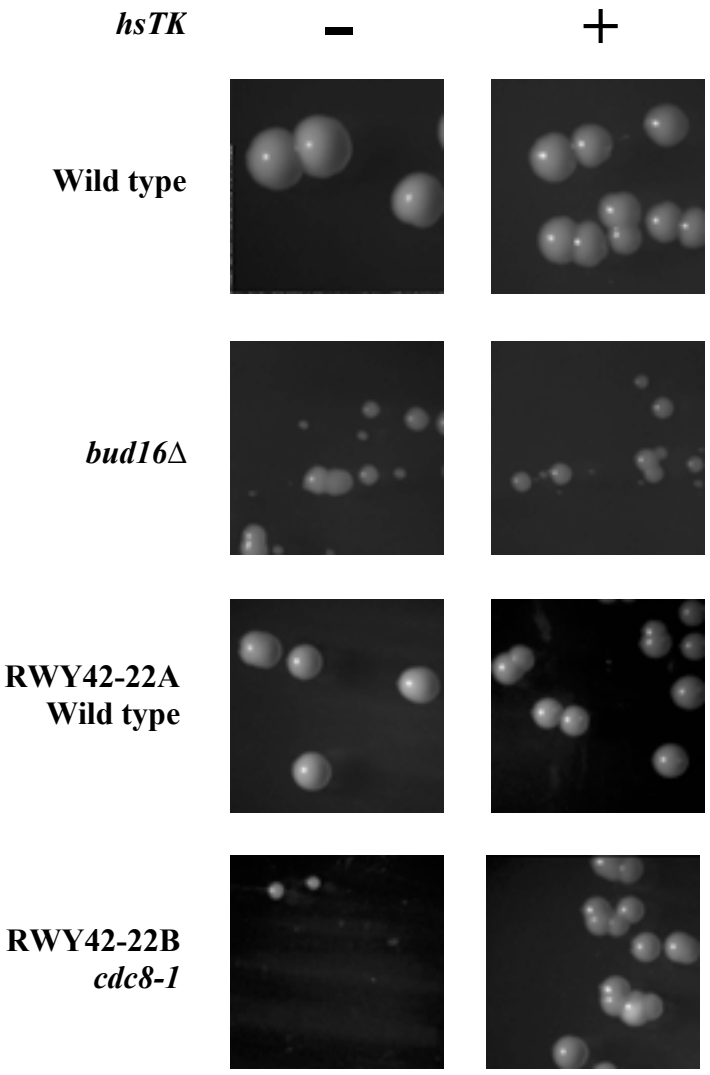

Figure 3-11

Figure 3-12

Timing of survivor formation in *pdx3Δ est1Δ* and *est1Δ* mutants.

Panels show Southern blot analysis for telomere length shortening of senescing strains over generations. Each panel compares telomere length of a strain deleted for *est1Δ* to a double deletion strain, *pdx3Δ est1Δ,* which was isolated from the same tetrad. Generations 35-75 represent senescing cultures. Generation 85 is showing appearance of survivors in senescent cultures. Generations 95 and 105 correspond to cultures entirely of survivors.

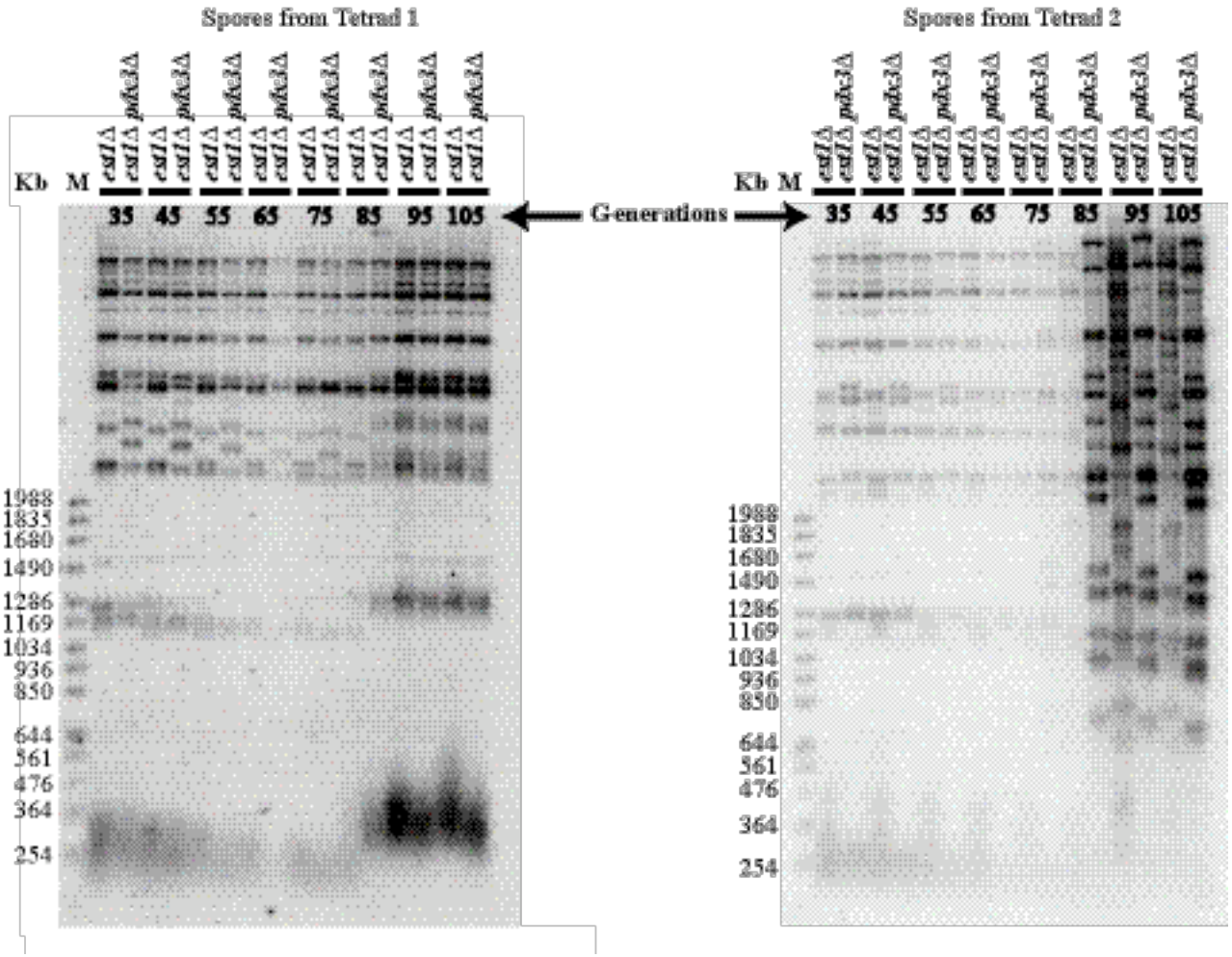
Spores from Tetrad 1
Spores from Tetrad 2
Kb M
csm1Δ csm1Δ pdc3Δ
35 45 55 65 75 85 95 105
Generations
1988
1835
1680
1490
1286
1169
1034
936
850
644
561
476
364
254

Figure 3-12

Figure 3-13

Both Type I and Type II survivors can form in a *est1*Δ *pdx3*Δ mutant.

Panels show Southern blot analysis with telomeric probe of two *est1*Δ *pdx3*Δ strains and one *est1*Δ single mutant. Generations 35 and 75 represent DNA from senescing cell cultures. Generation 85, 95, and 105 represent DNA from survivors. *Xho*1 and quadruple digests with *Alu*I/*Hae*III/*Hin*fI/*Msp*I differentiate between Type I and Type II survivors. M, marker.

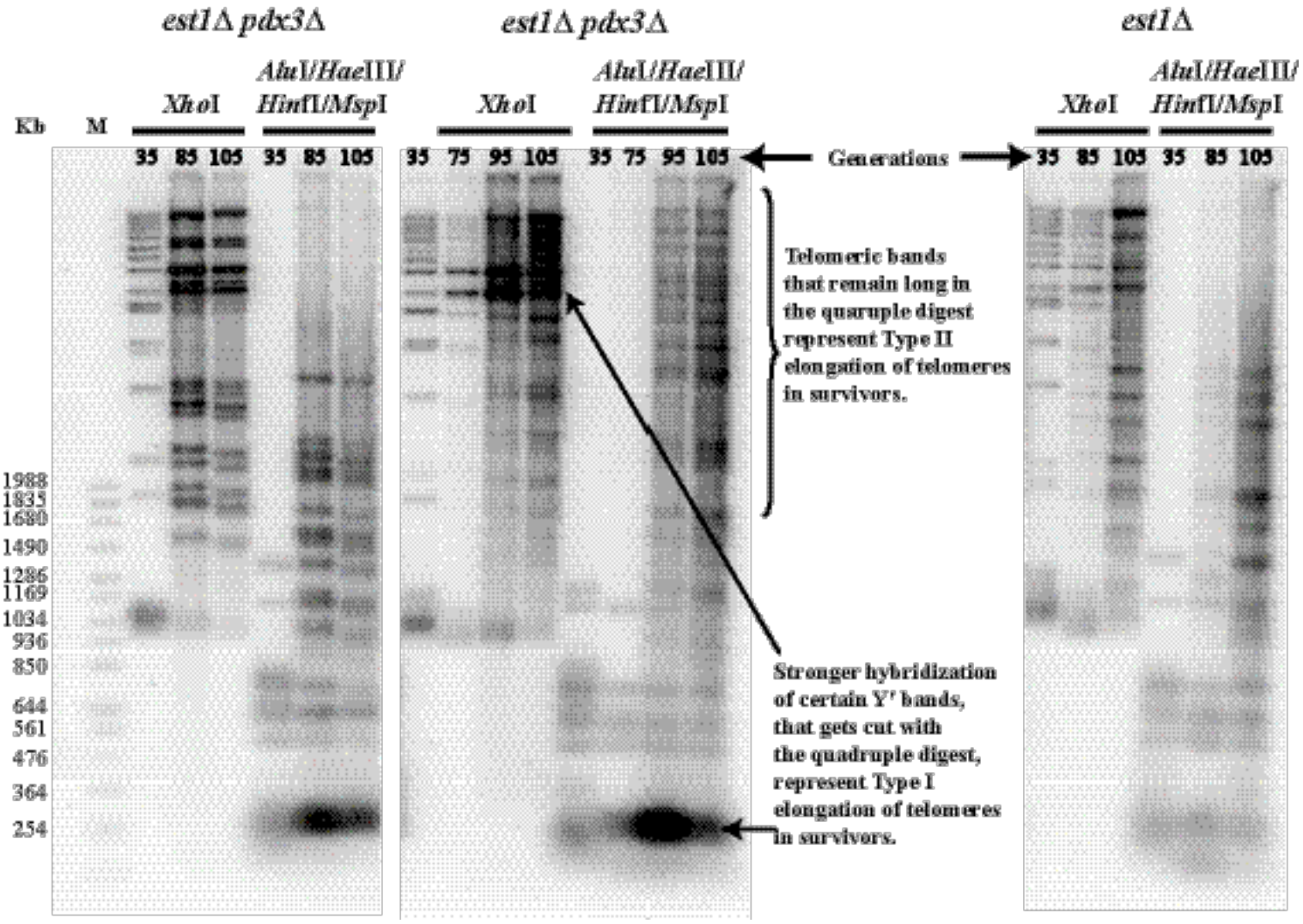
est1Δ pdx3Δ
est1Δ pdx3Δ
est1Δ
XhoI
AluI/HaeIII/ HinfI/MspI
Kb
M
35 85 105 35 85 105
35 75 95 105 35 75 95 105
Generations
35 85 105 35 85 105
1988
1835
1680
1490
1286
1169
1034
936
850
644
561
476
364
254
Telomeric bands that remain long in the quaruple digest represent Type II elongation of telomeres in survivors.
Stronger hybridization of certain Y' bands, that gets cut with the quadruple digest, represent Type I elongation of telomeres in survivors.

Figure 3-13

Figure 3-14

Vitamin B6 salvage pathway in *Saccharomyces cerevisiae*.

Figure illustrates the B6 salvage pathway of yeast in the light of the data presented and facts previously known. Bud16p is the bona fide PL/PN/PM kinase. The preferred route for utilization of PL may be via conversion to PN by PL reductase, Ypr127w. Pdx3p is essential for PN utilization. There is a PDX3-independent activity for conversion of PMP to PLP. Biosynthetic pathway does not require Bud16p, Ypr127wp, or Pdx3p.

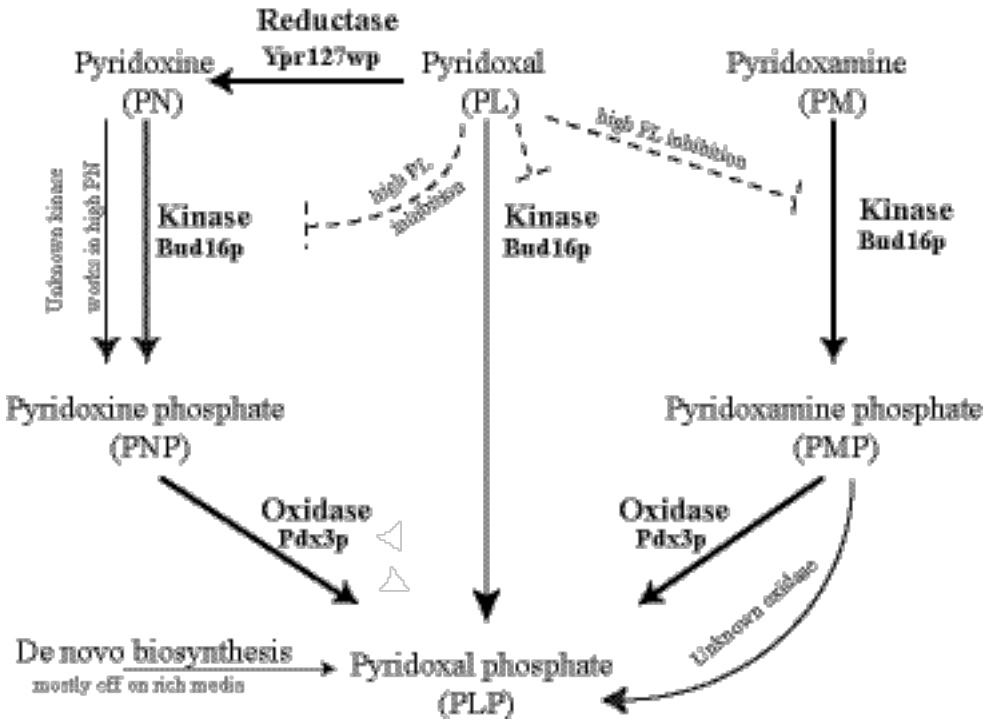
Reductase
Ypr127wp
Pyridoxine
(PN)
Pyridoxal
(PL)
Pyridoxamine
(PM)
Unknown kinase
works in high PN
Kinase
Bud16p
high PL inhibition
Kinase
Bud16p
high PL inhibition
Kinase
Bud16p
Pyridoxine phosphate
(PNP)
Pyridoxamine phosphate
(PMP)
Oxidase
Pdx3p
Oxidase
Pdx3p
Unknown oxidase
De novo biosynthesis
mostly off on rich media
Pyridoxal phosphate
(PLP)

Figure 3-14

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

Studies in yeast have contributed greatly to our current understanding of basic biological processes operating at telomeres (Teixeira and Gilson, 2005). Work presented here furthers this knowledge in two ways. A genome-wide screen, based on detection of telomere length in a deletion library of *Saccharomyces cerevisiae*, quadrupled the number of genes known to affect telomere length in this model system. We reported 109 single gene deletion strains that exhibit consistently shorter than wild type telomere lengths and 46 with consistently longer telomere lengths (Askree et al., 2004). Secondly, we have established a connection between telomere length and vitamin B6 metabolism in yeast. Deletion mutants for pyridoxal kinase, *bud16*Δ, and pyridoxine/pyridoxamine phosphate oxidase, *pdx3*Δ*,* harbor shorter telomere lengths than wild type. These enzymes convert precursors of vitamin B6 to the active form of the vitamin, pyridoxal phosphate or PLP. Our experiments show that the telomere phenotypes seen in vitamin B6 salvage mutants are due to a deficiency of PLP. A third achievement of this work was unrelated to telomere biology. This work has helped characterize the *S. cerevisiae* vitamin B6 salvage pathway (see Chapter 3).

One important direction for future research is in dissecting the mechanisms underlying telomere phenotypes of the novel gene candidates reported. These genes have very diverse known functions. For some, known functions can help in elucidating their connection to telomere metabolism. For example, 35 genes that affect telomere length have previously been implicated in chromatin remodeling and silencing, emphasizing chromatin modification at and near the

telomeres to affect length maintenance. In many instances several components of the same chromatin remodeling complex show similar telomere phenotypes when deleted (components of Rpd3, Paf1, Sum1p/Rfm1p/Hst1p, and Tho complexes). An exception was the yeast chromatin remodeler RCS complex where deletions of only one of several components, Rsc2p, had a short telomere phenotype. A recent study published after our report has shown Rsc2p binding to Mre11p (Shim et al., 2005). Mre11p is a component of the MRX complex with known functions in cellular response to DNA damage, telomere length maintenance, and meiotic recombination (Larrivee et al., 2004). Mre11p recruits the yeast chromatin remodeler RCS complex to double-strand breaks, and such an interaction may also apply for the common telomere function (Shim et al., 2005).

Many genes with telomere length defects have known functions in cellular processes with no obvious connection to telomere homeostasis. These are expected to affect telomere length by indirect mechanisms. Using a software called OSPREY, Edmonds *et al.* have generated a network that visualizes the known genetic and biochemical interactions among all novel gene candidates and genes with known telomere functions (Breitkreutz et al., 2003; Edmonds et al., 2004). This analysis has helped rationalize some of these links. For example, a gene with known vacuolar protein sorting function, *VPS9* interacts genetically with *SAP30*, a component of the Rpd3 histone deactylase complex (Hama et al., 1999; Sun and Hampsey, 1999). Deletions in either *VPS9* or *SAP30* led to similar short telomere length phenotypes, suggesting a possible common connection to telomere length homeostasis. Similarly, disruption of two genes encoding proteins that interact physically in a yeast two-hybrid screen, *CSM1* and *TAT2,* led to slightly longer than wild type telomeres (Askree et al., 2004; Uetz et al., 2000). These genes have completely unrelated known functions:: Tat2p is a permease for tryptophan and tyrosine

(Regenberg et al., 1999), and Csm1p is a nuclear protein that functions in meiotic chromosome segregation (Rabitsch et al., 2003).

Epistasis analysis can help categorize some of the genes indirectly affecting telomere length. Rog *et al.* applied this approach to several of the vacuolar sorting proteins (VPS) reported to affect telomere length (Rog et al., 2005). They showed that *VPS15*, *VPS34*, *VPS22*, *VPS23,* and *VPS28* affect telomeres via a single pathway. Their results place the telomere function of these genes in the same epistatic group as telomerase and Ku heterodimer proteins but not with Tel1p or Rif2p (Rog et al., 2005).

As discussed in Chapter 2, the method used in screening through the deletion library for telomere length mutants was efficient in avoiding false positives. However, false negatives are expected, especially with mutants exhibiting mild phenotypes that could be easily overlooked by our method. Slightly long telomeres (~50 bp) in deletions of three genes, *PLC1*, *IPK2,* and *KCS1* that function in inositol diphosphate signaling were missed in our screen and have been reported in a recent study (York et al., 2005). Also, *def1*Δ leads to a short telomere length phenotype that was not found in our efforts (Chen et al., 2005). Possibly, suppressor mutations or strain differences are responsible for these discrepancies.

We have demonstrated that defects in vitamin B6 salvage are responsible for the short telomere phenotypes of two of the mutants, *bud16*Δ and *pdx3*Δ. Other studies where a micronutrient was implicated in any way in telomere metabolism include (1) Vitamin C as preventing accelerated telomere shortening in human somatic cells caused by chronic oxidative stress (Furumoto et al., 1998; Kashino et al., 2003; Yokoo et al., 2004) and (2) Nicotinic Acid (vitamin B3) affecting silencing of subtelomeric genes via the action of NAD^+-dependent deacetylases Sir2p in yeast (Bitterman et al., 2002; Gallo et al., 2004; Sandmeier et al., 2002;

Sauve et al., 2005). Data indicating micronutrient deficiency as a cause of defects in telomere length have not been reported before. However, there has been compelling evidence of DNA damage from deficiencies in folic acid, vitamin B12 (Courtemanche et al., 2004a; Courtemanche et al., 2004b), iron (Walter et al., 2002) and zinc (Olin et al., 1993). The mechanism by which these nutrients cause DNA damage is best understood for folate. Figure 4-1 illustrates an outline of folate metabolism and its role in thymine and purine nucleotide synthesis. In human cells, deficiency in folate leads to incorporation of uracil in DNA due to deficiency in dTTP (Blount et al., 1997; Mashiyama et al., 2004). Vitamin B12 deficiency was also shown to correlate with uracil incorporation in bone marrow cells of healthy individuals and patients with megaloblastic anemia (Wickramasinghe and Fida, 1994). There is good evidence that excessive misincorporation of uracil causes DNA breaks that result in chromosome breakage. The occurrence of a higher number of erythrocytes with fragmented nuclei (micronuclei index) correlates with higher uracil in DNA from splenectomized individuals (Blount et al., 1997). Usually erythrocytes that reach the blood stream before enucleation are removed by the spleen. Folate metabolism is coupled with both B6- and B12-dependent reactions (Figure 4-1). Therefore, all three nutrients are thought to affect nucleotide metabolism and possibly lead to uracil incorporation into DNA (Ames, 2001).

All three vitamins, folate, B6, and B12, have also been implicated in homocysteine metabolism. Accumulation of homocysteine is a major risk factor for arterial endothelial dysfunction leading to heart disease (Refsum et al., 1998). Homocysteine is metabolized in the cell via folate-dependent conversion to methionine and PLP-dependent conversion to cysteine (Figure 4-1). High serum levels of homocysteine have been correlated with deficiency in folate and vitamin B6 (Rimm et al., 1998; Stabler et al., 1993; Stampfer and Rimm, 1996). In addition,

there is a correlation with higher micronuclei frequency in individuals with high serum homocysteine levels and low vitamin B12 levels that respond to intake of both vitamins (Fenech, 1999; Fenech et al., 1998). Interestingly, cardiovascular disease certainly has many correlations with telomere shortening. In vitro studies with mammalian vascular endothelial and smooth muscle cells, as well as cardiac myocytes, have implicated telomere shortening in functional deterioration, both age- and hypoxia-induced (Serrano and Andres, 2004).

Another possible connection between vitamin B6 and telomeres may be via its role in the first step of heme synthesis (Ferreira and Gong, 1995; Gong and Ferreira, 1995). Heme deficiency leads to mitochondrial decay, oxidative stress, and iron accumulation (Atamna, 2004; Han et al., 2001; Killilea et al., 2004), all of which are hallmarks of ageing or age-related disorders such as Alzheimer's disease (AD) (Atamna and Frey, 2004). Moreover, B6 has been recently discovered to be a potent antioxidant (Bilski et al., 2000; Chumnantana et al., 2005; Ehrenshaft et al., 1999; Kannan and Jain, 2004). It is possible that lack of B6 impacts telomeres via direct oxidative stress.

Having debated all these possibilities, it is still possible that the connection to telomere metabolism still lies outside nucleotide deficiency and DNA damage, in some other function of vitamin B6. Nonetheless, our findings in yeast certainly raise the question of whether accelerated shortening of telomeres from micronutrient deficiencies occurs in human cells. Recently, the Blackburn lab demonstrated that shorter telomeres, lower telomerase activity, and higher oxidative stress in blood mononuclear leukocytes of pre-menopausal women correlate with chronic life stress (Epel et al., 2004). Nineteen “control mothers” who were a biological parent of a healthy child were compared to 39 “caregiving mothers” of chronically ill children. Controlling for data that can be influenced by many environmental factors is not a simple task. However,

discovering a factor that can be manipulated or compensated for in a simple way, like dietary components, could prove promising. Impact of micronutrient deficiencies on telomere length in humans is one possible venue needing investigation.

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Figure 4-1

Folate metabolism.

Figure illustrates the role of folate in synthesis of nucleotides and the universal methyl-group carrier, S-adenosylmethionine (SAM). Reactions highlighted with “B6” and “B12” require these vitamins as cofactors. Purine synthesis involves two steps catalyzed by Formyltransferase (enzyme converting formyl tetrahydrofolate to tetrahydrofolate).

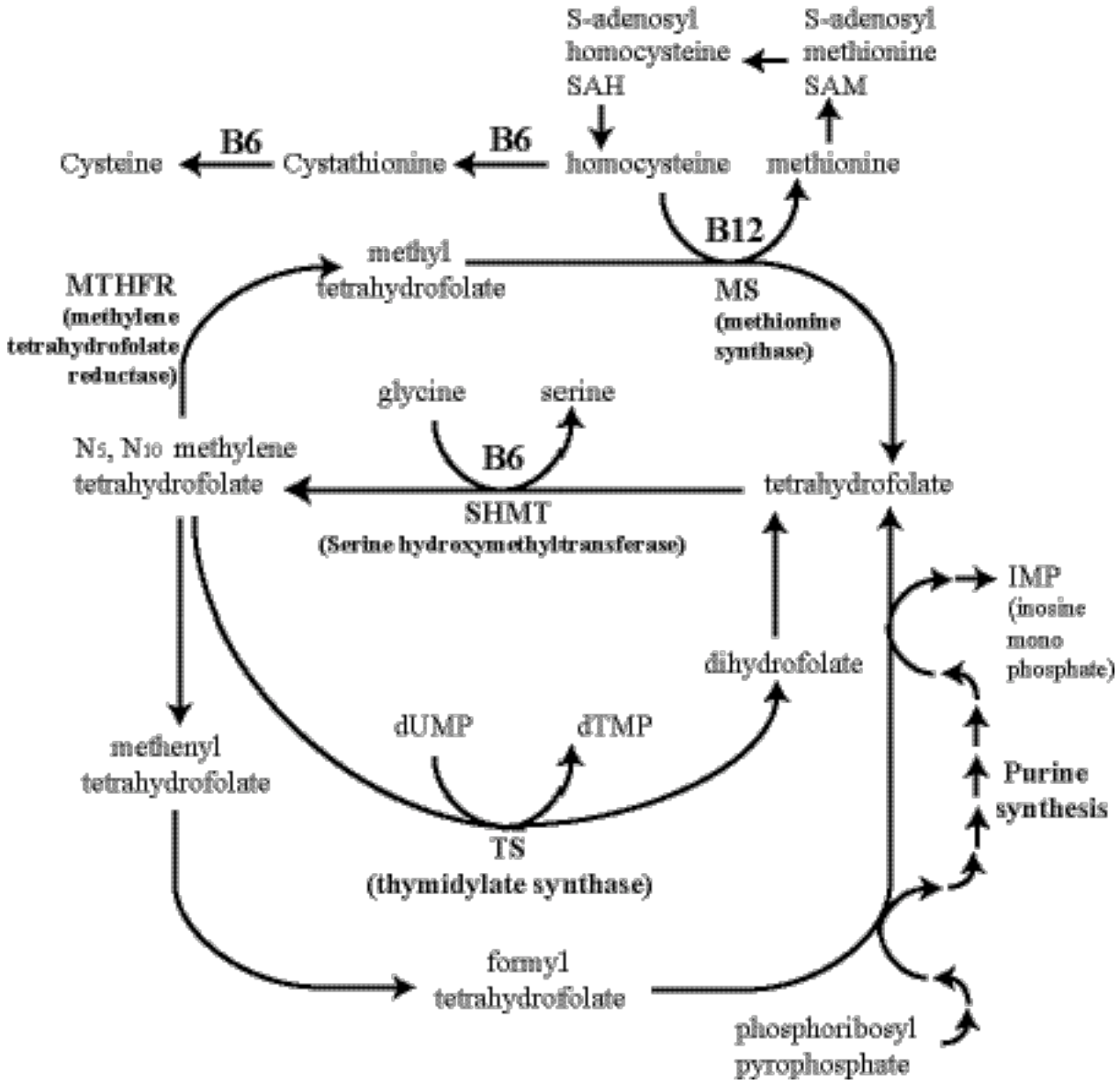
S-adenosyl homocysteine SAH
S-adenosyl methionine SAM
B6
B6
Cysteine
Cystathionine
homocysteine
methionine
B12
methyl tetrahydrofolate
MTHFR (methylene tetrahydrofolate reductase)
MS (methionine synthase)
glycine
serine
B6
N5, N10 methylene tetrahydrofolate
tetrahydrofolate
SHMT (Serine hydroxymethyltransferase)
IMP (inosine mono phosphate)
dihydrofolate
dUMP
dTMP
methenyl tetrahydrofolate
Purine synthesis
TS (thymidylate synthase)
formyl tetrahydrofolate
phosphoribosyl pyrophosphate

Figure 4-1