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A genome-wide screen for essential yeast genes that affect telomere length maintenance

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ABSTRACT

Telomeres are structures composed of repetitive DNA and proteins that protect the chromosomal ends in eukaryotic cells from fusion or degradation, thus contributing to genomic stability. Although telomere length varies between species, in all organisms studied telomere length appears to be controlled by a dynamic equilibrium between elongating mechanisms (mainly addition of repeats by the enzyme telomerase) and nucleases that shorten the telomeric sequences. Two previous studies have analyzed a collection of yeast deletion strains (deleted for nonessential genes) and found over 270 genes that affect telomere length (*T*elomere *L*ength *M*aintenance or *TLM* genes). Here we complete the list of *TLM* by analyzing a collection of strains carrying hypomorphic alleles of most essential genes (DAmP collection). We identify 87 essential genes that affect telomere length in yeast. These genes interact with the nonessential *TLM* genes in a significant manner, and provide new insights on the mechanisms involved in telomere length maintenance. The newly identified genes span a variety of cellular processes, including protein degradation, pre-mRNA splicing and DNA replication.

INTRODUCTION

Telomeres are the specialized DNA–protein structures at the ends of eukaryotic chromosomes. Telomeric DNA is composed of highly repetitive sequences [such as (TTA GGG)n in humans and ($C_{1-3}A/TG_{1-3}$)n in yeast (1)]. The telomeric structure performs a capping function by which it defines the end of the chromosome as a native edge, rather than the aberrant structure of a DNA double-strand break (DSB). This telomeric capping allows cells with linear chromosomes to function properly, while maintaining efficient mechanisms for DSB repair. When telomeric capping is abolished, activation of a DSB repair mechanism may cause telomere–telomere fusions resulting in chromosomal aberrations. Thereby, by performing their capping function telomeres carry out an essential role in maintaining chromosomal stability and integrity (2,3).

A linear DNA molecule cannot be completely replicated by the cellular DNA polymerase due to the 'end replication problem' (4). All DNA polymerases need a primer to initiate DNA replication. This primer is later removed from the DNA by the DNA polymerases arriving from the upstream direction. At telomeres no upstream fragment is present, resulting in a gap of unreplicated DNA (5). As a consequence, in most somatic cells, chromosomes lose information from their ends during each DNA replication cycle. This eventually leads to senescence and cell death (6). In germ cells, and some proliferating somatic cells, telomeres are maintained by telomerase, a cellular reverse transcriptase that copies a short template sequence within its own RNA into the telomeric sequence (6,7). When telomerase is inactivated, telomeres shorten from one division to the other leading eventually to cell death (8–10). Therefore telomerase performs an essential function, allowing proper replication of the chromosomes.

Telomerase elongates the telomere, adding one repeat at a time to the telomeric single-stranded substrate. This results in elongation of the single stranded DNA (ssDNA) at the end of the chromosome (7). Following telomerase action, the DNA polymerases can replicate the complementary strand, creating a double-stranded DNA (dsDNA) molecule (11,12). Addition of new sequences by telomerase is typically tightly regulated, resulting in the telomeres of many organisms being kept within particular size ranges. For example, while repetitive sequences in yeast telomeres span an average of 350 bp, human telomeres exceed several kilobases (kb) (1).

In all organisms studied, telomere length seems to be controlled by a dynamic equilibrium between elongating mechanisms (such as telomerase) and nucleases that shorten the telomeric sequences (13). Nevertheless, telomere length in all organisms tested tends to be remarkably

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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stable, indicating that telomere length is under strict genetic control. Two recent genome wide studies in yeast have identified genes that affect telomere length (*TLM*, or *t*elomere *l*ength *m*aintenance genes). A collection of 4750 yeast strains, each of them deleted for a particular gene, was screened by Southern blot analysis for genes that, when mutated, caused telomere lengthening or shortening (14,15). Together, these studies identified 272 *TLM* genes (representing ~5% of all nonessential genes); these include not only genes affecting expected categories, such as DNA and RNA metabolism, chromatin remodeling, etc., but also many genes affecting seemingly unrelated processes [e.g. vesicular transport (16)]. The large number of genes that affect telomere length homeostasis underscores the importance of this process. Many cellular functions seem to contribute to telomere length maintenance, and an initial unified model that integrates these activities has been recently proposed (17).

While the previous studies have focused on investigating the role of nonessential genes, two new methodologies have been developed lately to create a systematic collection of mutants defective in all essential genes in the yeast. The first collection consists of temperature-sensitive mutants (18): haploid cells can be grown at the permissive temperature, and only show their defective phenotype at the restrictive temperature. Here, we take advantage of a second collection, the Decreased Abundance by mRNA Perturbation library (DAmP) (19,20), which consists of hypomorphic alleles of most of the yeast essential genes. Screening this collection of 739 mutants, we have identified 86 genes that affect telomere length: 37 of them show long telomeres, whereas 49 show short telomeres (strikingly, a larger percentage of genes involved in the *TLM* system than that found in the nonessential gene set). Our results complete the list of telomere-affecting genes and add new insights on the mechanisms involved in telomere length maintenance.

MATERIALS AND METHODS

Yeast strain collection

The hypomorphic library developed by Weismann and co-workers (19,20) was used. Mutations were confirmed by PCR, and the relationship between the *tlm* phenotype and the *KanMX* insertion was confirmed by tetrad analysis of 20 individual mutants, after crosses to the isogenic BY4741/2 yeast strains. BY4741: (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*); BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). In all 20 cases, analysis of two tetrads showed a direct correspondence between the *tlm* phenotype and resistance to G418.

Telomere length measurement

Telomeric Southern blots were carried out as in (14). PCR fragments containing telomeric sequences and a genomic region that hybridizes to two size marker bands (2044 and 779 bp) were used as probes. The telomere length was measured with the GelQuant software using the size marker bands as reference. Telomere length was ≈1250 bp in wt cells [composed of the sub-telomeric region (≈900 bp) and the telomere repeats (≈350 bp)]. At least three independent colonies of each strain were analyzed by Southern blot to ensure reproducibility. Whenever discrepancies were observed, the strains were re-created and three additional independent colonies were tested.

PCR

The identity of a sample of 20 mutants was determined by PCR analysis, as described in (14).

Comparison with previous screens

We assembled protein–protein interaction (PPI) data from public databases and recent publications to construct a comprehensive PPI network of yeast (21–23). The PPIs were assigned confidence scores based on the experimental evidence available for each interaction using a logistic regression model adapted from (24).

To test the set of TLM genes identified by the DAmP screen, we examined their interactions with a set S of nonessential TLM genes discovered on previous screens using the hypergeometric P-value:

$$p\text{-value(A)} = \sum_{k=P(A,S)}^{\min\{|A|\times|S|,P(D,S)\}} \frac{\binom{P(D,S)}{k}\binom{|D|\times|S|-P(D,S)}{|A|\times|S|-k}}{\binom{|D|\times|S|}{|A|\times|S|}}$$

where D denotes the entire set of genes in the DAmP collection, and A denotes the genes on the DAmP collection that exhibited defects in telomere length. For two sets of proteins X_1 and X_2, we denote the number of interactions between them by $P(X_1, X_2)$. We use two definitions for P: (i) considering the entire set of interactions regardless of their confidence, and (ii) considering only interactions with confidence level above 0.5. Notably, the set of genes that were discovered on previous screens and have PPI information available contains a total of 250 genes [167 from Askree *et al.* (14), and 147 from Gatbonton *et al.* (15)].

RESULTS AND DISCUSSION

Telomere length maintenance is a complex trait, affected by a large number of genes. Two previous systematic genome-wide studies in the yeast *Saccharomyces cerevisiae* have uncovered 272 nonessential genes that, when mutated, cause either telomere lengthening or shortening (14,15). This large collection of mutants, encompassing ~5% of the yeast genome, is composed of haploid cells in which individual genes were deleted, one at a time. In order to complete the screening of all yeast genes, including those that are inviable when deleted, we took advantage of a collection of hypomorphic mutants, encompassing 842 out of the 1033 essential yeast genes (20). Briefly, in this strain collection a *KanMX* cassette (conferring resistance to the drug G418) was inserted within the 3′ UTR of all essential genes. In some cases, this insertion resulted in lethality (only 842 haploid strains could be created); however, a large proportion of the viable strains thus created exhibit scorable phenotypes,

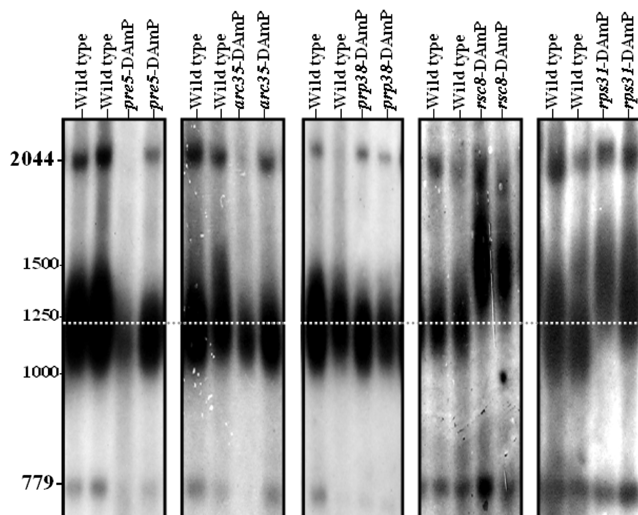

Figure 1. Tetrad analysis of DAmP *tlm* mutants. To confirm that the *tlm* phenotype observed was caused by the *KanMX* insertion, tetrad analysis was carried out after crossing to a wild-type yeast strain. In 20/20 cases, resistance to G418 (provided by the KanMX cassette) co-segregated with telomere length. Tetrad results of five representative mutants are shown. The white horizontal line represents the average wild-type telomere length.

suggesting that the KanMX insertion destabilized the expression of the disrupted gene (20).

Due to technical reasons (many strains failed to grow or to yield appropriate DNA preparations) we analyzed a total of 739 DAmP mutants for telomere length. At least three individual colonies of each strain in the DAmP collection were grown in rich medium, and their DNA was subjected to a telomeric Southern blot analysis. Each Southern blot also included triplicates of the wild-type isogenic strain. Marker PCR fragments, containing a genomic region that hybridizes to two bands (2044- and 779-bp long), were included in the labeled probes along with the telomeric probe. Telomere length in all strains was extremely reproducible, with a standard variation of <10% among repeats of the same strain. After an initial screen, all strains suspected as having a significant telomere length phenotype were re-tested, starting from fresh duplicate cultures. PCR analysis and tetrad analysis were used to confirm the identity of each individual mutant, and the co-segregation between telomere length and resistance to G418 (conferred by the *KanMX* allele at the DAmP allele). Figure 1 shows several examples of various tetrads thus analyzed. In total, our screen uncovered 86 essential genes whose DAmP allele leads to altered telomere length. Tables 1 and 2 show the list of genes that, when mutated, exhibit short and long phenotypes, respectively, divided by category. Figure 2A shows representative Southern blot analysis of various *tlm* DAmP alleles. Figure 2B shows all the *tlm* DAmP alleles sorted by relative length compared to the wild type. As in our previous screen (14) a slight bias is seen towards mutants exhibiting short telomeres (49 versus 37). It is not clear whether this represents a biological outcome (i.e. it is easier to cause telomere shortening by mutation than to cause telomere lengthening) or a technical bias (e.g. the probe used may underestimate long telomeres). For a discussion on these possibilities, see ref. 1). The frequency of *tlm* mutants in the DAmP collection (86/739 = 11.6%) is much higher than the one obtained among the non-essential mutants (250/4770 = 5.2%). This reflects the centrality of the telomeric pathways: *TLM* genes are enriched for essential functions. Notably, the frequency calculated for the essential collection is likely to be an under-estimate, as we have no efficient way of evaluating how well the DAmP methodology fares in creating hypomorphic alleles (19). See below for a discussion of this point.

The genes identified in our screen may be divided into various categories, according to their known functions (Tables 1 and 2). Below, we discuss the main categories individually:

Telomere maintenance and DNA replication

Our screen uncovered only a small number of the essential genes previously known to affect telomere length. This is most likely due to the fact that even hypomorphic mutations in these genes lead to continuous telomere shortening and to cell death. For example, the genes encoding the catalytic subunit of telomerase, Est2, and its two associated proteins Est1 and Est3 (25), were absent from our collection (due to their low cell viability), as did other essential genes encoding DNA polymerases. We did, however, find genes encoding some of the known components of telomeres: Cdc13, a telomeric ssDNA binding protein involved in telomere capping and protection (26) and its partner Stn1, which, together with Cdc13, helps in the recruiting of telomerase (27) (Table 2).

We also identified several genes involved in DNA replication: mutations in the *PRI1* primase gene, or in the *MCM3*/*MCM6* genes, encoding subunits of the replicative helicase, caused elongated telomeres. Similar results were observed for mutations in the ORFs YDL163w and YDL165w, which flank or overlap the gene *CDC9*, encoding the only essential DNA ligase of yeast. Thus, it appears that, as previously suggested (28), defects in some aspects of DNA replication may cause telomere elongation. This is consistent with the fact that the non-essential genes *RAD27, ELG1, PIF1* and *POL32*, also involved in various aspects of DNA replication, cause elongated telomeres upon deletion (14).

Proteasome

Mutations in eight genes encoding proteasomal subunits (*PRE2, PRE5, RPT3, RPT5, RPN5, RPN6, RPN7* and *RPN12*) lead to short telomeres [Table 1, Figures 1 and 2; results regarding *RPN6, RPN12* and *RPT3* have been reported in another publication (Yosef *et al*., in press)]. These mutations affect different substructures (e.g. base and lid), as well as functional portions (e.g. ATPases and non-ATPases) of the proteasome. In addition, mutations in components of the SCF machinery, required to add ubiquitin moieties to proteins in order to send them to degradation, cause the same phenotype (e.g. *HRT1* and *CDC34*). Therefore, our results suggest that proteasomal

Table 1. List of *S. cerevisiae* essential-DAmP genes that exhibit 'short' telomere phenotype

Gene	Function
Telomere-related	
TBF1	Binds to TTAGGG repeats within subtelomeric anti-silencing regions.
Proteasome/SCF	
PRE2	Beta 5 subunit of the 20S proteasome.
PRE5	Alpha 6 subunit of the 20S proteasome.
RPN5	Non-ATPase regulatory subunit of the 26S proteasome lid.
RPN6	Non-ATPase regulatory subunit of the 26S proteasome lid.
RPN7	Non-ATPase regulatory subunit of the 26S proteasome.
RPN12	Subunit of the 19S regulatory particle of the 26S proteasome lid.
RPT3	One of six ATPases of the 19S regulatory particle of the 26S proteasome.
RPT5	One of six ATPases of the 19S regulatory particle of the 26S proteasome.
HRT1	RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF).
CDC34	Ubiquitin-conjugating enzyme (E2) and catalytic subunit of SCF complex.
Splicing	
PRP4	Splicing factor, component of the U4/U6-U5 snRNP complex.
PRP22	RNA helicase of the DEAH-box family/ATP-dependent RNA helicase.
PRP31	Splicing factor, component of the U4/U6-U5 snRNP complex.
PRP38	Splicing factor, component of the U4/U6-U5 snRNP complex.
PRP43	RNA helicase of the DEAH-box family
NTR2	Involved in spliceosome disassembly, forms a complex with Prp43.
Arp2/3 complex	
ARP2	Essential component of the Arp2/3 complex.
ARP3	Essential component of the Arp2/3 complex.
ARC35	Essential component of the Arp2/3 complex.
ARC15	Essential component of the Arp2/3 complex.
Secretion and Golgi traffic	
TRS23	One of 10 subunits of the TRAPP complex (ER to Golgi traffic).
COG3	Component of the conserved oligomeric Golgi complex (vesicle to Golgi traffic).
YPP1	Cargo-transport protein involved in endocytosis.
RNA processing and Transcription	
FCP1	Essential for dephosphorylation of RNA polymerase II large subunit (Rpo21p).
RNA14	Cleavage and polyadenylation factor I (CF I) component.
RNA15	Cleavage and polyadenylation factor I (CF I) component
RPB7	RNA polymerase II subunit B16.
RGR1	Subunit of the RNA polymerase II mediator complex
YDR396W	overlaps NCB2:Subunit of the NC2 transcription regulator complex (with Bur6p).
rRNA processing/nucleolus	
FAL1	Nucleolar protein required for maturation of 18S rRNA.
POP7	Subunit of both RNase MRP (pre-rRNA processing), and RNase P (tRNA processing).
TSR1	Protein required for processing of 20S pre-rRNA.
NET1	Core subunit of the RENT complex (nucleolar).
MDN1	Midasin, remodeling and export of 60S ribosomal subunits.
NOC4	Nucleolar protein, forms a complex with Nop14p.
MTR3	3′5′ exoribonuclease, exosome subunit; nucleolar protein involved in export of mRNA.
tRNA processing	
SEN54	Subunit of the tRNA splicing endonuclease.
tRNA synthetases	
KRS1	Lysyl-tRNA synthetase.
ALA1	Cytoplasmic alanyl-tRNA synthetase.
APC/cyclosome	
APC4	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C).
DNA Replication	
ORC5	Subunit of the origin recognition complex.
PSF3	Subunit of the GINS complex, a putative helicase with a role in DNA replication.
Kinetochore	
TID3	Component of the evolutionarily conserved kinetochore-associated Ndc80 complex.
DAD2	Essential subunit of the Dam1 complex (aka DASH complex).
Miscellaneous	
SAM35	Mitochondrial outer membrane protein, member of the SAM complex.
SAH1	*S*-adenosyl-L-homocysteine hydrolase.
TUB2	Beta-tubulin; associates with alpha-tubulin (Tub1p and Tub3p) to form tubulin dimmer.
ILV5	Acetohydroxyacid reductoisomerase, mitochondrial protein.
NAM9	Mitochondrial ribosomal component of the small subunit

Descriptions are from the *Saccharomyces* genome database (SGD: http://www.yeastgenome.org/).

Table 2. List of *S. cerevisiae* essential-DAmP genes that exhibit 'long' telomere phenotype

Gene	Function
Telomere-related	
STN1	Telomere end-binding and capping protein.
CDC13	Telomere single-stranded DNA-binding protein.
DNA replication	
YDL163W	Overlaps CDC9: DNA ligase.
YDL165w	Overlaps CDC9: DNA ligase.
YOR218C	Overlaps RFC1: clamp loader, loads PCNA to initiate replication.
PRI1	Subunit of DNA primase.
MCM3	Replicative helicase.
MCM6	Replicative helicase.
APC/cyclosome	
CDC16	Subunit of the anaphase-promoting complex/cyclosome.
Transcription	
RPB5	RNA polymerase subunit ABC27.
SPN1	Protein that interacts with Spt6p and co-purifies with Spt5p and RNA polymerase II
NHP2	Nuclear protein related to mammalian high-mobility group (HMG) proteins.
SUMO/Ubiquitin	
SMT3	SUMO (small ubiquitin-like modifier).
RPS31	Ubiquitin.
Chromatin remodeling	
RSC4	Component of the RSC chromatin-remodeling complex.
RSC8	Component of the RSC chromatin-remodeling complex.
Secretion and Golgi traffic	
SEC20	Membrane glycoprotein, v-SNARE (Golgi to ER).
SEC63	Essential subunit of Sec63 complex (import to the ER).
TRS20	Component of the TRAPP complex (*cis*-Golgi).
YJL032W	Overlaps BET4: ER–Golgi transport.
MCD4	ER protein involved in GPI anchor synthesis.
GPI8	Subunit of the ER GPI transamidase complex.
CMD1	Calmodulin; Ca^{++}-binding protein. Required for vacuolar fusion.
Splicing	
SAD1	Conserved zinc-finger domain protein involved in pre-mRNA splicing, required for assembly of U4 snRNA into the U4/U6 particle.
AAR2	Component of the U5 snRNP, required for splicing of U3 precursors.
rRNA/tRNA processing	
YLR317W	Overlaps TAD3: Subunit of tRNA-specific adenosine-34 deaminase.
LSM8	RNA degradation, tRNA/rRNA modification and splicing.
CBF5	Pseudouridine synthase, catalytic subunit of box H/ACA snoRNPs.
YLR198C	Overlaps NOP56: Nucleolar component of the box C/D snoRNP complexes that direct 2′-*O*-methylation of pre-rRNA during its maturation.
Miscellaneous	
YLR230W	Overlaps CDC42: small Rho-like GTPase essential for cell polarity.
NCP1	NADP-cytochrome P450 reductase, ergosterol synthesis.
BBP1	Protein required for the spindle pole body (SPB) duplication
CDC19	Pyruvate kinase, last step in glycolysis.
HEM4	Heme biosynthetic pathway.
YLR339C	Overlaps RPP0: conserved ribosomal protein P0.
GCD1	Gamma subunit of the translation initiation factor eIF2B, the guanine-nucleotide exchange factor for eIF2.

Descriptions are from the *Saccharomyces* genome database (SGD: http://www.yeastgenome.org/).

function (and not individual subunits) plays an important role in telomere elongation. Similar conclusions were reached in a more elaborate network-based analysis of protein–protein interaction data (Yosef *et al*., in press). Lack of degradation of a protein with a role in telomere shortening (such as a nuclease or one of its regulators) may lead to higher abundance levels, causing increased telomere shortening. Alternatively, proteasome inactivation may lead to accumulation of a repressor of telomerase activity [such as Rif1, Rif2 (29) or Pif1 (30)], thus changing the equilibrium towards a shorter length.

Pre-mRNA splicing

Six genes with roles in pre-mRNA splicing lead to short telomeres when mutated: *PRP4, PRP22, PRP32, PRP31, PRP38, PRP43* and *NTR2* (Table 1).

Pre-mRNA splicing is a complex reaction involving dozens of proteins, and consists of two consecutive catalytic reactions. Interestingly, all the genes identified seem to play a role in the structural changes in the spliceosome associated with the second catalytical splicing reaction (Prp4, Prp31 and Prp38), or with the disassembly of the

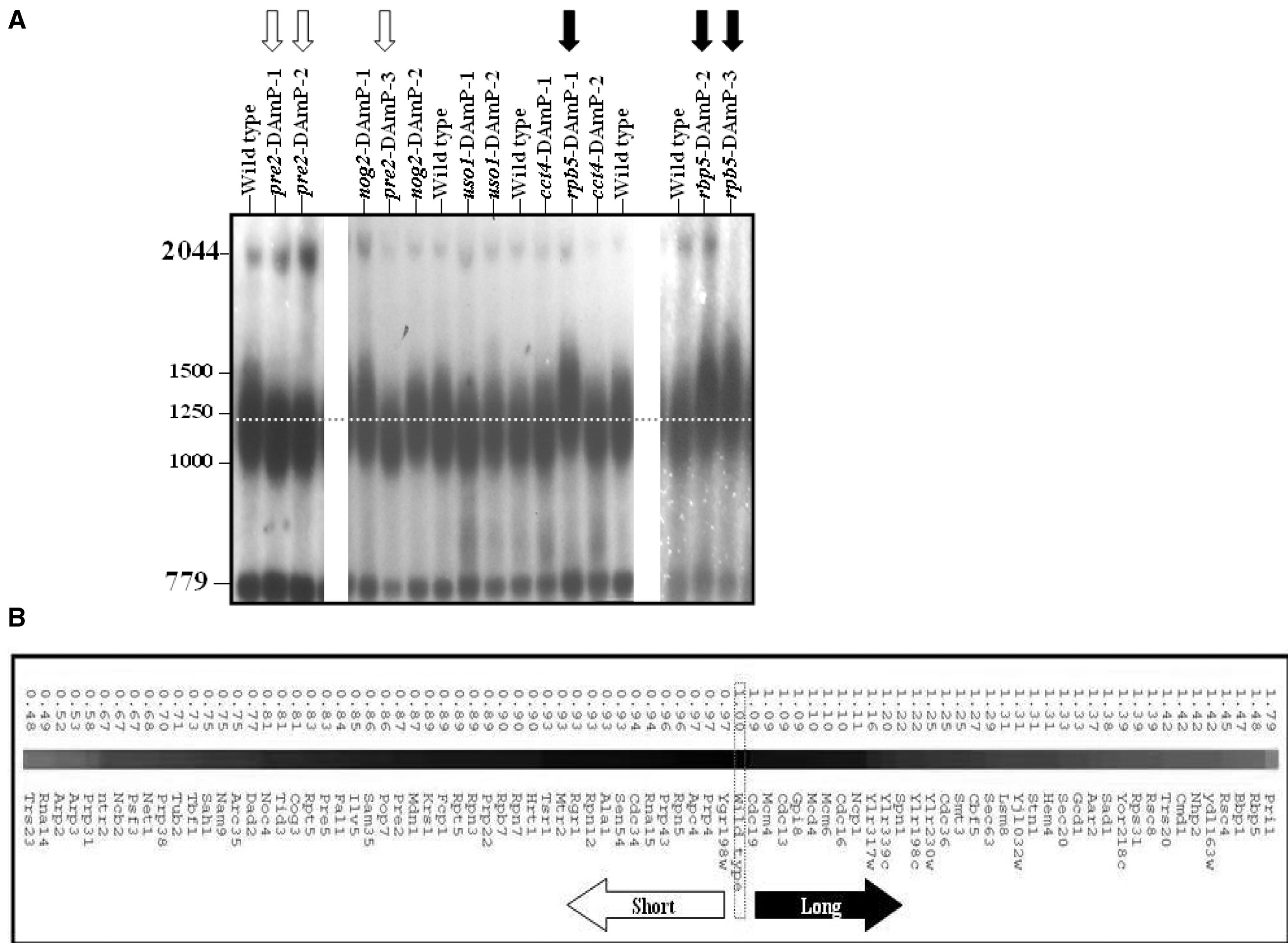

Figure 2. (**A**) Representative Southern blots of DAmP strains. The white horizontal line represents the average wild-type telomere length. The white arrow points to *pre2-DAmP* mutants, which shows short telomeres, whereas black arrow points to *rpb5-DAmP* mutant, which exhibit long telomeres. The DAmP alleles of *NOG2*, *USO1* and *CCT4* do not show *tlm* phenotypes. (**B**) *tlm* DAmP alleles sorted by relative length. The telomere length of all the *tlm* DAmP alleles was measured and compared to the wild-type length (here defined as 1.0). The wild-type telomere length is about 350 bp.

spliceosome after completing the reaction (Prp22, Prp43 and Ntr2). Notably, Prp22 and Prp43 (along with its auxiliary subunit Ntr2) are both DEAH-box RNA-dependent ATPase/ATP-dependent RNA helicases that mediate ATP-dependent mRNA release from the spliceosome, unwinding RNA duplexes (31,32). Thus, it appears that spliceosomal disassembly, but not assembly, or other stages of pre-mRNA splicing, affects telomere length. The significance of this observation is still unclear. Although pre-mRNA splicing is a global cellular mechanism, in yeast only about 250 genes carry introns, and specific effects of individual splicing components on the splicing of particular genes have been observed (33,34).

Transcription and RNA processing

Telomere length regulation requires a delicate balance between more then 270 nonessential genes (14,15) and close to 90 essential ones, as this paper suggests. Diminishing the RNA transcription or processing of Telomerase core components or their regulators may have an influence on telomere length. Several of these regulators may be present in limiting levels in the nucleus, and thus may be strongly affected by even slight variations in transcript levels. For example, previous work has shown that a subgroup of the *tlm* genes influences telomere length by affecting the abundance of *TLC1*, the telomerase RNA component (35). Two of the short *tlm* mutants identified in this study involve an RNA polymerase II subunit (Rpb7) or a regulator of RNA polymerase II activity (Fcp1) (36,37) (Table 1). Interestingly, mutations in a different RNA polymerase II subunit, Rpb5, lead to elongated telomeres (Table 2). In addition, we found that mutations in the *RNA14* and *RNA15* genes lead to a significant shortening of telomeres. *RNA14* and *RNA15* encode components of the cleavage and polyadenylation factor I (CF I) complex, required for mRNA end processing (38). It was previously shown that *RNA15* is required for poly(A) addition to the telomerase RNA (39). This gene plays also a role in the processing of TERRA (telomeric repeat-containing RNA), telomeric-specific transcripts of unknown function created by RNA polymerase II (40). Further work is needed to determine the precise mechanism by which mRNA processing affects telomere length.

The Arp2/3 complex

This complex is a highly conserved actin nucleation center required for the motility and integrity of actin patches (41). It is involved in endocytosis and in membrane growth and polarity processes. The Arp2/3 complex is a seven-protein complex containing two actin-related proteins, Arp2p and Arp3p, and five nonactin-related proteins, Arc15p, Arc18p, Arc19p, Arc35p and Arc40p. We have identified four of the genes encoding these proteins: *ARP2, ARP3, ARC15* and *ARC35* as short *tlm* DAmP mutants (Table 1). *ARC18* and *ARC19* were not present in our collection, and the DAmP allele of *ARC40* exhibited normal telomere length. Either this particular subunit is dispensable for the telomere-related function of the Arp2/3 complex, or, more likely, the DAmP allele of this gene is not tight enough to produce a visible phenotype. As in the case of the proteasome, it is likely that the whole complex is involved in a process that helps regulate telomere length. Among the many roles attributed to the Arp2/3 complex is the fusion of vacuoles during endocytosis, and internal cellular traffic (42). This may link the complex to the next category:

Vesicular traffic

One of the surprises that came out from the two genome-wide screens for nonessential *tlm* genes was that the largest category observed consisted of proteins involved in vesicular traffic (14,15). As expected, this category was also prominent among the essential genes, and included many proteins of the Golgi-ER traffic system. Surprisingly, however, whereas in the nonessential collection the *tlm* mutants in this category exhibited mainly short telomeres, among the DAmP collection most mutations (although not all) led to elongated telomeres (Tables 1 and 2). In this context, it is interesting to note that two members of the TRAPP complex, Trs20 and Trs23, involved in ER-Golgi traffic (43) exhibit opposite effects on telomere length (Tables 1 and 2). Several mechanisms have been proposed to explain how vesicular traffic may affect telomere length (16). For example, component(s) of the telomerase machinery may require vesicular traffic (in order to be modified, for example) to allow proper activity regulation (44). Alternatively, a yet-unknown structural relation may exist between vesicular traffic and telomere maintenance (e.g. through nuclear morphology). More experiments are required in order to solve this enigma.

Nucleolar proteins and rRNA processing components

A number of genes encoding proteins involved in nucleolar functions show a short telomere phenotype when mutated (Table 1). These include *POP7*, a member of the RNase MRP (45), and *TSR1* and *FAL1*, both required for the processing of rRNA. Additional nucleolar proteins include *NET1*, a subunit of the RENT complex, *NOC4*, important for the maturation and nuclear export of 40S ribosomal subunits, and *MTR3*, encoding a nucleolar nuclease that also plays a role in export of ribosomal subunits. Finally, YDR396w is a dubious ORF, but neighbors *UTP5*, a subunit of the SSU processome complex, involved in production of 18S rRNA and assembly of small ribosomal subunits (46). Interestingly, not all the proteins involved in rRNA processing, when mutated, give raise to short telomeres. Mutations in the *CBF5* and *NOP56* genes, both subunits of snoRNPs complexes with roles in modifying nucleolar RNA, produce elongated telomeres. *CBF5* encodes the catalytic subunit of a pseudouridine synthase enzyme, which modifies box H/ACA-containing small nucleolar ribonucleoproteins. *NOP56* is a component of the box C/D-containing snoRNP complexes and directs 2′-*O*-methylation of pre-rRNA during its maturation (46).

Telomerase functions as a ribonucleoprotein, containing an RNA moiety (7). In higher eukaryotes, telomerase RNA includes sequences of the box H/ACA snoRNA family and associates with box H/ACA core snoRNP proteins (47). In yeast, telomerase RNA is not a box H/ACA snoRNA, but rather associates with Sm proteins (48). This suggests that either an Sm-binding site or a box H/ACA motif can supply the functions required for this RNA, such as stability, localization and association with various factors. Our identification of mutants in genes encoding the snoRNA modifying enzymes Cbf5 and Nop56 as having a 'long Tlm' phenotype supports this hypothesis. Further support is provided by a recent study, which found that the non-essential snoRNA methyltransferase Tgs1 plays a role in the hypermethylation of yeast telomerase RNA (49).

Ubiquitin and SUMO

Interestingly, our screen uncovered both the gene encoding ubiquitin (*RPS31*) and the one encoding SUMO (*SMT3*). Mutations in these two essential protein modifiers lead to elongated telomeres. A similar phenotype was seen when nonessential components of the SUMO machinery were analyzed for telomere length [e.g. *NFI1/SIZ2* and *SIZ1* (50)].

However, mutations in known nonessential members of the ubiquitin-modifying machinery [e.g. *RAD6, BRE1* (15)], as well as mutations in the proteasome/SCF machinery required for ubiquitin-mediated protein degradation (Table 1), result in telomere shortening. This suggests that the *RPS31* mutation may play a regulatory role on ubiquitin metabolism. Alternatively, it is possible that the phenotype observed is due to a role of the ribosomal protein that is co-translationally synthesized with the ubiquitin moiety (51). Most nonessential proteins of the small subunit of the ribosome cause telomere elongation when deleted (14,15).

RSC complex

Two essential components of the RSC complex, involved in chromatin remodeling, Rsc4 and Rsc8, were uncovered as long telomere *tlm* mutants by our screen. RSC is an abundant complex composed of 10 essential and seven nonessential proteins, with roles in transcription regulation, sister chromatid cohesion and genomic stability (52). Deletion of genes encoding other nonessential components of the RSC complex (*HTL1, NPL6, LDB7*) led

to the same long *tlm* phenotype (14,15). Interestingly, a recent publication (53) that analyzed gene expression in the absence of RSC subunits found increased levels of transcription of genes involved in ER-Golgi traffic. Moreover, RSC mutants show resistance to brefeldin A, a compound that promotes Golgi disassembly (53). Thus, the RSC complex seems to act antagonistically to the vesicular traffic pathway, consistent with the opposing *tlm* phenotypes of most participants in these pathways.

Additional genes

Not all genes could be grouped by their known function. As with previous studies, we find a number of mutants with clear telomeric phenotype, whose alleged function is apparently unrelated to telomere biology. Further work is required to find out whether these represent proteins playing several independent roles, or whether connections may be found between telomere length maintenance and seemingly unrelated cellular pathways. For example, two members of the APC/cyclosome, the ubiquitin binding machinery in charge of controlling cell cycle progression, have been found: *APC4* (Table 1) and *CDC16* (Table 2). Interestingly, these two members of the same complex also exhibit opposite effects on telomere length.

Epistasis analysis

In order to start organizing the large number of *TLM* genes uncovered into functional pathways, we began an epistasis analysis of the DAmP mutants identified in the screen. We crossed several of the mutants to known telomere-affecting mutants, and created double mutants (at this stage of the same type, long or short). The telomere length of the double mutants was compared to that of each single mutant parent (obtained from the same cross), after an extensive number of generations, to ensure that telomeres can reach their final length. In principle, three types of interactions are expected (54,55): (i) epistasis: the phenotype of the double mutant is as severe as that of the most defective parent, the most likely explanation is that the two affected genes act in the same pathway of telomere length maintenance; (ii) additivity: the double mutant appears as the sum of the phenotypes of each parent – this result suggests that the two mutants affect independent pathways; and finally, (iii) synergism: the double mutant is markedly more defective than what would be expected from an additive relation. This category is harder to interpret, but it is usually assumed that the mutants affect competing pathways.

Figure 3 shows representative results of this analysis. Mutation of *PRI1* causes a severe elongation of the telomeres, comparable to that seen in *rif1, rif2* or *pif1* mutants. The *pri1 rif1* double mutant is no more defective than the single mutants, indicating epistatic relations, whereas *pri1* is additive to *rif2* and synergistic to *pif1*. Similar results were seen for other mutants affecting DNA replication, suggesting that Rif1 may play a role in the coordination between chromosomal replication and telomerase addition. Interestingly, mutations in SUMO (*smt3*) show a similar pattern of interactions

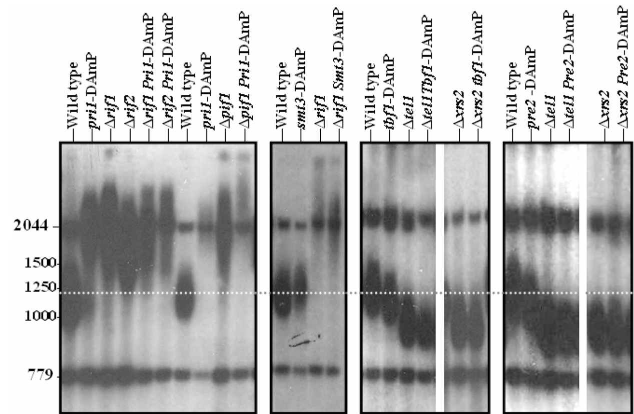

Figure 3. Representative Southern blots of telomere epistasis assay. The white horizontal line represents the average wild-type telomere length.

(Figure 3), indicating a possible role of SUMO modification in this pathway.

Figure 3 also shows an epistasis analysis of *pre2*, a component of the proteasome. It is possible to see that mutations in either *TEL1*, the yeast ATM ortholog (56), or in Xrs2 [a subunit of the MRX complex, (57)] are epistatic to proteasome defects, suggesting that the proteasome affects telomere length through the Tel1-MRX pathway (58). An attractive possibility is that a regulator in this pathway is degraded by the proteasome in a timely manner, to ensure proper telomere length maintenance. Alternatively, the effect may be indirect, as proteasome function may be needed for transcription of a regulator, or for signal transduction within the cell. Interestingly *tbf1*, a DNA-binding protein that plays roles in transcription and in silencing, but is also capable of telomere binding (59), affects telomere by the same pathway, as seen in Figure 3.Additional epistasis analysis of this kind should help us elucidate all the pathways affecting telomere length and their topology.

Comparison with previous screens

Examining the DAmP collection provides us with additional information on the regulation of telomere length, focusing on a set of genes, which were excluded on previous screens (14,15). Since the collection of genes analyzed in this paper and that of the combined previous studies are complementary and do not intersect, we used their relatedness in a protein-protein interaction network as a measuring rod to test whether the genes identified in the different studies are related. We discovered that proteins from the DAmP collection that exhibited a defect in telomere length show significantly more interactions with nonessential proteins discovered on the previous screens, compared with proteins from the DAmP collection that had no effect. Considering the entire available PPI data (see 'Materials and Methods' section), a protein from the library that exhibited a phenotype has on average 83% ($P < 1e$-5) more interactions with proteins discovered by Askree *et al.* (with an average of 0.9 interactions per protein with phenotype versus 0.49 interactions per protein

without phenotype) and 87% ($P = 1.1e$-5) more interactions with proteins discovered by Gatbonton *et al.* (with an average of 0.76 interactions per protein with phenotype versus 0.4 interactions per protein without phenotype). Considering only high confidence interactions ($P \geq 0.5$) we observe even more substantial differences: 89% more interactions ($P < 1e$-5) for the data set of Askree *et al.*, and 97% more for Gatbonton *et al.* ($P < 1e$-5, see 'Materials and Methods' section).

Revisiting the DamP methodology

Clearly, the DAmP collection is a useful tool for screening phenotypes in a genome-wide fashion. For example, the ts collection (18) requires one to grow each individual mutant at its highest permissive temperature, whereas DAmP mutants can be grown in batches at a single temperature. However, it is also clear that it has some disadvantages: first, as the *KanMX* insertion is likely to affect differently each individual gene, the collection is somewhat heterogeneous, with some genes being more affected than others. The exact level of expression of each hypomorphic allele in the collection is not known. For the same reason, only 739 haploid strains [out of the 1033 essential genes (20)] could be tested. This is likely due to the fact that in many cases, the 3′ UTR insertion destabilizes the transcript at a level that is below that required for survival. For example, although we obtained the two ORFs that flank the *CDC9* gene, encoding the yeast only replicative DNA ligase, the DAmP allele of that gene is apparently lethal.

Despite these disadvantages, the DAmP collection has allowed us to increase our knowledge on the cellular processes that affect telomere length regulation. A total of more than 350 genes, and possibly many more, affect the regulation of telomere length. Remarkably, many of these genes are additionally involved in a diverse array of other cellular functions (as evident from the large variety of processes involved), testifying to the extensively distributed nature of cellular processing in yeast. Somehow the cells are able to integrate this enormous amount of information into a final output that keeps length homeostasis constant. The mechanisms by which cells achieve such integration are the subject of intensive studies, and promise to unravel basic aspects of genetic expression and its regulation.

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