Distinct Roles of Non-Canonical Poly(A) Polymerases in RNA Metabolism

Salvatore San Paolo¹, Stepanka Vanacova², Luca Schenk³, Tanja Scherrer³, Diana Blank¹, Walter Keller¹*, André P. Gerber³*

¹ Department of Cell Biology, Biozentrum, University of Basel, Basel, Switzerland, ² National Center for Biomolecular Research, Masaryk University, Brno, Czech Republic, ³ Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zürich, Zürich, Switzerland

Abstract

Trf4p and Trf5p are non-canonical poly(A) polymerases and are part of the heteromeric protein complexes TRAMP4 and TRAMP5 that promote the degradation of aberrant and short-lived RNA substrates by interacting with the nuclear exosome. To assess the level of functional redundancy between the paralogous Trf4 and Trf5 proteins and to investigate the role of the Trf4-dependent polyadenylation in vivo, we used DNA microarrays to compare gene expression of the wild-type yeast strain of S. cerevisiae with either that of trf4Δ or trf5Δ mutant strains or the trf4Δ trf5Δ mutant expressing the polyadenylation-defective Trf4(DADA) protein. We found little overlap between the sets of transcripts with altered expression in the trf4Δ or the trf5Δ mutants, suggesting that Trf4p and Trf5p target distinct groups of RNAs for degradation. Surprisingly, most RNAs of which the expression was altered by the trf4Δ deletion were restored to wild-type levels by overexpression of TRF4(DADA), showing that the polyadenylation activity of Trf4p is dispensable in vivo. Apart from previously reported Trf4p and Trf5p target RNAs, this analysis along with in vivo cross-linking and RNA immunopurification-chip experiments revealed that both the TRAMP4 and the TRAMP5 complexes stimulate the degradation of spliced-out introns via a mechanism that is independent of the polyadenylation activity of Trf4p. In addition, we show that disruption of trf4 causes severe shortening of telomeres suggesting that TRF4 functions in the maintenance of telomere length. Finally, our study demonstrates that TRF4, the exosome, and TRF5 participate in antisense RNA-mediated regulation of genes involved in phosphate metabolism. In conclusion, our results suggest that paralogous TRAMP complexes have distinct RNA selectivities with functional implications in RNA surveillance as well as other RNA-related processes. This indicates widespread and integrative functions of TRAMP complexes for the coordination of different gene expression regulatory processes.

Introduction

Gene expression in eukaryotes depends on highly complex mechanisms for production of mature RNA molecules. Precursors of mRNAs, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nucleolar RNAs ( snoRNAs), and small nuclear RNA ( snRNAs) undergo stepwise processing and maturation, which includes 5'-capping, splicing, 3'-polyadenylation, endo- and exonucleolytic trimming, and base modifications. All these processes are error-prone and thus, RNA maturation has to be monitored by nuclear and cytoplasmic RNA quality control pathways to remove potentially harmful aberrant RNAs [1,2].

In the budding yeast Saccharomyces cerevisiae, nuclear RNA surveillance is mediated by the combined action of the Trf4p/5-Air1/2-Mtr4 (TRAMP) complex and the exosome that promote rapid degradation of nonfunctional RNAs [3–10]. The TRAMP complex consists of either one of the two paralogous non-canonical poly(A) polymerases Trf4p and Trf5p forming TRAMP4 and TRAMP5 complexes, respectively, the RNA binding proteins Air1 or Air2, and the RNA helicase Mtr4p [5–7]. In contrast to the canonical poly(A) polymerase Papl, which adds long poly(A) tails to the 3’-end of mRNAs that facilitates nuclear RNA export and increases the stability and translation of messages [11,12], the Trf proteins add short poly(A) tails to their substrate RNAs, which is assumed to trigger efficient decay of the RNAs by recruitment of the nuclear exosome complex [5–7].

Initially, Trf4 protein was identified as a key player in the surveillance and degradation of hypomethylated initiator tRNA ( tRNA(M) ) [4]. Further studies revealed more widespread roles of TRAMP complexes to assist the exosome-mediated degradation and trimming of several types of non-coding RNAs (ncRNAs) including precursors of rRNAs, tRNAs, snoRNAs, snRNAs, and of aberrant pre-mRNAs that are defective in 3’ end cleavage, splicing, or export to the cytoplasm [1,6–9,13–17]. Many of these RNA substrates are part of ribonucleoprotein (RNP) complexes and pre-ribosomes suggesting that most if not all newly synthesized nuclear RNPs are subject to quality control by TRAMP and the exosome. Another major class of potential RNA targets for TRAMP...
nuclear RNA degradation mutants such as [7]. CUTs are small, capped and fairly unstable transcripts that are complexes are the so-called ‘cryptic unstable transcripts’ (CUTs) [7]. CUTs are small, capped and fairly unstable transcripts that are expressed at such low levels that they can only be readily detected in nuclear RNA degradation mutants such as np64. Originally detected in some intergenic regions (IGRs) [7], the recent systematic exploration of CUTs by RNA sequencing and tiling arrays suggests the existence of hundreds of CUTs that preferentially originate from nucleosome-free 5’ promoter regions, or from the 3’-ends of protein-coding genes [18,19]. However, whether these CUTs have a biological role, or merely reflect transcriptional noise made from nucleosome-depleted regions is not known [18,19].

Most studies investigating the functions of TRAMP complexes focused on TRAMP4 and much less is known about TRAMP5 [20,21]. TrfHp and TrfSp share 56% amino-acid sequence similarity and loss of both pol(A) polymerases is lethal [22]. The conditional depletion of TrfSp in trf4Δ mutant cells increases the steady-state levels of specific RNAs, such as the 3’-extended forms of U114 snRNA, the 23S pre-rRNA and the CUT NEL025c that accumulate in either single mutant indicating that TrfHp and TrfSp have at least partially overlapping substrate specificities in vivo [7,15,20,23].

Besides RNA quality control and processing, Trf proteins may also participate in DNA-related processes. Originally, TRF4 was identified in a screen for mutations that are synthetically lethal with top1, which encodes the DNA topoisomerase I [24]. A top1 trf4-1 double mutant was defective in several mitotic events, such as sister chromatid cohesion, chromosome condensation at the rDNA loci, and chromosome segregation [22,24–26]. These defects were suppressed by overexpression of TRF5 suggesting that both TrfHp and TrfSp have roles in DNA metabolism and heterochromatin formation [22]. Moreover, TrfHp as well as the orthologous protein Cid14 in S. pombe stimulate the RNA-mediated silencing of heterochromatic transcripts and control rDNA copy numbers [24,27–32]. Hence, it was postulated that RNA-mediated recruitment of TrfHp and TrfSp may promote chromatin remodeling through regulation of histone modifying enzymes at specific chromatin loci [23].

Although the above mentioned studies revealed some substrates and functions for complexes containing Trf4 (TRAMP4) and Trf5 (TRAMP5), a comprehensive view of the substrate specificities and potential functional implications of the different TRAMP complexes is still lacking. We therefore wished to obtain a global picture of the RNA substrates that are regulated by the TRAMP4 and TRAMP5 complexes. To this end, we have used DNA microarrays to systematically map the RNA targets of TrfHp and Trf5. Surprisingly, we found that the different TRAMP complexes per se regulate only marginally overlapping sets of RNAs in the cell. Furthermore, the polyadenylation-defective form of TrfHp (TrfHp-DADA) suppressed most of the altered expression pattern as seen in the trf4Δ mutant cells suggesting that the TRAMP polyadenylation activity is not essential for RNA regulation. We further demonstrate that TrfHp and to a lower extent TrfSp promotes the degradation of a group of introns through an exosome-dependent but polyadenylation-independent mechanism. Moreover, TrfHp but not TrfSp stimulates RNA degradation mechanisms that are functionally linked to telomere maintenance and to antisense RNA-mediated regulatory pathways of gene expression. These results suggest widespread and distinct roles of different TRAMP complexes in the regulation of gene expression.

Results

Trf4p and Trf5p Modulate the Expression of Different Sets of Genes

TRAMP complexes promote the exosome-assisted degradation of diverse ncRNAs and aberrant or nonfunctional RNAs [4–7,14–16]. To identify additional specific RNA targets for the TRAMP4 and TRAMP5 complexes, we measured the relative changes of gene expression of S. cerevisiae cells lacking either trf4 (trf4Δ) or trf5 (trf5Δ) compared to wild-type (WT) cells using yeast oligo microarrays that contained features representing all annotated yeast ORFs, ncRNAs, introns, rRNA precursors, as well as some intergenic regions (IGRs) and tiled regions downstream of a few genes (see Materials and Methods). To this purpose, total RNA isolated from exponentially growing cells was reverse transcribed with a mixture of random nonamers and oligo(dT) primers. Cy5 fluorescently labeled cDNAs derived from total RNA isolated from either the trf4Δ or the trf5Δ mutants were then competitively hybridized with Cy3 labeled cDNAs from WT cells. To define a list of arrayed features determining transcripts that significantly changed expression in the trf4Δ, the trf5Δ and the trf4Δ/Trf4pΔ/Trf5Δ (DADA) mutants (which are explained below), we arbitrarily selected those features that changed relative expression at least 2-fold (average of three biological replicates) with false discovery rates (FDRs) of less than 5% [33] (Figure 1A; a list of the selected features is provided in Table S1). Similar results were obtained by statistical analysis with Cyber-T [34] followed by selection of those features with a p-value of less than 0.05 [34] (for a comparison of FDRs and p-values see Table S2). To further visualize the relation among the 715 features selected by this analysis, we hierarchically clustered the features and experiments (Figure 1A). To identify common themes among the differentially expressed mRNAs in the trf4Δ and trf5Δ mutants, we searched for common Gene Ontology (GO) annotations among the 550 transcripts for which GO annotations were available at the Saccharomyces Genome Database (SGD; Table S3).

Not surprisingly, most of the 715 selected features showed increased steady-state levels in trf mutants (691 features, 97%), which is in agreement with the idea that TrfHp and TrfSp promote RNA degradation and their depletion hence leads to the accumulation of RNAs that are normally targeted by these proteins (Figure 1A). More interestingly, although deletions of trf4 and trf5 are synthetically lethal, which may suggest common
functions and targets, we found that the vast majority of the affected transcripts overlapped only marginally though significantly (Figure 1B). Only 33 transcripts changed expression in both the trf4Δ and the trf5Δ mutants; among these, 27 transcripts were selectively increased (Figure 1B) and represented rRNA processing intermediates, mRNAs encoding chaperones (SSB1, HSP130, and TIR2) or enzymes involved in glucose metabolism (TDH2, TDH3, ADH2, and PDC1). These results suggest that trf4 and trf5 specifically affect very different groups of transcripts in vivo. However, we wish to point out that the comparison of gene expression profiles of single trf4 and trf5 deletion mutants may not reveal the full spectra of the in vivo targets; particularly in cases where either functional Trf4 or Trf5 proteins can fully substitute the absence of the other paralog.

Consistent with previous reports [6,7,14–16], 72 of the 422 features (17%) that accumulated at least 2-fold in the trf4Δ mutant were ncRNAs, such as snoRNAs (27 features) and RNAs derived from intergenic regions (IGRs; 20 features), or autonomously replicating sequences (ARSs; 4 features; Figure S1A, Figure S2, and Table S1). Interestingly, 13 of the 20 IGRs overlap with CUTs that have been recently mapped by massive sequencing of RNAs bound to the nuclear cap-binding protein Cbp20p isolated from conditional trf4Δ np6Δ double mutants [10, or that were accumulated in np6Δ mutants and identified with tiling arrays [19]. (A comparison of selected IGRs and CUTs is provided in a separate worksheet of Table S1.)

Ty1 retrotransposons represented the second most abundant class of transcripts with altered expression in the trf4Δ strain (Figure S1), as 68 out of the 98 probes specific for TyA and TyB exhibited an average 4-fold increase in relative expression levels compared to the WT strain (Figure S3A). This result was further confirmed by quantitative real-time PCR (qRT-PCR) analysis with primers specific for a sequence overlapping the TyA and TyB boundary (Figure S3B). In contrast to trf4Δ cells, the expression of the Ty1 elements was slightly decreased in the trf5Δ mutant (~1.5-fold) and unchanged in cells lacking the exosome subunit Rrp6p (np6Δ) (Figure S3B). Ty1 transcription and transposition is regulated by the trans acting antisense regulatory RTL-RNA that is transcribed divergently to TyA from an internal promoter and is degraded by the 5′ to 3′ exoribonuclease Xrn1p [27]. In agreement with a previous report [27], we found that the steady state levels of RTL-RNA were unaffected in np6 mutants. Conversely, RTL-RNA levels were slightly increased (>1.5-fold) in the trf4Δ mutant and decreased (~1.5-fold) in the trf5Δ mutant (Figure S3B). Since both the Ty1 elements and the negative regulator RTL-RNA were simultaneously increased in the trf4Δ mutant and decreased in the trf5Δ mutant, but remained unchanged in the exosome mutant np6Δ, we speculate that the TRAMP/exosome pathway is not involved in either the degradation of the TyA and TyB mRNAs or of the antisense regulatory RTL-RNA. However, the opposite effect of trf4 or trf5 deletions on the levels of the TY1- and RTL-RNAs suggests that TRAMP4 and TRAMP5 likely act through a yet uncharacterized mechanism to regulate the expression of the TY1 locus.

Deletion of TRF5 caused the accumulation of only 11 ncRNAs (4%) out of the 269 features (representing 220 GO annotated genes) for which we measured significantly altered expression levels (Figure S1B). This includes one snoRNA (SNR668), and four IGRs likely representing two CUTs (CUT857, CUT195) and the stable untranscribed transcript SUT180 [19]. (A comparison of selected IGRs and CUTs is provided in a separate worksheet of Table S1.)

Conversely, RTL-RNA levels were slightly increased (~1.5-fold) in the trf4Δ mutant and decreased (~1.5-fold) in the trf5Δ mutant (Figure S3B). Since both the Ty1 elements and the negative regulator RTL-RNA were simultaneously increased in the trf4Δ mutant and decreased in the trf5Δ mutant, but remained unchanged in the exosome mutant np6Δ, we speculate that the TRAMP/exosome pathway is not involved in either the degradation of the TyA and TyB mRNAs or of the antisense regulatory RTL-RNA. However, the opposite effect of trf4 or trf5 deletions on the levels of the TY1- and RTL-RNAs suggests that TRAMP4 and TRAMP5 likely act through a yet uncharacterized mechanism to regulate the expression of the TY1 locus.

Deletion of TRF5 caused the accumulation of only 11 ncRNAs (4%) out of the 269 features (representing 220 GO annotated genes) for which we measured significantly altered expression levels (Figure S1B). This includes one snoRNA (SNR668), and four IGRs likely representing two CUTs (CUT857, CUT195) and the stable untranscribed transcript SUT180 [19]. (A comparison of selected IGRs and CUTs is provided in a separate worksheet of Table S1.)
episomally expressed in \textit{trf4A} cells under the control of the \textit{NOP1} promoter (see Materials and Methods). Trf4p-DADA contains two aspartate to alanine mutations in the poly(A)/polymerase catalytic site which renders the enzyme inactive [5]. Similar to the microarray experiments with the \textit{trf4A} and the \textit{trf4A} mutants, we compared transcript levels of the \textit{trf4A}/\textit{TRF4-DADA} mutant to that of the WT strain harboring the empty vector (BY4741/\textit{nNOPPATA1}).

Surprisingly, the expression levels of more than 90% of transcripts that were significantly altered in \textit{trf4A} mutant cells were almost fully restored to WT levels by the overexpression of the \textit{TRF4-DADA} allele (Figure 1, Figure S1C). Only 37 transcripts were more than 2-fold enriched (FDR<5%) in cells expressing the \textit{TRF4-DADA} allele as compared to WT (Figure 1B). 18 of them (32%) are ncRNAs (Figure S1C). Although the relative abundance of most SGD annotated rRNA intermediates, snoRNAs, and IGRs was reduced in \textit{trf4A}/\textit{TRF4-DADA} compared to the \textit{trf4A} mutant cells (Table S1), 31 ncRNAs (7 rRNA intermediates, 15 SNRs, and 9 IGRs) still exhibited increased steady-state levels (on average between 1.5-fold and 4-fold; FDR<5%) relative to the WT cells (Figure S2, Table S1). Similar results were found for the RNA component \textit{RME1} of RNase MRP and for a group of short dubious ORFs (\textit{YJL047C-A}, \textit{YBR072C-A}, \textit{YGR121W-A}, and \textit{YBR182C-A}) that were also enriched in \textit{np6A} mutants [19] and, hence, most likely do not encode proteins but rather correspond to CUTs. Some of these ncRNAs are highly abundant such as rRNAs and snoRNAs. It therefore appears that highly expressed and structured RNAs strongly depend on the polyadenylation activity of Trf4p although they represent only a minor fraction (10%) of Trf4 targets. However, this fraction may recruit a considerable amount of Trf4 complexes in vivo and thus, a substantial fraction of the total RNA turnover mediated by Trf4p may depend upon Trf4 catalytic activity.

\textbf{Trf4p Stimulates the Degradation of a Subgroup of Introns}

In addition to ncRNAs such as rRNA intermediates, snoRNAs, and IGR RNAs/CUTs, our microarray data showed that a group of introns, some of which containing snoRNAs, were specifically accumulated (2- to 8-fold; FDR<5%) in either the \textit{trf4A} mutant (Trf4-dependent introns) or the \textit{trf4A} mutant (Trf5-dependent introns; Figure 2A, Table S1). To rule out that accumulation of these introns simply reflects increased transcript levels of the pre-mRNAs, we compared the relative changes of intron abundance with that of the corresponding pre-mRNAs and mature mRNAs as revealed with arrayed probes that specifically detect intron-exon junctions and exons (Figure 2B and 2C). We found that unlike introns, the corresponding pre-mRNAs and mature mRNAs were not significantly changed in the \textit{trf4A} mutant compared to WT cells. Likewise, the cognate pre-mRNAs and mature mRNAs of the Trf4-dependent introns were also unchanged in the \textit{trf4A} and \textit{trf4A}/\textit{TRF4-DADA} mutants (Figure 2B, Table S1). Thus, this analysis indicates that only spliced-out introns, such as those of the \textit{RPS9A}, \textit{RPL7B}, and \textit{GCR1} genes, specifically accumulate in the \textit{trf4A} mutant. To validate this finding, we carried out qRT-PCR experiments with primers either specific for the introns, the pre-mRNAs, or the mRNAs of \textit{RPS9A}, \textit{RPL7B}, and \textit{GCR1}. Consistent with our microarray data, the levels of introns but not those of pre-mRNAs or mature mRNAs were increased in the \textit{trf4A} mutant compared to WT cells (Figure S4 and results not shown).

As Trf4p promotes the exosome-mediated degradation of targeted RNAs through its polyadenylation activity [1,5–7], we also analyzed the steady-state levels of introns in the \textit{np6A} exosome mutant and in \textit{trf4A}/\textit{TRF4-DADA} mutant cells. Similarly to what has been observed in the \textit{trf4A} mutant, introns accumulated in cells lacking \textit{np6} but no significant change in the expression of the pre-mRNAs and mature mRNAs of \textit{RPS9A}, \textit{RPL7B}, and \textit{GCR1} was detected (Figure S4 and data not shown). Conversely, overexpression of the \textit{TRF4-DADA} allele in \textit{trf4A} cells restored WT levels for nine of the 13 Trf4-dependent introns, or reduced their abundance to values slightly above 1.5 fold the WT levels (Figure 2A, Figure S4, Table S1, and results not shown). This indicates that the polyadenylation-defective Trh4-DADA protein also participates in the regulation of “normal” steady-state levels of introns in vivo.

Unlike to what we observed in the \textit{trf4A} mutant, however, the increased levels of the Trf5-dependent introns (Figure 2A; \textit{RPL16A-INT} and \textit{RPL40A-INT} in Figure S4) coincided with similar amounts of the related pre-mRNAs and mature mRNA transcripts (Figure 2C, Table S1, and data not shown), strongly suggesting that accumulation of introns in the \textit{trf3A} mutant reflects increased relative abundance of unspliced pre-mRNAs.

To test whether Trf3p could efficiently target spliced-out introns in the absence of Trf4p, we analyzed intron accumulation by qRT-PCR experiments upon conditional \textit{TRF4} or \textit{TRF5} depletion. In this experiment, total RNA was isolated from a \textit{trf4A} \textit{trf3A} double mutant strain complemented with a plasmid either expressing \textit{TRF4} or \textit{TRF5} under the control of the \textit{GAL1} promoter (for details see Materials and Methods). As shown in Figure 2D, an one hour shift of cells to media supplemented with glucose to repress expression of \textit{TRF4} or \textit{TRF5}, led to an enrichment of all the introns tested. Conversely, no change in the abundance of introns was observed in control experiments performed with total RNA purified from WT cells that were transformed with the empty vector (BY4741/pYC6-CT; results not shown).

Taken together, these results suggest that Trf3p likely promotes the exosome-mediated degradation of a group of spliced-out introns through a mechanism that is not dependent on polyadenylation. In addition, as depletion of \textit{TRF5} in \textit{trf4A} cells caused intron accumulation in vivo, we infer that Trf4p and Trf3p are functionally redundant for intron decay (Figure 2D).

\textbf{TRAMP4 Interacts with Introns In Vivo}

To identify RNAs associated with TRAMP4, we performed in vivo cross-linking and ribonucleoprotein-immunopurification experiments followed by microarray analysis of bound RNAs (X- RIP-Chip). Cells expressing recombinant tandem-affinity purification (TAP)-tagged Trf4 protein were cross-linked with formaldehyde, and Trf4-containing ribonucleoprotein complexes were recovered by affinity selection on IgG-coupled beads (see Materials and Methods). Cells expressing TAP-tagged Trf4 proteins fully restore Trf4 functions and were previously used to purify functional TRAMP complexes [5]. As a control for non-specifically enriched RNAs, the same experiment was done with untagged WT cells and cells expressing Fpr1-TAP, a peptidyl-prolyl-cis-trans-isomerase not expected to bind RNA. About 70% of Trf4-TAP and 60% of Fpr1-TAP was captured from the whole cell extract (WCE) as shown by dot-blot analysis (Figure 3A, left panel). Moreover, Air2p, a well-known component of the TRAMP4 complex [5,6], co-purified with crosslinked Trf4-TAP but was absent in control purifications performed with untagged WT cells (Figure 3A, right panel).

We isolated total RNA from extracts (input) and from the immunopurified samples and labeled cDNAs derived from the RNAs with Cy3 and Cy5 fluorescent dyes, respectively. The differentially labeled samples were mixed and competitively hybridized on yeast oligo arrays. In this assay, the ratio of the two RNA populations at a given array element provides a measurement for enrichment of the respective RNA with the TRAMP4 complex.
Figure 2. Trf4p promotes polyadenylation-independent degradation of introns. (A) Microarray data for 18 introns that changed more than 2-fold (FDRs < 5%) in trf4Δ or trf5Δ mutants. Overexpression of Trf4p-DADA in trf4Δ mutant cells reduced steady-state levels of most of these introns to WT levels. Microarrays are the same as shown in Figure 1. Introns containing snoRNAs (snR) are indicated. (B) Relative changes of the expression of RPL7B (left panel) and RPS9A (right panel) introns, pre-mRNAs, and mature mRNAs as revealed by exon (ex), intron (int), and intron-exon junction probes (pre) present on the microarrays. Int-1 and int-2 refer to the first and the second intron of the RPL7B gene; pre-1 and pre-2 refer to the respective intron-exon junction probes of the RPL7B pre-mRNA. The height of the bar represents average log2 ratios from triplicate microarray data (Dataset S1); error bars show the standard deviation. (C) Relative changes of expression of RPL16A (left panel) and RPL40A (right panel) introns (int), pre-mRNAs (pre) and mature mRNAs (ex). Data was extracted from triplicate microarray data as described above. (D) Depletion of Trf4p or Trf5p promotes intron stability in vivo. Total RNA was purified from trf4Δ trf5Δ mutant strains either expressing TRF4 or TRF5, which were transcribed under the pGAL1 promoter. Cells were initially grown in a galactose containing medium (TRF4 Gal; TRF5 Gal) and then shifted to a glucose containing medium (YPD) at 30°C (TRF4 Glc; TRF5 Glc) for 1 h. Accumulation of introns of RPS9A and RPL40A and of the first intron of RPL7B was determined by qRT-PCR with intron-specific primers. The steady-state levels of introns in each sample was calculated as log2 of normalized ratios relative to the t₀ time point, which corresponds to the cultures immediately before the galactose to glucose shift (for details see Materials and Methods). The values represent averages from two independent qRT-PCR analyses.

doi:10.1371/journal.pgen.1000555.g002
Figure 3. Selective enrichment of introns in TRAMP4 affinity-isolates. (A) RNA–protein complexes were purified from extracts of cells expressing recombinant Trf4-TAP protein after in vivo cross-linking with formaldehyde (Trf4-TAP-X). Extracts of BY4741 cells and of cells expressing Fpr1-TAP were used as control (BY4741-X; Fpr1-TAP-X). Affinity purification of the Trf4-TAP protein complex or Fpr1-TAP from the WCEs was monitored by dot blot (left panel) and immunoblot (right panel) analyses with antibodies detecting the calmodulin-binding region of the TAP-tag (α-TAP) or the Air2p subunit of the TRAMP4 complex (α-Air2). Input and supernatant correspond to cross-linked WCEs before and after immunopurification, respectively. Affinity purified TAP-tagged Trf4 protein complex from non cross-linked WCEs was used as a positive control for the purification procedure (Trf4-TAP). A molecular weight marker is shown next to the immunoblot; * denotes likely Air2p degradation products. (B, C) Percentile rank analysis of immunopurified RNA preparations. RNAs enriched by TAP-tag affinity purification was comparatively analyzed to total RNA purified from WCEs with DNA microarrays. The enrichment profiles of introns (B) and the respective ORFs (C) were created by binning average percentile ranks from three biological replicates of Trf4-TAP or control IPs (two replicates of Fpr1-TAP and one untagged cells) into 0.05 unit bins. P-values to estimate the difference between Trf4 and control data were calculated with the Kolmogorov-Smirnov test. (D) Northern analysis with intron- and exon- specific probes in RNA surveillance mutants (trf4-ts, trf4-DADA, trf5-ts, and rrp6-ts) and in the debranching enzyme dbr1-ts mutant strain. Strains were grown at 30°C in YPD, except SC311 and trf4-ts/trf5-ts [25] that were shifted to 37°C for the indicated times prior to RNA extraction. doi:10.1371/journal.pgen.1000555.g003
Because of the relatively high variation of array data between biological replicates, we ranked ordered the data and determined percentile ranks for each analyzed feature (0, no enrichment; 1 high enrichment; Dataset S2).

In agreement with known functions of TRAMP4 on ncRNAs, we found that many small and stable ncRNAs such as snoRNAs and tRNAs were highly enriched in purified cross-linked TRAMP4 complexes. However, these transcripts were also strongly enriched in the control isolates and thus, only limited conclusions can be drawn from this analysis. Nevertheless, despite the high background from small ncRNAs in these experiments, we found that spliced-out introns were selectively enriched in the Trf4-TAP RNA isolates when compared to control isolates (Kolmogorov-Smirnov test; \( p = 2.5 \times 10^{-7} \); Figure 3B, data for all intron probes are shown in Table S4). The corresponding expression levels for 48 introns that were significantly changed in either the trf4A or trf5A mutants (Figure 2A) and for which X-RIP-Chip data were available, we found higher ranking with TRAMP4 complexes (Table S1, Figure 4B, and Figure S5A). In particular, the subtelomeric transcripts originate from telomeres that either contain (TEL02L, TEL04R, and TEL08R) or lack (TEL116) the Y` sequence element (Figure 4B). Similar to what has been reported previously [30], they are commonly oriented in the 5` to 3` direction towards the centromere (results not shown). Neither subtelomeric RNAs transcribed from the opposite strand towards the telomeres nor telomeric TERRA RNAs [41] were detected (results not shown). Although overexpression of TRF4-DADA reduced the abundance of these subtelomeric RNAs in trf4A mutant cells, their steady-state levels were still about 1.5-2.2 fold higher than in WT cells (Figure 4B). This decrease in the abundance of subtelomeric RNAs seen in the trf4A/TRF4-DADA mutant indicates that the polyadenylation activity of Trf4p may enhance the degradation of these RNA molecules in vivo (Figure 4B). Increased expression for two of these RNAs (YDR543C and YKL225W) was also found in strains deficient of trf5 as shown by qRT-PCR experiments (Figure 4B). Interestingly and in contrast to trf4A mutants, the trf5A mutant exhibited also changes in the relative expression of factors that positively (SIR2, SIR3, and MCM10) or negatively (SAD5) regulate chromatin silencing (Figure S5B). Thus, it could be that accumulation of subtelomeric RNAs in the trf5A mutant reflects defects in pathways other than RNA turnover.

Telomere Shortening Correlates with Increased TLC1 Expression in trf4A Mutants

To test whether any correlation existed between subtelomeric RNA accumulation and the structural integrity of telomeres, we performed Southern blot experiments with Xhol digested genomic DNA to determine the length of Y` containing telomeres (Figure 5A and 5B). Y` telomeres were on average shortened by ~120 bp in the trf4A mutant and ~40 bp in the trf5A exosome mutant (Figure 5A). Conversely, the length of Y` telomeres was similar to that of the WT strain in the trf5A mutant or in trf4A mutant cells complemented with a plasmid (pNOPPATA1L) carrying the WT allele of TRF4 (Figure 5A). Intriguingly, shortening of telomeres in the trf4A mutant was also strongly suppressed by overexpression of TRF4-DADA, where telomeres were about 40 bp shorter than in the WT strain (Figure 5B). These results showed that telomere shortening in trf4A cells is a reversible event achieved by reintroduction of episomally expressed Trf4p proteins. In addition, our results strongly suggest that Trf4p exerts a role in telomere maintenance mainly through a mechanism that is independent of polyadenylation.

The results from these Southern blotting experiments do not indicate any straightforward correlation between telomere shortening and accumulation of the subtelomeric RNA molecules. In fact, although subtelomeric RNAs were more abundant in the trf4pA mutant compared to the trf4A or trf4A/TRF4-DADA mutant strain (Figure 4B), only disruption of trf4 resulted in a severe shortening of the telomeres. Moreover, some subtelomeric RNAs (YDR543C and YKL225W; Figure 4B) also highly accumulated in the trf5A mutant, which did not show any recognizable change in telomere length (Figure 5A).

To investigate whether misregulation of the telomerase components could be the cause of the telomere shortening, we carried out qRT-PCR experiments with primers specific for the TLC1, EST1, EST2, and EST3 subunits (Figure 5C) [42,43]. In the
rp6Δ mutant, the steady-state levels for the mRNAs encoding these telomerase subunits were more than 2-fold increased compared to the WT strain, suggesting a consequent increase in the activity of the holoenzyme in this mutant. In contrast, only the TLC1 RNA was 2.6-fold increased in the trf4Δ mutant, whereas no change was detected for EST1, EST2, and EST3 mRNAs levels (Figure 5C). It was reported that overexpression of TLC1 causes telomere shortening in yeast because of the specific sequestration of the telomeric factors yKu70 and yKu80, which promote telomerase recruitment [42]. Thus, the imbalance in the expression level between TLC1 and the other subunits of the telomerase may in part account for the telomere shortening observed in the trf4Δ mutant. This hypothesis is further supported by the observation that overexpression of the TRF4-DADA allele not only suppressed the telomeric defect of the trf4A mutant, but also coincided with the restoration of expression of the TLC1 RNA subunit to WT levels (Figure 5C). Furthermore, these results indicate that Trf4p promotes TLC1 turnover through a polyadenylation-independent mechanism. Consistent with the proposed connection between TLC1 overexpression and telomere shortening, we found no change of TLC1 RNA abundance in the trf5Δ mutant (Figure 5C). It is noteworthy, however, that the unaltered telomere length found in the trf5Δ mutant might also reflect increased expression levels (>3-fold) of factors such as EST3, CST6, and MET18, which participate in the maintenance of telomeres in vivo (Figure 5C) [44].

Trf4p and the Exosome Promote Degradation of Antisense RNAs

Besides the many mRNAs for which relative expression levels were significantly increased in strains devoid of Trf4p or Trf5p, the expression of a few genes including those coding for proteins involved in phosphate metabolism (PHO3, PHO5, PHO11, PHO12, and PHO89) were significantly decreased (Table S1). The relative abundance of these mRNAs was almost fully restored to WT levels by the overexpression of the TRF4-DADA allele as shown by microarray and qRT-PCR experiments (Figure 6, Figure S6, and Table S1).

Such reduced expression of the PHO genes has also previously been observed in nuclear exosome mutants [16]. Particularly for PHO84 and PHO5, it was shown that Rrp6p affects the stability of corresponding antisense RNAs involved in the transcriptional control of their cognate sense mRNAs [19,29,45]. To assess whether the decreased levels of PHO5, PHO11, and PHO89 mRNAs in trf4A, trf5A, and rp6Δ exosome mutants correlates with increased levels of corresponding antisense RNAs, we carried out strand-specific qRT-PCR experiments with primers specific for antisense RNAs that span across the PHO promoter regions (Figure 6B, Figure S6). Antisense RNAs could be detected in all the strains tested, including the WT strain, however, their levels were more than 2-fold increased in trf4A and rp6Δ but not in trf5A mutants. The abundance of the antisense RNAs was decreased to WT levels by overexpression of TRF4-DADA (Figure 6B, data for PHO5 and PHO89 are shown in Figure S6). Therefore, similar to PHO84 [29], the expression of PHO5, PHO11, and PHO89 is likely modulated by antisense RNAs, the degradation of which is promoted by the exosome and the Trf4 protein, and does not require the polyadenylation activity of Trf4p. In addition, although loss of Trf5p did not cause any change in the expression of the PHO antisense transcripts, the reduced steady state level of the PHO5, PHO11, and PHO89 mRNAs suggests that Trf5p, through an yet unknown mechanism,
Figure 5. Trf4p promotes telomere maintenance and *TLC1* regulation through a polyadenylation-independent mechanism. (A, B) Southern blot analyses of Y'-type telomeres was carried out with *Xho*I-digested DNA of RNA surveillance mutants (*trf4Δ*, *trf4Δ/TRF4-DADA*, *trf5Δ*, and *rrp6Δ*) of *S. cerevisiae* probed with telomeric sequences. Mutant strains are indicated at the top: wt corresponds to the wild-type BY4741 strain; *trf4Δ/vector* represents the *trf4Δ* mutant strain transformed with pNOPPATA1L. DADA(1) and DADA(2) represent two different clones of the *trf4Δ/TRF4-DADA* mutant. A marker (M) for DNA sizes in kilobases is shown to the left. Solid black bars flanked by Y' shows the *Xho*I-digested Y' DNA fragments, where white bars refer to the position of average size fragments. Numbers in brackets refer to the average shortening of the Y'-containing telomeres in the *trf4Δ* and *trf4Δ/TRF4-DADA* mutants relative to wt. (C) qRT–PCR analysis examining the levels of specified mRNAs and ncRNAs (*TLC1*) in four RNA surveillance mutants (*trf4Δ*, *trf4Δ/TRF4-DADA*, *trf5Δ*, and *rrp6Δ*). RNA amounts were normalized to *ACT1* mRNA and compared to the isogenic WT strain. Relative changes of transcript abundances (log2 ratio scale) represent averages from two independent qRT-PCR analyses. The same RNA was used for the microarray analysis presented in Figure 1A.
doi:10.1371/journal.pgen.1000555.g005
may also participate with Trf4p and the exosome in fine tuning the expression of the \textit{PHO} genes.

**Discussion**

Identification of RNAs Regulated by Trf4p and Trf5p

Trf4p and Trf5p are non-canonical poly(A) polymerases that activate RNA turnover and quality control pathways by targeting aberrant and short-lived RNA substrates to the nuclear exosome for degradation [1,4–7,15,20]. Trf4 and trf5 are synthetically lethal and depletion of Trf5p strengthens the defects in RNA maturation of \textit{trf4} mutants, suggesting that Trf4p and Trf5p have partially overlapping functions in vivo [6,7,20]. To globally investigate the extent of functional redundancy and to systematically identify Trf4p- and Trf5p-specific RNA targets, we used microarrays to compare RNA expression profiles of \textit{S. cerevisiae} mutant strains lacking Trf4p or Trf5p with that of WT cells (Figure 1, Table S1). We found that almost all (>90%) of the 715 features that were at least 2-fold changed, were selectively increased in either the \textit{trf4}\textDelta or the \textit{trf5}\textDelta mutants. This finding is in agreement with known functions of these proteins in RNA degradation and their depletion is therefore expected to lead to the accumulation of RNA targets [1,5–7,20]. However, in contrast to the proposed functional redundancy of Trf4p and Trf5p, we found that \textit{trf4} and \textit{trf5} deletion affected barely overlapping sets of transcripts (Figure 1A). Such heterogeneity of the genes with altered expression was previously reported for different mutants of the exosome complex, possibly reflecting differential target specificities by the different subunits of the complex [16].

Interestingly, the \textit{trf4}\textDelta and the \textit{trf5}\textDelta mutants differed in the number of ncRNAs that accumulated in the cell. ncRNAs represented 17% and 4% of the transcripts that were selectively increased (>2-fold, FDR<5%) in \textit{trf4}\textDelta and \textit{trf5}\textDelta mutants, respectively. However, our microarrays cover only a fraction of the experimentally defined CUTs derived from intergenic regions (IGRs) [18,19]. Moreover, functional antisense RNAs are also not
detected with our oligo arrays including the antisense RNAs spanning the promoter region of different PHO genes (Figure 6, Figure S5) [29,45]. Nevertheless, application of qRT-PCR with antisense-RNA specific primers suggests that both TRAMP4 and TRAMP5 complexes as well as the exosome participate in RNA-mediated regulatory mechanisms to modulate the expression of several PHO genes [29] but that only TRAMP4 triggers the exosome-mediated degradation of regulatory antisense PHO RNAs in vivo.

In conclusion and consistent with previous reports [6,7,14–16], our experiments support a major role for Trf4p in the exosome-mediated degradation of mRNAs and suggest that TRAMP4 and TRAMP5 may function on specific subsets of RNAs in vivo. However, it remains to be further investigated how the TRAMP4 and the TRAMP5 complexes achieve specificity for their selective targets. TRAMP4 and TRAMP5 consist of structurally similar protein complexes [1,5,7], therefore specificity could be conferred by protein-protein interactions that are engaged by Trf4p or Trf5p and by the Air1p or Air2p subunits [5–8,20]. Misfolding of the RNPs or the association of proteins with aberrant RNAs may act as selectivity factors that eventually favor the recruitment of either TRAMP4 or TRAMP5 to the RNP target.

**The Polyadenylation Activity of Trf4p Promotes Degradation of a Subset of RNAs**

Several groups have previously demonstrated that the polyadenylation activity of Trf4p stimulates the exosome-mediated degradation of different RNA species in vivo and in vitro [4–6]. Consistently, RNA processing intermediates, snRNAs, snoRNAs, and a few CUTs accumulate as non-polyadenylated molecules in the *trf4 Δ* or the *trf5 Δ* mutant strains [6,7,14,15,20]. Intriguingly, even though polyadenylation activity is required for the degradation of highly structured RNAs in vitro, it was reported that a polyadenylation-defective form of Trf4p (Trf4p-DADA) can also activate degradation of RNAs by the exosome [9,30]. Moreover, a polyadenylation-defective *trf4* mutation can rescue the lethality of *trf4* and *trf5* double mutants [7]. These findings lead to a model, which proposes that the polyadenylation activity of Trf4p may not generally be necessary to guide RNA to the exosome for degradation. However, the universality of this model and whether there might be sets of RNAs that differentially depend on polyadenylation activity has not been addressed so far. Surprisingly, we found that Trf4p-DADA almost fully suppressed the altered gene expression profile of the *trf4 Δ* mutant upon overexpression (Figure 1, Table S1). This finding generally supports and extends the model introduced above: Since Trf4p-DADA only partially rescues the accumulation of selected RNAs in the *trf4 Δ* mutant, we suggest that the polyadenylation activity of Trf4p enhances the degradation of most target RNAs by the exosome, but this function is not essential. Polyadenylation in combination with the helicase activity of Mtr4p, which has a marked preference for binding to poly(A) RNAs [46], may be required for digestion of highly structured RNAs. This may be exemplified by the higher fraction of non-coding RNAs among the RNAs that remained accumulated in *trf4 Δ* mutants overexpressing TRF4-DADA (Figure S1).

However, additional mechanisms may account for the suppression of the *trf4* mutation by Trf4p-DADA. For instance, since the TRAMP complexes share common subunits, an intriguing speculation is that Trf4p-DADA, in the context of TRAMP4, recruits Trf5p to target RNAs. Trf5p then adds poly(A) tails to facilitate exosome-mediated degradation. In agreement with this idea is the finding that deletion of *trf5* in the polyadenylation-defective *trf4-236* mutant enhanced the defect in the degradation of CUTs compared to either single mutant [6]. Although this model could explain some of the observed effects in our system (Figure 2D), it cannot account for the observation that Trf4p-DADA rescues the lethality of *trf4* *trf5* double mutants [7].

**Trf4p and Trf5p Stimulate the Degradation of Introns**

Whereas the mechanism of splicing has been extensively investigated, very little is known about the degradation of spliced-out introns [38,39,47]. In this work, we showed by combined crosslinking-RNA-immunopurification experiments that TRAMP4 likely interacts directly with introns in vivo (Figure 3). We also provide experimental evidence supporting a role for TRAMP4 in the degradation of spliced-out introns, which is largely independent of the polyadenylation activity of Trf4p (Figure 2, Figure S4). However, we could not find a simple correlation between the introns that were highly associated with TRAMP4, and the relative changes of expression in single *trf4 Δ* or *trf5 Δ* mutants. Moreover, because the expression levels of some introns became exclusively affected in *trf4* *trf5* double mutants, Trf5p may promote the breakdown of introns in the absence of Trf4p suggesting functional redundancy between Trf4p and Trf5p in intron decay. Further experiments are required to unravel the contributions of different TRAMP complexes in intron decay and to delineate the exact extent of functional redundancy.

We envisage that after splicing, intron lariats are rapidly converted into linear forms by the debranching enzyme Dbn1p. Subsets of specific linear introns are then captured by TRAMP complexes to be eventually degraded by the nuclear exosome. Additional pathways may also exist, which involve the 5′ to 3′ exoribonuclease Rat1 and the endonuclease RnaseIII. In fact, lariats that contain RnaseIII binding sites can also undergo internal cleavage by RnaseIII irrespective of the Dbn1-mediated debranching, generating cleavage products that are eventually degraded by exoribonucleases [38].

**Trf4p Functions in Subtelomeric RNA Silencing and Telomere Maintenance**

Transcription at heterochromatin regions was recently reported to occur in *S. cerevisiae* and *S. pombe* cells that lack Trf4p or Rrp6p [30,48]. Consistent with these reports, we detected the accumulation of a number of RNAs originating from silent mating type cassettes and subtelomeric transcripts in the *trf4 Δ* and *rtp8 Δ* mutants, and to a lower extent in *trf5 Δ* cells (Figure 5, Figure S5). This activity is partially dependent on the polyadenylation activity of Trf4p and on a functional exosome (Figure 4B). Although further experiments are needed to elucidate how Trf4p and the exosome contribute to the silencing of heterochromatin domains, we hypothesize that during degradation of subtelomeric RNAs, TRAMP4, and the exosome modulate the interaction or the accessibility of chromatin remodeling factors such as Sir2 and Set1 [49,50] within sites of heterochromatin formation [49,50]. There is a growing body of evidence that suggests interactions of Trf4p and chromatin remodeling factors (reviewed in [23]).

Transcription of heterochromatin regions can regulate important physiological pathways. In *S. cerevisiae* and *S. pombe* strains with mutations in TRAMP or exosome components, accumulation of heterochromatic CUTs has been linked to changes in rDNA copy numbers [30,31]. Likewise, high levels of telomeric repeat-containing RNAs (TERRA) were shown to act in telomere maintenance in mammalian cells [51] and yeast [41]. In addition to alteration in the rDNA copy number [30], we discovered that the *trf4* mutant of *S. cerevisiae* exhibits a severe shortening of telomeres and that telomeres were only mildly reduced in the *rtp8 Δ* mutant (Figure 5A). Similarly to what was reported for the regulation of the rDNA repeats [30], telomere maintenance was
not strictly dependent on the polyadenylation activity of Trf4 (Figure 5B). Although accumulation of subtelomeric RNAs may perturb the chromatin integrity at the telomeres and negatively affect the telomerase activity, additional mechanisms probably account for the severe shortening of chromosome ends in the trf4Δ mutant. In fact, our results do not provide any strong evidence of a direct link between the extent of subtelomeric RNA accumulation and the severity of telomere shortening. Rather it emerged that the telomeric phenotype of the trf4Δ mutant can in part reflect imbalances in the expression level between the protein subunits Est1p, Est2p, Est3p, and the RNA component TLC1 of the telomerase. We propose that Trf4p stimulates the exosome-mediated degradation of TLC1 through a polyadenylation-independent mechanism. However, in contrast to what happens in cells defective in rpp6, trf4 deletion causes only high levels of TLC1, whereas the expression of EST1, EST2, and EST3 remains unchanged. It was previously demonstrated that recruitment of the telomerase holoenzyme is mediated by the heterodimeric Ku70/80 complex, which binds the chromosome ends and interacts with the telomerase via a small stem loop region of TLC1 [42]. Thus, the excess of TLC1 in the trf4Δ mutant could interfere with the recruitment of the telomerase at the chromosome ends and ultimately lead to telomere shortening.

To conclude, in this work we provide experimental evidence demonstrating that in addition to RNA surveillance, Trf4p and Trf5p participate in post-transcriptional regulatory networks that connect RNA degradation with DNA metabolism and gene regulation (Figure 7). Although the polyadenylation activity of Trf4p clearly enhances the efficiency of degradation of a broad variety of RNAs via the TRAMP4/exosome complex, expression of the Trf4 protein rather than its polyadenylation activity emerged to be essential for the maintenance of effective post-transcriptional regulatory pathways in the cell.

Materials and Methods

Yeast Strains and Plasmid Construction

Manipulations of S. cerevisiae strains were performed by standard procedures. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose), or in synthetic minimal medium (0.67% Bacto-yeast nitrogen base without amino acid, 2% glucose, and amino acid supplements as required) at 30°C. Temperature shifts to 37°C were done in a shaking water bath. Yeast strains are described in Table S5. The trf5Δ and rpp6Δ mutant strains were purchased from Open Biosystems. Replacement of the trf5 and the rpp6 genes with the kanMX6 cassette was confirmed by PCR following the manufacturer’s instructions. The strain with C-terminal TAP-fusion of FPR1 (YNJ135C) was purchased from BioCat (Heidelberg, Germany). The correct integration of the TAP-tag was verified by PCR. The trf4Δ strain is a derivative of BY4741 in which the trf4 gene was replaced with the natMX4 marker by homologous recombination as previously reported [52]; primer sequences can be provided upon request.

To complement trf4Δ with the wild type allele of trf4 the coding region of trf4 was PCR amplified from S. cerevisiae BY4741 genomic DNA with primers XmaI-trf4-Fw (5’-GTCCGGGCTATGTTGATTATTTAGAATC-3’) and trf4-Rev-Sall (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’) and restriction sites are underlined. The insert was cloned in pGEMT-easy vector (Promega), digested with XmaI and Sall restriction enzymes and ligated into the same sites on the pNOPPATA1l vector to generate pNOPPATA1l/jNop1::TRF4. Trf4Δ cells were transformed with pNOPPATA1l/jNop1::TRF4 and transformants were selected for leucine prototrophy in synthetic medium at 30°C. The correct integration of the TRF4 gene was verified by sequencing. Control trf4Δ/pNOPPAT1 and BY4741/ pNOPPATA1l strains were selected for leucine prototrophy onto synthetic medium after transformation of trf4Δ and BY4741 cells with the pNOPPATA1l plasmid.

To express trf4 or trf5 from an inducible pGAL1 promoter the coding regions of trf4 and trf5 were PCR amplified from S. cerevisiae BY4741 genomic DNA with primers SacI-trf4-Fw (5’-GTCGAGTCTCATATGGCTGCTTGATGGATATTTAGAATC-3’) and trf4-Rev-Xhol (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’) or BamHI-trf5-Fw (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’) and trf5-Rev-Xhol (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’) or BamHI-trf5-Fw (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’) and trf5-Rev-Xhol (5’-AAGGATCTGGATGATGAGTGGAGATGTTACC-3’). These fragments were cloned into pGEMT-easy vector, digested with SacI-Xhol or with BamHI-Xhol and ligated into the same sites on pYC6/CT (Invitrogen) to generate pSAL1 (pGAL1::TRF4) or pSAL2 (pGAL1::TRF5), respectively. trf4Δ trf5Δ mutant cells complemented with wild type TRF4 by the pRS416-TRF4 plasmid were successively transformed with either pSAL1 or pSAL2. Transformants were selected onto YPD supplemented with blasticidin (InvivoGen) 150 μg/ml at 30°C. To induce the loss of the pRS416-TRF4 plasmid, blasticidin resistant cells were inoculated three times on synthetic medium supplemented with 2% galactose, blasticidin (15 μg/ml) and 5-fluoro-orotic acid (1 μg/ml; Zymo Research) at 30°C. Transformation with pSAL1 or pSAL2 and loss of pRS416-TRF4 was confirmed by restriction digestion with SacI-Xhol or with BamHI-Xhol of plasmid DNA preparations purified from clones that were uracil auxotrophic, blasticidin resistant, and glucose sensitive.

RNA Purification

Total RNA was extracted with the hot phenol extraction method. Single yeast colonies were inoculated in 5 ml YPD or YPGal medium supplemented with the appropriate amount of selective drug (G418, 200 μg/ml; clonNat, 100 μg/ml) and incubated overnight at 30°C (pre-cultures). Pre-cultures were diluted to an OD600 of 0.1 in 50 ml of fresh YPD or YPGal medium without drugs and grown at 30°C to an OD600 of 0.7.

Figure 7. Combined view on the current knowledge of the different roles of the TRAMP4 complex. PHO: mRNAs encoding proteins involved in phosphate metabolism.
Cells were collected by centrifugation for 5 min at 3,000 g and resuspended in AE buffer (50 mM Na-aceate, 10 mM EDTA, pH 5.3) with 1% SDS. After adding one volume of phenol (pH 5.3), the suspension was vigorously shaken for 1 min and incubated at 65°C for 4 min in a heating block (Thermomixer comfort, Eppendorf). The aqueous phase was separated from the phenol phase by centrifugation at 20,000 g and extracted again with phenol (pH 5.3) and then with chloroform. Total RNA was precipitated from the aqueous phase by the addition of 2.6 volumes of ice-cold ethanol and of 1/10 volume of 1 M Na-aceate (pH 5.3) for 20 min on ice. The precipitated RNA was recovered by centrifugation at 20,000 g for 30 min at 4°C, the pellet was washed with 70% ethanol and resuspended in DEPC-treated water.

To remove contaminating DNA, RNA preparations were treated with DNA-freeTM (Ambion) according to the manufacturer’s instructions. The integrity of RNA samples was routinely checked by gel electrophoresis (1.2% agarose - 6% formaldehyde) in 1 x HEPES buffer (50 mM HEPES [pH 7.8], 10 mM EDTA) and RNA was quantified by UV-spectrophotometry (A260).

In Vivo Depletion of Trf4 or Trf5 Proteins

Single colonies of the trf4A trf5A/pSAL1 or of the trf4A trf5A/pSAL2 mutant strains were inoculated in YPGal liquid medium supplemented with blasticidin (150 μg/ml; InvivoGen) at 30°C and grown overnight. The following day, cultures were diluted to an OD600 of 0.1 in 300 ml of fresh YPGal medium and grown at 30°C to an OD600 of 0.7. Cultures were equally split into three tubes and cells were collected by centrifugation at RT for 5 min at 3,000 g. After centrifugation, one-third of the culture was rapidly frozen in liquid nitrogen and stored at −70°C (t0 time point); one-third was resuspended in YPGal and incubated at 30°C for 1 h (t1 Gal time point), while the rest of the culture was inoculated in YPD at 30°C for 1 h (t2 Gclidean point). Cells were collected by centrifugation and stored at −70°C. Total RNA was purified from the cell pellet as described above.

Microarray Analysis

DNA microarrays contained 10,944 oligo probes (70-mers) from the Array-Ready Oligo Set Version 1.1 representing 6,388 S. cerevisiae ORFs, and the Yeast Brown Lab Oligo Extension Version (YBOX vers. 1.0) with 3,454 probes to detect ncRNAs, rRNA precursors, introns, exon-intron and exon-exon junctions, other sequences predicted to be expressed, additional probes for genes with high cross-hybridization potential and controls for array quality measurements and normalization. Details of oligonucleotide selection and probe sequences are available from the Operon website (www.operon.com). All microarray data are available at the Stanford Microarray Database (SMD) or at the Gene Expression Omnibus (GEO) with accession number GSE16107.

The probes were printed on epoxy coated glass slides (Nexeterion slide E) at the Center for Integrative Genomics, University of Lausanne, Switzerland. Oligo arrays were blocked in 5 x SSC, 0.1 mg/ml BSA, 0.1% SDS for 1 h at 42°C, and subsequently washed three times in 0.1 x SSC for 5 min at RT, rinsed in water for 30 s, and dried by centrifugation (13,500 g for 2 min). The slides were used the same day.

Microarray analysis was performed by competitive hybridization of Cy3 and Cy5 fluorescein labeled cDNA. Total RNA (25 μg) was reverse transcribed with SuperScript RT II (Invitrogen) in the presence of 2.5 mM aminononyl-dUTP (Sigma) and dNTPs, with a 1:1 mixture of dT20 and random nonamer (N9) primers (5 μg of each, Sigma). After first strand cDNA synthesis, RNA was hydrolyzed with 0.1 M NaOH and 0.1 M EDTA at 65°C for 15 min, and samples were neutralized with 0.35 M HEPES (pH 8.0). Clean up of the reaction mix was performed in Microcon YM30 (Millipore) filled with distilled water. Amino-allyl containing cDNA was eluted with 100 μM NaHCO3 (pH 9.0) and covalently linked to either fluorescent Cy3 or Cy5 NHS-monoster (GE Healthcare). Thereby, cDNAs derived from wild-type control cells were labeled with Cy3, the ones derived from mutant cells with Cy5. Unincorporated dyes were removed with the QIAquick PCR Purification Kit (Qiagen). The samples were mixed in standard formamide based hybridization buffer (Orium Biosolution Hybridization Solution, Cat. No. 1150-000010) supplemented with 1 mg/ml poly(A) in a final volume of 20 μl, and competitively hybridized to yeast oligo arrays in a sealed hybridization chamber (Corning) at 42°C for 12–16 h. Arrays were successively washed in three buffer chambers filled with 2× SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0), 0.2% SDS; 2× SSC; and 0.2× SSC. The first wash was performed at 42°C for 12 min, the subsequent washes at RT for 12 min. After briefly rinsing in ethanol, microarrays were scanned with an Axon Instruments Scanner 4200A (Molecular Devices). Scanning parameters were adjusted to give similar fluorescent intensities in both channels. Data were collected with GenePix Pro 5.1 (Molecular Devices) and spots with abnormal morphology were excluded from further analysis. Array data were exported to Acuity 4.0 (Molecular Devices) and normalized to the mean of ratio of medians = 1 excluding the signals from control features.

We collected three biological replicates each for determining the relative changes of transcript levels in the trf4A, trf5A and trf4A/ TRF4-DADA mutant cells compared to the respective wild-type cells (total of 9 arrays). Data were filtered in Acuity for regression correlation (Rgn2>0.5), signal to noise ratio >2.5 in both channels, and only features that met these criteria in >60% of arrays were considered for further analysis (total 7481 features; Dataset S1). Data were exported into Microsoft Excel to determine percentile ranks and to perform SAM (version 3.0 [28]). We used the web interface for Cyber-T (http://cybert.microarray.ics.uci.edu/) to employ statistical analyses based on regularized t-tests that use a Bayesian estimate of the variance among gene measurements within an experiment [34].

The 715 unique features (9.5% of all analyzed features) that were on average at least 2-fold changed with an FDR<5% in either the trf4A, trf5A, or trf4A/TRF4-DADA replicates were compiled (Table S1). The genes and arrays were hierarchically clustered based on Pearson correlations with Cluster 3.0 [53] and the result was visualized as a heatmap with Java TreeView 1.0. [54] (Figure 1A). Commonly enriched GO terms among list of genes were retrieved with GO Term Finder that uses a hypergeometric distribution with Multiple Hypothesis Correction (i.e., Bonferroni Correction) to calculate p-values (SGD; www.yeastgenome.org).

Quantitative Real-Time PCR

qRT-PCR was performed with an ABI Prism 7000 Sequence Detection System (ABI Prism) and the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The first strand cDNA was synthesized with 5 μg of total RNA, 50 μM oligo(dT)20, 0.1 μM random hexamers and SuperScript RT III (Invitrogen). RNA was subsequently hydrolyzed with 125 mM NaOH and 10 mM EDTA at 65°C for 15 min. The mix was neutralized with 400 mM Tris-HCl (pH 8.0) and loaded onto a Microcon YM30 (Millipore) concentrator column filled with 10 mM Tris-HCl (pH 8.0). After centrifugation for 8 min at 13,500 g the microcon was filled again with 10 mM

RNA Targets of TRAMP Complexes

In Vivo
Tris-HCl (pH 8.0). This step was repeated twice. After elution, the cDNA was used as template for PCR with the following conditions: 95°C for 10 min; 40 cycles at 95°C for 15 s, and 60°C for 1 min. Transcript abundance was calculated as log of normalized ratios with the Pfaffel method of relative quantification [55]. Data were normalized to actin mRNA levels. (All primers used for quantitative real time PCR analysis and for strand specific reverse transcription are listed in Table S6.) Primer sequences for the strand specific synthesis of the antisense-Ty1 (RTL) cDNA and of the antisense-PHO3, -PHO11, and -PHO89 cDNAs can be provided upon request.

**In Vivo Cross-Linking and Ribonucleoprotein-Immunopurification-Chip Analysis (X-RIP-Chip)**

1 L of fresh YPD medium was inoculated with an overnight pre-culture of yeast cells (OD600 = 0.1) that were further cultured at 30°C to an OD600 = 0.7. RNAs and proteins were cross-linked with 1% formaldehyde that was added directly to the culture for 7 min at RT. The cross-linking reaction was quenched by the addition of 125 mM glycine (pH 7.0) for 5 min at RT. During cross-linking and quenching reactions the cultures were maintained under constant shaking at 100 rpm. Cells were harvested by centrifugation (1,500 g) at 4°C, washed twice with 50 ml of ice-cold PBS, and lysed in 5 ml of ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 140 mM KCl, 1.8 mM MgCl2, 0.1% NP-40, 0.1 mM DTT, 10% glycerol, 0.2 mg/ml heparin, 0.5 mM EDTA, 10 mM DTT, 140 mM KCl, 5% glycerol, 0.01% BSA). WCE was added to the blocked IgG beads and mixed on a rotator overnight at 4°C. TAP-tagged proteins were then recovered by spinning down the IgG beads at 72 g for 2 min at 4°C.

Cross-linked TAP-tagged proteins were captured from the WCE as follows: 300 μl rabbit IgG-coupled agarose beads (Sigma) were equilibrated at 4°C in lysis buffer supplemented with 5% BSA. WCE was added to the blocked IgG beads and mixed on a rotator overnight at 4°C. TAP-tagged proteins were then recovered by spinning down the IgG beads at 72 g for 2 min at 4°C. Beads were thoroughly washed three times with ice-cold lysis buffer supplemented with increasing concentrations of NaCl (100 mM, 200 mM, and 350 mM). RNP complexes were digested in 1 ml of elution buffer (50 mM Tris-HCl [pH 7.0], 0.1% SDS, 5 mM EDTA, 10 mM DTT, 140 mM KCl, 5% glycerol, 0.01% NP-40) with 100 μl Proteinase K (4 mg/ml) for 30 min at 37°C. Formaldehyde cross-linking was reversed by incubation of the eluate at 70°C for 45 min in a gently shaken heating block. Immuno purified RNA (IP-RNA) was isolated by extraction with PCI and isopropanol precipitation. The RNA pellet was washed twice with 70% ethanol and resuspended in DEPC-water.

For the microarray analysis of the IP-RNA, 5 μg of total RNA (input RNA) and 500 ng of IP-RNA were converted into Cy3 and Cy5 fluorescently labeled cDNA, respectively, and samples were competitively hybridized on yeast oligo arrays as described above. We collected data from three biological replicates with Trf1-TAP, from two replicates with Fpr1-TAP and from one untagged control (BY4741 strain) sample. Array data were filtered in Acuity for signal to noise ratio >3 for the channel with the input RNA (Cy3), and percentile ranks for filtered data were calculated based upon the log of the Cy3/Cy3 ratio in each experiment with Excel (Dataset S2).

**Northern Blot Analysis**

Northern blotting experiments were performed as previously described [56,57]. Briefly, 35 μg of total RNA in RNA loading buffer (50% formamide, 6% formaldehyde, 50 mM HEPES [pH 7.0], 0.25% xylene cyanol, 0.25% bromophenol blue, 10% glycerol) was loaded on a 1.5% agarose-6% formaldehyde gel and fractionated in 1× HEPES buffer (50 mM HEPES [pH 7.8]; 10 mM EDTA) at 50–60 Volts for 15 h. After washing of the gel in distilled water for 15 min, the RNA was partially cleaved with 75 mM NaOH for 15 min. The gel was neutralized in a solution comprised of 5 M Tris-HCl (pH 7.0) and 1.5 M NaCl for 15 min, and equilibrated in 10× SSC for 20 min. Capillary transfer of the RNA to Hybond-N+ membranes (Amersham) was performed in 10× SSC over night. RNA was UV-crosslinked to the membrane in a UV Stratalinker 1800 (Stratagene) with 1200 μJ.

DNA probes for hybridization were prepared by random incorporation of α-[32P] dATP with the Random Prime DNA Labeling Kit (Roche). Unincorporated α-[32P] dATP was removed by MicroSpinTM G-25 Columns (GE Healthcare). DNA templates for the preparation of the randomly labeled probes were produced by PCR amplification with primer pairs rps24A-int-Fw (5’-AGAAATHTGATGTTAAAAAGGTGTCAATGTG-3’) and rps24A-int-Rev (5’-CAGCCGGTCGACTGAGAAAAA C-3’) or rpl2B-int-Fw (5’-CGGATAATATGCGAATTGTTA GTAAGG-3’) and rpl2B-int-Rev (5’-GGAATAACTACTCTCCTTGTT AACATTAGG-3’) to detect the RPS24A and RPL2B introns, and primer pairs rps24A-ex-Fw (5’-CTGTGAGCTGTACTAC TATCGGTCTA-3’) and rps24A-ex-Rev (5’-AATCGCGGTCGTTAAGCGAATACCT-3’) or rpl2B-ex-Fw (5’-CAGACAGG TTAAGAACAGGTGTCT-3’) and rpl2B-ex-Rev (5’-GAAC CACGTAATAACGCGTTCCTTT-3’) to detect the RPS24A or RPL2B mRNAs. The DNA template for the preparation of the randomly labeled PGK7 probe was previously described [56]. Hybridization was carried out in rolling tubes in hybridization buffer containing 50% formamide, 5× SSPE (750 mM NaCl, 50 mM NaH2PO4, 5 mM EDTA, pH 7.4), 5× Denhardts solution, 1% SDS, and 200 μg/ml salmon sperm DNA according to standard procedures. The result of the hybridization was visualized with a Phosphor Imager.

**Western and Dot Blot Analysis of Immunopurified Protein Complexes**

Two μl of protein samples were directly spotted onto the nitrocellulose membrane (Whatman) for dot blot analysis. For Western blots, proteins were separated on 12% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with antibodies indicated in the figure legends. The anti-TAP antibodies were previously described [5]. To generate anti-Air2 antibodies a C-terminal fragment of Air2p (comprising amino acids 210–344 of Air2p) was cloned in pET22b (Novagen) and affinity purified on Ni2+-NTA agarose (Sigma) under denaturing conditions as described [5]. After further purification on reverse phase chromatography (GE Healthcare) in FPLC, approximately 100 μg of the purified protein was used for three injections into a rabbit (Eurogentec).
Peroxidase-conjugated swine anti-rabbit antibodies (DAKO) served as secondary antibodies for detection of the primary antibodies with the ECL Plus Western blotting detection system (Amersham).

Telomere Length Measurement

Telomere length measurement was carried out as previously described [44]. Genomic DNA was prepared from yeast cells grown in YPD and according to standard procedures. DNA from each strain was digested overnight with the restriction enzyme XhoI and fractionated by 1% agarose gel electrophoresis in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 40 Vols for 15 h. DNA was transferred to a Hybond-N+ membrane (Amersham) and Southern blot was performed by hybridization with a telomeric probe (26G; 5'-TCTGGGCTGTCGTTGTTG-3') that was end-labeled with \( \gamma^{32P} \) ATP and T4 polynucleotide kinase (Biolabs). All hybridizations were done in 200 mM Na2HPO4, 1 mM EDTA, 2% SDS, 1% BSA and 50 µg/ml salmon sperm DNA. The average telomeric length for each lane was estimated by a 1 kb DNA ladder (peqLab) that was run in a lane next to the XhoI digested genomic DNA. The ladder was probed with the same DNA ladder after \( \gamma^{32P} \) end-labeling.

Supporting Information

Figure S1 Classes of RNAs that accumulate in trf4, trf5, and trf4A/TRF4-DADA mutants. Pie chart classifying the transcripts with more than 2-fold (FDRs <5%) increased expression in the trf4A (A), the trf5A (B), and the trf4A/TRF4-DADA (C) mutant strains as determined by microarray analysis. Microarrays contained 10,944 oligo probes (70-mers) representing 6,388 snoRNAs, 274 IGRs/CUTs, 62 rRNA precursors, INTs, Ty1 retrotransposon elements in RNA surveillance mutants (sir4A, snr10, sno5A, snr27, sno45, snr48, snr49) and some IGRs (IGR67, IGR130) strongly accumulated in the trf4A mutant, but not in the trf5A mutant. In addition, most of the snoRNAs (SNR10, SNR11, SNR65, SNR2, SNR72, SNR45, SNR48, and SNR49) and some IGRs (IGR67, IGR130) showed an increase of more than 1.5-fold relative to WT cells.

Microarrays are the same as shown in Figure 1.

Found at: doi:10.1371/journal.pgen.1000555.s001 (2.19 MB TIF)

Figure S2 Expression profiles of SnoRNAs and IGRs (CUTs) in RNA surveillance mutants. Microarray analysis of trf4A, trf5A, and trf4A/TRF4-DADA (DADA) mutants showing relative changes for a sample of snoRNAs (14 out of 27; SNR2 and IGRs/CUTs) in RNA surveillance mutants (sir4A, snr10, trf4A/TRF4-DADA, trf5A, and snr6A). Relative changes of transcript abundances (log2, ratio scale) correspond averages from two independent qRT-PCR analyses. The RNA was also used for the microarray analysis presented in Figure 1A.

Found at: doi:10.1371/journal.pgen.1000555.s002 (1.66 MB TIF)

Figure S3 Retrotransposon Ty1 elements accumulate in the trf4A Mutant and are restored to wt levels by Trf4p-DADA overexpression. (A) Bar diagram representing relative changes of Ty1 retrotransposon RNAs as found by microarray analysis of the trf4A, the trf5A and the trf4A/TRF4-DADA (DADA) mutants. The values are averages of the levels of Ty1 retrotransposon transcripts as displayed by 68 out of 96 Ty1 retrotransposon probes showing more than 2-fold increase (FDR <5%) in the trf4A mutant. Microarrays are the same as shown in Figure 1. (B) Bar diagrams show the results of the qRT-PCR analysis for the Ty1 retrotransposon elements in RNA surveillance mutants (trf4A, trf4A/TRF4-DADA, trf5A, and snr6A). The scheme above the bar diagram represents the Ty1 retrotransposon locus: grey arrow (UP) indicates the position of the UP-Ty1 primer used for strand specific synthesis of anti-sense Ty1 (RTL) cDNA; convergent solid arrows (\( \gamma^{32P} \)TyF and \( \gamma^{32P} \)TyR) indicate the primer pairs anti-Ty-Fw and anti-Ty-Rv used for the quantification of the RTL cDNA; convergent grey arrowheads (TyF and TyR) show the location of Ty-Fw and Ty-Rv primers used for the quantification of the TyA/B cDNA. Consistent with the microarray analysis the expression of Ty1 retrotransposon is restored to WT levels by the overexpression of Trf4p-DADA in trf4A mutant cells. RNA amounts were normalized to ACT1 mRNA and are compared to the isogenic WT strain. Relative changes of transcript abundances (log2, ratio scale) represent averages from two independent qRT-PCR analyses. The RNA was also used for the microarray analysis presented in Figure 1A.

Found at: doi:10.1371/journal.pgen.1000555.s003 (1.39 MB TIF)

Figure S4 Intron expression profiles in RNA surveillance mutants. Bar diagrams show the results of qRT-PCR analysis for a group of introns (RPS9A-INT; RPL16A-INT, RPL7B-INT1, GCR1-INT, and RPL40A-INT) in RNA surveillance mutants (sir4A, trf4A/TRF4-DADA, trf5A, and snr6A). qRT-PCR analysis was performed with intron-specific primers. Overexpression of Trf4p-DADA in trf4A mutant cells abolished the accumulation of the first intron of RPL7B (RPL7B-INT1) and of GCR1 (GCR1-INT) and reduced by 3.6-fold (log2) the abundance of the intron of RPS9A (RPS9A-INT). RNA amounts were normalized to ACT1 mRNA and are compared relative to the isogenic wild-type strain. Relative changes of transcript abundances (log2, ratio scale) represent averages from two independent qRT-PCR analyses. The RNA was also used for the microarray analysis presented in Figure 1A.

Found at: doi:10.1371/journal.pgen.1000555.s004 (0.76 MB TIF)

Figure S5 Expression profiles of transcripts derived from the silenced HML/HMR cassettes and of genes involved in chromatin silencing. (A) Bar diagram representing the results of the qRT–PCR analysis for HML1x1 and ARS318 in RNA surveillance mutants (trf4A, trf4A/TRF4-DADA, trf5A, and snr6A). Whereas overexpression of Trf4p-DADA restored the abundance of HML1x1 to WT levels, ARS318 transcripts still exhibited a 2-fold increase in trf4A/TRF4-DADA mutant cells. Both HML1x1 and ARS318 RNA strongly accumulated in the snr6A mutant strain. RNA levels were normalized to ACT1 mRNA and compared to the relative expression in isogenic wild-type strain. Relative changes of transcript abundances (log2, ratio scale) correspond to the average from two independent experiments. The RNA was also used for the microarray analysis presented in Figure 1A. (B) Bar diagram representing the levels of SIR2, SIR3, SIR4, SIR5, and MCM10 mRNAs in the trf4A and the trf5A mutant strains quantified qRT-PCR. RNA amounts were normalized to ACT1 mRNA and are compared relative to the isogenic wild-type strain. Relative changes of transcript abundances (log2, ratio scale) represent averages from two independent qRT-PCR analyses. The same RNA was used for the microarray analysis shown in Figure 1A.

Found at: doi:10.1371/journal.pgen.1000555.s005 (1.14 MB TIF)

Figure S6 Expression profiles of PHO5, PHO89, anti-PH05, and anti-PHO89 RNAs in RNA surveillance mutants. Strand-specific qRT–PCR analysis examining the steady-state levels of PHO5, anti-PHO5 (A), PHO89, and anti-PHO89 (B) RNAs in RNA surveillance mutants (trf4A, trf4A/TRF4-DADA, trf5A, and snr6A). RNA amounts were normalized to ACT1 mRNA and compared relative to the isogenic wild-type strain. Relative changes of transcript abundances (log2, ratio scale) represent averages from
two independent qRT–PCR analyses. The RNA was also used for the microarray analysis presented in Figure 1A.

Found at: doi:10.1371/journal.pgen.1000555.s006 (1.15 MB TIF)

Table S1  List of genes with 2-fold altered expression (FDR < 5%) by deletion of either trf4 or trf5 or by overexpression of Trf4p-DADA in the trf4A mutant strain. Columns indicate the following (from left to right): Clone ID; gene name; systematic name; Probe sequence on the array (70-mer); GO annotations for biological process, function, and cellular compartment; Operon description of the gene product; average log2 ratio in trf4A mutants; average log2 ratios in trf4A/TRF4-DADA1 mutants; average log2 ratio in trf5A mutants; FDRs trf4A; p-value trf4A; FDR trf4A/TRF4-DADA; p-value trf4A/TRF4-DADA microarrays; FDR trf5A; p-value trf5A; p-value trf4A vs. trf4A/TRF4-DADA; cyberT test of trf4A vs. trf5A microarrays; FDR trf4A vs. trf4A/TRF4-DADA; FDR trf4A vs. trf5A microarrays.

Found at: doi:10.1371/journal.pgen.1000555.s007 (5.12 MB XLS)

Table S2  Statistics of the 2-fold changed features. Columns indicate the following (from left to right): mutant strain; number of features exhibiting 2-fold up- or downregulation; number of features exhibiting 2-fold up- or downregulation after CyberT test (p < 0.05); number of features exhibiting 2-fold up- or downregulation after SAM analysis (FDR < 0.05%).

Found at: doi:10.1371/journal.pgen.1000555.s008 (0.02 MB XLS)

Table S3  Significantly enriched GO terms among transcripts that are significantly increased in the trf4A and the trf5A mutants. Columns indicate the following (from left to right): Mutant strain; category; gene ontology (GO) term; number of genes with annotations; number of genes in the genome with annotation; p-value.

Found at: doi:10.1371/journal.pgen.1000555.s009 (0.02 MB XLS)

Table S4  Percentile ranks of introns from X-RIP-Chip data. Columns are the same as for Dataset S2. Changes of relative expression (average log2 ratios) in trf4A and trf5A mutants are shown in separate columns (values are taken from Dataset S1).

Found at: doi:10.1371/journal.pgen.1000555.s010 (2.57 MB XLS)

Table S5  Yeast strains used in this work. Columns indicate the following (from left to right): S. cerevisiae strain; genotype; reference.

Found at: doi:10.1371/journal.pgen.1000555.s011 (0.03 MB XLS)

Table S6  Oligonucleotide primer sequences for qRT–PCR analyses. Columns indicate the following (from left to right): Oligonucleotide name; target RNA; oligonucleotide sequence in the 5’ to 3’ direction.

Found at: doi:10.1371/journal.pgen.1000555.s012 (0.03 MB XLS)

Dataset S1  Normalized data from DNA microarray experiments with trf4A, trf5A, and trf4A/TRF4-DADA mutants. Columns indicate the following (from left to right): Oligo ID (Operon); gene name; systematic name; yeast ORF (compatible with SGD); GO annotations for process, function, and cellular compartment; Operon description of the gene product; log2 ratio trf4A mutants (triplicates); average log2 ratio trf4A mutants; log2 ratio trf4A/TRF4-DADA mutants (triplicates); average log2 ratio trf4A/TRF4-DADA mutants; log2 ratio trf5A mutants (triplicates); average log2 ratio trf5A mutants; FDRs trf4A microarrays; p-values trf4A microarrays; FDRs trf4A/TRF4-DADA microarrays; p-values trf4A/TRF4-DADA microarrays; FDRs of trf5A microarrays; p-values trf5A microarrays; p-values trf4A vs. trf4A/TRF4-DADA microarrays; p-values trf4A vs. trf5A microarrays; FDRs trf4A vs. trf4A/TRF4-DADA microarrays; FDRs trf4A vs. trf5A microarrays.

An annotation key to the oligos and a comparison of IGRs to recently mapped CUTCs [18,19] is given in separate worksheets.

Found at: doi:10.1371/journal.pgen.1000555.s013 (4.36 MB XLS)

Dataset S2  Percentile ranks from X-RIP-Chip data. Columns indicate the following (from left to right): Oligo ID (Operon); gene name; yeast ORF (compatible with SGD); GO annotations for process, function, and cellular compartment; Operon description of the gene product; percentile rank in Trf4 affinity isolations (3 biological replicates); average percentile rank of Trf4 affinity isolations; percentile rank of Fpr1 control affinity isolations (2 biological replicates); percentile rank of WT mock control isolations (BY4741); average percentile rank of control isolates (Fpr1, BY4741).

Found at: doi:10.1371/journal.pgen.1000555.s014 (1.99 MB XLS)

Acknowledgments

We are very grateful to Georges Martin, Christiane Rammelt, Sophie Jäger, and Mihaela Zavolan for discussion and comments on the manuscript. We thank Hubert Rehblauer from the Functional Genomics Center Zurich for help with statistical analysis and Joachim Liniger for generously communicating unpublished results.

Author Contributions

Conceived and designed the experiments: SSP SV WK APG. Performed the experiments: SSP SV LS. Analyzed the data: SSP LS APG.

Contributed reagents/materials/analysis tools: TS DB WK AG. Wrote the paper: SSP SV WK APG.

References

1. Vanacova S, Stefl R (2007) The exosome and RNA quality control in the nucleus. EMBO Rep 8: 651–657.
2. Houseley J, Tollervey D (2009) The many pathways of RNA degradation. Cell 136: 763–776.
3. Hilleren P, McCarthy T, Rosbash M, Parker R, Jensen TH (2001) Quality control of mRNA 3’ end processing is linked to the nuclear exosome. Nature 413: 538–542.
4. Kedzierska S, Kueger A, Trice T, Krecic AM, Himanen SG, et al. (2004) Nuclear surveillance and degradation of hypomodified initiator tRNAMet in S. cerevisiae. Genes Dev 18: 713–724.
5. Vanacova S, Wolf J, Martin G, Blank D, Dettwiler S, et al. (2005) A new yeast poly(A) polymerase complex involved in RNA quality control. PLoS Biol 3: e189. doi:10.1371/journal.pbio.0030189.
6. LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, et al. (2005) RNA degradation by the exosome is promotored by a nuclear polycadenylation complex. Cell 121: 715–724.
7. Wyers F, Rougemaille M, Badis G, Rousselle JC, Dufour ME, et al. (2005) Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell 121: 725–737.
8. Des C, Houssely J, Tollervey D (2008) Surveillance of nuclear-preced dedosines within a subnucleolar region of Saccharomyces cerevisiae. EMBO J 25: 1534–1546.
9. Rougemaille M, Gudiapati RK, Olesen JR, Thomsen R, Seraphin B, et al. (2007) Dissecting mechanisms of nuclear mRNA surveillance in THO/sub2 complex mutants. EMBO J 26: 2317–2326.
10. Martin G, Keller W (2007) RNA-specific ribonucleotidyl transferases. RNA 13: 1834–1849.
11. Edmonds M (2002) A history of poly A sequences: from formation to factors to function. Prog Nucleic Acid Res Mol Biol 71: 203–389.
12. Minvielle-Sebastia L, Keller W (1999) mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. Curr Opin Cell Biol 11: 352–357.
13. Kadaba S, Wang X, Anderson JT (2006) Nuclear RNA surveillance in Saccharomyces cerevisiae: TRF1p-dependent polyadenylation of nascent hypomethylated RNA and an aberrant form of 5S rRNA. RNA 12: 508–521.

14. Davis CA, Ares M Jr (2006) Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rep51p in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 103: 3262–3267.

15. Egecioglu DE, Henras AK, Chanfreau G (2006) Contributions of TRF1p and TRF2p-dependent polyadenylation to the processing and degradative functions of the yeast nuclear exosome. RNA 12: 26–32.

16. Houalla R, Devaux F, Fatica A, Kafé J, Barrass D, et al. (2006) Microarray detection of novel nuclear RNA substrates for the exosome. Yeast 23: 439–454.

17. Houseley J, Tollervey D (2008) RNA-quality control by the exosome. Nat Rev Mol Cell Biol 7: 529–539.

18. Neil H, Malabat C, d’Aubenton-Carafa Y, Xu Z, Steinmetz LM, et al. (2009) Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature 457: 1030–1042.

19. Xu Z, Wei W, Gagnier J, Poreco F, Clauder-Munster S, et al. (2009) Bidirectional promoters generate pervasive transcription in yeast. Nature 457: 1033–1037.

20. Houseley J, Tollervey D (2006) Yeast TRF5p is a nuclear poly(A) polymerase. EMBO Rep 7: 205–211.

21. Wery M, Ruidant S, Schillewaert S, Lepore N, Lafontaine DL (2009) The nuclear poly(A) polymerase and Exosome cofactor TRF5 is recruited cotranscriptionally to nuclear RNA surveillance. RNA 15: 406–419.

22. Castano IB, Heath-Pagliuso S, Sadoff BU, Fitzhugh DJ, Christman MF (1996) A cryptic unstable transcript mediates bidirectional promoter function, and functionally, suggesting a role for polymerase epsilon in sister chromatid separation. Nucleic Acids Res 24: 2404–2410.

23. Houseley J, Tollervey D (2008) The nuclear RNA surveillance machinery: The link between mRNA and genome structure in budding yeast? Biochem Biophys Acta 1779: 239–246.

24. Castano IB, Brzoska PM, Sadoff BU, Chen H, Christman MF (1996) Mitotic chromosome condensation in the rDNA requires TRF4p and DNA topoisomerase I. EMBO J 26: 4996–5006.

25. Edwards S, Li CM, Levy DL, Brown J, Snow PM, et al. (2003) Saccharomyces cerevisiae DNA polymerase epsilon and polymerase sigma interact physically and functionally, suggesting a role for polymerase epsilon in sister chromatid cohesion. Mol Cell Biol 23: 2733–2748.

26. Berretta J, Pinskaya M, Morillon A (2008) A cryptic unstable transcript mediates transcriptional trans-silencing of the Yl1 retrotransposon in S. cerevisiae. Genes Dev 22: 615–628.

27. Pandit S, Lynn B, Rymond BC (2006) Inhibition of a spliceosome turnover pathway suppresses splicing defects. Proc Natl Acad Sci U S A 103: 13700–13705.

28. Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, et al. (2004) A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc Natl Acad Sci U S A 101: 8658–8663.

29. Houseley J, Tollervey D (2008) The nuclear RNA surveillance machinery: The link between mRNA and genome structure in budding yeast? Biochem Biophys Acta 1779: 239–246.

30. Castano IB, Brzoska PM, Sadoff BU, Chen H, Christman MF (1996) Mitotic chromosome condensation in the rDNA requires TRF4 and DNA topoisomerase I. EMBO J 26: 4996–5006.

31. Wang Z, Castano IB, De Las Penas A, Adams C, Christman MF (2000) Pol kappas: A DNA polymerase required for sister chromatid cohesion. Science 289: 774–779.

32. Edwards S, Li CM, Levy DL, Brown J, Snow PM, et al. (2003) Saccharomyces cerevisiae DNA polymerase epsilon and polymerase sigma interact physically and functionally, suggesting a role for polymerase epsilon in sister chromatid cohesion. Mol Cell Biol 23: 2733–2748.

33. Berretta J, Pinskaya M, Morillon A (2008) A cryptic unstable transcript mediates transcriptional trans-silencing of the Yl1 retrotransposon in S. cerevisiae. Genes Dev 22: 615–628.

34. Buhler M, Spies N, Bartel DP, Moazed D (2007) RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatin gene silencing. Cell 131: 706–717.

35. Buhler M, Haas W, Gygi SP, Moazed D (2007) RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatin gene silencing. Cell 131: 706–717.

36. Camblong J, Iglesias N, Fickentscher C, Dieppeis G, Stutz F (2007) Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylase recruitment. J Biol Chem 283: 4930–4942.

37. Uhler JP, Hertel C, Swejstrup JQ (2007) A role for noncoding transcription in activation of the yeast PHO5 gene. Proc Natl Acad Sci U S A 104: 8011–8016.

38. Dichtl B, Blank D, Sadowski M, Hubner W, Weiser S, et al. (2002) Yhh1p/9h1p, an essential activator of the yeast PHO5 gene. Proc Natl Acad Sci U S A 103: 13700–13705.

39. Dichtl B, Blank D, Sadowski M, Hubner W, Weiser S, et al. (2002) Yhh1p/9h1p, an essential activator of the yeast PHO5 gene. Proc Natl Acad Sci U S A 103: 13700–13705.

40. Yamada M, Hayatsu N, Matsura A, Ishikawa F (1998) Y′-Help1, a DNA helicase encoded by the yeast subtelomeric Y′ element, is induced in survivors defective for telomerase. J Biol Chem 273: 33360–33366.

41. Luke B, Panza A, Redon S, Iglesias N, Li Z, et al. (2008) The Ratlp1p 5′ to 3′ Exonuclease Decreases Telomeric Repeat-Containing RNA and Promotes Telomere Elongation in Saccharomyces cerevisiae. Mol Cell 32: 465–477.

42. Peterson SE, Stellwagen AE, Diele SJ, Singer MS, Haimberger ZW, et al. (2001) The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. Nat Genet 27: 64–67.

43. Smogorzewska A, de Lange T (2004) Regulation of telomerase by telomeric proteins. Annu Rev Biochem 73: 177–208.

44. Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, et al. (2004) A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc Natl Acad Sci U S A 101: 8658–8663.

45. Pandit S, Lynn B, Rymond BC (2006) Inhibition of a spliceosome turnover pathway suppresses splicing defects. Proc Natl Acad Sci U S A 103: 13700–13705.

46. Saudubray JM, Reichenbach P, Khoriua L, Giulotto E, Lingner J (2007) The nuclear poly(A) polymerase and Exosome cofactor Trf5 is recruited cotranscriptionally to nuclear RNA surveillance. Yeast 23: 439–454.

47. Dror V, Winston F (2004) The Swi/Snf chromatin remodeling complex is required for ribosomal DNA and telomeric silencing in Saccharomyces cerevisiae. Mol Cell Biol 24: 8227–8235.

48. Dichtl B, Blank D, Sadowski M, Hubner W, Weiser S, et al. (2002) Yhh1p/9h1p, an essential activator of the yeast PHO5 gene. Proc Natl Acad Sci U S A 103: 13700–13705.