The EMBO Journal (2007) 26, 2317–2326 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE EMBO JOURNAL

Dissecting mechanisms of nuclear mRNA surveillance in THO/sub2 complex mutants

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The nuclear exosome is involved in numerous RNA metabolic processes. Exosome degradation of rRNA, snoRNA, snRNA and tRNA in *Saccharomyces cerevisiae* is activated by TRAMP complexes, containing either the Trf4p or Trf5p poly(A) polymerase. These enzymes are presumed to facilitate exosome access by appending oligo(A)-tails onto structured substrates. Another role of the nuclear exosome is that of mRNA surveillance. In strains harboring a mutated THO/Sub2p system, involved in messenger ribonucleoprotein particle biogenesis and nuclear export, the exosome-associated 3′→5′ exonuclease Rrp6p is required for both retention and degradation of nuclear restricted mRNAs. We show here that Trf4p, in the context of TRAMP, is an mRNA surveillance factor. However, unlike Rrp6p, Trf4p only partakes in RNA degradation and not in transcript retention. Surprisingly, a polyadenylation-defective Trf4p protein is fully active, suggesting polyadenylation-independent mRNA degradation. Transcription pulse–chase experiments show that *HSP104* molecules undergoing quality control in THO/sub2 mutant strains fall into two distinct populations: One that is quickly degraded after transcription induction and another that escapes rapid decay and accumulates in foci associated with the *HSP104* transcription site.

The EMBO Journal (2007) **26,** 2317–2326. doi:10.1038/sj.emboj.7601669; Published online 5 April 2007
Subject Categories: RNA
Keywords: mRNA surveillance; nuclear exosome; RNA degradation; RNA retention; TRAMP

Introduction

Processing of RNA polymerase II (RNAPII) transcripts and their assembly with proteins into export-competent messenger ribonucleoprotein particles (mRNPs) are error-prone processes challenged by nuclear quality control systems. Such control occurs early and sometimes even before mRNP release from the gene (Saguez *et al*, 2005). This is needed because most factors involved in mRNA processing, packaging and export act cotranscriptionally (for reviews see Jensen *et al*, 2003; Vinciguerra and Stutz, 2004; Buratowski, 2005; Fasken and Corbett, 2005). One example is provided by the *Saccharomyces cerevisiae* THO complex composed of four components (Hpr1p, Mft1p, Tho2p and Thp1p). The THO complex affects mRNP assembly and export via its interaction with mRNA export factors Sub2p and Yra1p in the TREX (TRanscription/EXport) complex and is required for efficient recruitment of Sub2p to nascent RNA (Strasser *et al*, 2002; Zenklusen *et al*, 2002). Deletion or mutation of any of the THO/TREX components results in rapid nuclear accumulation of polyadenylated mRNA and sequestration of heat-shock (hs)-RNAs, *HSP104* and *SSA4*, in transcription site-associated foci (dots) at elevated temperatures (Jensen *et al*, 2001a; Libri *et al*, 2002; Strasser *et al*, 2002; Zenklusen *et al*, 2002; Thomsen *et al*, 2003; Vinciguerra *et al*, 2005). This hs-RNP retention requires the 3′→5′ exonuclease Rrp6p, an auxiliary component of the nuclear exosome (Jensen *et al*, 2001a; Libri *et al*, 2002; Vinciguerra *et al*, 2005). Steady-state levels of full-length hs-RNAs are also strongly diminished in THO deletion and *sub2* mutant strains, a defect which is restored upon *RRP6* deletion (Libri *et al*, 2002). This suggests that in addition to its effect on retention of aberrant hs-RNP, Rrp6p is also involved in degrading hs-RNA when export is compromised or inefficient. The mechanistic relationship between these two functions of Rrp6p has not yet been elucidated.

In addition to mRNA quality control, Rrp6p and the nuclear exosome also participate in a wide range of RNA-degradative reactions, including processing and complete degradation of stable RNAs (rRNA, snoRNA, snRNA and tRNA) as well as degradation of so-called cryptic unstable transcripts (CUTs) (van Hoof and Parker, 1999; Mitchell and Tollervey, 2000; Butler, 2002; Kadaba *et al*, 2004; Wyers *et al*, 2005; Davis and Ares, 2006). Purified exosome only shows weak exonucleolytic activity *in vitro*, a feature that was recently used to identify an exosome activating complex coined TRAMP (Trf4p/Air2p/Mtr4p polyadenylation) (LaCava *et al*, 2005). The TRAMP complex harbors a poly(A) polymerase (Trf4p or its relative Trf5p) that belongs to the same polymerase family as the conventional Pap1p enzyme (LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005). However, Trf4p lacks the RNA-binding domain of Pap1p, an activity presumably provided by Air2p, or its relative, Air1p. Adenylation-triggered RNA decay is widespread in prokaryotes, and thus TRAMP is believed to ease exosome access onto difficult substrates (e.g. highly structured ones) by adding an unstructured tag (Dreyfus and Regnier, 2002; LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005). TRAMP-assisted exosomal decay might

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Received: 27 September 2006; accepted: 7 March 2007; published online: 5 April 2007

subsequently occur through multiple rounds of adenylation/degradation. Finally, it has been shown that Trf4p/Trf5p-dependent RNA degradation in eukaryotes is not only restricted to complete decay but also takes place in RNA processing pathways (Egecioglu *et al*, 2006).

Recently, Trf4p has been linked to nuclear processes involving authentic mRNAs. In yeast, removal of Trf4p increased levels of *NRD1* RNA, which in a wild-type (wt) context is kept low by autoregulated premature transcription termination of the *NRD1* gene (Arigo *et al*, 2006). Furthermore, after depletion of human Rrp6 (PM/Scl100) from HeLa cells, accumulation of mRNA degradation intermediates harboring adenylated 3′ends was observed reminiscent of TRAMP-mediated exosome decay (West *et al*, 2006).

In this paper, we present direct evidence that TRAMP complexes harboring Trf4p are involved in nuclear mRNA quality control in *S. cerevisiae*. However, in contrast to Rrp6p, Trf4p is not required for retention in nuclear foci of either the inducible *HSP104* RNA or the constitutively expressed *PDR5* RNA. Transcription pulse–chase experiments of the *HSP104* gene reveal a partitioning of molecules into a pool undergoing rapid decay and a pool of stable RNAs retained in transcription site foci. On the basis of our results, a model for nuclear mRNA surveillance is discussed.

Results

Polyadenylation-independent stimulation of HSP104 RNA degradation by Trf4p

To assess directly the involvement of Trf4p in nuclear mRNA surveillance, we deleted the *TRF4* gene in cells lacking either one of the two THO components Mft1p or Hpr1p, and in cells carrying the previously described temperature-sensitive (ts) *sub2-201* allele (Jensen *et al*, 2001a). Compared with a wt strain, *HSP104* RNA levels are severely decreased in *mft1*Δ, *hpr1*Δ and *sub2-201* single mutants, after a brief 15 min transcription induction at 37°C, as a result of Rrp6p-dependent degradation from the 3′end (Libri *et al*, 2002). We therefore used these conditions and conducted *HSP104* RNA Northern blotting and quantitative RT–PCR analysis utilizing a Northern probe and PCR primer pairs specifically targeting the *HSP104* 3′end (Figure 1A). Deletion of the *TRF4* gene

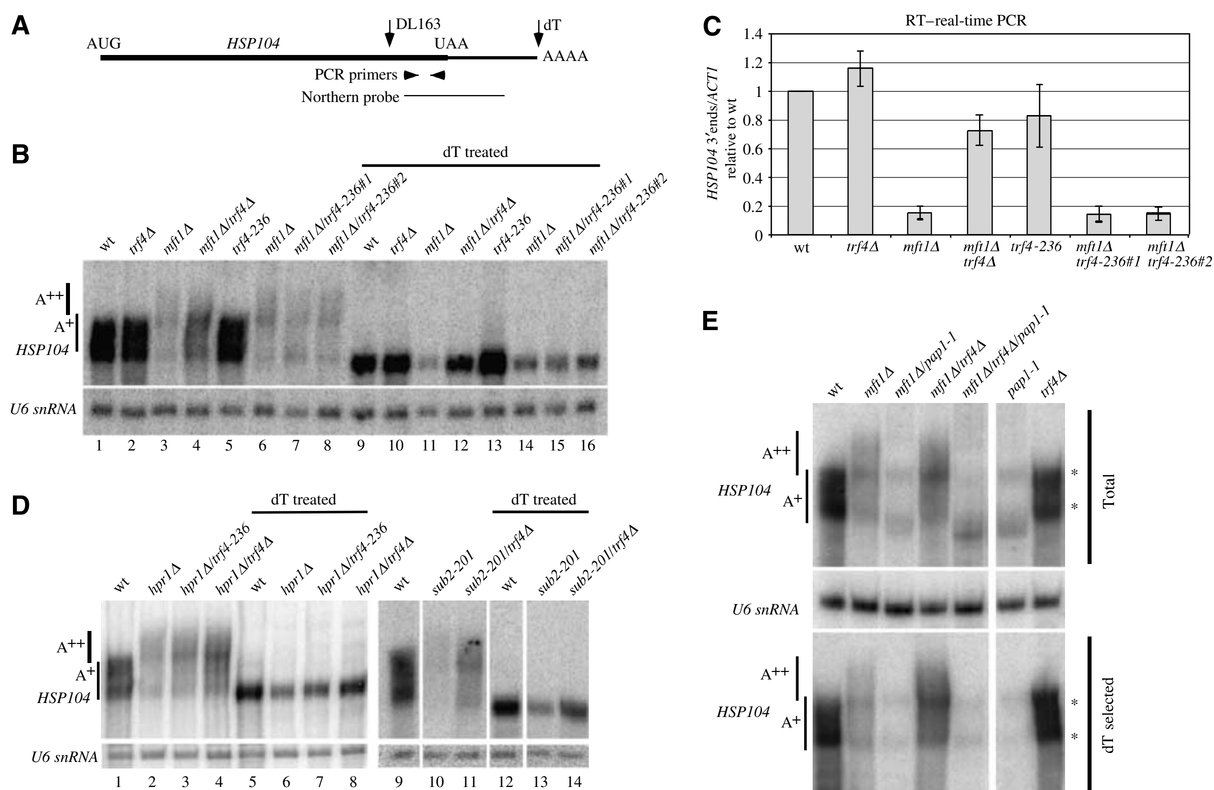

Figure 1 Trf4p participates in *HSP104* RNA nuclear surveillance. (**A**) Schematic representation of the assayed *HSP104* transcript showing the approximate positions of the utilized RNaseH cleaving DNA oligoes (DL163 and dT), the RT–real-time PCR primers and the Northern probe. (**B**) *HSP104* Northern analysis of RNA harvested from the indicated yeast strains after a 15 min temperature shift from 25 to 37°C. RNAs visualized in lanes 1–8 and 9–16 (denoted 'dT-treated') were RNaseH-cleaved by DNA oligonucleotides DL163 and DL163/dT, respectively, before gel loading. *U6* snRNA was probed as a control for sample loading. The migration of *HSP104* molecules harboring wt-length poly(A) tails (A^+), as well as hyperadenylated poly(A) tails (A^{++}), is denoted to the left of the image. The strains *mft1*Δ/*trf4-236#1* and *mft1*Δ/*trf4-236#2* represent two independent spores arising from the cross between *mft1*Δ and *trf4-236* single mutant strains. (**C**) Quantitative RT–real-time PCR analysis of *HSP104* RNA 3′ends from some of the RNA samples described in (B). *HSP104* RNA levels were normalized to *ACT1* RNA, which was unaffected by the relevant mutations. The *HSP104/ACT1* RNA ratio from wt samples was set to 1. *HSP104* PCR primers are depicted in (A). Averages and standard deviations are calculated from three experiments. (**D**) *HSP104* Northern analysis of RNA harvested from the indicated yeast strains after a 15 min heat pulse at 37°C. Sample treatments and notations of RNAs as described in (B). Lanes 9–14 are all taken from the same PhosphorImage scan. (**E**) *HSP104* Northern analysis of RNA harvested from the indicated yeast strains after a 15 min heat pulse at 37°C. Samples denoted 'dT selected' were passed over oligo(dT) columns before loading. Sample treatments and notations of RNAs are as in (B). The two bands denoted by asterisks are of unknown origin. All lanes are taken from the same PhosphorImage scan.

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caused a significant increase in *HSP104* transcript levels in all three *mft1*Δ, *hpr1*Δ and *sub2-201* mutant backgrounds (Figure 1B, compare lanes 3 and 4; and D, left panel, compare lanes 2 and 4, and right panel, compare lanes 10 and 11). This was even more evident when polyadenylated 3′-ends were enzymatically removed by oligo(dT)/RNaseH treatment (Figure 1B and D, panels labeled 'dT treated'). Real-time RT–PCR assays confirmed the Northern blot results (Figure 1C). This effect of *TRF4* deletion was nearly as prominent as that of deleting *RRP6* (Supplementary Figure 1) and strongly implies that the function of the nuclear exosome in aberrant mRNA decay is stimulated by Trf4p.

It has been suggested that the polyadenylation activity of Trf4p stimulates substrate degradation by the exosome (Vanacova *et al*, 2005). To test the involvement of polyadenylation by Trf4p in *HSP104* degradation, we constructed the *mft1*Δ/*trf4-236* and *hpr1*Δ/*trf4-236* mutant strains where deletion of the *HPR1* or *MFT1* genes is associated with a polyadenylation-defective, catalytic site mutant allele of *TRF4* (Wyers *et al*, 2005). These strains were analyzed for their *HSP104* RNA content after a 15 min heat shock at 37°C. Surprisingly, the Trf4-236p protein substitutes fully for wt Trf4p in the degradation of *HSP104* RNA in *mft1*Δ and *hpr1*Δ cells (Figure 1B, lanes 6–8 (and 14–16); C and D compare lanes 2 and 3 (and 6 and 7)). Therefore, the polyadenylation activity of Trf4p is not required for the degradation of *HSP104* transcripts.

Adenylated forms of HSP104 are produced by Pap1p

Consistent with previously published data, RNaseH/oligo(dT)-directed removal of poly(A) sequences reveals that virtually all detectable complete *HSP104* RNAs in *mft1*Δ, *hpr1*Δ and *sub2-201* strains are polyadenylated (Figure 1B and D; Libri *et al*, 2002). Interestingly, a fraction of these RNAs harbors poly(A) tails that are slightly longer than those of the wt control (denoted A^{++} in Figure 1B and D). *TRF4* deletion does not lead to removal of these *HSP104* species, arguing that this poly(A) polymerase is not responsible for the adenylation (Figure 1B and D). To analyze poly(A) addition requirements in the *mft1*Δ background in more detail, we constructed *mft1*Δ/*pap1-1* and *mft1*Δ/*trf4*Δ/*pap1-1* mutant strains and subjected them to *HSP104* RNA biochemical analysis. Introduction of the *pap1-1* mutation clearly decreased the extent of polyadenylated *HSP104* RNAs in both *mft1*Δ and *mft1*Δ/*trf4*Δ mutant contexts (Figure 1E, upper panel). Consistently, very little *HSP104* RNA was purified after oligo(dT) selection of total RNA from these strains (Figure 1E, lower panel). We conclude that polyadenylation in the *mft1*Δ strain background of *HSP104* RNAs with both normal and hyperadenylated poly(A) tails, is carried out by Pap1p.

Trf4p is not required for mRNA retention in nuclear foci

HSP104 RNAs accumulate in Rrp6p-dependent transcription site-associated foci in THO/TREX mutant strains (Jensen *et al*, 2001a; Libri *et al*, 2002; Thomsen *et al*, 2003; Vinciguerra *et al*, 2005). To evaluate the effect of *TRF4* deletion on *HSP104* RNA retention, the *mft1*Δ/*trf4*Δ, *hpr1*Δ/*trf4*Δ and *sub2-201*/*trf4*Δ strains were subjected to *HSP104* RNA fluorescent *in situ* hybridization (FISH) analysis. As controls, single mutants *mft1*Δ, *hpr1*Δ and *sub2-201* as well as double mutants *mft1*Δ/*rrp6*Δ, *hpr1*Δ/*rrp6*Δ and *sub2-201*/*rrp6*Δ were examined simultaneously. As previously reported, all three *mft1*Δ, *hpr1*Δ and *sub2-201* single mutants retain *HSP104* RNA in nuclear foci after a 15 min shift to the non-permissive temperature of 37°C, whereas codeletion of *RRP6* in all three contexts abolishes *HSP104* RNA retention (Figure 2A; Jensen *et al*, 2001a; Libri *et al*, 2002). Strikingly, *TRF4* deletion has no effect on *HSP104* RNA localization in neither of the three mutant backgrounds.

To learn more about the generality of mRNA surveillance in THO/sub2 mutants, we examined RNA expressed from the *PDR5* gene, which we have identified as a THO complex target in a genome-wide study (Rougemaille *et al*, in preparation). *PDR5*-FISH probes detected a weak dot signal in up to 20% of wt cells (Figure 2B, left panel), which presumably stems from nascent RNA transcribed off the 4.5 kb long *PDR5* gene. In *hpr1*Δ cells, signal intensity increased and 95% of analyzed cells contained the *PDR5* RNA dot. As observed for hsRNAs, co-deletion of *TRF4* had no dramatic effect on dot intensity, whereas *RRP6* deletion resulted in a decrease back to wt levels (Figure 2B, left panel). At the level of RNA decay, the *PDR5* RNA also phenocopied that of hsRNAs: low *PDR5* 3′end levels in *hpr1*Δ are restored both by *TRF4* and *RRP6* deletion (Figure 2B, right panel). We conclude that nuclear mRNA surveillance in THO/sub2 mutants is not restricted to hsRNAs.

Finally, we also investigated the effect of deleting or mutating *TRF4* in the *mft1*Δ background on the localization and levels of general poly$(A)^+$ RNA using an oligo(dT_{20}) LNA-modified FISH probe (Figure 2C; Thomsen *et al*, 2005). In the *mft1*Δ strain, a robust granular poly$(A)^+$ RNA signal, overlapping the DAPI-stained chromatin region, was detected, which neither deletion of *TRF4* nor addition of the *trf4-236* allele changed significantly (Figure 2C, right panel). Thus, transcripts detected by the oligo(dT_{20}) probe behave similar to *PDR5*- and *HSP104*-RNAs. Consistent with previous observations, this probe detects a low level of poly$(A)^+$ RNA in wt cells (Figure 2C, left panel; Thomsen *et al*, 2005). This signal does not overlap that of the DAPI stain and is believed to stem from polyadenylation of stable nucleolar RNAs or from random occurrence of A stretches. In contrast to *TRF4* removal, introduction of the *pap1-1* allele reduced the intensity of the chromatin-associated poly$(A)^+$ signal considerably (Figure 2C, right panel, lower row). Thus, also at the level of RNA poly$(A)^+$-FISH, it is clear that Pap1p provides the major adenylation activity to chromatin-restricted mRNAs.

The entire TRAMP complex operates in mRNA surveillance

Besides Trf4p, the TRAMP complex also harbors one of the two RNA-binding proteins Air1p or Air2p (LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005). A third TRAMP component is the RNA helicase Mtr4p, which also shares functions with the nuclear exosome (de la Cruz *et al*, 1998). To evaluate the context requirements for Trf4p function in mRNA surveillance, we constructed the *mft1*Δ/*air1*Δ/*air2*Δ triple mutant and subjected it to *HSP104* RNA-FISH and Northern analysis. Deletion of *AIR1* and *AIR2* in the *mft1*Δ context largely phenocopied the deletion of *TRF4*: there was only a slight decrease in the number of *HSP104* RNA dot-positive cells compared with the *mft1*Δ single mutant (Figure 3A), and *HSP104* RNA 3′-end levels were restored

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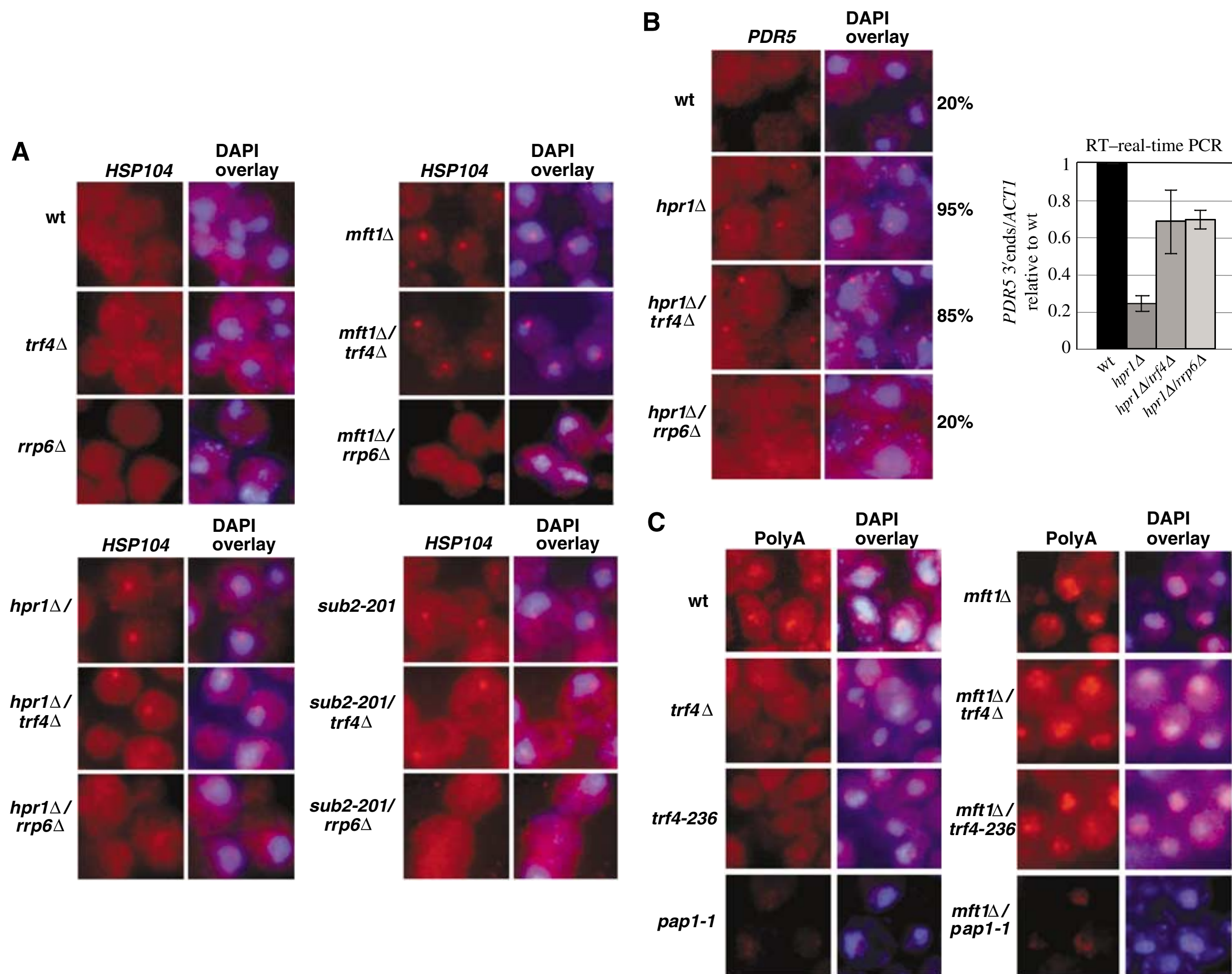

Figure 2 Nuclear retention of mRNA in THO/sub2 mutants in the absence of *TRF4*. (**A**) *HSP104* RNA-FISH on fixed samples of the indicated yeast wt and mutant strains. Cells were grown at 25°C followed by a temperature shift to 37°C for 15 min before fixation. *HSP104* RNA was detected using a mixture of three Cy3-labeled oligonucleotide probes directed against the 3′end of the transcript. DNA was stained with DAPI. Retained *HSP104* RNA was detected in >95% of the *mft1*Δ, *mft1*Δ*/trf4*Δ, *hpr1*Δ, *hpr1*Δ*/trf4*Δ, *sub2-201* and *sub2-201/trf4*Δ cells in a given field of view. (**B**) Left panel: *PDR5* RNA-FISH on fixed samples of the indicated yeast wt and mutant strains after a temperature shift to 37°C for 15 min. *PDR5* RNA was detected by a mixture of three Cy3-labeled oligonucleotide probes directed against the transcript. DNA was stained with DAPI. The fraction of cells with detectable *PDR5* RNA dot signals are shown to the right of the images. Right panel: quantitative real-time RT–PCR analysis of *PDR5* RNA 3′ends from RNA samples harvested from cultures shown in the left panel. *PDR5* RNA levels were normalized to *ACT1* RNA and the *PDR5/ACT1* RNA ratio from wt samples was set to 1. Averages and standard deviations are calculated from three experiments. (**C**) Poly(A)$^+$ RNA-FISH on the indicated fixed samples of yeast cells after a temperature shift to 37°C for 15 min. Poly(A)$^+$ RNA was detected using an LNA-modified dT_{20} probe (Thomsen *et al*, 2005). DNA was stained with DAPI.

(Figure 3B, compare lane 7 with 8 and 9). We also tested the impact of removing the Rrp6p-interacting protein, Rrp47p (Mitchell *et al*, 2003), in the *mft1*Δ context, and found that while *HSP104* RNA 3′-end levels were restored (Figure 3B, compare lane 2 with lanes 3 and 4), the number of *HSP104* dot-positive cells were 40%, with a concomitant more diffuse FISH signal (Figure 3A). Thus, although not as penetrant, the effect of Rrp47p removal mimics that of an *RRP6* deletion.

Finally, we analyzed the effect of decreasing cellular Mtr4p levels on *HSP104* RNA surveillance. This was achieved by driving expression of the genomic *MTR4* gene by a tetracycline repressible promoter (Tet-*MTR4*) in a wt or an *mft1*Δ background. After 6 h of incubation under repressive- (+doxycycline) or permissive (−doxycycline) conditions, cultures were shifted to 37°C for 15 min and subsequently harvested and processed for *HSP104*-RNA-FISH or real-time RT–PCR analysis. *HSP104* 3′-end levels in the *mft1*Δ/Tet-*MTR4* strain were not decreased as prominently compared with the wt control as in *mft1*Δ and wt strains carrying the endogenous *MTR4* gene (Figure 3B, right panel; compare with Figure 1C). This is presumably due to altered *MTR4* expression from the Tet promoter (data not shown). In any case, repressing Mtr4p production restored *HSP104* RNA 3′ends back to wt levels. Moreover, lowering Mtr4p levels in the *mft1*Δ context also resulted in a significant decrease in *HSP104* dot containing cells (Figure 3A, right panel), suggesting that these conditions impact nuclear exosome activity.

Taken together, these data strongly indicate that TRF4p functions in the context of TRAMP.

Lack of RRP6 still leads to release of aberrant mRNA in the absence of TRF4

Previously, transcription site retention of *HSP104* RNA in THO/sub2 mutants always correlated with *HSP104* transcript

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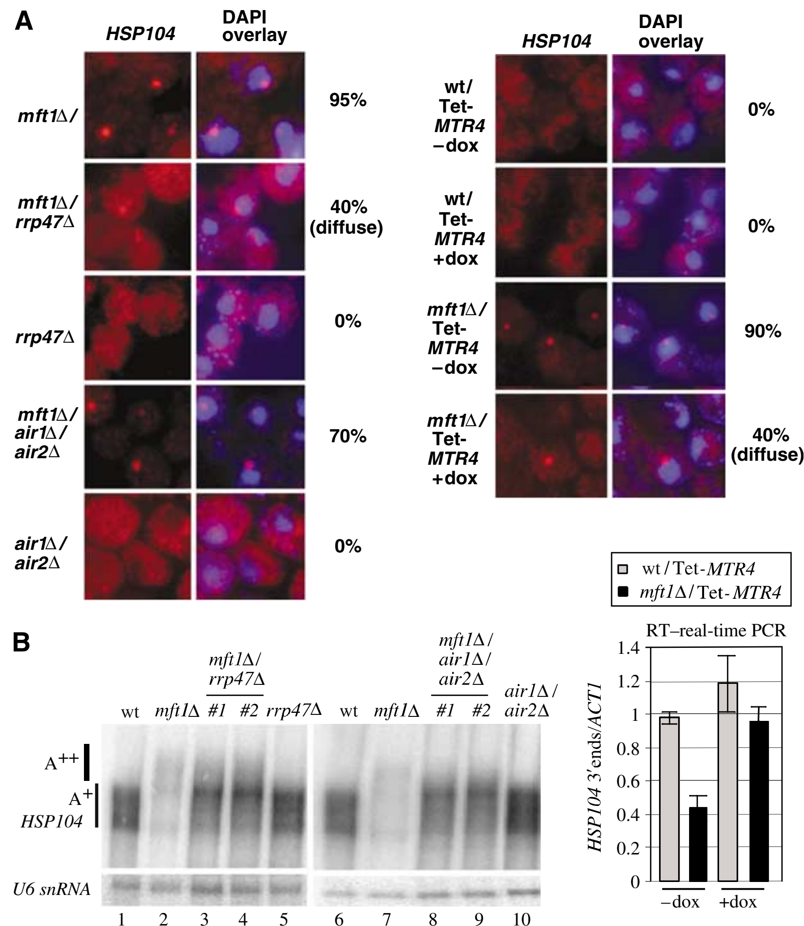

Figure 3 *HSP104* RNA surveillance characteristics of TRAMP and nuclear exosome components. (**A**) *HSP104* RNA-FISH on fixed samples of the indicated yeast strains after a temperature shift to 37°C for 15 min. *HSP104* RNA was detected as described in legend to Figure 2A. DNA was stained with DAPI. The fraction of cells with detectable *HSP104* RNA dot signals are shown to the right of the images. For experiments involving the pTET-repressible *MTR4* construct, cultures were either treated with 10 μg/ml of doxycycline, or not, for 6 h before the temperature shift. (**B**) *HSP104* Northern (left) or quantitative RT–real-time PCR analysis (right) of RNA harvested from the indicated yeast strains after a 37°C temperature shift for 15 min. Experimental details are as described in legends to Figure 1B and C. The strains *mft1Δ/rrp47Δ#1* and *mft1Δ/rrp47Δ#2* as well as *mft1Δ/air1Δ/air2Δ♯1* and *mft1Δ/air1Δ/*air2Δ*#2* represent two independent spores arising from the relevant crosses.

instability. Accordingly, transcripts are both released and stabilized upon *RRP6* deletion. Thus, the lack of correlation between these two events upon deletion of TRAMP components in a THO/sub2 mutant was surprising, especially in light of the multiple physical and functional connections of the TRAMP complex with the exosome. The mechanism by which deletion of *RRP6* promotes transcript release in, for example, an *hpr1Δ* strain is unknown and might involve, directly or indirectly, Trf4p. It is possible for instance that the large amounts of Trf4p-dependent polyadenylated rRNAs and sno/snRNAs, which accumulate in *rrp6Δ* cells interfere with the retention process (Kuai *et al*, 2004; Wyers *et al*, 2005; Davis and Ares, 2006; Egecioglu *et al*, 2006). Perhaps this 'excess' nuclear polyadenylated species titrates away factor(s) required for transcript retention in THO/sub2 mutants, thus provoking mRNP release in for example, an *hpr1Δ/rrp6Δ* mutant background. Transcript release in this context would then be an indirect effect of the *RRP6* deletion and should require Trf4p.

To evaluate this possibility, we constructed the triple mutant *hpr1Δ/rrp6Δ/trf4Δ* and subjected it to RNA-FISH analysis. However, we first verified the presence of a prominent oligo (dT_{20}) FISH signal in *rrp6Δ* single mutant cells (Figure 4A). In line with the reported polyadenylation of stable nucleolar RNA species (Kuai *et al*, 2004; Wyers *et al*, 2005; Davis and Ares, 2006; Egecioglu *et al*, 2006), this signal was clearly separated spatially from the DAPI stain (Figure 4A, 'DAPI overlay'), and partly overlapped that of the nucleolar antigens Nop1p and Nsr1p (Figure 4B, 'PolyA/NOP1' and data not shown). Consistently, a similar poly(A)$^+$ RNA localization was observed in a strain lacking the *RRP47* gene (Supplementary Figure 2 and data not shown). Moreover, as expected for a Trf4p-dependent polyadenylation process, deletion of *TRF4* (but not inactivation of Pap1p) significantly decreased the nucleolar dT signal observed in *rrp6Δ* cells (Figure 4A). The low residual poly(A)$^+$ RNA level in the nucleolus of *rrp6Δ/trf4Δ* cells is most likely produced by a Trf5p containing TRAMP complex, which was recently reported to adenylate rRNAs (Houseley and Tollervey, 2006).

Decreasing the nucleolar poly(A)$^+$ signal by *TRF4* removal does not 're-install' the *HSP104* dot in *hpr1Δ/rrp6Δ/trf4Δ* triple mutant cells (Figure 4C). Thus, by these criteria, release of mRNA from chromatin upon *RRP6* removal is not a consequence of increased nucleolar poly(A)$^+$ RNA load. These experiments also more generally indicate that Trf4p is not required for mRNP release in the *hpr1Δ/rrp6Δ* strain.

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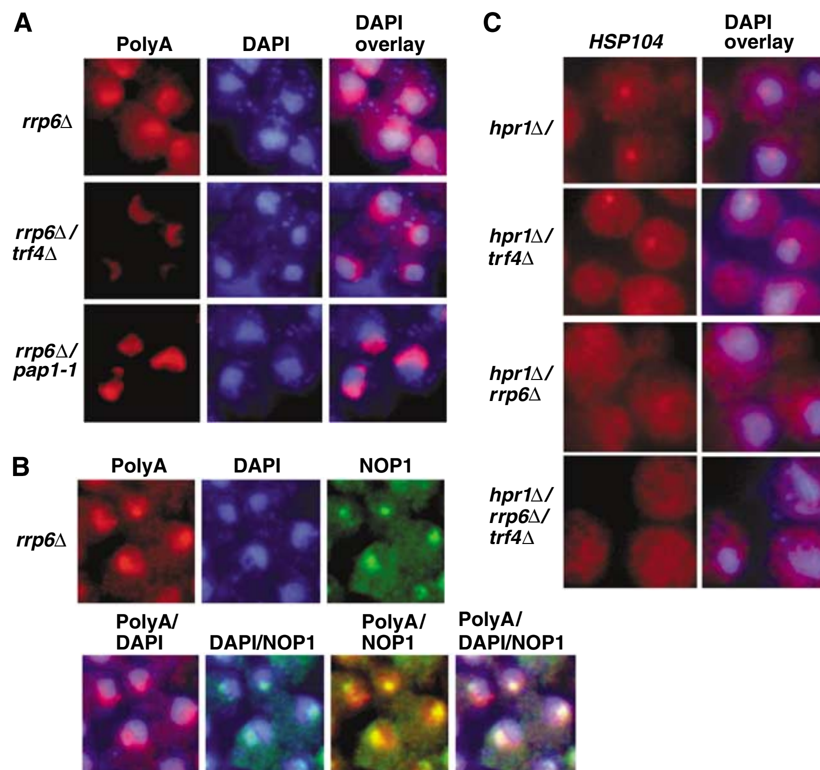

Figure 4 Deletion of *TRF4* does not restore mRNA retention in Δ*hpr1*/*rrp6*Δ cells. (**A**) Poly(A)$^+$ RNA-FISH on the indicated fixed samples of *rrp6*Δ, *rrp6*Δ/*trf4*Δ and *rrp6*Δ/*pap1-1* cells after a temperature shift to 37°C for 30 min. Poly(A)$^+$ RNA and DNA was visualized as described in legend to Figure 2B. (**B**) Dual poly(A)$^+$ RNA-FISH and Nop1p immunolocalization analysis on fixed *rrp6*Δ cells temperature shifted to 37°C for 30 min. Nop1p was detected using a monoclonal anti-Nop1p antibody followed by an FITC-conjugated secondary antibody. (**C**) *HSP104* RNA-FISH on fixed samples of Δ*hpr1*Δ, *hpr1*Δ/*trf4*Δ, *hpr1*Δ/*rrp6*Δ and *hpr1*Δ/*rrp6*Δ/*trf4*Δ cells as indicated. Strains were temperature shifted to 37°C for 15 min before fixation. *HSP104* RNA and DNA was stained as described in legend to Figure 2A. Retained *HSP104* RNA 'dots' were detected in >90% of the *hpr1*Δ and *hpr1*Δ/*trf4*Δ cells and <10% of the *hpr1*Δ/*rrp6*Δ and *hpr1*Δ/*rrp6*Δ/*trf4*Δ cells in a given field of view.

Persistence of nuclear HSP104 RNAs in the sub2-201 mutant in the absence of transcription

So far, it is unclear whether the *HSP104* RNAs present in THO/sub2 mutant cells after a 15 min heat pulse represent species that are in the process of being degraded or molecules that have escaped nuclear degradation. Some or all of these molecules might be in transcription site foci, although, it is not clear whether degradation is actually taking place at these sites. To investigate this issue, we analyzed localization and turnover of *HSP104* RNAs in wt and *sub2-201* cells after a 15 min transcription pulse at 42°C, as well as at different time points in a subsequent chase period. Transcription was shut off by rapidly decreasing the temperature of heat-shocked cells to 25°C and simultaneously adding the transcription inhibitor thiolutin. *HSP104* RNA was analyzed by Northern blotting, real-time RT–PCR and FISH analysis. The *sub2-201* mutant strain and the 42°C temperature were chosen because of the more robust build-up of *HSP104* RNA in transcription site foci under these conditions (data not shown).

As shown by both Northern and RT–PCR analyses, *HSP104* RNA was gradually turned over in a wt strain in the absence of transcription (Figure 5A and B). In marked contrast, the lower amount of *HSP104* RNA observed in *sub2-201* cells after a 15 min heat shock remained constant for at least an hour after addition of thiolutin. This was paralleled by the persistence of *HSP104* RNA-FISH dots in *sub2-201* cells after transcription shut off (Figure 5C). These experiments suggest that both total and transcription site-restricted *HSP104* RNAs in *sub2-201* cells are remarkably stable in the period following a 15 min heat shock. Such a result is intriguing because the low level of *HSP104* RNAs in THO/sub2 mutants, at the 15 min time point, is restored to wt levels upon deletion of *RRP6*, suggesting that the majority of *HSP104* RNAs are degraded extremely rapidly in single THO/sub2 mutants (Libri *et al*, 2002).

The different half-lives of steady-state *HSP104* RNAs in *sub2-201* and wt cells suggest that these RNAs have different fates and, notably, that in mutant cells, they do not undergo translation and ensuing cytoplasmic decay. Consistently, impairment of the major cytoplasmic decay pathway by deletion of the 5′→3′ exonuclease *XRN1* strongly affected the pseudo-pulse–chase kinetics of *HSP104* molecules in the wt but not in the *sub2-201* background (Supplementary Figure 3). This indicates that these *HSP104* RNAs in *sub2-201* cells are dead-end products, which do not experience a cytoplasmic phase.

To demonstrate that the low amount of *HSP104* RNA present in the *sub2-201* strain is not due to decreased transcription, nuclear run on (NRO) experiments were performed on nuclei isolated in these conditions from either wt or *sub2-201* cells. As shown in Figure 5D, NRO signals were indistinguishable between the two strains at every assayed position of the *HSP104* gene, which indicates that RNAPII activity is not affected by mutation of Sub2p. Thus, the simplest interpretation of these experiments is that *HSP104* RNAs are rapidly degraded shortly after transcriptional induction.

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Surprisingly, however, transcripts that evade this decay have dramatically increased half-lives.

Discussion

Several phenotypes have been associated with mutation or deletion of individual subunits of the THO/TREX complex, including defects in transcription, genomic stability as well as mRNP biogenesis and nuclear export (Chavez *et al*, 2000; Jensen *et al*, 2001a; Libri *et al*, 2002; Strasser *et al*, 2002; Zenklusen *et al*, 2002; Huertas and Aguilera, 2003). Although the precise function of THO/TREX is still unresolved, THO/sub2 mutant strains provide good systems to study nuclear mRNA surveillance as these cells phenotypically illustrate the two major hallmarks of this process: (i) retention and (ii) elimination of aberrant mRNAs. In this study, we have taken advantage of THO/sub2 mutant cells to show the involvement of the TRAMP complex in the degradation-leg of nuclear mRNA surveillance. Furthermore, we have probed the relationship between transcript retention and decay, and reached the surprising conclusion that the *HSP104* transcripts remaining in mutants after a 15 min transcription induction, of which a considerable fraction is presumably transcription site associated, are remarkably stable. This is in sharp contrast to the majority of *HSP104* molecules, which undergo rapid decay during the first minutes of transcription induction.

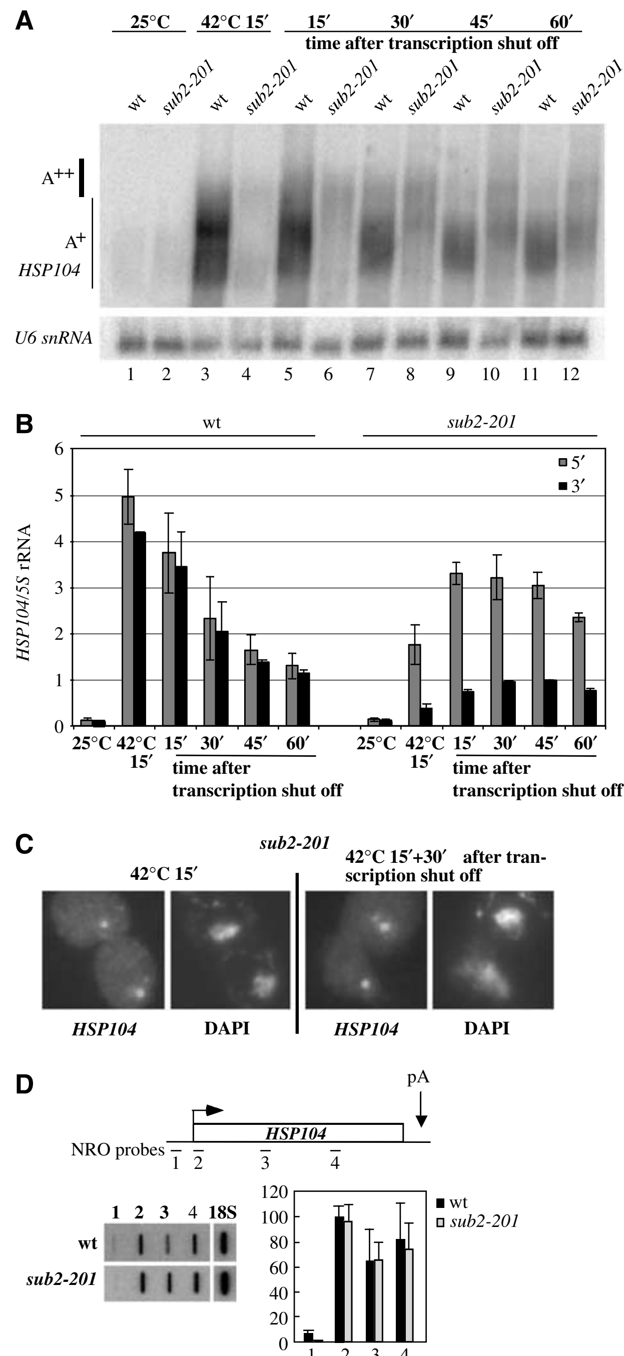

Figure 5 *HSP104* RNAs detected in *sub2-201* mutant cells after a 15 min heat shock are remarkably stable. (**A**) *HSP104* Northern analysis of RNA harvested from the indicated wt or *sub2-201* strains incubated at 25°C (lanes 1 and 2), heat shocked for 15 min at 42°C (lanes 3 and 4) or heat shocked for 15 min at 42°C followed by a 'chase period' of the indicated length (lanes 5–12). RNAs were RNaseH-cleaved using DNA oligonucleotide DL163 before gel loading. *U6* snRNA was probed as a control for sample loading. The migration length of *HSP104* molecules harboring wt length poly(A) tails (A^{+}), and hyperadenylated poly(A) tails (A^{++}), is denoted to the left of the phosphorImage. (**B**) Quantitative RT–real-time PCR analysis of *HSP104* RNA 5′ends and 3′ends from RNA samples described in (A). *HSP104* RNA levels were normalized to *5S* rRNA, which was unaffected by the different conditions. Averages and standard deviations are calculated from two experiments. (**C**) *HSP104* RNA-FISH on fixed samples of *sub2-201* cells heat shocked for 15 min at 42°C (left panel), or heat shocked for 15 min at 42°C followed by a 'chase period' of 30 min. *HSP104* RNA was detected as described in legend to Figure 2A. DNA was stained with DAPI. Retained *HSP104* RNA was detected in >80% of cells in both conditions. (**D**) Nuclear run on (NRO) analysis of nuclei harvested from wt or *sub2-201* cells after 15 min heat shock at 42°C. Radioactive RNA samples were hybridized to DNA oligonucleotide NRO probes complementary to the approximate positions of the *HSP104* as indicated on top. Hybridization to an *18S* rRNA probe was used as an internal control. *HSP104* NRO signals were quantitated by normalizing to the *18S* rRNA signal and setting the value of probe 2 in the wt strain to 100 (lower right).

Trf4p, but not its poly(A) polymerase activity, is required for efficient degradation of HSP104 RNAs in THO/sub2 mutants

As a pool of newly synthesized *HSP104* RNAs in THO/sub2 mutants, and in mutants of other mRNA export factors, are hyperadenylated (Jensen *et al*, 2001b; Libri *et al*, 2002), we reasoned that these RNAs might be nuclear exosome degradation intermediates tagged by the TRAMP complex that was recently discovered as an exosome activator for degradation and maturation of nuclear RNAs (Kadaba *et al*, 2004; LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005; Egecioglu *et al*, 2006). However, the long-sized poly(A) tails are unaffected by deletion of *TRF4*, but rather disappear after inactivation of the canonical poly(A) polymerase Pap1p. Nonetheless, Trf4p is required for efficient degradation of *HSP104* RNAs as the amount of these rises to near wt levels in double *mft1Δ/trf4Δ*, *hpr1Δ/trf4Δ* and *sub2-201/trf4Δ* mutant strains. These observations therefore identify Trf4p (in the context of TRAMP) as a nuclear mRNA surveillance factor.

Importantly, *HSP104* transcript degradation does not depend on the poly(A) polymerase activity of Trf4p, that is, the catalytic site mutant *trf4-236* substitutes fully for wt

TRF4. This allows the somewhat surprising conclusion that polyadenylation is not an obligatory event for the TRAMP complex to stimulate the nuclear exosome. Other recent data also suggest that polyadenylation might not be of universal importance for TRAMP activity. For instance, *trf4-236* only partially phenocopies the effect of a *TRF4* gene deletion on the degradation of CUTs (Wyers *et al*, 2005). Moreover, artificial addition of an oligo(A) tail to a synthetic structured RNA substrate *in vitro* is not sufficient *per se* to promote TRAMP-independent degradation by the exosome (LaCava *et al*, 2005). Consistently, Trf4p, but not its poly(A) polymerase activity, is essential in the absence of its paralogue Trf5p (Wyers *et al*, 2005). The mechanism by which TRAMP promotes polyadenylation-independent stimulation of exosome activity is so far unknown, but it may include the helicase activity of Mtr4p, or the RNA-binding activity of the Air1/2p proteins, to favor exosome recruitment to the substrate.

Although TRAMP is required for efficient degradation of the bulk of *HSP104* RNAs in THO/sub2 mutants, lack of TRAMP components Trf4p, Air1p and Air2p does not affect the transcription site-associated retention of *HSP104* RNA in these mutants. This phenotype contrasts that of an *RRP6* deletion where *HSP104* transcripts in THO/sub2 mutants are both stabilized and released. The finding implies that either transcript retention is not mechanistically coupled to degradation, or that the Rrp6p degradation activity that mediates retention is Trf4p-independent. As retained transcripts are remarkably stable (see below), it is possible that TRAMP is only required for the exosome to access malformed RNAs that have been released into the nucleoplasm.

A model for nuclear mRNA surveillance in THO/sub2 mutants

HSP104 transcription pulse–chase experiments in the *sub2-201* mutant show only minor decay of the total transcript pool in the chase period. Moreover, these RNAs do not have a cytoplasmic phase as demonstrated by their insensitivity to impairment of the major cytoplasmic decay pathway. The fraction of *HSP104* RNAs, retained in transcription site foci, is also stable, as the RNA-FISH signal persists for at least 30 min after transcription shut off. Thus, these data demonstrate the existence of nuclear mRNAs, in THO/sub2 mutants, with very low turnover rates. However, our data also strongly suggest that the major fraction of *HSP104* transcripts produced in *sub2-201* cells before starting the chase are rapidly degraded. This is because after the 15 min transcription pulse, complete *HSP104* RNAs in the *sub2-201* strain only constitute roughly 20% of wt levels, despite indistinguishable transcription activities in wt and *sub2-201* cells. Furthermore, deletion of *RRP6* or *TRF4* leads to restoration of *HSP104* RNA levels in the *sub2-201* background, indicating that the 'missing' fraction of these transcripts (approximately 80%) was rapidly degraded. Taken together, these observations are best explained by a model in which stable *HSP104* RNAs in *sub2-201* cells must have escaped early degradation and now persist in transcription site-associated foci or elsewhere in the nucleus where destruction is occurring at a lower rate. Perhaps stochastic failure to access nuclear degradation exposes these molecules to protective coating by RNA-binding proteins or perhaps sequestration in certain nuclear regions makes them inaccessible to the nuclear exosome, or to TRAMP.

What then is the nature of the Rrp6p-dependent mechanism that retains RNAs? First, Rrp6p, and possibly the exosome, might physically tether malformed mRNPs and *RRP6* deletion might break these links, thus allowing release. Although this explanation is consistent with the fact that the exosome can be crosslinked to the *HSP104* locus in a transcription-dependent manner (Andrulis *et al*, 2002; Hieronymus *et al*, 2004; our unpublished results), it is at odds with the fact that we were unable to detect an increase in exosome occupancy in THO/sub2 mutants (data not shown). This implies that the exosome is recruited to chromatin independent of mRNP surveillance. Second, deletion of *RRP6* could release foci-retained *HSP104* molecules because of indirect effects, for example, accumulation of polyadenylated stable RNAs in Rrp6p-minus cells could potentially titrate away factors required for transcription site retention. As abolishing a large share of stable RNA polyadenylation by deletion of *TRF4* did not restore *HSP104* RNA retention in the *hpr1*Δ/*rrp6*Δ strain we can rule out this latter possibility. We cannot however exclude the existence of other indirect effects. As an important corollary, Rrp6p and Rrp47p have previously been assigned roles in mRNA export based on the detection of a nuclear poly(A)$^+$ RNA-FISH signal in *rrp6*Δ and *rrp47*Δ cells (Hieronymus *et al*, 2004). However, data presented here argue against this interpretation and instead suggest that the reported observation reflects the accumulation of poly(A)$^+$ RNA in the nucleolus rather than retention of mRNA in the nucleus.

A third possibility, and the one we favor, is that an export-antagonizing event, involving Rrp6p, negatively affects *HSP104* RNA export and triggers the building up of transcripts detected by FISH (Figure 6). The export-promoting activity challenged by Rrp6p could be an aspect of the mRNA 3′end formation process where transcripts with exposed 3′ends would be desired targets of the enzyme. Furthermore, major mRNP remodeling steps occur at the 3′ends of genes, which could be slowed by Rrp6p competition. Regardless the precise molecular event, we suggest that in wt conditions, the export-promoting activity is strong enough to out-compete Rrp6p. However, in a THO/sub2 mutant context, diminished/slowed transcript release allows Rrp6p to attack kinetically disfavored mRNPs. Most of these are rapidly degraded in the first wave of decay (requiring TRAMP for complete degradation). A minor fraction (approximately 20%) of the molecules, represented by the stable pool of *HSP104* RNA in nuclear dots, is engaged in a non-productive degradation pathway despite the fact that they also failed to productively enter export. Perhaps these mRNPs escaped rapid decay because of local exhaustion of decay factors; however, they also missed the export pathway because of insufficient 'release signals' (e.g. THO/Sub2p), or because they simply missed the right kinetic window within which export competence can be achieved. An important feature of this model is that Rrp6p is involved in mRNA dot creation rather than maintenance.

The factors that ultimately define export competence as well as the dynamics of factor exchange around the nascent RNA molecule in the late phases of transcription are still elusive. Their definition will be of paramount importance to

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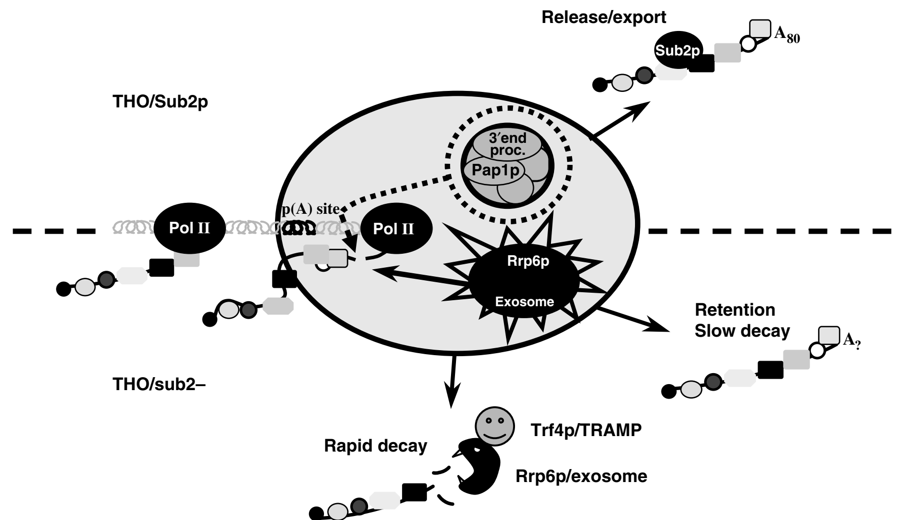

Figure 6 Model of transcription site-associated mRNA surveillance in THO/sub2 mutants. Upper half: in wt conditions, cotranscriptional mRNA packaging and 3′end formation occur efficiently and the mRNP particle is released for export. Lower half: in a THO/sub2 mutant context, a less efficient assembly/processing pathway leads to Rrp6p/exosome intervention and rapid RNA decay. A minor fraction of molecules escape nuclear degradation but cannot enter a productive export pathway. See text for further details.

unveil the precise mechanism underlying quality control of mRNA at the site of transcription.

Materials and methods

Yeast strains and manipulations

Yeast strains used in this study are all derived from W303 and are described in Supplementary Table S1. Crosses were performed using standard laboratory procedures. All temperature shifts were performed by the addition of an equal volume of prewarmed medium to a 25°C culture, followed by incubation at 37 or 42°C for the appropriate times. For experiments involving the transcription inhibitor thiolutin, cells were heat shocked at 42°C for 15 min, rapidly pelleted and resuspended in medium containing 50 μg/ml of thiolutin at 25°C. Aliquots were taken at different time points for RNA analyses. It was verified that addition of thiolutin before heat shock efficiently inhibited *HSP104* transcription activation (data not shown).

For experiments with cells containing the *MTR4* gene under control of the Tet promoter, cultures were grown for 6 h in the presence of 10 μg/ml doxycyclin to repress *MTR4* expression before *HSP104* induction.

RNA-FISH analysis

RNA-FISH and dual RNA-FISH/protein immunolocalization analyses were performed as previously described (Jensen *et al*, 2001b; Thomsen *et al*, 2003, 2005). *HSP104*- and *PDR5*-RNAs were detected using a mixture of Cy3 body-labeled oligonucleotide probes. *HSP104* probes THJ203, THJ204 and THJ206 have previously been described (Jensen *et al*, 2001b). For *PDR5* detection, the following probe mix was used (X denotes an Amino-C6-dT modification disposed for Cy3-labeling): PDR5-1 (5′-CAGAGXCCTTGCCAG XTTTTGGATXCGAGCTTCXGTATGCTCAXCGAACC), PDR5-2 (CCX GGTCTACCXAAAACGACTAGCAAXTCACCTGGGXTTAGGCAACCAX CC) and PDR5-3 (CCCXATCGACACCCXTGATACGGXTCTGTG GGGXTTTCAACCTCGCXACTG). Poly(A)$^+$ RNA was detected by an LNA-modified dT_{20} probe 5′end labeled with Cy3 (Thomsen *et al*, 2005). Nsr1p, Nop1p and Nsr1p monoclonal antibodies (EnCor) were used for immunostaining following the manufacturer's recommendations.

RNA preparations and analysis

RNA was prepared by the hot phenol method as described previously (Libri *et al*, 2002). Reverse transcription was performed with MMLV reverse transcriptase (Invitrogen) and cDNA synthesis was primed with random hexamers, oligo dT and specific primers directed against *U4* snRNA. The reaction was diluted ten times before real-time PCR analysis. Amplifications were performed in duplicate using a LightCycler (Roche) with no-reverse transcriptase controls to estimate the contribution of contaminating DNA. Amplification efficiencies were measured for each primer pairs and every set of amplification reactions. Amplification primers have the following sequences: *HSP104*-3′: DL528 (sense): 5′-GTTCTAC CAAATCACGAAGC and DL529 (antisense): 5′-TCTAGGTCATCAT CAATTTCC; *HSP104*-5′: DL258 (sense): 5′-ATATGAACGACCA AACGC and DL259 (antisense): 5′-AGATCATAGTCGTAACGGC); *ACT1*: DL377 (sense): 5′-ATGTTCCCAGGTATTGCCGA and DL378 (antisense): 5′-ACACTTGTGGTGAACGATAG; *PDR5*: DL795 (sense): 5′-ACTGACACCTGTAGTTTCTGTC and DL796 (antisense): 5′-TTCT CCATCTCTCACTGTAGA. At least three independent experiments were performed to calculate averages and standard deviations. RNaseH/ Northern blot analysis was performed as described previously (Libri *et al*, 2002).

Transcription analysis

NRO analysis on the *HSP104* gene was performed as described previously (Jensen *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Dorthe Riishøj and Jocelyne Boulay for excellent technical assistance, F Lacroute for help with yeast crosses and H Klein, A Aguilera and A Lebreton for gift of strains and also thank members of the DL and THJ laboratories for stimulating discussions, and Michael Rosbash, Claire Moore, Bjarne Bonven and Sylvie Camier for critical reading of the manuscript. This work was supported by La Ligue Contre le Cancer (BS, Equipe labilisée 2005), the CNRS (DL), the ANR (programme CUTs) (DL), the foundation pour la Recherche Medicale (DL, Equipe labilisee FRM 2007), the Danish National Research Foundation (Grundforskningsfonden) (DL and THJ) and the Novo Nordisk Foundation (THJ). MR is a recipient of a fellowship from the French Ministry of Research.

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