RNA polymerase II (RNAP II)–associated factors are recruited to tRNA loci, revealing that RNAP II– and RNAP III–mediated transcriptions overlap in yeast

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In yeast (Saccharomyces cerevisiae), the synthesis of tRNAs by RNA polymerase III (RNAP III) down-regulates the transcription of the nearby RNAP II–transcribed genes by a mechanism that is poorly understood. To clarify the basis of this tRNA gene–mediated (TGM) silencing, here, conducting a bioinformatics analysis of available ChIP-chip and ChIP-sequencing genomic data from yeast, we investigated whether the RNAP III transcriptional machinery can recruit protein factors required for RNAP II transcription. An analysis of 46 genome-wide protein-density profiles revealed that 12 factors normally implicated in RNAP II–mediated gene transcription are more enriched at tRNA than at mRNA loci. These 12 factors typically have RNA-binding properties, participate in the termination stage of the RNAP II transcription, and preferentially localize to the tRNA loci by a mechanism that apparently is based on the RNAP III transcription level. The factors included two kinases of RNAP II (Bur1 and Ctk1), a histone demethylase (Jhd2), and a mutated form of a nucleosome-remodeling factor (Spht6) that have never been reported to be recruited to tRNA loci. Moreover, we show that the expression levels of RNAP II–transcribed genes downstream of tRNA loci correlate with the distance from the tRNA gene by a mechanism that depends on their orientation. These results are consistent with the notion that pre-tRNAs recruit RNAP II–associated factors, thereby reducing the availability of these factors for RNAP II transcription and contributing, at least in part, to the TGM-silencing mechanism.

RNAs in yeast are transcribed using three RNA polymerases, RNAP I, II, and III, which catalyze the synthesis of specific subsets of RNAs. For example, RNAPs I, II, and III transcribe rRNAs (except 5S rRNA), mRNAs, and tRNAs, respectively. The active synthesis of tRNAs by RNAP III can act as a transcriptional repressor of the neighboring RNAP II–transcribed genes by an unknown mechanism, referred to as tRNA gene-mediated (TGM) silencing or tRNA position effect (1–3). The 275 genes encoding tRNAs in yeast are scattered throughout the genome, but most of them are spatially clustered in the nucleoli (4), where most pre-tRNA processing occurs (5). The clustering of tRNA genes in or near the nucleolus requires their active transcription (4) and appears to be a prerequisite for TGM silencing (6, 7). In fact, both TGM silencing and the nucleolar clustering of tRNA genes are reduced by defective mutations in subunits of condensin (8, 9) and cohesin (10), two protein complexes that play a central role in chromosome organization and segregation (11).

Nucleolar clustering of the tDNAs and the tRNA synthesis are not the only factors affecting TGM silencing. The integrity of pre-tRNA also appears to play a role in TGM silencing, considering that its alteration by transcript truncation relieves silencing effects in vivo (12), even if half-tRNA molecules have the ability to inhibit RNAP II transcription better than the intact pre-tRNAs in vitro (13). A mutant of the small nucleolar ribonucleoprotein protein Cbf5 that relieves TGM silencing also dissociates the pre-tRNA and tRNA genes from the nucleolus, suggesting a possible role of the pre-tRNA subnuclear localization in the silencing mechanism (7). Moreover, the TGM silencing mechanism appears independent of tRNA gene orientation and does not imply simple steric blockage of RNAP II (1). Remarkably, the gene deletions and histone amino acid substitutions affecting TGM silencing are different from those affecting other types of gene silencing, indicating a distinct mechanism from the other silencing phenomena (6, 14).

In this work, we report that some protein factors implicated in the termination stage of RNAP II transcription appear to be significantly recruited at RNAP III–transcribed loci by a mechanism that is positively correlated with their RNA-binding properties and with the RNAP III occupancy. The results suggest a new antagonistic model for TGM silencing in which the tRNA transcripts contribute to the silencing mechanism by reducing the availability of factors required for the normal RNAP II transcription.

Results

Twelve proteins implicated in mRNA transcription exhibit significantly higher enrichment at the tRNA than at the mRNA loci

To investigate the mechanistic basis of TGM silencing, we evaluated the possibility that RNAP III transcriptional machinery could recruit specific RNAP II–associated factors. Large datasets related to the ChIP-chip and ChIP-sequencing...
Recruitment of RNAP II–associated factors to tRNA loci

Table 1
ChIP-chip and ChiP-seq datasets
Proteins significantly more enriched at tRNA than at mRNA loci are indicated by bold text: *, p value (t test) < 0.00002; **, p value (t test) < 0.0002; ***, p value (t test) < 0.02.

| Dataset | A* | B* | C* | D* | Protein name | Description |
|---------|----|----|----|----|--------------|-------------|
| √       |    |    |    |    | Nab2*        | Nuclear polyadenylated RNA-binding protein |
| √       |    |    |    |    | Sub2*        | ATP-dependent RNA helicase |
| ✓       | ✓  | ✓  | ✓  | ✓  | Yra1*        | Nuclear polyadenylated RNA-binding protein |
| ✓       | ✓  | ✓  | ✓  | ✓  | Pcf11*       | Component of the cleavage and polyadenylation factor IA |
| ✓       | ✓  | ✓  | ✓  | ✓  | Cfp1*        | Component of the cleavage and polyadenylation factor IA |
| ✓       | ✓  | ✓  | ✓  | ✓  | Rat1*        | Nuclear 5’ to 3’ single-stranded RNA exonuclease |
| ✓       | ✓  | ✓  | ✓  | ✓  | Nrd1**       | RNA-binding subunit of Nrd1 complex |
| ✓       | ✓  | ✓  | ✓  | ✓  | Jhd2*        | JmJ/C domain-containing histone demethylase |
| ✓       | ✓  | ✓  | ✓  | ✓  | Bur1*        | Cyclin-dependent serine/threonine protein kinase |
| ✓       | ✓  | ✓  | ✓  | ✓  | Ctk1*        | Catalytic subunit of C-terminal domain kinase I |
| ✓       | ✓  | ✓  | ✓  | ✓  | Spt6ΔCTD*    | Nucleosome remodeling protein CTD deleted |
| ✓       | ✓  | ✓  | ✓  | ✓  | Npl3***      | mRNA-polymerase III largest subunit |
| ✓       | ✓  | ✓  | ✓  | ✓  | Rnc160*      | RNA polymerase III subunit |
| ✓       | ✓  | ✓  | ✓  | ✓  | Ssa7         | Transcription initiation factor TFIIH |
| ✓       | ✓  | ✓  | ✓  | ✓  | Kim28        | Protein kinase subunit of transcription factor TFIIH |
| ✓       | ✓  | ✓  | ✓  | ✓  | Gbp2         | Single-stranded telomeric DNA-binding/mRNA-binding protein |
| ✓       | ✓  | ✓  | ✓  | ✓  | Hpr1         | Subunit of THO/TREX complexes |
| ✓       | ✓  | ✓  | ✓  | ✓  | Hrb1         | poly(A−) RNA-binding protein |
| ✓       | ✓  | ✓  | ✓  | ✓  | Mif1         | Subunit of the THO complex |
| ✓       | ✓  | ✓  | ✓  | ✓  | Tho2         | Subunit of the THO complex |
| ✓       | ✓  | ✓  | ✓  | ✓  | Cof1         | RNA 3′−triphosphatase involved in mRNA 5′ capping |
| ✓       | ✓  | ✓  | ✓  | ✓  | Elf1         | Transcription elongation factor |
| ✓       | ✓  | ✓  | ✓  | ✓  | Pafl         | Component of the PAF1 complex |
| ✓       | ✓  | ✓  | ✓  | ✓  | Sptn1        | Transcription factor involved in RNA polymerase II regulation |
| ✓       | ✓  | ✓  | ✓  | ✓  | Spt16        | Subunit of FACT complex |
| ✓       | ✓  | ✓  | ✓  | ✓  | Spt4         | Sp4/5 (DSIF) transcription elongation factor complex subunit |
| ✓       | ✓  | ✓  | ✓  | ✓  | Spt5         | Sp4/5 (DSIF) transcription elongation factor complex subunit |
| ✓       | ✓  | ✓  | ✓  | ✓  | Spt6         | Nucleosome-remodeling protein |
| ✓       | ✓  | ✓  | ✓  | ✓  | Tfg1         | Transcription initiation factor TFIIH |
| ✓       | ✓  | ✓  | ✓  | ✓  | H3K4me3      | Histone H3 trimethylated at lysine 4 |
| ✓       | ✓  | ✓  | ✓  | ✓  | H3K4me3 (Δh2d) | Histone H3 trimethylated at lysine 4 in Jhd2-deleted strain |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-rp2-Ser2P | pSer-2–phosphorylated RNA polymerase II |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-p5-Ser5P | pSer-5–phosphorylated RNA polymerase II |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-rp2-Ser7P | pSer-7–phosphorylated RNA polymerase II |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-p2       | RNA polymerase II |
| ✓       | ✓  | ✓  | ✓  | ✓  | Rbp3         | RNA polymerase II subunit |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-p2-CTD   | C-terminal domain of RNA polymerase II |
| ✓       | ✓  | ✓  | ✓  | ✓  | RNA-rp2 (Δh2d) | RNA polymerase II in Jhd2-deleted strain |

* Data are from Meinel et al. (22) and Reuter et al. (17).
* Data from Mayer et al. (38).
* Data from Blair et al. (39).
* Data from Kim et al. (51) and Johnson et al. (52).

Genomic profiles of protein factors involved in the transcription by RNAP II have been produced and made available in recent years. These data have been principally used to establish where and when the RNAP II–associated factors act during the mRNA synthesis. In contrast, the possible interaction of these factors with the RNAP III transcriptional machinery has scarcely been considered. Yet, in some cases, the high signals of these factors detected in the RNAP III–transcribed tDNAs have been considered as artifacts of the technical procedures and deemed not worthy of particular attention (15, 16).

Here, we examined the genome-wide occupancy at RNAP III genes of protein factors with different functions and acting at different stages of the RNAP II transcriptional process, as illustrated in Table 1. We analyzed four datasets covering a total of 46 samples. To reduce the possible differences in ChIP profiles due to the use of various technical procedures, each dataset was characterized by samples acquired and processed by a single research group and was analyzed separately using at least one internal control constituted by ChIP profiles of RNAP II subunits. We considered an RNAP II–associated factor as significantly enriched at the tRNA loci if two conditions were met: first, its average ChIP signal at the tRNA loci had to be statistically higher than at the mRNA loci; second, to exclude false-positive results due to systematic technical artifacts, the RNAP II ChIP signals in samples within the same dataset had to be statistically higher at tRNA than at mRNA loci.

Among the RNAP II–associated factors investigated here, Nab2 and Nrd1 were previously shown to bind tRNA transcripts (17–21). Moreover, Nab2 was also shown to directly interact with RNAP III and have an important role in an efficient RNAP III transcription initiation (17, 21). For these reasons, the genomic ChIP profiles of Nab2 and Nrd1 were taken as positive controls for the recruitment of RNAP II factors to tRNA loci.

Fig. 1 illustrates our typical results for protein factors with a high and low preference for tRNA loci. For each transcription unit of tRNA (red squares) and mRNA (gray circles), Fig. 1 compares the average read counts of RNAP II with those of Nab2, Sub2, Hpr1, and the RNAP III subunit Rnc160, taken from dataset A (17, 22). As expected, the signal of RNAP II is significantly higher at the mRNA than at tRNA loci, and the signal of RNAP III is higher at tRNA than at mRNA loci (p value (t test) < 0.000001). The RNAP II–associated factors Nab2, which was previously reported to interact with RNAP III and RNAP III
transcripts (17, 21), and Sub2, an RNA helicase involved in the splicing, exporting, and 3'-end processing of mRNA (23–25), appear to on average prefer tDNAs to mRNA sites ($p$ value ($t$ test) $< 0.000001$) (Fig. 1). As illustrated in Fig. 2, the co-occupancy of Sub2 and Nab2 is also supported by the strong correlation between their average ChIP counts at the tRNA loci (Pearson correlation coefficient ($r_p$) = 0.79, $n = 275, p < 0.0001$). In contrast to Sub2 and Nab2, the genomic distribution of Hpr1, a subunit of the THO complex operating in coupling transcription elongation with mRNA export, is noticeably higher at the mRNA sites than at tDNAs ($p$ value ($t$ test) $< 0.000001$) (Fig. 1), consistent with its preference for mRNA loci.

Among the proteins of dataset A (Table 1), which included the Sub2, Hpr1, Nab2, Rpc160, and RNAP II examined above, two other RNAP II–associated factors exhibited a significant preference for tDNAs, Yra1 ($p$ value ($t$ test) $< 0.000001$) and, with weaker evidence, Npl3 ($p$ value ($t$ test) = 0.018) (Fig. S1). The remaining RNAP II–associated factors of dataset A (Gbp2, Hrb1, Mft1, and Tho2) exhibited a clear preference for the mRNA loci (Fig. S1).

The above analysis of the read counts distribution between tRNA and mRNA loci was performed on all ChIP profiles of the four datasets listed in Table 1 (see Fig. S1). As a final result, we found that 13 of the 46 proteins investigated here exhibited a preferential localization at the tDNAs rather than the mRNA loci: the subunit of RNAP III Rpc160, eight RNAP II–associated factors (Nab2, Sub2, Yra1, Pcf11, Clp1, Rat1, Nrd1, and Npl3), one histone demethylase (Jhd2), two protein kinases (Bur1 and Ctk1), and a mutant of the nucleosome remodeling factor Spt6 lacking the 202 C-terminal residues (Spt6ΔCTD). It should be noted that, although not clearly evident and relatively weaker than the other factors, the occupancy of Nrd1 at tRNA sites satisfies the criterion of being statistically higher than at mRNA sites ($p$ value ($t$ test) $< 0.0002$). The high levels of recruitment to the tRNA loci of Jhd2, Bur1, Ctk1, and Spt6ΔCTD (Fig. S2) are surprising because, to our knowledge, they have never been mentioned before either as genuine signals nor as artifacts. The results from the four datasets were consistent with each other; the enrichment at tDNAs of Pcf11, Sub2, and Yra1 was coherently validated by more than one dataset (see Table 1), and as expected by unbiased data, all the 11 RNAP II subunits examined here were richer at the mRNA than at the tRNA loci.

Because dataset A also includes the ChIP profiles of the RNAP III subunit Rpc160 (17, 22), we used this dataset to determine whether the enrichment of RNAP II–associated factors at tDNAs correlated with the level of RNAP III. Among the 10 samples of dataset A, only those proteins with a highly significant signal at the tRNA loci (Nab2, Sub2, and Yra1) significantly and positively correlated with the RNAP III occupancy at tDNAs ($r_p = 0.67, 0.76, and 0.45$, respectively, $p < 0.0001$) (Fig. S3). It is noteworthy that the Pearson partial correlation between the occupancy of RNAP III and Yra1 at the tRNA loci, after controlling for the Sub2 occupancy, was slightly negative and statistically insignificant ($partial r_p = -0.038, p = 0.53$), suggesting that the recruitment of Yra1 to tDNA sites could be mediated by Sub2.

It has previously been reported that Nab2 occupies the tRNA sites and the other RNAP III genes: RDN5, SNR6, SNR52, RPR1, RNA170, ZOD1, and SCR1 (17). Here, we found that the occupancies of Sub2 and Yra1 at the RNAP III genes others than the tDNAs positively correlated with the occupancy of Nab2 ($r_p = 0.92$ and 0.61, respectively, $p < 0.05$). Moreover, as shown above for the tRNA loci, we found that the read counts of RNAP III at the RNAP III genes other than the tDNAs positively correlated with those of Nab2, Sub2, and Yra1 ($r_p = 0.82, 0.91, and 0.78$, respectively, $p < 0.005$).

**Deletion of the C terminus triggers Spt6 recruitment to tRNA loci**

Spt6 is an essential RNAP II transcription elongation factor (26) that coordinates chromatin structure and histone modifications (27). Its structure contains several domains, including an S1 RNA-binding domain and, at the C terminus, a tandem
Recruitment of RNAP II–associated factors to tRNA loci

SH2 (tSH2) domain that binds the phosphorylated Rpb1 subunit of RNAP II (28). Spt6 does not typically occupy the tRNA loci (29), and the deletion of its C terminus, which contains the RNAP II-binding domain, has partial effects on its occupancy at coding regions (30). Here, we analyzed the effects that the C-terminal deletion of Spt6 caused on its distribution between the mRNA and tRNA loci.

As shown in Fig. 3, the occupancy of native Spt6 at tDNAs is low, as consistent with previous results (29). Surprisingly, Spt6ΔCTD is significantly more enriched at the tDNAs than at the mRNA sites. This selective enrichment at tRNA loci upon the C-terminal deletion is illustrated in Fig. 4, where the occupancy at mRNA and the mRNA loci of Spt6 and Spt6ΔCTD are directly compared. Remarkably, the deletion of the C terminus only marginally reduced the correlation between Spt6 and RNAP II at the mRNA sites (rp(Spt6, RNAP II) = 0.97 and rp(Spt6ΔCTD, RNAP II) = 0.84).

We also analyzed an additional independent ChIP dataset (30) that confirmed the significant increase of the Spt6 signal at the tRNA loci upon the deletion of the C terminus (Fig. S4).

Comparison of the Spt6 and Spt6ΔCTD results indicates that a structural change, apparently independent from the ChIP procedure, can strongly increase the ChIP signal at the tRNA loci and can alter the relative distribution of protein factors between RNAP II and RNAP III genes. This is consistent with a possible antagonism between the RNAP II and RNAP III transcriptional complexes in the recruitment of common interactors. Spt6ΔCTD likely can bind analogous constituents of the RNAP II and RNAP III transcription complexes; for example, the precursor RNAs by the S1 RNA-binding domain, through a mechanism that emerges when its interaction with RNAP II, is weakened by the deletion of the tSH2 domain. The result suggests that tRNA genes may act as a sink for multiple factors, especially when their normal protein interactions are disrupted.

RNAP II–associated factors enriched at the tDNAs commonly present RNA-binding properties and participate in the termination stage of the mRNA transcription

To clarify the origin of the factor recruitment to the tRNA sites, we evaluated the binding properties of the factors analyzed here in relation to their preference for tRNA loci. Among the 46 ChIP samples analyzed here, 23 include RNAP II and RNAP III subunits, histones, kinases, histone demethylases, and Spt6ΔCTD. The remaining 23 samples include factors recruited to the RNAP II transcriptional complex for its normal activity. We found that eight of these 23 RNAP II factors were more enriched at the tRNA than at the mRNA loci: Sub2, Yra1, Clp1, Nab2, Pcf11, Rat1, Nrd1, and Npl3. These eight factors enriched at the tRNA loci were all annotated with the Gene Ontology (GO) term “RNA binding” (GO:0003723). In contrast, only six of the 15 factors preferring mRNA loci were annotated with the RNA binding GO term. This is a statistically significant difference (Fisher’s exact p value (one-tailed) = 0.0061). The remaining native factors enriched at the tDNAs, i.e. the histone demethylase Jhd2 and the protein kinases Bur1 and Ctk1, have been recently reported to bind RNA (31, 32). Considering that the Spt6ΔCTD contains the S1 RNA-binding domain, we can conclude that all 12 proteins that prefer tRNA to mRNA loci have RNA-binding properties.

In contrast, seven RNAP II factors were annotated with the GO term DNA binding (GO:0003677), but none of them were enriched at the tRNA loci (Fisher’s exact p value (one-tailed) = 0.0262). Finally, the RNAP II factors annotated as “protein binding” (GO:0005515) were uniformly distributed between those that prefer tDNAs and mRNA loci. These results strongly suggest that the pre-tRNAs could be the main targets of the RNAP II–associated factors enriched at the tDNAs. If so, the tRNA transcripts should be mapped by in vivo experiments of protein–RNA cross-linking.

For this reason, we analyzed an additional dataset of photoactivatable ribonucleoside–enhanced cross-linking and immuno precipitation (PAR-CLIP) experiments, including 23 RNAP II–associated factors that bind pre-mRNA (33). PAR-CLIP is a method used for detecting the RNA sites targeted by RNA-binding proteins. An important advantage of the PAR-CLIP method is that it can detect RNA sites cross-linked to proteins because mutations occur in specific bases in the RNA that are directly cross-linked to the protein when cross-linking is reversed (34). We found high PAR-CLIP signals at the tRNAs for all 23 RNAP II factors that recognize pre-mRNA elements in vivo. Although the high PAR-CLIP signals at tRNAs have not been mentioned and discussed in the original paper (33)
and their origin is unclear, this is the result to be expected if the tRNA transcripts have the ability to recruit the RNAP II–associated factors with RNA-binding properties in vivo.

The GO term analysis also showed that all eight RNAP II factors that are enriched at the tDNAs (Sub2, Yra1, Clp1, Nab2, Pcf11, Rat1, Nrd1, and Npl3) participate in the processes associated with the termination stage of RNAP II transcription: polyadenylation, 3′-end–processing, and export of mRNA. At the same time, none of the six RNAP II factors annotated with GO terms associated with the transcription initiation (Kin28, Sua7, Tfg1, Paf1, SpI6, and Spt16) were found to be enriched at the tRNA loci.

**Distance between the 3′-ends of tRNA and downstream RNAP II genes in the opposite strand is correlated with RNAP II transcription level**

As reported above, the RNAP II–associated factors that show a preference for tRNA genomic loci are RNA-binding proteins and participate in the termination stage of RNAP II transcription. This biased recruitment should lead to an asymmetric occupancy profile of the 12 factors at tRNA loci and may affect the expression of the RNAP II genes in a way that depends on their position and orientation with respect to the tRNA genes.

In fact, as shown in the Fig. 5A, the peak at the tRNA sites of the average occupancy of the 12 factors presents a higher shoulder on the 3′-side, consistent with the RNA-binding property of the 12 proteins. Because the upstream elements nearest to tRNA genes are prevalently long-terminal repeats (LTR), whereas the protein-coding genes primarily occupy tDNA downstream regions (Fig. 5B), we investigated the expression level of the RNAP II genes downstream to tRNA genes in relation to their orientation and distance from 3′-end of tRNA gene.

As illustrated in Fig. 6 (see data in Table S1), the distance between the 3′-tDNA and the 3′-end of the downstream RNAP II genes in the opposite strand is highly correlated with the expression level of the RNAP II gene (Spearman correlation coefficient (rs) = 0.55, p < 0.0005). In contrast, no significant correlation was found between 3′-tDNA and the 5′-end of the downstream RNAP II genes on the same strand. Therefore, in the downstream region of tRNA genes, the relationship between RNAP II expression level and the distance from the tRNA gene does not appear to be random with respect to the gene orientation. The correlation coefficients reported above were estimated considering the 118 isolated tRNA loci that do not present any other tRNA gene within a distance of 15,000 nucleotides (cutoff distance = 15,000). To determine whether the other tRNA loci interfere with the relationship between the tRNA gene and the expression of its downstream RNAP II gene, we investigated the effects on the correlation coefficient of varying the cutoff distance. We found that the shortening of the cutoff distance leads to a decrease of the correlation coefficient. For cutoffs of 20,000, 15,000, 10,000, and 5000 nucleotides, we found rs = 0.64, 0.55, 0.38, and 0.33, respectively (p < 0.005 for all). In fact, a strong correlation between distance and RNAP II expression appears to be detectable only in highly-isolated tRNA genes, suggesting that the combined effects of more than one tRNA locus can affect the transcription of an RNAP II gene.

**High ChIP signal at the tRNA loci is not compatible with the location of the hyper-ChIPable sites**

A list of 238 genomic sites that exhibit a systematic ability to produce ambiguous high ChIP signals, termed “hyper-ChIPable” sites, has been published (16). Because this list includes 145 regions overlapping with 154 tRNA genes, we
Recruitment of RNAP II–associated factors to tRNA loci

Figure 7. Average occupancy of protein enriched at the tRNA loci compared with the RNAP II occupancy. For each protein, the graph compares the average occupancy at mRNA loci and at the tRNA loci within and outside the hyper-ChIPable sites. The error bars indicate S.E.

evaluated the possibility that our observed high ChIP signal at the tRNA loci could be related to the hyper-ChIPability. In the original paper (16), the high ChIP signal observed at the hyper-ChIPable sites has been considered a possible artifact due to a technical issue with the immunoprecipitations. One reason to consider hyper-ChIPability as an artifact was that the Sir proteins exhibited high signals at hyper-ChIPable sites even though they were not expected to occupy these regions (16).

We analyzed the enrichment data reported in the tables of the original paper on hyper-ChIPability (16). The analysis did not find any significant difference between the occupancy of RNAP II and Sir proteins at hyper-ChIPable sites containing tDNAs, which is consistent with the hyper-ChIPability also affecting the ChIP signals of RNAP II. In contrast, as shown in Fig. 7, the occupancy at the tRNA loci of hyper-ChIPable regions of the 12 factors that we found to be enriched at the tDNAs is significantly higher than that of RNAP II. Moreover, with the exclusion of the Spt6ΔCTD, the occupancy of the RNAP II factors that we found to be enriched at the tDNAs exhibited no significant difference between the tRNA loci located within or outside the hyper-ChIPable regions (t test, p > 0.05 with Bonferroni correction) (Fig. 7).

Additionally, as reported above, the Spt6ΔCTD ChIP signal was strongly higher than the Spt6 signal at the tRNA loci (p value (t test) < 0.000001), although they were not significantly different at the hyper-ChIPable sites that do not contain tRNA loci (p value (t test) >0.05). These results show that the high signal at the tRNA sites observed in this work is not consistent with the location of the hyper-ChIPable sites.

Discussion

Active tRNA synthesis down-regulates the RNAP II transcription of the nearby genes (1, 2). Although this phenomenon, termed TGM silencing, was first observed in 1991 (2), its origin is not yet clear. In this work, we proposed and tested a new hypothesis for the origin of TGM silencing based on a possible antagonistic ability of the RNAP III transcriptional machinery to recruit RNAP II–associated factors and thus reduce their availability for mRNA synthesis. In the possible model proposed here, the recruitment of RNAP II–associated factors at tRNA genes could contribute to TGM silencing by reducing locally the number of free factors accessible to RNAP II polymerase. This possible phenomenon could be favored by the structure of the nucleolus, where most tRNA genes are localized, and by the high level of tRNA transcription.

To that end, we analyzed the genome-wide ChIP profiles of 46 samples and found that 12 proteins associated with the RNAP II transcription exhibited a significantly higher ChIP signal at the tRNA than at the mRNA loci. Experimental evidence reported in the literature supports the role of each of these 12 factors in the RNAP II transcription. Conversely, excluding the case of Nab2 (17, 21), the role of these RNAP II–associated factors in the tRNA genesis is unknown, encouraging the idea that their high ChIP signals at RNAP III–transcribed genes could originate from experimental or technical bias. In fact, most of the tRNA sites are included in a list of genomic regions showing an ambiguous and systematic high level of ChIP signals, termed hyper-ChIPable sites (16). Different causes of hyper-ChIPability have been proposed, including technical issues with immunoprecipitation (16), site mappability (35), GC content (36), sequence uniqueness (15), and cross-linking time (37). All these proposed causes of hyper-ChIPability are inherent in technical procedures or in the sequence of genomic targets and appear not to be primarily dependent on the structural characteristics of the protein factor. In this work, consistent with a marginal role of systematic artifacts due to the technical procedures or the nature of genomic targets, we found that different samples acquired in the same laboratory can exhibit very different levels of ChIP signals at the tRNA loci. Moreover, we found that the results of different datasets are consistent with each other, although they were obtained in different laboratories. For example, all 11 subunits of RNAP II, as well as the two histones, show low ChIP signal at tDNAs. Moreover, all the analyses of data from independent sources of Sub2
TGM silencing could explain why pre-tRNA truncation relieves RNA-binding proteins involvement of pre-tRNA also explains why the TGM silencing and why a CBF5 mutation that relieves TGM silencing also dissociates the pre-tRNA and tRNA genes from the nucleolus (16). The results reported here indicate that the high signal of the 12 RNAP II factors detected at the tDNA loci does not arise from possible artifacts associated with the hyper-ChIPability of the tRNA loci.

Our results from the analysis of dataset A show a positive and significant correlation of the RNAP III occupancy at tDNAs only with those protein factors that exhibited a highly-significant preference for the tDNAs (Nab2, Sub2, and Yra1), indicating that their recruitment to the tRNA loci could be physically associated with the RNAP III transcription complex. The binding properties of the protein factors analyzed here strongly suggest that the pre-tRNAs could be the preferred target for the factors enriched at the tRNA loci. In fact, we observed, with statistical significance, that all eight RNAP II-associated factors enriched at the tRNA loci (Sub2, Yra1, Clp1, Nab2, Pcf11, Rat1, Nrd1, and Npl3) are annotated RNA-binding proteins that participate in the termination stage of RNAP II transcription. Consistent with this, we found a higher average occupancy of the 12 RNAP II factors at the 3’- than at the 5’-side of tRNA loci (Fig. 5A). Moreover, we detected a clear polarity in the transcriptional activity of RNAP II genes downstream to the tRNA loci that is consistent with silencing effects more active on RNAP II genes that are oriented with their 3’-end toward the tRNA loci.

A possible role of the RNA-binding property in generating the high ChIP signal at the tRNA loci is also supported by our analysis of a PAR-CLIP dataset (33) showing a high level of direct cross-linking between the tRNA transcripts and 23 pre-mRNA-binding proteins.

The high subnuclear concentration of the tRNA transcripts at the nucleolus, due to the combined effect of their compartmentalization (4, 5) and their high level of synthesis (39), could favor the TGM silencing by promoting the recruitment of the RNA-binding proteins to the pre-tRNA. The possible role in TGM silencing of the tRNA transcripts and their localization in the nucleolus could explain why pre-tRNA truncation relieves TGM silencing in vivo (12), why TGM silencing requires nucleolar clustering and active transcription of tRNA genes (1, 6), and why a CBF5 mutation that relieves TGM silencing also dissociates the pre-tRNA and tRNA genes from the nucleolus and leads to their dispersal in the nucleoplasm (7). Consistently, defective mutations in all five subunits of condensin that lead to loss of TGM silencing also cause pre-tRNA dispersion throughout the nucleoplasm or to the nuclear periphery (8). The direct involvement of pre-tRNA also explains why the TGM silencing mechanism does not imply the presence of a steric blockade of RNAP II (1).

Moreover, consistent with a possible antagonistic model of RNAP II silencing based on the recruitment of RNA-binding factors that act at the terminal stage of RNAP II transcription, we found that the distance between the 3’-ends of tRNA and RNAP II genes oriented tail-to-tail positively correlates with the expression level of the RNAP II genes. Consistent with the antagonistic model, we also noticed that such a relationship between the distance from tRNA and the expression level of RNAP II genes appears affected by the genomic distance from the other tRNA genes.

It should be noted that the mRNA genes upstream to tRNAs have been previously reported to be on average less transcribed than the downstream genes and that the level of transcription of the upstream genes within 400 bp does not present significant differences due to gene orientation (3). It appears that the tRNA positional effect on upstream and downstream regions could be different, likely reflecting more than one down-regulation mechanism associated with their high level of expression.

The antagonistic model of the silencing mechanism proposed here, in which the pre-tRNA competes with the RNAP II transcription machinery for the recruitment of proteins factors, is also consistent with our results on the genomic occupancy data of Spt6 and Spt6ΔCTD. The structure of Spt6 presents an S1 RNA-binding domain (42) and a tSH2 domain that binds the Rpb1 subunit of RNAP II (28). We observed that the native Spt6 preferentially occupies the RNAP II–transcribed genes rather than tRNA loci. In contrast, Spt6ΔCTD, which lacks the RNAP II-binding domain but conserves the RNA-binding region, prefers tRNA over mRNA loci, consistent with a competitive recruitment of Spt6 by RNAP II and RNAP III transcriptional complexes. It is likely that the Spt6 could bind the RNA precursors of RNAP II and RNAP III transcription processes through a competitive mechanism that emerges in Spt6ΔCTD when its interaction with the RNAP II is weakened by the removal of the tSH2-binding domain.

RNAs, including tRNAs, have the ability to block the binding of the DNA template in vitro by a mechanism that requires direct interaction with the RNAP II (13). However, whereas half of the pre-tRNA molecules have a better ability than intact pre-tRNA to inhibit RNAP II transcription through a direct interaction with the polymerase in vivo (13), they completely relieve TGM silencing in vivo (12), suggesting that a possible direct RNAP II–pre-tRNA interaction is not a main cause of TGM silencing.

Based on the antagonistic hypothesis proposed here, in which tRNA transcripts recruit RNAP II factors, we should expect that molecules that are involved in the tRNA genesis and physically interact with pre-tRNAs could affect TGM silencing. Consistent with this assumption, it has been previously shown
that deletion of Mod5, a tRNA isopentenyltransferase that modifies a subset of tRNAs, relieves TGM silencing without altering the tRNA sub-nucleolar localization (12, 43). Mod5 binds both substrate and nonsubstrate pre-tRNAs and is physically associated with RNAP III complex proteins in vitro (12, 44). Moreover, the subcellular localization of Mod5, which includes the nucleolus (45), is altered in mutants of MAF1 (46), a RNAP III transcriptional repressor. This ability of MAF1 mutants to delocalize Mod5 may explain the paradox of MAF1 deletion, which increases tRNA transcription and relieves TGM silencing (47, 48). In addition to this RNAP II–associated factors reported here, the TATA-binding protein (TBP) was previously found strongly enriched at the tRNA loci (49). TBP is a component of the preinitiation transcription complex that is shared by the three RNA polymerases RNAP I, II, and III (50). A possible antagonistic mechanism between RNAP II and RNAP III could also involve TBP. However, its involvement in the silencing process cannot explain why, for example, tRNA truncation in vivo completely relieves TGM silencing (12), or as observed in the present work, the 3’–3’ distance between tRNA and downstream RNAP II genes in the opposite strand correlates with the expression level of the RNAP II genes.

In conclusion, the results reported here show that 12 protein factors with a role in RNAP II transcription, typically characterized by RNA-binding properties and implicated in the termination stage of RNAP II transcription, occupy the genomic tRNA loci by a mechanism that, at least in the cases of Sub2, Yra1, and Nab2, positively correlates with the level of RNAP III. The tRNA transcripts appear to be the main components of the RNAP III transcription complex responsible for the recruitment of the RNAP II factors to the tDNA sites. Because of their high local compartmentalization, the tRNA transcripts can compete favorably with the RNAP II transcriptional complexes in the recruitment of RNA-binding factors. As a possible consequence of this antagonism, the transcriptional efficiency of the RNAP II close to the tRNA genes decreases, contributing, at least in part, to the mechanism of TGM silencing. Consistent with this hypothesis, we show that the expression level of the RNAP II genes downstream to tRNA loci correlates positively with the distance from tRNA when the 3’-ends of tRNA and RNAP II genes point toward each other. As a secondary effect, such a mechanism could favor the RNAP III transcription itself, as a down-regulation of RNAP II synthesis should increase the availability of those cellular resources that are shared by the RNA polymerases. Although our results are consistent with the involvement of a possible antagonistic mechanism in the TGM silencing, additional experimental data will be required in the future to validate the new antagonistic model.

**Experimental procedures**

The genome annotation of the *Saccharomyces cerevisiae* S288C used in this work was the release R64 (Saccharomyces_cerevisiae_R64-2-1_20150113.gff) deposited at the Saccharomyces Genome Database (http://www.yeastgenome.org). The processed genome-wide profile data of the ChIP with microarray or sequencing (ChIP-chip or ChIP-seq) of dataset A (accession numbers E-MTAB-1400 and E-MTAB-3700) (17, 22) and dataset B (accession number E-TABM-1033) (38) were downloaded from the ArrayExpress (www.ebi.ac.uk/arrayexpress) (56). The processed data of dataset C (accession number GSE67212) (31) and dataset D (accession numbers GSE23960 and GSE30706) (51, 52) were taken from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). PAR-CLIP transcriptome profiles of 23 mRNP biogenesis factors were downloaded from GEO (accession number GSE59676) (33). All downloaded ChIP data were originally normalized against input or mock control.

The average read counts of the ChIP signal related to mRNA and tRNA loci were computed using genomic coordinates of features annotated as mRNA and tRNA. Conversion between the different versions of yeast genomic coordinates was performed using the Perl script convert_yeast_genome_version.pl from BioToolBox libraries version 1.17 (https://github.com/tjparnell/biotoolbox). The GO annotations for the GO term analysis were retrieved using AmiGO 2 tools (http://amigo.geneontology.org) (53, 57–59). The correlation and partial correlation analyses were performed by calculating the rp between the average read counts of mRNA and tRNA. The correlation analysis was validated using the nonparametric rs to overcome the stringent conditions of linearity and normality and the effects of possible outliers.

The correlation analysis between the RNAP II expression level and the distance between tRNAs and the protein-coding gene was performed using the genomic coordinates of elements annotated as gene and tRNA gene that were located within a distance of 501 nucleotides. The transcriptome and UTR data were obtained from the works of Yassour et al. (54) and Pelechano et al. (55), respectively.

The average occupancy profile of the 12 factors enriched at tRNA loci was calculated after normalization of the occupancy values of each factor from 0 (at −1000 nucleotides from 5′-tRNA) to 100 (at maximum peak value).

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Recruitment of RNAP II–associated factors to tRNA loci

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