A cryptic unstable transcript mediates transcriptional *trans*-silencing of the Ty1 retrotransposon in *S. cerevisiae*

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Cryptic unstable transcripts (CUTs) are synthesized from intra- and intergenic regions in *Saccharomyces cerevisiae* and are rapidly degraded by RNA surveillance pathways, but their function(s) remain(s) elusive. Here, we show that an antisense *TY1* CUT, starting within the Ty1 retrotransposon and encompassing the promoter 5' long terminal repeat (LTR), mediates RNA-dependent gene silencing and represses Ty1 mobility. We show that the Ty1 regulatory RNA is synthesized by RNA polymerase II, polyadenylated, and destabilized by the cytoplasmic 5' RNA degradation pathway. Moreover, the Ty1 regulatory RNA represses Ty1 transcription and transposition in *trans* by acting on the de novo transcribed *TY1* RNA. Consistent with a transcriptional regulation mechanism, we show that RNA polymerase II occupancy is reduced on the Ty1 chromatin upon silencing, although TBP binding remains unchanged. Furthermore, the Ty1 silencing is partially mediated by histone deacetylation and requires Set1-dependent histone methylation, pointing out an analogy with heterochromatin gene silencing. Our results show the first example of an RNA-dependent gene *trans*-silencing mediated by epigenetic marks in *S. cerevisiae*.

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Recent data have shown that cryptic unstable transcripts (CUTs) are RNA polymerase II (RNAPII)-dependent non-coding RNAs (ncRNAs) corresponding to inter- and intragenic regions of the genome and may represent 10% of intergenic transcripts in *Saccharomyces cerevisiae* (Wyers et al. 2005). Under normal conditions, CUTs are almost undetectable, as they are rapidly degraded by the activity of Rps6 and Trf4, members of the nuclear exosome and the TRAMP complex, respectively (LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005). In addition to Trf4 and Rps6, the cytoplasmic 5'–3' exoribonuclease Xrn1 also plays an important role in the turnover of CUTs, supporting the idea that some of these transcripts escape the nuclear quality control and might have a cytoplasmic residency (Thompson and Parker 2007).

Despite these observations, the function(s) of CUTs remain(s) poorly characterized. Cryptic transcription has been widely described from yeast to human and qualified as “transcriptional noise.” Interestingly, it has been proposed that cryptic transcription allows RNA polymerase-dependent chromatin changes but not the produc-

tion of functional RNA molecules, as those are immediately degraded (Struhl 2007). In agreement with this model, CUT transcription has been shown to interfere with promoters of coding regions and hence regulates gene expression in *S. cerevisiae* (Martens et al. 2005; Hongay et al. 2006; Uhler et al. 2007). However, alternative models emerged, providing a direct function for cryptic transcripts. Indeed, recent reports have shown that the processing of siRNAs in *Schizosaccharomyces pombe* is mediated by homologs of the TRAMP and exosome subunits (Buhler et al. 2007; Nicolas et al. 2007), strongly indicating that the fission yeast's siRNAs might originate from CUTs. It is tempting to hypothesize that CUTs in *S. cerevisiae* are linked to an ancient mechanism that would control gene expression in an RNA-dependent manner.

One of the RNAi functions is to limit mobile genetic element (MGE) proliferation in the genome (Weiner 2002). MGEs encompass viruses, transposons, and retrotransposons and are dispersed from bacteria to mammalian cells (Capy 2005). The paroxysm of mobile elements has been described in human cells whereby up to 40% of the genome is composed of repeated sequences (Lander et al. 2001) that play potentially deleterious functions in chromosome rearrangements and gene expression. Therefore, each step of the transposition cycle is tightly

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controlled. In *S. cerevisiae*, the Ty1 retrotransposon is subjected to a cosuppression regulation (Jiang 2002) that presents similarities with the transcriptional cosuppression mediated by siRNAs in plants (Jorgensen 1995). This observation strongly suggests that a yet-uncharacterized RNAi-like mechanism might exist in budding yeast, despite the absence of the entire RNAi pathway in this organism (Aravind et al. 2000).

In this study, we address the existence of an RNA-dependent gene regulation mechanism in *S. cerevisiae*. By analogy with transposon regulation in higher eukaryotes, we hypothesized that regulatory RNAs might provide the silencing signal to repress Ty1 expression. With the use of strains defective for the 5'–3' and 3'–5' RNA decay pathways, we show the existence of a *TY1* unstable antisense RNA that triggers Ty1 transcriptional silencing in *trans*. We present evidence that the Ty1 silencing is partially suppressed by *in vivo* treatment with the histone deacetylase inhibitors nicotinamide (NAM) and Trichostatin A (TSA). In addition, the Ty1 silencing is controlled by the histone methyltransferase (HMT) Set1 and the histone H3 Lys 4 methylation, providing an interesting parallel with heterochromatic gene silencing. We propose that CUTs contribute to establish a specialized chromatin domain over repetitive elements in *S. cerevisiae* to limit their dispersion.

Results

The 5'–3' exoribonuclease *Xrn1* maintains *Ty1* expression and mobility and destabilizes a *Ty1* antisense RNA

To address the question whether regulatory RNAs can be detected within the Ty1 transposon, we analyzed, by Northern blot, *TY1* RNAs in strains mutated for 5' and 3' RNA decays that accumulate high levels of CUTs. Thus, total RNAs from yeast strains deleted for *XRN1*, *RRP6*, *TRF4*, or *TRF5* encoding genes were extracted, loaded on 1% agarose gel, and transferred onto a nitrocellulose membrane. A *tec1Δ* strain, deleted for a Ty1 transcription activator, was used as a control characterized by negligible levels of *TY1* RNA (Laloux et al. 1994; Baur et al. 1997; Morillon et al. 2000). We probed the membrane with sense and antisense riboprobes corresponding to different regions of Ty1 (schematized in Fig. 1A) and normalized the signals with the *scR1* RNA level. In the *xrn1Δ* strain, quantifications revealed a dramatic 10-fold accumulation of a >400-nucleotide (nt)-long antisense RNA, mapping to the 5'-end long terminal repeat (LTR) region, and that we named *RTL* (Fig. 1B). In contrast, *rrp6Δ* and *trf4Δ* showed a modest twofold increase of the *RTL* RNA, with a slight additive effect in the *rrp6Δtrf4Δ* strain (2.5-fold increase). It is interesting to note that the *tec1Δxrn1Δ* strain has similar levels of the *RTL* RNA as the *xrn1Δ* strain, suggesting that *RTL* transcription is *Tec1*-independent. Remarkably, the *TY1* full-length sense RNA (*TY1FL*) showed a fivefold decrease in the *xrn1Δ* strain, similarly to the *tec1Δ* strain (Fig. 1B). In addition, we noticed the presence of a sense-

Figure 1. The 5'–3' exoribonuclease *Xrn1* prevents Ty1 antisense RNA accumulation and maintains Ty1 expression and transposition. (A) Schematization of different Ty1 transcripts (dashed lines/arrows), with their respective coordinates. Positions of riboprobes specific to the *RTL*, *TY1FL*, and *TY1SL* RNAs are represented by solid line/arrows. Note that the probe in the *TYB* region hybridizes the *TY1SL* and *TY1FL* RNAs. (B) Northern analysis of Ty1 full-length (*TY1FL*), Ty1 5' short-length (*TY1SL*), and 5'-LTR antisense Ty1 RNA (*RTL*). *scR1* and *ACT1* were used as loading controls. Numbers represent *TY1FL/scR1* and *RTL/scR1* RNA ratios. Strains are YAM92 (WT W303), YAM591 (*tec1Δ*), YAM460 (*tec1Δxrn1Δ*), YAM97 (*xrn1Δ*), YAM456 (*trf4Δ*), and YAM458 (*xrn1Δtrf4Δ*). YAM118 (WT BY4741), YAM125 (*trf4Δ*), YAM123 (*rrp6Δ*), YAM126 (*trf5Δ*), and YAM128 (*rrp6Δtrf4Δ*). (C) Growth assay of strains bearing *TY1[ML2]::URA3* on plates containing complete synthetic media and +5FOA. The strains are YAM698 (WT), YAM700 (*xrn1Δ*), YAM783 (*tec1Δ*), YAM784 (*ste12Δ*), and YAM166 (*ura+*). (D) Transposition rate of *TY1HIS3AI*-containing strains, expressed as a rate of *HIS+* cells per total number of cells (see Materials and Methods). Strains are YAM359 (WT), YAM641 (*xrn1Δ*), and YAM601 (*ste12Δ*). The transposition rate represents the mean of three independent experiments with standard deviations.

orientated short-length Ty1 RNA (*TY1SL*) in the *xrn1Δ* strain. Further characterization with a probe spanning the 5' end of the *TYA* region failed to reveal the *TY1SL* RNA, suggesting that *TY1SL* is a 5'-end-truncated form of the *TY1FL* RNA (data not shown). Interestingly, the *tec1Δ xrn1Δ* double mutant showed an additive decrease of the *TY1FL* RNA, suggesting that Xrn1 and Tec1 regulate *TY1FL* through two independent pathways. In contrast, *TRF4*, *TRF5*, and *RRP6* deletions only resulted in slight increases of the *TY1FL* steady-state RNA levels. Taken together, these results show that the cytoplasmic 5'-exonuclease Xrn1 up-regulates the *TY1FL* RNA but strongly represses the accumulation of two unstable *TY1* RNAs: *RTL* and *TY1SL*.

To confirm the importance of Xrn1 in Ty1 expression, we constructed a strain carrying the *URA3* gene in frame with the highly expressed Ty1(ML2) on chromosome XIII (Morillon et al. 2002). The expression of *TY1(ML2)::URA3* was determined by assessing the resistance of cells to the 5FOA drug (see Materials and Methods). We performed a growth assay dispensing serial 10× cell dilutions of wild-type, *ste12Δ*, *tec1Δ*, and *xrn1Δ* strains carrying *Ty1(ML2)::URA3*, on 5FOA plates (Fig. 1C). As expected, the *Ty1(ML2)::URA3 xrn1Δ* strain was more resistant than the wild type to the 5FOA drug, similar to what is observed in strains mutated for the Ty1 transcription activators Tec1 and Ste12 (Laloux et al. 1994; Baur et al. 1997; Morillon et al. 2000). This result shows that Xrn1 is required for the expression of a single and highly transcribed Ty1 element.

Next, we aimed to test whether Xrn1 influences Ty1 mobility with the use of the *Ty1HIS3(AI)* strain carrying the *HIS3(AI)* gene inserted into a chromosomal Ty1 retrotransposon (see Materials and Methods). We observed

that the transposition rate of *Ty1HIS3(AI)* was reduced >500-fold in the absence of Xrn1 compared with the wild type, similar to the rate in the *ste12Δ* strain (Fig. 1D), suggesting that Xrn1 is required for Ty1 mobility.

Altogether these results show that the 5'-3' exoribonuclease Xrn1 maintains high levels of Ty1 expression and transposition. In addition, Xrn1 controls shorter length *TY1* RNAs species, in particular the Ty1 anti-sense *RTL* RNA.

RTL RNA is a *CUT* initiating within the *Ty1* coding region

To further characterize the *RTL* RNA, we extended our analysis to a wider variety of RNA decay mutants. In addition to the *xrn1Δ* mutant, deletions of *DCP1* and *DCP2* genes encoding the decapping activity (Coller and Parker 2004) showed a 10-fold and fivefold increase of the *RTL* RNA, respectively (Fig. 2A). In contrast, *RTL* RNA levels were modestly increased in *pat1Δ*, *lsm1Δ*, and *lsm7Δ* strains, defective for the RNA decapping enhancers (Coller and Parker 2004). Similarly, mutants defective for the No-Go-Decay (*dom34Δ*) (Doma and Parker 2006), for the nonsense-mediated decay (*upf1Δ*, *upf2Δ*, *upf3Δ*) (Muhlrad and Parker 1994), for the RNA-deadenylation pathway (*ccr4Δ*) (Daugeron et al. 2001), and for the endoribonuclease III *RNT1* (*rnt1Δ*) (Danin-Kreisel et al. 2003) showed wild-type levels of the *RTL* RNA. Interestingly, when comparing the levels of *RTL* and *TY1FL* RNAs, all mutants can be divided into four classes (summarized in Fig. 2B). Class I consists of cytoplasmic 5'-3' RNA decay mutants and is characterized by a large decrease of the *TY1FL* RNA (more than

Figure 2. Effect of different RNA degradation pathways on Ty1 RNAs. (A) Extended Northern analysis of transcript levels in strains bearing mutations in different RNA surveillance pathways. The membrane is identical as in Figure 1B, but mutants have been separated for presentation convenience. The probes are the same as in Figure 1B. In the left panel, the W303 strains are YAM92 (WT W303), YAM591 (*tec1Δ*), YAM97 (*xrn1Δ*), YAM460 (*tec1Δxrn1Δ*), YAM456 (*trf4Δ*), YAM458 (*xrn1Δtrf4Δ*), YAM93 (*upf1Δ*), YAM94 (*upf2Δ*), and YAM95 (*upf3Δ*). W303 strains in the right panel are YAM92 (WT W303), YAM584 (*ccr4Δ*), YAM586 (*dom34Δ*), YAM115 (WT W303), (YAM119), YAM141 (*dcp2Δ*), YAM142 (*dcp1Δdcp2Δ*), YAM92 (WT W303), and YAM96 (*dcp1Δ*). The S288C strains are YAM31 (WT S288C), YAM34 (*lsm1Δ*), YAM33 (*lsm7Δ*), and YAM32 (*pat1Δ*). The BY4741 background strains are YAM118 (WT BY4741), YAM125 (*trf4Δ*), YAM123 (*rrp6Δ*), YAM126 (*trf5Δ*), and YAM128 (*rrp6Δtrf4Δ*). (B) Table summarizing the effects of the four classes of mutants on Ty1 RNA accumulation. *RTL* and *TY1FL* RNA changes were classified by the following rule: (+++) more than fivefold increase; (+) between twofold and fivefold increase; (-) more than twofold decrease; (NSC) no significant change (less than twofold).

twofold) concomitant with the appearance of high levels of *RTL* (more than fivefold). Class II is represented by mutants defective for decapping enhancing activities and nuclear RNA processing and that show a significant reduction of *TY1FL* (twofold) but without appearance of the *RTL* RNA (less than twofold). In contrast, class III mutants, defective in the 3' nuclear surveillance pathway, have no changes in *TY1FL* but accumulate intermediate levels of *RTL* (up to fivefold). Finally, class IV is composed of mutants altered in translation-coupled RNA degradation that have very little effect on any Ty1 RNAs.

According to this classification, we conclude that the cytoplasmic 5'–3' RNA decay is the main pathway controlling the *RTL* RNA stability but independently of translational-related and RNA deadenylation-coupled RNA decay pathways. Interestingly, the 3' RNA nuclear surveillance machinery plays a minor role in *RTL* stability except when the 5'–3' RNA decay is strongly impaired. Finally, we noted that *RTL* accumulation systematically correlates with the decrease of the steady-state levels of *TY1FL* RNA, suggesting a causal link for the fate of these two transcripts.

Ty1 RTL RNA initiates within Ty1, is synthesized by RNAPII, and degraded by Xrn1

To define more precisely the *RTL* RNA, we performed Rapid Amplification of CDNA Ends (5'-RACE) followed by sequencing analysis. It defined several transcription start sites in the *TYA* coding region with a major starting point at the +661 position from the beginning of the *LTR* sequence (schematized in Fig. 1A). No open reading frame was identified, suggesting that the *TY1 RTL* RNA is noncoding.

Furthermore, Northern blot analysis of polyadenylated [poly(A)⁺] RNAs showed that the poly(A)⁺ *RTL* RNA is still detectable in the *xrn1Δ* strain (Fig. 3A). Moreover, *RTL/ACT1* ratios from total RNAs and poly(A)⁺ RNAs extracted from the *xrn1Δ* strain suggest that the majority of the *RTL* RNA is polyadenylated (66%) (Fig. 3A). Unexpectedly, we noted equivalent amounts of poly(A)⁺ *RTL* extracted from the *trf4Δ xrn1Δ* mutant (64%). This observation suggests that the additive accumulation of the *RTL* RNA in the *trf4Δ xrn1Δ* mutant is not related to the Trf4-dependent poly(A) polymerization activity, consistent with previous observations showing that Trf4 has additional roles in RNA stability (Wyers et al. 2005).

Next, to confirm that the *RTL* RNA is produced by RNAPII, deletion of *XRN1* was performed in a thermo-sensitive *rpb1ts* mutant strain (*rpb1-1*), mutated for *RPO21*, encoding the main subunit of RNAPII (see Materials and Methods). As shown in Figure 3B, after 60 min at 37°C, *RTL* was no longer visible in the *xrn1Δrpb1-1* strain (see quantification in Supplemental Fig. 1), similarly to the RNAPII-dependent RNA *ACT1*. In contrast, levels of the RNAPII-dependent rRNA and the RNAPIII-dependent *scR1* RNA remained unchanged, suggesting that RNAPII synthesizes the *RTL* RNA. In addition, the relatively rapid decrease of *RTL* levels in

Figure 3. Characterization of the Ty1 antisense RNA. (A) Northern blot analysis of transcript levels in strains bearing mutations in the RNA surveillance pathways, purified on oligo(dT) beads [poly(A)⁺ RNA, right panel] or not (total RNA, left panel). The probes are the same as in Figure 1B. The strains are YAM92 (WT), YAM97 (*xrn1Δ*), YAM456 (*trf4Δ*), YAM458 (*xrn1Δtrf4Δ*), and YAM587 (*trp6Δ*). (B) Northern blot analysis of total RNA upon RNAPII inactivation. The time is in minutes after a 37°C heat shock. The probes are the same as in Figure 1B. The strains are YAM9 (*xrn1Δ*) and YAM538 (*xrn1Δrpb1-1*). (C) Northern blot analysis of 1 µg of total RNA treated (+) or not (–) with 0.3 µg of recombinant Xrn1p (rXrn1p). The probes are the same as in Figure 1B. The strains are YAM92 (WT) and YAM97 (*xrn1Δ*).

the *xrn1Δrpb1-1* strain suggests that another pathway is involved in *RTL* degradation, consistent with an additive role of the 3' nuclear surveillance pathway in *RTL* stability.

Finally, we addressed the question whether Xrn1 directly degrades the *RTL* RNA or controls an activator of *RTL* transcription. It has been shown that Xrn1 preferentially degrades RNA with 5' monophosphate extremity but not RNA with a capped extremity in vivo and in

vitro (Muhlrad et al. 1995). Thus, if *RTL* is subjected to a Xrn1-dependent degradation in vivo, it should present an uncapped extremity when accumulating in the *xrn1Δ* strain. To test this hypothesis, we treated total RNAs extracted from the *xrn1Δ* strain with purified recombinant yeast rXrn1p and analyzed the degradation profile by Northern blot (Fig. 3C). We observed that the *RTL* RNA was degraded by rXrn1p with the same efficiency as the uncapped 18S ribosomal RNA (rRNA) (fivefold and sevenfold reduction, respectively). In contrast, the capped *ACT1* mRNA was resistant to rXrn1p activity. This result indicates that the *RTL* RNA detected in the *xrn1Δ* strain is mainly uncapped, confirming that the high accumulation of the *RTL* RNA is a consequence of RNA stabilization and not transcriptional activation in this strain.

Taken together, our data indicate that *RTL* is synthesized by RNAPII initiating within the Ty1 coding region and polyadenylated. The Ty1 *RTL* RNA is then subjected to decapping followed by 5'–3' RNA degradation performed by Xrn1. *RTL* destabilization is reinforced by the nuclear Trf4-dependent RNA surveillance pathway.

TY1 RTL RNA controls *TY1* expression in trans

As *RTL* accumulation coincides with down-regulation of Ty1 expression, we next asked whether the *RTL* RNA directly controls Ty1 expression and mobility. We cloned the *RTL* sequence under the *GAL1*-inducible promoter (*pGRTL*). By switching the carbon source from glucose to galactose in the growth media, we assessed the effect of *RTL* overexpression on Ty1 mobility. As shown in Figure 4A, the transposition rate of the *Ty1HIS3(AI)* element was reduced from fourfold to sevenfold in two different yeast genetic backgrounds, W303 and S288C, respectively, upon transcriptional activation of *RTL* when compared with an empty plasmid. In contrast, upon repressive conditions in glucose, the *Ty1HIS3(AI)* transposition rate was identical in the strain containing the *pGRTL* and the strain containing the empty plasmid. We conclude that the *RTL* RNA inhibits Ty1 retrotransposition in *trans*.

To get insights into the minimal regulatory region(s) of *RTL*, we cloned, under the control of *GAL1* promoter, *RTL* sequences lacking the U3R (*pGRTL^{U3RΔ}*), the LTR (*pGRTL^{LTRΔ}*), and the TYA (*pGRTL^{TYAΔ}*) (see Supplemental Fig. 2 for a schematic view of the truncated *RTL* versions). Interestingly, expression of *RTL^{U3RΔ}*, *RTL^{LTRΔ}*, or *RTL^{TYAΔ}* failed to fully repress (less than twofold) Ty1 mobility (Fig. 4B), showing that the integrity of the *RTL* RNA is required to silence Ty1 transposition. It is interesting to note that the minimal U3R region, corresponding to the Ty1 promoter and 5' untranslated region of the Ty1 RNA, is required for the Ty1 silencing, suggesting that the *RTL* RNA acts in *trans* to control transcription.

Furthermore, to test whether *RTL* represses a single genomic and highly expressed Ty1 element, we performed a growth test assay on 5FOA plates with the *Ty1(ML2)::URA3* strain transformed with the *pGRTL* or an empty plasmid (Fig. 4C). We observed that

Figure 4. *Trans*-silencing of Ty1 expression in the presence of the Ty1 *RTL* RNA. (A) Transposition repression fold of *TY1HIS3AI* strains in the presence of *pGRTL*. The repression fold is expressed as a ratio between the transposition rate measured in a strain bearing *pGRTL* (*pAM134*) and a strain bearing an empty plasmid (*pAM80*) under the same growth conditions. The strains are YAM359 (S288C) and YAM358 (W303), grown in glucose (glu) or galactose (gal). The repression fold represents the mean of three independent experiments with standard deviations. (B) Transposition repression fold of *TY1HIS3AI* strains in the presence of truncated versions of *RTL*. The strains are the same as in A but with YAM359 (WT) grown in galactose and bearing *pGRTL* (*RTL*), *pGRTL^{U3RΔ}* (*U3RΔ*), *pGRTL^{LTRΔ}* (*LTRΔ*), or *pGRTL^{TYAΔ}* (*TYAΔ*). (C) Growth ability of *TY1(ML2)::URA3* strains containing *pGRTL* (*pAM167*) or an empty plasmid (*pAM20*). Galactose plates contain complete synthetic media without leucine supplemented with 5FOA (5FOAgal) or not (gal). Cells were dropped after serial 10× dilutions. The strains are YAM698 (WT) and YAM784 (*ste12Δ*). (D) Northern blot analysis of total RNAs in a *P_{GAL1}:ste12* strain (YAM799) bearing *pGRTL* (*pAM134*) or an empty plasmid (*pAM80*). The probes are the same as in Figure 1B. The time is in hours of growth in galactose.

Ty1(ML2)::URA3 strain grew better on 5FOA⁺ galactose plates in the presence of *pGRTL* when compared with the *TY1(ML2)::URA3* strain containing an empty

plasmid, but with a lower efficiency than a *Ty1(ML2)::URA3 ste12Δ* strain, fully 5FOA-resistant. This result confirms that *RTL* down-regulates the highly expressed *Ty1(ML2)* in *trans* by controlling *Ty1* expression at transcriptional or/and post-transcriptional steps.

Finally, to kinetically monitor *RTL*-dependent repression on the newly synthesized *TY1* RNA, we designed a *P_{GAL1}ste12* strain containing the *STE12* gene, encoding the *Ty1* transcription activator, under the control of the *GAL1* promoter. The *P_{GAL1}ste12* strain was transformed with the *pGRTL* plasmid or an empty vector. In glucose-containing medium, repressing *STE12* transcription, we observed very low levels of the *TY1FL* RNA, confirming that *STE12* is required for *Ty1* transcription (Fig. 4D, first lane in left panel). As expected, upon shift to galactose-containing media and in the presence of the empty vector, we observed progressive increase of the *TY1FL* RNA levels (Fig. 4D, left panel; see quantification in Supplemental Fig. 3). In contrast, coexpression of *RTL* impaired the *Ste12*-dependent *TY1* activation, suggesting that the *RTL* RNA represses the accumulation of neo-synthesized *TY1* RNA (Fig. 4D, right panel; see quantification in Supplemental Fig. 3).

Altogether, these results strongly suggest that the *RTL* RNA silences the *Ty1* expression in *trans* through an RNA-dependent mechanism acting on the de novo synthesized *TY1* RNA.

Xrn1 controls Ty1 expression at a post-TBP recruitment step

In an attempt to define the RNA-dependent regulatory mechanism that controls *Ty1* expression, we performed chromatin immunoprecipitation (ChIP) on formaldehyde-cross-linked cells and recovered the coimmunoprecipitated DNA. The DNA was subjected to real-time PCR by using *Ty1*-specific probes (schematized on Fig. 5A) to measure changes in the relative occupancy of chromatin-associated factors. When immunoprecipitating Rpb1, the largest subunit of RNAPII, we found that RNAPII occupancy decreased from twofold to threefold within the *Ty1* elements in *xrn1Δ* and *ste12Δ* strains when compared with the wild type (Fig. 5B). In contrast, the unrelated constitutive *POL1* gene showed equivalent levels of RNAPII occupancy in wild-type, *xrn1Δ*, and *ste12Δ* strains (Supplemental Fig. 4), strongly suggesting that, in the *xrn1Δ* strain, *Ty1* transcription is severely impaired and reaches background levels observed in the *ste12Δ* strain.

The transcription process is controlled at the level of initiation, elongation, and termination (Svejstrup 2004). To discriminate which transcription step is controlled upon *Ty1* silencing, we performed ChIP experiments using wild-type and *xrn1Δ* strains carrying the HA-tagged TBP, which is associated with the preinitiation complex for RNAPII-dependent regulation (Kuras and Struhl 1999). Remarkably, TBP occupancy was not significantly affected in *xrn1Δ* and *ste12Δ* strains (Fig. 5C), suggesting that TBP recruitment on the *Ty1* promoter is not a limiting step when *Ty1* is silenced.

Figure 5. *Xrn1* controls *Ty1* transcription initiation at a post-TBP recruitment step. (A) Positions of different *Ty1* amplicons on the *Ty1* chromatin for real-time PCR analysis. (B) Real-time PCR quantification of RNAPII using primers over *Ty1* chromatin. Percentage of immunoprecipitation signals relative to input, normalized with the *tRNA* region. The strains are YAM198 (WT), YAM775 (*xrn1Δ*), and YAM778 (*ste12Δ*). (C) Real-time PCR quantification of HA-TBP using primers over *Ty1* chromatin. The same normalization as in B. The strains are YAM208 (WT), YAM238 (*xrn1Δ*), and YAM756 (*ste12Δ*).

Altogether, our results suggest that the *RTL* RNA promotes *Ty1* gene silencing through a transcriptional mechanism occurring after TBP recruitment.

Histone deacetylation and HMT Set1 mediate Ty1 gene silencing

Gene silencing is a key feature of heterochromatin domains and is marked by epigenetic signals such as histone H3 and H4 lysine deacetylation. In addition, HMT Dot1 and Set1 controlling H3K79me and H3K4me, respectively, create a boundary between euchromatin and heterochromatin through a mechanism that might restrict localization of the silencing regulators Sir3 and/or Sir2 (van Leeuwen et al. 2002; Santos-Rosa et al. 2004). To test whether the *Ty1* silencing mechanism involves heterochromatin regulators, we first treated wild-type and *xrn1Δ* strains with histone deacetylase inhibitors NAM and TSA, repressing nicotinamide adenine dinucleotide (NAD)-dependent and class I or II histone deacetylases, respectively (Bernstein et al. 2000; Bitter-

man et al. 2002). Cells treated with 5 mM NAM or 10 μ M TSA were harvested at different time points to extract total RNAs. Northern blot analysis (Fig. 6A) showed that, in the *xrn1* Δ strain, the *TY1FL* RNA levels increased up to threefold after 240 min of NAM treatment (see quantification in Supplemental Fig. 5A). In addition, TSA treatment showed a dramatic eightfold increase of the *TY1FL* levels in the *xrn1* Δ strain after 240 min (see quantification in Supplemental Fig. 5B). Furthermore, we noted that the *RTL* RNA levels were twofold decreased upon NAM treatment (see quantification in Supplemental Fig. 5C), suggesting that a NAD-dependent histone deacetylase(s) mediate(s) the Ty1 silencing, putatively by controlling the *RTL* accumulation. In contrast, upon TSA treatment, the *RTL* levels increased up to twofold (see quantification in Supplemental Fig. 5C), strongly supporting that a class I or a class II histone deacetylase controls the Ty1 silencing at a step following

RTL expression. This analysis suggests that deacetylation of histones is a key regulation step of Ty1 silencing.

Next, to further determine whether Ty1 silencing resembles heterochromatin silencing, *set1* Δ and *dot1* Δ mutants were combined with *XRN1* deletion, and total RNAs were probed for *TY1FL* and *RTL* RNAs (Fig. 6B). We observed that, in *dot1* Δ *xrn1* Δ , the *TY1FL* remained at the same level as in the *xrn1* Δ strain (Fig. 6B). In contrast, a null allele of *SET1* inserted in the *xrn1* Δ strain restored wild-type *TY1FL* levels, strongly suggesting that Set1 is required to silence Ty1. However, as with the NAM treatment, we observed a slight decrease of *RTL* in *set1* Δ *xrn1* Δ compared with the *xrn1* Δ strain, suggesting that the *TY1FL* derepression might be due to the decrease of the *RTL* RNA levels.

To further test the role of Set1 in Ty1 silencing, we asked whether Set1 controls the *RTL*-dependent silencing in *trans*. The *SET1* gene was deleted in the strain containing the *Ty1HIS3(AI)* to measure the Ty1 mobility in the presence of *pGRTL* upon galactose induction. As shown in Figure 6C, the *set1* Δ strain containing the *pGRTL* showed a modest twofold repression of the Ty1 transposition rate when compared with the sevenfold repression in wild type. As the *set1* Δ strain showed mild change in the *pGRTL* induction (data not shown), we conclude that *RTL* mainly represses the Ty1 expression via Set1.

Taken together, these results show that Ty1 silencing is mediated by epigenetic signals established by Set1 and histone deacetylases and presents strong similarities with heterochromatin silencing.

Figure 6. Histone deacetylation and HMT Set1 mediate Ty1 silencing. (A) Northern blot analysis of total RNAs in the presence of histone deacetylase inhibitors. The probes are the same as in Figure 1B. The strains YAM250 (WT) and YAM97 (*xrn1* Δ) were treated with 5 mM NAM or 10 μ M TSA, and cells were collected at the indicated time points. (B) Northern analysis of total RNAs. The probes are the same as in Figure 1B. The strains are YAM250 (WT), YAM97 (*xrn1* Δ), YAM249 (*set1* Δ), YAM448 (*set1* Δ *xrn1* Δ), YAM248 (*dot1* Δ), YAM449 (*dot1* Δ *xrn1* Δ), YAM247 (*set1* Δ *dot1* Δ), and YAM444 (*set1* Δ *dot1* Δ *xrn1* Δ). (C) Repression fold of transposition rates of *Ty1HIS3AI* strains bearing the *pGRTL* and empty plasmids. The strains are YAM359 (WT) and YAM702 (*set1* Δ). The same conditions as in Figure 4A.

Ty1 silencing is controlled by H3K4me

To get further insights into the Ty1 silencing mechanism, we investigated the role of Set1 activity on Ty1 expression. The HMT Set1 catalyzes the addition of one, two, or three methyl groups on the H3K4 (H3K4me1, H3K4me2, and H3K4me3, respectively) and plays multiple roles in DNA repair, transcription, and heterochromatin regulation (Dehe and Geli 2006). In addition, H2B ubiquitylation on Lys 123 (H2BK123-Ub) is required for H3K4me2 and H3K4me3, but not for tethering Set1 on chromatin (Sun and Allis 2002; Dehe et al. 2005; Shahbazian et al. 2005).

As Set1 is required for Ty1 silencing, we first tested, by ChIP experiments, whether H3K4me1, H3K4me2, or H3K4me3 would change on Ty1 chromatin upon Ty1 silencing. Interestingly, the methylation states of H3K4 showed no difference in the *xrn1* Δ and wild-type strains (data not shown), suggesting that H3K4 methylation marks have an indirect or transient effect on Ty1 silencing. Alternatively, Set1 might control Ty1 silencing in a histone-independent manner.

To discriminate between these two possibilities, we asked whether H3K4 methylation is required for the Ty1 silencing. We performed Northern blot analysis with RNAs extracted from strains bearing point mutations at H3 Lys 4. As a control, we used a mutation at H3 Lys 79, targeted by the HMT Dot1. As expected, the

*H3K79A**xrn1* Δ mutant had similar *TY1FL* RNA levels as *xrn1* Δ , confirming that Dot1 catalytic activity is not involved in the Ty1 silencing mechanism (Fig. 7A). In contrast, the *H3K4A* mutant, preventing Set1-dependent H3K4 methylation, showed wild-type levels of *TY1FL* RNAs when combined with the *xrn1* Δ mutant, supporting the idea that H3K4 methylation promotes Ty1 silencing in the strain lacking Xrn1. In addition, we noted that the *H3K4A* mutant showed higher levels of the

TY1FL RNA than the wild type, in contrast to the *set1* Δ strain (Fig. 6B). This observation suggests that Lys 4 on histone H3 has a residual repressive activity on Ty1 expression even when it is unmethylated.

To confirm that Set1 has a histone methylation-dependent activity in Ty1 silencing, we analyzed Ty1 RNA levels in a *H2BK123R* strain controlling the histone-dependent Set1 activity without impairing its chromatin recruitment. Thus, we combined *xrn1* Δ and *H2BK123R* mutations and performed a Northern blot analysis (Fig. 7B). Our result showed that the *TY1FL* RNA level was sixfold increased in *H2BK123R**xrn1* Δ strain compared with *xrn1* Δ , suggesting that H2BK123-dependent histone methylation controls Ty1 silencing. Furthermore, as the *H2BK123R* strain is only devoid of H3K4me2 and H3K4me3, we propose that Ty1 silencing is mediated by H3K4me2 and/or H3K4me3.

These data support the idea that Set1 controls Ty1 silencing through its histone methylation activity, directly or indirectly.

Figure 7. H3K4me controls Ty1 silencing. (A) Northern blot analysis of total RNAs. The probes are the same as in Figure 1B. The strains are YAM212 (WT), YAM762 (*xrn1* Δ), YAM216 (H3K4A), YAM764 (H3K4A*xrn1* Δ), YAM214 (H3K79A), and YAM673 (H3K79A*xrn1* Δ). (B) Northern blot analysis of total RNAs. The probes are the same as in Figure 1B. The strains are YAM166 (WT), YAM167 (*xrn1* Δ), YAM835 (H2BK123R), and YAM836 (H2BK123R*xrn1* Δ). (C) Model of RNA-dependent Ty1 transcriptional gene silencing indirectly controlled by Set1. If not destabilized by the cytoplasmic exonuclease Xrn1, the *RTL* RNA targets an unknown silencing factor that inhibits Ty1 transcription through histone deacetylation. Furthermore, the silencing factor is restricted on Ty1 chromatin by Set1-dependent histone methylation, as suggested for heterochromatic domains. In the absence of Set1, the silencing factor spreads on adjacent chromatin, allowing Ty1 transcription to be derepressed. (D) Model of RNA-dependent Ty1 transcriptional gene silencing directly controlled by Set1. Same as in C but the *RTL* RNA activates Set1-dependent histone methylation on Ty1 embedded nucleosomes. Histone methylation is then recognized by a putative silencing factor that represses Ty1 transcription through histone deacetylation.

Discussion

Our results support the existence of an RNA-dependent gene regulation mechanism in *S. cerevisiae* controlling the Ty1 retrotransposon transcription and mobility. This regulation is mediated by an antisense *TY1* RNA, initiating within the *TYA* region and encompassing the 5'-LTR domain, and necessitates histone deacetylation and Set1-dependent histone methylation, two important epigenetic features of heterochromatin domains. Importantly, the Ty1 silencing is antagonized by the cytoplasmic 5'-3' exonuclease Xrn1 that prevents accumulation of the cryptic regulatory RNA.

Identification of a novel CUT

In this study, we characterized a novel CUT not identified previously, as repeated sequences were eliminated from the microarray analysis (Wyers et al. 2005). CUTs were shown to accumulate when RNA surveillance pathways are defective, in particular when the Rps6 component of the exosome is inactive (Wyers et al. 2005). Some CUTs escape the nuclear surveillance pathway but are degraded by the cytoplasmic 5'-3' decay (Thompson and Parker 2007). This is the case of the Ty1 *RTL* CUT, identified in this study, which is not subjected to the nuclear surveillance pathway but is very sensitive to the activity of the 5'-3' decay factors Xrn1, Dcp1 and Dcp2. The nuclear Xrn1-homolog Rat1 has a very modest impact on *RTL* stability (data not shown), suggesting that *RTL* processing and degradation are mainly cytoplasmic. The deadenylation coupling RNA degradation factors Ccr4, Pat1, and Lsm proteins have a minor effect on *RTL* stability, suggesting that *RTL* might be processed and degraded independently of its poly(A) tail. However, in the absence of the cytoplasmic 5'-3' RNA degradation pathway, Trf4 plays an important role in the *RTL* destabilization process as we noted an addi-

tive accumulation of the *RTL* RNA in *xrn1Δ trf4Δ*, implying that these two pathways communicate with each other despite functioning in two different compartments of the cell.

CUTs mediate gene regulation

The function of CUTs remains elusive, but cryptic transcription was proposed to mediate chromatin changes controlling promoter activity and gene expression. In *S. cerevisiae*, several examples have already been described, and the current model proposes that the CUT transcription interferes with the gene transcription (Martens et al. 2005; Hongay et al. 2006; Uhler et al. 2007). This model implies that the RNA molecule itself has a minor function as being immediately degraded by the nuclear surveillance pathway in normal conditions. Our data show that *RTL* silences Ty1 expression in *trans* by acting on the de novo synthesized *TY1* RNA. Furthermore, attempts to define a minimalist Ty1 regulatory RNA showed that the *TYA* and *U3/R* regions are required for Ty1 silencing, suggesting that the integrity of the regulatory RNA is important. Consistent with this observation, we failed so far to detect shorter, processed regulatory *TY1* RNAs that could be involved in the Ty1 silencing in vivo. To our knowledge, this is the first example of RNA-dependent gene *trans*-silencing in *S. cerevisiae*.

Transcriptional gene silencing

Gene *trans*-silencing has been well characterized in plants and mammalian cells, where it mainly acts at a post-transcriptional level, controlling cognate mRNA translation or degradation. Here, we show that the Ty1 *trans*-silencing affects transcription at early steps. Indeed, two lines of evidence support a transcriptional gene silencing model. First, Ty1 silencing is characterized by a significant reduction of RNAPII occupancy over the Ty1 chromatin, which might lead to a large decrease in Ty1 expression and mobility. Furthermore, we show that histone deacetylation and Set1-dependent histone methylation control Ty1 repression, suggesting that Ty1 silencing is a nuclear event occurring at the chromatin level.

However, we cannot rule out the possibility that Ty1 is silenced additionally at post-transcriptional step(s). This idea is reinforced by the large difference in the transposition repression fold observed in the *xrn1Δ* strain (500-fold) and in a strain in which *RTL* transcription is induced from a plasmid (sevenfold in presence of *pGRTL*). This observation suggests that Xrn1 either destabilizes other Ty1 regulatory RNAs or controls post-transcriptional steps during the transposition cycle. For instance, previous studies revealed a structural role of Xrn1 and P-body components in the assembly of gypsy-like Ty3 VLPs (Beliakova-Bethell et al. 2006). One could hypothesize that this function is conserved for the assembly process of Ty1 VLPs as well. This defect would

add up to the transcriptional defect we observed in the *xrn1Δ* strain and would provide an explanation for the low transposition rate in this mutant.

The transcriptional silencing model implies that the regulatory *TY1* RNA shuttles back to the nucleus. This dynamic model is supported by very few examples in *S. cerevisiae*. For instance, only some tRNAs have been shown to cycle back to the nucleus after delivering their amino acids under stress conditions (Dahlberg and Lund 2005). Alternatively, we can hypothesize that the Ty1 regulatory RNA is retained and processed in the nucleus, despite Xrn1 and Dcp1/2 proteins being mainly cytoplasmic. In support of this idea, decapping activity was shown to occur in the nucleus when mRNA export was blocked (Kufel et al. 2004). Further experiments on localization of the Ty1 regulatory RNA will provide insights into mechanisms of the Ty1 silencing as well as RNA transport.

Chromatin-mediated gene silencing

Ty1 silencing is mediated by epigenetic signals that control heterochromatin domains. In particular, we showed that the Set1-dependent histone methylation has a crucial role. Set1 has been extensively characterized over the last 5 years (Dehe and Geli 2006). It is part of the eight-subunit SET1C complex that mono-, di-, and trimethylates histone H3 and nonhistone proteins (Roguev et al. 2001; Zhang et al. 2005). H3K4me3 is a mark of active genes, but, surprisingly, no major transcription defect has been linked with the absence of H3K4me3 (Santos-Rosa et al. 2002). Originally, Set1 was characterized to establish subtelomeric HM and rDNA silencing (Nislow et al. 1997). Current models propose that H3K4 methylation restricts silencing factors such as Sir3 to telomeric regions (Santos-Rosa et al. 2004). Indeed, in the absence of Set1, Sir3 spreads into the euchromatin, fading the silencing at the heterochromatin loci. Similarly to heterochromatin silencing, we present evidence that Set1 and H3K4me2 and/or H3K4me3 are also involved in Ty1 silencing. Two alternative models of Ty1 silencing can be proposed.

By analogy with heterochromatin, Set1-dependent H3K4 methylation, flanking the transposon, might restrict a Ty1 putative silencing factor on the transposon. The absence of Set1 and H3K4 methylation would release the silencing factor from the Ty1 chromatin and derepress the Ty1 expression (see model in Fig. 7C). As in heterochromatin domains, histone deacetylases would deacetylate Ty1 chromatin and contribute to Ty1 silencing. In favor of this model for an indirect role of Set1, no change in H3K4 methylation was observed on Ty1 chromatin upon silencing (data not shown).

The second model is based on a direct role of histone H3K4 methylation in Ty1 silencing. The *RTL* regulatory RNA could transiently target or activate Set1, allowing temporally H3K4 methylation of the Ty1 chromatin that might be subsequently recognized by a specific silencing regulator (see model in Fig. 7D). As for the previous model, histone deacetylation would reinforce the Ty1

silencing. Despite the fact that H3K4me3 and H3K4me2 are marks of actively transcribed chromatin, recent data suggest that they might also have inhibitory effects as well. Indeed, chromatin repressing factors, such as histone deacetylase complexes, were shown to bind directly to the trimethylated H3K4 via the plant homeodomain (PHD) (Mellor 2006; Sims and Reinberg 2006). Moreover, upon silencing induction, H3K4 has been shown to be transiently mono- or dimethylated on the silenced HM regions in *S. cerevisiae*, revealing a putative direct role of these marks during heterochromatin formation (Katan-Khaykovich and Struhl 2005).

Interestingly, both models involve histone deacetylation. In *S. cerevisiae*, heterochromatin establishment and maintenance is mainly regulated by the NAD-dependent histone deacetylase Sir2 (Shore et al. 1984), but also by class I and II histone deacetylases (Rundlett et al. 1996). Further investigation will allow us to determine the histone substrate and histone deacetylase(s) involved in Ty1 silencing (Hickman and Rusche 2007).

Ancient form of RNA-dependent gene silencing

Long or short regulatory RNAs have been widely described in prokaryotes and eukaryotes to control gene expression. Following our observations, several similarities between eukaryotic RNA-dependent gene silencing and the *RTL*-dependent Ty1 silencing can be drawn.

Long ncRNAs control gene expression and can initiate chromatin modifications on specific regions. Xist ncRNA and the *Hox* antisense ncRNAs (*HOTAIR* RNA) silence the entire X chromosome and the *HOX* genes' expression, respectively (Masui and Heard 2006; Rinn et al. 2007). The relationships between the Xist RNA and chromatin modifications are still poorly characterized. But recently, the *HOTAIR* regulatory RNA was shown to directly interact with the HMT PRC2 to tether and induce histone H3K27 methylation over the *Hox* locus and hence to control, in *trans*, gene expression and chromatin organization (Rinn et al. 2007). This direct interaction between a regulatory RNA and a chromatin modifier is fundamental and provides an important step to understand targeted chromatin modifications. Our results show that Set1 is involved in the RNA-dependent Ty1 silencing, and one could speculate that the *RTL* long ncRNA would directly interact with Set1 protein, targeting its activity to the Ty1 chromatin for silencing.

Concerning short regulating RNAs, we could not provide any data that support a Ty1 silencing mechanism mediated by siRNAs. Nevertheless, it is interesting to note that two genetic screens in *Caenorhabditis elegans* showed that the Set1 homolog regulates transposon silencing in an RNAi-dependent manner, through a mechanism that could be similar to the Set1-mediated Ty1 silencing described in this study (Grishok et al. 2005; Robert et al. 2005). More strikingly, in rat testis, small regulatory RNAs, such as rasiRNAs and piRNAs, were recently found to control transposable element transcription (O'Donnell and Boeke 2007). Interestingly, these RNAs were proposed to originate from long anti-

sense ncRNA (Vagin et al. 2006) that could resemble the *RTL* RNA regulating Ty1 expression. Although most of the RNAi features are absent in *S. cerevisiae*, our work might have uncovered a primitive and ancestral pathway of RNA-dependent gene regulation.

Materials and methods

Yeast strains and plasmid construction

The strains used in this study are described in Supplemental Table 1. The experiments were mostly performed in the W303 background. Gene deletions and epitope tags were introduced by transformation of PCR fragments generated with specific primers (sequences can be obtained upon request) using the appropriate plasmids listed in Supplemental Table 2 (Longtine et al. 1998).

The yeast strain carrying *ML2::URA3* is a derivative of W303 in which we introduced *URA3* in frame with *TYA(ML2)* by single-step recombination. The recombination sites correspond to *TYA*(967–1007) and to a region located downstream from Ty1(*ML2*) (chrXIII coordinates 202532–202571) resulting in the deletion of the *TYB(ML2)* region. Plasmid *pAM134* is a derivative of *pAM80* (pYESdest52; Invitrogen) in which we introduced the Ty1 sequence amplified by PCR from *pAM22* (661–1 nucleotides) following the manufacturer's instructions. Similarly, partially truncated *RTL* sequences were cloned to produce *pAM182* (*U3RA*, 661–290, *pGRTL^{U3RA}*), *pAM184* (*TYAΔ*, 334–1, *pGRTL^{TYAΔ}*), and *pAM183* (*LTRAΔ*, 661–334, *pGRTL^{LTRAΔ}*). Ty1 sequences were amplified by PCR from *pAM3*. *pAM167* is a derivative of *pAM134* with *LEU2* instead of *URA3*. All constructs were subsequently sequenced.

Media and culture conditions

Growth media and plates were prepared with standard methods using YPDA and CSM media (Gibco) supplemented as indicated and containing 2% glucose or 2% galactose. 5FOA experiments were carried out as follows: Transformed yeast cells were grown on appropriate selective media and were dropped on selective CSM plates containing 1 mg/mL 5FOA (Melford laboratory). Histone deacetylase inhibitors were added to YPDA media at 5 mM for NAM (Sigma) and 10 μM for TSA (Sigma).

RNA extraction, poly(A)⁺ RNA purification, and Northern blotting

Total RNA was extracted using the hot phenol extraction procedure. Poly(A)⁺ RNAs were purified on oligo(dT) Dynabeads (Invitrogen). Otherwise stated, 5 μg of total or poly(A)⁺ RNAs were loaded on denaturing 1% agarose gel and transferred to nitrocellulose membranes. Membranes were UV-cross-linked and hybridized overnight at 65°C with either ³²P-labeled DNA probes or single-stranded riboprobes in Ultrahyb buffer (Sigma). Blots were washed twice with 0.5× SSC and 1% SDS for 20 min at 65°C. DNA probes were obtained by random-primed labeling (Stratagene) of specific DNA fragments generated by PCR. Single-stranded riboprobes were obtained by SP6 in vitro transcription of gene-specific PCR fragments containing the SP6 promoter (Ambion). PCR primers are available upon request. *ACT1* and *scR1* RNAs were used as loading controls.

Transposition test

In the Ty1HIS3AI strain, *HIS3* was inserted in the *TYB* region of a Ty1 element in the antisense orientation (Curcio and Garfinkel 1991). *HIS3* RNA synthesis was independent of the

Ty1 transcription but was interrupted by an artificial intron only spliced during *TY1* RNA transcription. The colony became His^+ when the Ty1 $\text{HIS3}[\text{AI}]$ transposed resulting in a newly inserted Ty1 HIS3 with a functional *HIS3* gene. Thus, the transposition rate corresponded to the number of His^+ over the total number of colonies. Three independent cultures of strains—YAM359 (WT), YAM627 (*xrn1* Δ), and YAM601 (*ste12* Δ)—were grown in YPDA overnight at 30°C. Cells were diluted 1:100 in YPDA media and grown 48 h at 20°C. An equal volume of 2× YPDA broth was added, and cultures were grown for an additional 24 h at 20°C. Aliquots of each culture were plated on YPDA and CSM-HIS plates and incubated during 3–4 d at 30°C. The rate of Ty1 his3AI transposition is the number of His^+ colonies divided by the total number of colonies plated (on YPDA plates) (Curcio and Garfinkel 1991).

In the *trans*-silencing transposition assay with *RTL*-dependent repression, three independent transformants of plasmids *pAM80* or *pAM134* in strains YAM359 (S288C) and YAM358 (W303) were grown in CSM-Ura 2% glucose broth at 30°C. Cells were diluted 1:100 in CSM-Ura 2% galactose broth and grown for 48 h at 20°C. Glucose was added to reach 2% final, and cells were grown for an additional 24 h at 20°C. Aliquots of each culture were plated on CSM-Ura 2% glucose agar and CSM-Ura-His 2% glucose agar and incubated at 30°C. The frequency of Ty1 his3AI transposition is the number of His^+ Ura $^+$ colonies divided by the total number of Ura $^+$ colonies plated.

ChIP

ChIPs were performed essentially as described previously. Yeast strains were grown to $\text{OD}_{600} = 0.5$ either in YPDA or CSM minimum media at 30°C, and cross-linked for 20 min by the addition of formaldehyde to a final concentration of 1.2%. The cross-linking reaction was quenched by adding glycine at 0.3 M final. Chromatin was sonicated to obtain 400–500 nt of DNA fragment, and 150 μL (at 1.25 $\mu\text{g}/\mu\text{L}$) of sonicated chromatin was immunoprecipitated overnight in the presence of specific antibodies against the N-terminal domain of Rpb1 (Y80, Santa Cruz Biotechnologies), the HA epitope (fp7, Santa Cruz Biotechnologies) for the TBP HA-tagged strain. Immunoprecipitated chromatin was purified with 5 mg of protein A-Sepharose (Amersham Pharmacia). All immunoprecipitations were repeated at least twice with different chromatin extracts. Immunoprecipitated DNA was quantified by real-time PCR using the LightCycler 480 (Roche), with primer pairs available upon request. Signals are expressed as the percentage of input DNA relative to the *tf(GAA)P2* (tRNA region in ChrXVI, coordinates 622628–622537). Error bars correspond to standard deviations.

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