Trans-acting antisense RNAs mediate transcriptional gene cosuppression in *S. cerevisiae*

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Homology-dependent gene silencing, a phenomenon described as cosuppression in plants, depends on siRNAs. We provide evidence that in *Saccharomyces cerevisiae*, which is missing the RNAi machinery, protein coding gene cosuppression exists. Indeed, introduction of an additional copy of *PHO84* on a plasmid or within the genome results in the cosilencing of both the transgene and the endogenous gene. This repression is transcriptional and position-independent and requires *trans*-acting antisense RNAs. Antisense RNAs induce transcriptional gene silencing both in *cis* and in *trans*, and the two pathways differ by the implication of the Hda1/2/3 complex. We also show that *trans*-silencing is influenced by the Set1 histone methyltransferase, which promotes antisense RNA production. Finally we show that although antisense-mediated *cis*-silencing occurs in other genes, *trans*-silencing so far depends on features specific to *PHO84*. All together our data highlight the importance of noncoding RNAs in mediating RNAi-independent transcriptional gene silencing.

[**Keywords:** Antisense RNA; *cis* and *trans* transcriptional gene silencing; *PHO84*; cosuppression; RNAi-independent TGS; noncoding RNA; *S. cerevisiae*]

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Eukaryotic gene expression is a complex process regulated at multiple steps from transcription initiation to protein degradation. For years, transcription factors recognizing specific DNA-binding sites have been considered the main regulators of gene expression. More recently, the discovery of the histone code revealed that transcription is also tightly regulated by histone modifications (Strahl and Allis 2000; Mellor 2006; Mellor et al. 2008). Importantly, these modifications not only regulate transcription within euchromatin but also participate in transcriptional gene silencing (TGS) at heterochromatic loci. Heterochromatic gene silencing has been most extensively described in *Schizosaccharomyces pombe*, where siRNAs originating from these regions target the RITS complex to induce repressive histone modifications (Volpe et al. 2002; for review, see Buhler and Moazed 2007). Although, the complete RNAi machinery is absent in *Saccharomyces cerevisiae* (Aravind et al. 2000), RNA-mediated TGS within euchromatin has been

recently reported (Camblong et al. 2007; Berretta et al. 2008; Houseley et al. 2008). In particular, we showed that stabilization of *PHO84* antisense RNAs, escaping degradation by the nuclear exosome in the absence of the Rrp6 exoribonuclease or during chronological aging, leads to *PHO84* TGS via a mechanism implicating the Hda1/2/3 histone deacetylase (Camblong et al. 2007). In addition, histone modifications within heterochromatic rDNA regions were also shown to be influenced by the accumulation of noncoding RNAs from the non-transcribed rDNA spacer regions (NTS) (Houseley et al. 2007; Vasiljeva et al. 2008). Together these observations suggest the existence of an alternative, potentially conserved pathway for RNA-dependent chromatin modifications in *S. cerevisiae*.

Earlier work in *S. pombe* indicated that siRNAs primarily act in *cis* to target heterochromatic TGS (Buhler et al. 2006). Similarly, the fact that in *S. cerevisiae* antisense RNA accumulation in the progeny of old cells is paralleled by a decrease in Rrp6 occupancy on *PHO84* suggested the view that antisense RNAs are degraded by this exosome component at their site of synthesis, and might consequently act in *cis* (Camblong et al. 2007). However, recent data from *S. pombe* indicate that siRNAs produced in *trans* can also be effective in TGS but still require antisense RNA to be produced in *cis* (Iida et al.

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2008). Moreover, like in higher eukaryotes, Ty1 expression and mobility are regulated by *trans*-acting antisense RNAs in *S. cerevisiae* (Berretta et al. 2008). Related mechanisms may therefore control retrotransposon spreading in all these organisms, whether or not they harbor the RNAi machinery (Girard and Hannon 2008). This finding suggests the interesting possibility that, like in higher eukaryotes, *trans*-acting noncoding RNAs may mediate homology-dependent silencing of protein coding genes in budding yeast.

To address this issue, we used *PHO84* as a model gene to test whether antisense RNAs mediating *PHO84* TGS in *cis* (Camblong et al. 2007) may also have the ability to act in *trans*. We found that expression of *PHO84* antisense RNAs from an ectopic *PHO84* gene copy is sufficient to trigger transcriptional silencing of both the endogenous and the ectopic genes. This robust reciprocal silencing is analogous to transcriptional cosuppression initially described in plants (Matzke and Matzke 1995a) and provides strong evidence that RNA-mediated protein coding gene cosuppression can occur through an RNAi-independent pathway in *S. cerevisiae*.

Results

An additional *PHO84* gene copy triggers *PHO84* cosuppression

In the course of our studies on the mechanism underlying transcriptional silencing of the *PHO84* gene, encoding a high-affinity phosphate transporter (Persson et al. 1998), we cloned *PHO84* on a centromeric plasmid (plasmid N°1) (Fig. 1A). When transformed in $\Delta pho84$ strains, *PHO84* on plasmid behaved as the endogenous gene (Supplemental Fig. 1; Camblong et al. 2007). However, unexpectedly, transformation of plasmid N°1 into a wild-type strain led to a strong decrease in *PHO84* sense transcripts paralleled by an increase in *PHO84* antisense RNA levels (Fig. 1B). Plasmid loss from the transformed cells followed by transformation of an empty vector into the cured strains restored *PHO84* sense RNA levels, demonstrating that the silencing is reversible and not due to the acquisition of mutations or gene conversions during the transformation process (Fig. 1C). These observations suggested that the presence of the *PHO84* plasmid leads to *PHO84*

Figure 1. A *PHO84* extra copy leads to cosuppression of *PHO84* mRNA expression. (A) Map of the *PHO84* locus. Arrows indicate the orientation of *PHO84* sense, *TUB3* sense, and *PHO84* antisense transcripts. *YML122C* described as a hypothetical ORF contains four Pho4-binding sites (black circles) and encompasses the *PHO84* upstream activating sequence (UAS). The line below indicates the *PHO84* sequence N°1 subcloned into the centromeric vector YCpLac111. The size of the insert is indicated on the right. Position 1 lies 105 bp upstream of the beginning of hypothetical ORF *YML122C* (725 bp upstream of *PHO84* ATG), and position 2651 is located at +166 bp downstream from the *PHO84* stop codon. Filled rectangles indicate promoter (P), 5' and 3' regions of *PHO84* amplified in ChIP analyses (Fig. 2). The dotted line corresponds to the 3' region spanned by the ssRNA probe used in the blots below. (B) Presence of an extra gene copy induces *PHO84* cosuppression. Northern analysis of total RNA extracted from wild-type cells transformed with YCpLac111 empty vector (V) or YCpLac111 containing insert N°1 (N°1). After transformation cells were exponentially grown in –LEU media for 24 h. Total RNA was extracted and *PHO84* sense and antisense RNAs detected with riboprobes mapping to *PHO84* 3' end. Membranes were rehybridized with an *ACT1* random labeled probe to control for equal loading. (C) *PHO84* gene silencing is extra gene copy-dependent and reversible. Wild-type cells containing an empty vector (V) or plasmid N°1 were exponentially cultivated in –LEU for 24 h. A fraction of the culture was diluted into YEPD medium to lose vector (V) and N°1 and the

rest collected for RNA extraction. Cured cells were retransformed with empty vector followed by cultivation in –LEU and RNA extraction. *PHO84* sense and antisense RNA levels from cells containing vector (V) or N°1 (lanes 1,2) were compared with those of cured cells retransformed with the empty vector (lanes 3,4) as in B. (D) *PHO84* gene silencing is position-independent. Wild-type and $\Delta pho84$ strains, each containing a *PHO84* ectopic copy replacing either the *LYS2* (*lys::PHO*), the *YML023* (*yml::PHO*), or the *GUD1* (*gud::PHO*) loci, were transformed with an empty vector (V) or *PHO84* construct N°1 and cultivated in –LEU medium followed by RNA extraction. Membranes were probed as in B. The chromosomal positions of endogenous (white square) and ectopic (black squares) *PHO84* gene copies are diagrammed on the left. (E) An extra gene copy induces silencing in diploid cells. *PHO84* sense and antisense RNA levels in haploid and diploid wild-type cells transformed with an empty vector (V) or plasmid N°1 (N°1) were analyzed as in B. In B–E, the numbers within the box indicate the number of *PHO84* gene copies in the genetically modified and transformed strains.

cosuppression, a phenomenon originally described in transgenic plants and referring to homology-dependent gene silencing (Jorgensen 1995; Matzke and Matzke 1995b; Meyer and Saedler 1996).

To address whether *PHO84* silencing would also occur following integration of a second *PHO84* gene in the genome, an ectopic *PHO84* copy was integrated at three different locations in a wild-type or a $\Delta pho84$ strain, either in place of *LYS2* (*lys::PHO*), *YML023* (*yml::PHO*) or *GUD1* (*gud::PHO*) (Fig. 1D). All three ectopic genes were expressed to similar or slightly lower levels compared with endogenous *PHO84* when inserted in a $\Delta pho84$ strain. However, when inserted in a wild-type strain, the presence of a second *PHO84* gene in the genome resulted in *PHO84* silencing, showing that one *PHO84* extra copy is necessary and sufficient to trigger silencing. Note that in all cases, sense repression correlates with higher levels of antisense RNA (Fig. 1D, cf. lanes 3 and 5, lanes 7 and 9, and lanes 11 and 13). Interestingly, transformation of plasmid N°1 into the $\Delta pho84$ strains containing just one *PHO84* ectopic copy at the *LYS2*, *YML083*, or *GUD1* loci also led to *PHO84* sense mRNA silencing (Fig. 1D, cf. lanes 3 and 4, lanes 7 and 8, and lanes 11 and 12), demonstrating that this silencing does not depend on *PHO84* subtelomeric position. Consistently, loss of Ku70, Esc1, Sir2, and Hst1 involved in telomere silencing did not affect cosuppression (Supplemental Fig. 2; Supplemental Table 3; Taddei et al. 2004).

Finally, we compared expression levels in wild-type haploid and diploid cells. In the presence of the empty vector, *PHO84* mRNA levels in wild-type diploid cells were about three times those of wild-type haploid cells. Importantly, the presence of plasmid N°1 resulted in a sixfold reduction of *PHO84* mRNA levels (Fig. 1E). These results indicate that silencing does not occur in the presence of two *PHO84* copies in the context of a duplicated genome but is triggered by an extra gene copy, supporting the idea of a dose-dependent cosuppression.

Silencing in trans is transcriptional and Hda1/2/3-independent

To address at which level *PHO84* expression is cosuppressed, chromatin immunoprecipitation (ChIP) experiments were performed to measure RNA polymerase II (RNA Pol II) occupancy at the 5' and 3' ends of *PHO84*. Strikingly, RNA Pol II levels were dramatically reduced in the presence of plasmid N°1 (Fig. 2A), indicating that the silencing is transcriptional. The repression is at the level of transcription initiation as TATA-binding protein (TBP) occupancy at the *PHO84* promoter was strongly reduced (Fig. 2B). RNA Pol II and TBP occupancy was not further decreased in the absence of the *Pho4* transcription factor, indicating that the *PHO84* extra copy drastically affects transcription initiation (Fig. 2A,B).

Previously, we reported that *PHO84* TGS resulting from chronological aging or from inactivation of the nuclear exosome is triggered by the Hda1/2/3 HDAC complex (Camblong et al. 2007). In order to address whether this HDAC is also implicated in the cosuppres-

Figure 2. Silencing occurs at the level of transcription initiation and is Hda1/2/3-independent. (A) ChIP analysis of RNA Pol II over the *PHO84* gene. Wild-type and $\Delta pho84$ strains transformed with an empty vector (V) or plasmid N°1 (N°1) were grown in –LEU medium. RNA Pol II immunoprecipitated DNA was quantified by real-time PCR with *PHO84*-specific primers (Fig. 1A; Supplemental Table 2). The relative enrichment of the gene segments was expressed as the *n*-fold increase with respect to a nontranscribed intergenic sequence. Values derive from three independent experiments. (B) ChIP analysis of TBP at the *PHO84* promoter. The same chromatin extracts were immunoprecipitated with α -TBP antibodies. The DNA was quantified by real-time PCR with primers specific for *PHO84* promoter and normalized as in A. ChIP is measuring the level of association of both endogenous and plasmidic *PHO84* with these factors. (C) The Hda1/2/3 complex is not required for *trans*-silencing. *PHO84* sense and antisense RNAs in wild-type, $\Delta hda1$, and $\Delta hda2$ strains transformed with an empty vector (V) or plasmid N°1. Cell cultures and Northern blotting were performed as in Figure 1B. *PHO84* sense mRNAs were quantified and normalized to *ACT1*. For each strain, the levels of sense transcripts detected in presence of plasmid N°1 were expressed as a percentage of those detected in presence of empty vector (bottom line).

sion mechanism, plasmid N°1 was transformed in $\Delta hda1$ and $\Delta hda2$ strains. Importantly, *PHO84* sense RNA levels were similarly reduced in wild-type and the two mutant strains, indicating that the Hda1/2/3 complex is not required for *PHO84* cosuppression (Fig. 2C; Supplemental Fig. 3).

Cosuppression requires a full PHO84 sequence from the upstream activating sequence (UAS) to the stop codon

To further characterize the cosuppression mechanism, we aimed at defining the minimal *PHO84* homologous sequence to be inserted in the plasmid copy for silencing to occur. Various *PHO84* 5' and 3' truncations of plasmid N°1 were cloned (Fig. 3A) and first transformed into a $\Delta pho84$ strain to define their expression in the absence of the endogenous *PHO84* gene (Fig. 3B). While plasmids

Figure 3. A *PHO84* sequence containing both the UAS and ORF is required to trigger silencing. (A) Map of the *PHO84* gene. The lines below indicate all *PHO84* portions (N°1–N°6 and UAS) subcloned into the centromeric vector YCpLac111 or other expression vectors (see below). The size of each insert is indicated on the right. Position 1 lies 105 bp upstream of YML122C (725 bp upstream of *PHO84* ATG), and position 2651 is located at +166 bp downstream from the *PHO84* stop codon. Dotted lines correspond to 5' and 3' regions spanned by the ssRNA probes. *PHO84* sense and antisense RNA expression in $\Delta pho84$ (B) or in wild-type cells (C) transformed with *PHO84* plasmids N°1–N°6. In both cases, sense RNAs were detected with a probe mapping to *PHO84* 3' end. Antisense RNAs were revealed with a probe mapping to *PHO84* 3' end in B, or specific for *PHO84* 3' end or 5' end (common to all six constructs) in C. (D) Deletion of 120 bp within the *PHO84* terminator (Δter strain) leads to the production of a long *PHO84-TUB3* read-through transcript. Wild-type and Δter strains transformed with empty vector (V) or plasmid N°1 were analyzed as in Figure 1B. (E) Expression of the *PHO84-TUB3* read-through transcript in a Δter strain transformed with empty vector (V) or plasmids N°1–N°6.

N°1 and N°2 produce high levels of *PHO84* sense mRNA, the latter makes a slightly longer transcript, presumably due to usage of an alternate 3' processing signal in the vector. Transcripts of similar length but much lower abundance are encoded by plasmids N°4 and N°5 as these constructs lack the UAS. No sense transcripts could be detected in presence of plasmids N°3 and N°6 using the 3' end-specific probe, but were revealed for N°3 with a 5' end-specific probe (Fig. 3A; data not shown). When transformed into a wild-type strain, both plasmids N°1 and N°2 induced a strong decrease in *PHO84* sense RNA expression. Although plasmid N°3 had a detectable effect, it was much weaker. In contrast, constructs N°4–N°6,

missing the *PHO84* UAS within YML122C, had no effect (Fig. 3C). Notably, all constructs produced detectable amounts of antisense RNAs and those derived from plasmids N°1 and N°2 were comparable in size to the endogenous transcripts (Fig. 3C, lanes 1–3).

To confirm these data, we generated a new strain in which the endogenous *PHO84* sense transcripts are distinguishable from those produced by the transformed plasmid. This strain, called Δter , contains a 120-base-pair (bp) deletion in the 3' untranslated region (UTR) of the endogenous *PHO84* locus, leading to a defect in transcription termination and production of long chimeric *PHO84-TUB3* sense RNAs (Fig. 3D). Transformation of the Δter strain with plasmids N°1 or N°2 led to the disappearance of the *PHO84-TUB3* RNA, whereas plasmids N°3–N°6 did not substantially affect the levels of these transcripts, confirming the previous observations (Fig. 3C,E). These data therefore indicate that if antisense RNAs were players in *trans*-silencing (see below), they would have to be long and contain the region corresponding to the *PHO84* UAS.

Antisense RNA production is necessary for silencing in trans

The silencing of *PHO84* gene expression following transformation of plasmids N°1 and N°2 was paralleled by increased *PHO84* antisense RNA levels (Fig. 3B,C), suggesting that antisense RNAs could not only trigger TGS in *cis*, but also mediate cosuppression in *trans*. To demonstrate the direct involvement of antisense RNAs in the *PHO84* cosuppression phenomenon, the *PHO84* sequence from plasmid N°1 was subcloned behind a GAL promoter (GAL-N°1), such that antisense RNA production can be induced by shifting carbon source (Fig. 4). To test the efficiency of this system, we first asked whether we could manipulate silencing in *cis* by regulating antisense expression. Thus, $\Delta pho84$ cells transformed with plasmid GAL-N°1 were cultivated overnight in glucose or galactose-containing media. While cells cultivated in glucose expressed *PHO84* sense RNAs, those grown in galactose did not, suggesting that antisense RNAs driven by the GAL promoter are efficient in silencing sense transcription in *cis* (Fig. 4A, lanes 1,2). Consistent with the previously described role of Hda1/2/3 in *cis*-silencing, *PHO84* sense RNA expression was maintained in cells grown in galactose but lacking Hda2 (Fig. 4A, lanes 5,6). Notably, antisense RNAs were detectable neither in $\Delta pho84$ nor in $\Delta pho84 \Delta hda2$ strains. Since endogenous antisense RNAs are degraded by Rrp6, we also transformed the GAL-N°1 plasmid into a $\Delta pho84 \Delta rrp6$ strain and checked for antisense RNA accumulation in cells grown in glucose versus galactose. In the absence of Rrp6, antisense RNAs were stabilized in strains cultivated in galactose but not glucose (Fig. 4A, lanes 3,4), demonstrating that antisense transcripts are made in galactose but are very unstable. In *Drosophila*, the exosome is directed to RNA pol II within transcription hot-spots (Andrulis et al. 2002). Thus, increased instability of antisense transcripts driven by the strong GAL promoter could be due to increased recruitment of the exosome.

Figure 4. GAL-driven *PHO84* antisense RNAs silence endogenous *PHO84* gene expression in *trans*. (A) *PHO84* cis-silencing is recapitulated on plasmid. *PHO84* sense and antisense RNA levels in $\Delta pho84$, $\Delta pho84\Delta rrp6$, and $\Delta pho84\Delta hda2$ strains transformed with a *PHO84* plasmid in which antisense RNAs are driven by a galactose-inducible promoter (*GAL-N¹*) as drawn at the top. Cells were exponentially cultivated 24 h in $-LEU$ medium containing 2% glucose (GLU) or 2% galactose (GAL) and *PHO84* sense and antisense RNAs produced from the plasmid were detected as in Figure 1B. (B) GAL-inducible antisense RNAs promote TGS in *trans*. (Top) Map of the *PHO84 + HIS5* endogenous gene. The *S. pombe HIS5* gene was inserted within *PHO84* in reverse orientation and is flanked by TEF promoter and terminator sequences (black triangles). Arrows indicate the orientation of the sense transcripts expressed from this locus. Dotted lines correspond to the position of *PHO84* and *HIS5* probes used below. (Bottom) Northern analysis of RNA from the *PHO84+HIS5* strain transformed with empty vector (V) or plasmid *GAL-N¹*. Transformants were exponentially grown for 24 h in $-LEU$ media containing 2% glucose (GLU) or 2% galactose (GAL). (Top panels) *PHO84 + HIS5* and *HIS5* sense transcripts were detected with *HIS5* antisense- and sense-specific probes. (Middle panels) *PHO84 + HIS* and *PHO84* plasmid transcripts were also detected with a *PHO84* sense-specific probe. *GAL1* and *ACT1* mRNAs were detected with random primed probes and served, respectively, as galactose induction and loading controls. (C) TGS by GAL-induced *PHO84* antisense RNAs is rapid. The *PHO84 + HIS5* cells transformed with plasmid *GAL-N¹* were grown in $-LEU$ medium containing 2% glucose. After 24 h, cells were spun and resuspended in $-LEU$ media containing either 2% glucose (GLU) or 2% galactose (GAL) and grown for an additional 20 min.

Since the regulation of antisense RNA expression with the GAL promoter allows to control silencing in *cis*, we used this construct to test whether *PHO84* cosuppression is mediated by *trans*-acting antisense RNAs. Plasmid *GAL-N¹* was transformed into a strain containing a *HIS5* cassette in the middle of *PHO84* in reverse orientation such that endogenous antisense RNAs are

prematurely terminated and silencing in *cis* is impaired (Camblong et al. 2007). While cells grown in glucose expressed both the endogenous and the plasmid *PHO84* genes, distinguishable in size because of the *HIS5* cassette, cells cultivated in galactose exhibited silencing of both *PHO84* copies (Fig. 4B). Therefore, antisense RNAs produced from the plasmid can silence plasmid *PHO84* gene expression in *cis* and endogenous *PHO84* gene expression in *trans*. In addition, the silencing is very fast as 20 min of galactose induction was sufficient to repress endogenous *PHO84* gene expression (Fig. 4C). Considering that *PHO84* sense mRNA half-life is about 8 min (Wang et al. 2002), this last result supports the view that antisense RNA-mediated *PHO84* gene cosuppression is very rapid and penetrant. Finally, the fact that the *HIS5* cassette expressed a *HIS5* sense RNA in both glucose and galactose conditions indicated that *HIS5* transcription is not silenced (Fig. 4B). Thus, *PHO84* cosuppression is mediated by *trans*-acting antisense RNAs, which have the ability to target and restrict silencing to a specific sequence; i.e., the *PHO84* promoter.

Cleavage of antisense RNA is sufficient to impair cis- and trans-mediated silencing

In some experiments with the GAL-inducible antisense transcripts described above, the GAL promoter turned out to be leaky, resulting in partial silencing of the endogenous *PHO84* gene even in glucose (data not shown; see below). To confirm the importance of antisense RNA in *trans*-silencing, we developed an alternate approach aimed at cleaving antisense transcripts rather than regulating their transcription. Based on *Pho84* structure predictions, a 51-nucleotide hammerhead ribozyme (Rz) was inserted in frame in the middle of *PHO84* ORF so that the *Pho84* protein structure should not be dramatically disturbed (Fig. 5A). The Rz sequence was inserted in the reverse orientation to specifically induce self-cleavage of antisense RNA. To test the efficiency of the ribozyme, we performed an aging experiment with strains in which the endogenous *PHO84* gene had been replaced by a *PHO84* gene containing either the wild-type (*PHO84Rz*) or a mutant ribozyme (*PHO84Rzm*) harboring a single base change in its catalytic site that abolishes self-cleavage (Fig. 5B; Samarsky et al. 1999; Lacadie et al. 2006). As shown earlier, *PHO84* expression was silenced in the progeny of 25-d-old wild-type cells (Fig. 5B, lanes 1,2). In contrast, the insertion of Rz, but not Rzm, led to reduced antisense RNA levels and maintenance of *PHO84* sense expression in aged cells (Fig. 5B, lanes 3,4). Although Northern analyses failed to detect shortened *PHO84* antisense transcripts, these results strongly support the view that loss of *PHO84* silencing results from antisense RNA cleavage in presence of Rz.

To further confirm that *PHO84* cosuppression also depends on antisense RNAs produced in *trans*, two new plasmids similar to *N¹* but containing the Rz or Rzm sequences were constructed (*N¹ Rz* and *N¹ Rzm*) and transformed into the Δter strain, which produces a long *PHO84* read-through transcript distinguishable from

Figure 5. *PHO84* antisense RNA self-cleavage blocks silencing in *cis* and *trans*. (A) Map of the *PHO84*-ribozyme chimeric gene. A *PHO84* gene containing a 51-mer wild-type (Rz) or mutant (Rzm) hammerhead ribozyme sequence was integrated in place of the endogenous *PHO84* gene (strains *PHO84Rz* and *PHO84Rzm*) or inserted within the plasmid *PHO84* N°1 (N°1Rz and N°1Rzm). Arrows indicate *PHO84* sense and antisense transcripts. (B) Cleavage of endogenous *PHO84* antisense RNA blocks *PHO84* silencing in aged cells. Northern analysis of *PHO84* sense and antisense RNAs in the progeny 25-d-old wild-type, *PHO84Rz*, and *PHO84Rzm* strains and the progeny of 2-d-old wild-type cells. (C) Cleavage of plasmid-encoded antisense RNA blocks silencing of endogenous *PHO84*. Δter cells (Fig. 3D) transformed with empty vector (V), plasmid N°1 (N°1) as such or containing wild-type (N°1Rz) or mutant ribozyme (N°1Rzm) were analyzed as in Figure 1B. (D) Cleavage of endogenous *PHO84* antisense RNAs does not affect silencing in *trans*. The *PHO84Rz* strain (see A,B) transformed with empty vector or plasmid N°1 (N°1) was analyzed as in Figure 1B.

plasmid-derived *PHO84* sense RNAs (Fig. 3D,E). While transformation of plasmids N°1 and N°1 Rz triggered silencing of endogenous *PHO84* transcripts, plasmid N°1 Rz failed to do so (Fig. 5C). Thus, as concluded previously from the experiments using the GAL promoter, antisense RNAs can silence *PHO84* gene transcription both in *cis* and in *trans*.

To address whether *PHO84* cosuppression requires endogenous antisense RNAs, plasmid N°1 was transformed into the strain containing the integrated *PHO84*-Rz gene (Fig. 5D). This transformation led to the silencing of *PHO84* gene expression, indicating that antisense RNAs produced in *trans* from the plasmid are sufficient. This conclusion is consistent with the observation that silencing in *trans* is also not impaired when endogenous antisense transcripts are blocked by insertion of the *HIS5* cassette (Fig. 4B).

Set1 influences the silencing in *trans* by promoting production of antisense RNAs

Recently, it was proposed that Ty1 antisense RNAs escaping cytoplasmic degradation act in *trans* to repress Ty1 sense RNA transcription through a still poorly defined

mechanism involving the Set1 histone methyltransferase (Berretta et al. 2008). Notably, Set1 dimethylates and trimethylates H3K4 in the middle of the *PHO84* ORF (Shukla et al. 2006), raising the question of whether Set1 may also participate in *PHO84* cosuppression. To address this possibility, an empty vector and plasmid N°1 were transformed into the $\Delta set1$ strain. In addition, these strains were complemented with an empty or *SET1*-containing plasmid. Transformation of plasmid N°1 failed to induce efficient silencing unless $\Delta set1$ was complemented, suggesting that Set1 is implicated in the mechanism of silencing in *trans* (Fig. 6A, lanes 5,6). Set1 contains two RNA Recognition Motifs (RRM1 and RRM2), which could be required to recognize antisense RNAs and induce silencing. To test this possibility, the $\Delta set1$ strain containing plasmid N°1 was transformed with *SET1* plasmids in which the RRM1 or RRM2 motifs were mutated (Tresaugues et al. 2006). These Set1 mutants were still able to restore silencing, indicating that the RNA recognition motifs of Set1 are not implicated (Fig. 6A, lanes 6–8).

Notably, *PHO84* antisense RNA levels in the $\Delta set1$ strain were reduced in the noncomplemented condition as compared with the three complemented conditions (Fig. 6A; see below), suggesting that Set1 may influence silencing through the regulation of antisense RNA production. To address whether Set1 acts upstream of or downstream from antisense RNAs in *PHO84* silencing, wild-type and $\Delta set1$ strains were transformed with an empty vector or plasmid GAL-N°1 (Fig. 6B). Induction of antisense RNAs from the heterologous galactose-inducible promoter led to sense RNA silencing even in the absence of Set1 (Fig. 6B, cf. lanes 2,4 and 6,8). This observation supports the view that Set1 acts upstream of *PHO84* antisense RNAs and triggers silencing primarily by stimulating the production of *trans*-acting antisense RNAs. Note that the decreased *PHO84* sense expression in the wild-type strain containing GAL-N°1 grown in glucose (Fig. 6B, lane 3) is due to promoter leak and production of sufficient antisense RNA to trigger partial silencing.

A role for Set1 in antisense production was also observed when comparing *PHO84* transcript levels in wild type and $\Delta set1$ cells grown on plates for 2 d. In these conditions, loss of Set1 results in reduced antisense and slightly increased sense RNA levels, indicating that Set1 contributes to silencing in *cis* (Supplemental Fig. 4). The absence of Set1, however, did not impact on *PHO84* mRNA levels in aged cells, suggesting that Set1 is required at early stages and that additional chromatin-modifying activities may take over to silence *PHO84* in aging cells (Katan-Khaykovich and Struhl 2005). The same phenotypes were observed in the H3K4A mutant. These data together are consistent with the view that Set1 H3K4 methylation participates in *trans*-silencing by promoting antisense RNA production.

Antisense RNA levels and homology requirements for target gene silencing

The observation that only the longest *PHO84* constructs N°1 and N°2 were able to induce silencing in *trans* (Fig.

Figure 6. Antisense RNAs are stimulated by Set1 and require both 5' and 3' sequences to mediate silencing. (A) *SET1* is implicated in *PHO84* gene cosuppression. $\Delta set1$ cells containing an empty YCpLac33 vector (V), or complemented with plasmids encoding wild-type *SET1* or the *rrm1* and *rrm2* mutant forms, were transformed with a YCpLac111 vector (V) or the *PHO84* plasmid N°1 and analyzed as in Figure 1B. (B) *PHO84* antisense RNAs driven by a GAL promoter bypass the requirement for Set1 to trigger *PHO84* cosuppression. Wild-type and $\Delta set1$ cells transformed with empty vector or plasmid GAL-N°1 were grown for 24 h in –LEU medium containing 2% glucose (GLU) or 2% galactose (GAL). *PHO84* sense and antisense RNA levels were measured as in Figure 1B. (C) Efficient silencing requires long antisense RNAs containing both *PHO84* 3' end and UAS sequences. The W303 wild-type strain was transformed with the TetO₇ pCM252 vector alone (V) or containing the *PHO84* portions UAS, N°3, N°5, N°2, N°1 (Fig. 3A). Cells were exponentially grown for 16 h in –TRP media without (–) or with (+) doxycycline (20 μ g/mL) to induce antisense RNAs. *PHO84* sense and antisense RNAs were detected with single-stranded probes specific for *PHO84* 5' end (Fig. 3A). *ACT1* mRNA served as loading control. (D) Diagram of the chromosomal *pho84::URA3* gene. The *PHO84* ORF (codons 1 to stop) was replaced by the *K. lactis* *URA3* ORF (codons 1 to stop), so that *URA3* transcription is driven by the *PHO84* UAS promoter region. Total RNA extracted from the $\Delta pho84::URA3$ strain containing an empty vector (V) or plasmid N°1 was probed with probes specific for *URA3* sense and *PHO84* antisense transcripts, and *ACT1* for loading control.

3B) suggested that antisense transcripts had to be long and contain sequences corresponding to the UAS (Fig. 3C). To confirm these results and address whether shorter antisense transcripts would be able to induce silencing if overexpressed, the *PHO84* portions N°1, N°2, N°3, and N°5, as well as the upstream region corresponding to *PHO84* UAS (positions –1 to –725), were cloned inverted behind a TetO₇ promoter (Fig. 3A). Although large amounts of shorter antisense RNAs were produced from the UAS, N°3, and N°5 constructs, they induced no or only modest *PHO84* silencing in case of N°3. In contrast,

very potent *PHO84* gene repression was observed in the presence of the Tet-N°1 and Tet-N°2 constructs (Fig. 6C), confirming that full-length antisense RNAs containing both *PHO84* 3' end and UAS complementary sequences are necessary to mediate *PHO84* cosuppression. Note that although very efficient, antisense RNAs from Tet-N°1, as from GAL-N°1 (Fig. 4A), are unstable and hard to detect. Moreover Tet-N°1 and Tet-N°2 were able to induce silencing even in the absence of doxycycline (Supplemental Fig. 3; data not shown), indicating that very low amounts of these long antisense RNAs, resulting from the leaky TetO₇ promoter, are sufficient to induce silencing.

As a complementary approach to define the minimal homology region between these long antisense transcripts and the target gene required for silencing, strain $\Delta pho84::URA3$ was constructed in which the *PHO84* coding region (from codon 1 to stop) was replaced by the *Kluyveromyces lactis* *URA3* coding sequence. In this strain, the *URA3* gene is driven by *PHO84* UAS promoter and followed by *PHO84* 3'UTR sequences (Fig. 6D). Interestingly, transformation of plasmid N°1 induced a reduction in *URA3* mRNA levels, indicating that a homology restricted to the *PHO84* UAS promoter between the *trans*-acting antisense RNAs and the target gene is sufficient to trigger silencing, consistent with an effect on transcription initiation (Fig. 2A,B).

Antisense production does not necessarily promote silencing in trans of other genes

An important question is whether other antisense RNA-producing genes can mediate transcriptional cosuppression. Candidate genes (*VTC3*, *YJR129c*, and *GYP5*) were identified based on recently published tiling array analyses comparing sense and antisense RNAs in wild-type versus $\Delta rrp6$ strains (Xu et al. 2009). Gene-specific RT-qPCR analyses confirmed that stabilization of *PHO84*, *VTC3*, *YJR129c*, and *GYP5* antisense RNAs leads to sense RNA repression (Fig. 7A), suggesting that antisense RNA-mediated TGS in *cis* is not a rare phenomenon. In addition, these analyses revealed that several pathways might exist as *PHO84* and *VTC3* silencing, but not *GYP5* and *YJR129c* silencing, depends on Hda2 (Fig. 7A).

Next, to address whether these antisense RNAs may trigger cosuppression in *trans*, a wild-type strain was transformed with an empty vector, with centromeric plasmids containing each gene expressed from its own promoter, or with centromeric plasmids with each gene cloned upstream of a *GAL* promoter, allowing overexpression of antisense transcripts in galactose. The levels of sense transcripts expressed in the transformed strains were determined by RT-qPCR using primers specific for each gene (Fig. 7B). None of the strains transformed with the *VTC3*, *YJR129c*, and *GYP5* plasmids showed a decrease in the corresponding sense RNA levels compared with those detected in presence of the empty vector. Thus *trans*-silencing is not a general feature of antisense-producing genes.

As mentioned earlier, expression of the *PHO84* + *HIS5* transcript can be silenced in *trans* by galactose-induced

Figure 7. Analysis of candidate genes regulated by antisense transcripts like *PHO84*. (A) RT-qPCR analysis of *PHO84*, *VTC3*, *YJR129c*, and *GYP5* sense and antisense RNA levels in wild-type, $\Delta rrp6$, $\Delta hda2$, and $\Delta hda2\Delta rrp6$ strains exponentially grown in SC medium + 2% glucose. Values are represented as fold change of sense or antisense RNA levels in $\Delta rrp6$ versus wild type and in $\Delta hda2\Delta rrp6$ versus $\Delta hda2$. (B) *VTC3*, *YJR129c*, and *GYP5* antisense RNAs do not trigger silencing in *trans*. The W303 wild-type strain was transformed with empty vector (V), plasmids containing the three candidate genes with their own regulatory sequences (gene) or plasmids containing the three candidate genes behind a GAL promoter such that antisense RNAs are induced in galactose (GAL antisense). Cells were exponentially grown in $-LEU + 2\%$ glucose (vector and gene) or in $-LEU + 2\%$ galactose (vector and GAL antisense). Total RNA was subjected to RT-qPCR analyses with gene-specific probes. Values are expressed as fold change versus the vector control in the two different conditions. (C) Model shows antisense RNAs mediate *cis* and *trans* transcriptional gene silencing. Set1 H3K4 methylation promotes antisense RNA production. In *cis*, antisense RNAs escaping degradation by the nuclear exosome can trigger transcriptional gene silencing in an Hda1/2/3-dependent (*PHO84* and *VTC3*) or Hda1/2/3-independent (*GYP5* and *YJR129c*) way. In *trans*, *PHO84* antisense RNAs silence the homologous target sequence at the transcriptional level due to a homology region with the UAS promoter sequence. We propose that this requires an unidentified silencing factor coating the *PHO84* gene (named A). Importantly, while high amounts of short *trans*-acting antisense RNAs (Tet-N³) are weak in inducing silencing by A, small amounts of long antisense RNAs originating from the 3' end of *PHO84* gene are very effective (plasmid N¹). We speculate that a silencing factor (named B) might be recruited by these long RNAs and would interact with the prebound repressor A enhancing its silencing activity (A*). The same hypothesis might explain why other antisense RNAs are ineffective in *trans* (*VTC3*, *GYP5*, and *YJR129c*).

PHO84 antisense RNAs (Fig. 4B). This repression, however, does not affect the expression of the *S. pombe* *HIS5* gene inserted in the reverse orientation within *PHO84*. To address whether the context of the *PHO84* gene may

favor *trans*-silencing, we tested whether overexpression of *HIS5* antisense transcripts from a TetO₇ promoter could silence the *HIS5* gene located within the *PHO84* + *HIS5*. Due to the leakiness of the promoter, *HIS5*

antisense transcripts were detected already in the absence of induction but strongly increased following overnight treatment with doxycycline. Interestingly, antisense RNA accumulation correlated with some decrease in *HIS5* sense transcripts encoded by the *PHO84-HIS5* chimeric gene (Supplemental Fig. 5). These observations suggest that, while antisense-mediated *trans*-silencing is not a general feature (Fig. 7B), antisense RNAs can induce silencing in *trans* if they target a sequence in the context of the *PHO84* gene. However, as observed with construct N°3 (Fig. 6C), silencing was not very potent despite the accumulation of large amounts of antisense RNA.

Discussion

Recently, high-resolution transcriptome analysis in budding yeast (Neil et al. 2009; Xu et al. 2009) and in mammals (Affymetrix/Cold Spring Harbor Laboratory ENCODE Transcriptome Project 2009) reported the existence of a growing family of long noncoding RNAs. Among these, several have been described to regulate transcription of protein coding genes in *cis* (Martens et al. 2004; Hongay et al. 2006; Camblong et al. 2007; Uhler et al. 2007; Houseley et al. 2008). In the present study, we show that *PHO84* antisense RNAs induce transcriptional cosuppression in *trans*, highlighting that homology-dependent gene silencing is not the exclusivity of organisms containing the RNAi machinery.

Antisense RNAs trigger PHO84 TGS in cis and in trans

We previously proposed that, in *S. cerevisiae*, antisense RNAs accumulated in *cis* target TGS through histone deacetylation (Camblong et al. 2007). In the present study, we show that induction of antisense RNAs from a plasmid can bypass the requirement of natural *PHO84* antisense RNAs and direct endogenous *PHO84* gene silencing in *trans* (Fig. 4B) prior to cytoplasmic export (Supplemental Fig. 6). Consistently, self-cleaved antisense RNAs were inefficient in inducing silencing not only in *cis* but also in *trans* (Fig. 5B,C). Moreover, *trans*-acting antisense RNAs are very potent (Fig. 4C) and act rapidly at the transcription initiation level, completely blocking TBP recruitment to the *PHO84* promoter (Fig. 2B). However, the effects of *cis* and *trans*-acting antisense RNAs differ by the requirement of the Hda1/2/3 histone deacetylase complex. While Hda1/2/3 dependency reflects the *cis* mechanism (Fig. 4A; Camblong et al. 2007), *trans*-acting antisense RNAs induce silencing independently of this HDAC (Fig. 2C; Supplemental Fig. 3). Thus RNA-mediated *PHO84* TGS relies on at least two alternative pathways. Together, these findings provide the first evidence in *S. cerevisiae* that antisense RNAs can act in *trans* to silence transcription of a protein coding gene. Moreover, the data show that the same gene can be silenced by antisense RNAs acting in *cis* or in *trans*. These observations strengthen the view that antisense RNAs are central players in directing TGS in budding yeast.

A role for Set1 in gene regulation by promoting antisense production

Among all the mutants disrupted for chromatin-related functions that we tested so far (Supplemental Table 3), $\Delta set1$ was the only one to show a defect in *PHO84 trans*-silencing. Our data indicate that the Set1 H3K4 methyltransferase participates in cosuppression by promoting *PHO84* antisense RNA production (Fig. 6A,B). Remarkably, Set1 activity has also been linked to heterochromatin formation at the HM (Katan-Khaykovich and Struhl 2005) and rDNA (Briggs et al. 2001) loci. In addition, noncoding RNAs subjected to degradation by the nuclear exosome have been identified recently within rDNA silenced regions (Houseley et al. 2007; Vasiljeva et al. 2008). Since H3K4 trimethylation correlates with active chromatin states (Noma and Grewal 2002), the silencing defects observed in the absence of Set1 could reflect its role in facilitating the production of noncoding RNAs, which in turn may target silencing factors to corresponding DNA regions. An earlier study showed that *PHO84* and *GAL1-10*, two genes that were shown recently to produce and to be regulated negatively by antisense transcripts (Camblong et al. 2007; Houseley et al. 2008; Xu et al. 2009), become derepressed in the absence of Set1 (Carvin and Kladde 2004). Notably, a recent genome wide study showed that in *S. cerevisiae*, H3K4 methylation is not only promoter-associated but also present within ORFs and at the 3' end of a number of genes (Kirmizis et al. 2007). Moreover, a number of these genes become derepressed in the absence of Set1 and express antisense transcripts (F Holstege and V Géli, pers. comm.). These observations together indicate that Set1 is a key player in regulating antisense RNA-mediated TGS. Whether H3K4 methylation by Set1 during transcription influences RNA PolII processivity, increases antisense RNA stability, and/or facilitates the recruitment of silencing factors are important questions for the future.

Antisense RNA features required for TGS

To address the generality of antisense-induced *trans*-silencing, we selected, based on recently published genome-wide analyses (Xu et al. 2009), three candidate genes exhibiting antisense RNA accumulation and sense RNA repression in the absence of Rrp6 (Fig. 7A). Despite a mode of regulation similar to *PHO84*, none of these genes was able to induce cosuppression, whether expressed from a plasmid with their own promoter or with a GAL promoter driving antisense transcription (Fig. 7B). Thus, besides antisense production, additional, yet unidentified, *PHO84*-specific features are required for cosuppression to take place.

Our studies show that antisense-induced silencing in *trans* occurs at the transcriptional level (Fig. 2). Although very modest *trans*-silencing can be observed when truncated antisense RNAs containing UAS sequences are overexpressed, potent cosuppression is achieved only when long antisense RNAs, containing sequences complementary to both *PHO84* UAS and 3' end, are produced (Fig. 6C). These long antisense transcripts are effective

even when present in very low amounts (Figs. 4B, 6C; Supplemental Fig. 3), suggesting that these RNAs are merely required to initiate rather than to maintain the silenced state. Interestingly, the long *PHO84* antisense transcripts are able to promote transcriptional silencing of the *pho84::URA3* target gene, whose homology with plasmid encoded antisense RNAs is restricted to the *PHO84* UAS region (Fig. 6D). One possible scenario could be that factors implicated in TGS are recruited to the antisense RNA at early phases of transcription to subsequently facilitate promoter homology-driven silencing events (see the model in Fig. 7C). Finally, we show that a *S. pombe* *HIS5* gene embedded within the *PHO84* gene is down-regulated, albeit not very efficiently, when *HIS5* antisense transcripts are overexpressed in *trans* (Supplemental Fig. 5). These observations suggest that the *PHO84* gene may provide a chromatin environment prone to silencing by *trans*-acting antisense RNAs.

Analogies with RNA-mediated TGS and X-chromosome inactivation (XCI) in metazoa

Similar to antisense-induced *PHO84* promoter *trans*-silencing, ectopic expression of antisense RNAs in mammalian cells triggers TGS at targeted promoters, provided these are transcribed in the sense orientation (Weinberg et al. 2006; Han et al. 2007). Although likely, a role in promoter silencing has not yet been demonstrated for naturally occurring mammalian antisense transcripts. In contrast, HOTAIR, and Xist are two natural long non-coding RNAs involved in *trans*-repression of the mammalian HOXD locus and XCI, respectively (Rinn et al. 2007; Erwin and Lee 2008). Our finding that *PHO84* *trans*-silencing requires a 2.5-kb-long sequence containing the complete *PHO84* ORF and the upstream UAS (Fig. 3A,B) raises parallels with XCI and the possibility that the *PHO84* ectopic and endogenous copies physically interact. Indeed, in XCI, expression of *trans*-acting *Tsix* and *Xite* noncoding RNAs from, respectively, 3.7- and 5.6-kb-long sequences promotes X chromosome pairing and initiates Xist-dependent inactivation of one of the two copies (Xu et al. 2006).

How closely *PHO84* cosuppression relates to these phenomena, how extra copies are sensed and whether gene repression results from transient pairing of homologous sequences are important open questions. Future experiments aim at identifying the factors implicated in antisense-mediated *trans*-silencing, at defining the *PHO84* gene features enabling antisense RNAs to trigger silencing in *trans*, as well as understanding the physiological relevance of this regulation. The genetic dissection of this pathway in the budding yeast may provide important new insights into RNAi-independent mechanisms in higher eukaryotes.

Materials and methods

Plasmid constructions

PHO84 sequences N°1–N°6 were cloned as Sall–BamHI PCR fragments (see Supplemental Table 2 for primers) into

YCpLac111 and correspond, respectively, to *PHO84* positions –725 to +165 (N°1, pFS3178), –725 to stop (N°2, pFS3181), –725 to 770 (N°3, pFS3182), –200 to +165 (N°4, pFS3183), –200 to stop (N°5, pFS3184), and –200 to 770 (N°6, pFS3185). Plasmid GAL–YCpLac111 (pFS1932) was obtained by transferring the GAL1 UAS promoter region together with a multiple cloning site as an EcoRI–HindIII fragment from the high-copy galactose-inducible YEplac111 vector (Lee et al. 1996) into YCpLac111. Construct GAL–N°1 (pFS3247) was obtained by subcloning the Sall–BamHI *PHO84* fragment N°1 in the inverse orientation behind the GAL promoter of plasmid GAL–YCpLac111 cut with Sall and BamHI. pCM252–N°1 (pFS3132), pCM252–N°2 (pFS3133), pCM252–N°3 (pFS3478), and pCM252–N°5 (pFS3481) were generated by inserting the *PHO84* sequence N°1–N°3 and 5 in the reverse orientation behind the TetO₇ promoter of vector pCM252 as BamHI–StuI PCR fragments. pCM252–UAS (pFS3211) was obtained by cloning the region upstream of *PHO84* (position –1 to –725) as a BamHI–StuI fragment behind the TetO₇ promoter such that deoxycytidine induces *YML122c* antisense RNA. To express *S. pombe* *HIS5* antisense transcripts, a BamHI–StuI PCR fragment containing the coding and terminator but not the promoter sequences was generated using pUG27 (Gueldener et al. 2002) as template and cloned into pCM252 to obtain pFS3482. *VTC3*, *YJR129c*, and *GYP5* were cloned as BamHI PCR fragments into YCpLac111 to obtain pFS3465, pFS3466, and pFS3469; the same genes were cloned as BamHI fragments in reverse orientation behind the GAL promoter of YCpLac111–GAL (Stutz laboratory) to obtain pFS3462, pFS3463, and pFS3468.

The *PHO84Rz* and *PHO84Rzm* chimeric genes, containing a wild-type and a mutant 51-bp hammerhead ribozyme sequence, respectively, were obtained using a two-step PCR strategy and initially cloned into the integrating vector pRS406 cut with XhoI and SacI. Briefly a 5' fragment was generated with a 5' Sall primer (OFS1249) and a Rz or Rzm reverse primer (OFS1248 or OFS1299); a 3' fragment was generated with a Rz or Rzm forward primers (OFS1247 or OFS1298) and the 3' SacI primer (OFS1250). The 5' and 3' PCR fragments were mixed and reamplified with OFS1249 and OFS1250 external primers to generate the full chimeric *PHO84Rz* and *PHO84Rzm* genes followed by insertion as a Sall–SacI fragment into pRS406 cut with XhoI + SacI to generate pFS3154 and pFS3231. The ribozyme containing genes were transferred as SacI–PvuII fragments from pRS406 into YCpLac111 cut with SacI + SmaI to generate pFS3218 and pFS3348. To insert *PHO84* at other genomic locations, *PHO84* was first cloned as a Sall PCR fragment next to the *HIS5* marker in pUG27 (Gueldener et al. 2002) in either the forward or the reverse orientation to generate, respectively, pUG27-*PHO84* for (pFS3328) and pUG27-*PHO84* rev (pFS3329).

Yeast strain constructions

All yeast strains used in this study are described in Supplemental Tables 1 and 3. Gene deletions in W303 background were generated either by PCR amplification of the deleted locus in BY4741 (Euroscarf) with –500 and +500 primers and transformation into W303 or by PCR amplification of deletion cassettes as described (Longtine et al. 1998). The W303 strain containing a *PHO84* + *HIS5* gene (FSY3332) was constructed as described previously (Camblong et al. 2007). Strain *PHO84Δter* (FSY3657) was obtained by deleting *PHO84* terminator sequences (position +50 to +170) through insertion of a LoxP-*HIS5*-LoxP cassette followed by excision of *HIS5* by expression of Cre recombinase (Gueldener et al. 2002). The Rz and Rzm strains (FSY3346 and FSY3568) were obtained using a pop-in/pop-out gene replacement strategy. Briefly, the pRS406-*PHO84Rz* and pRS406-*PHO84Rzm* integrative plasmids (pFS3154 and pFS3231)

were linearized with *Ava*I and transformed into W303. URA⁺ colonies were streaked on 5-FOA to select for loss of one of the two *PHO84* gene copies. Retention of the ribozyme-containing gene was identified by PCR. The insertion of a *PHO84* gene copy instead of *LYS2*, *YML083*, or *GUD1* was obtained by PCR amplification of pFS3329 (for *LYS2*) and pFS3328 (for *YML083* and *GUD1*) to generate *PHO84-HIS5* fragments with sequences complementary to the target genes. W303 and Δ *pho84* strains transformed with the PCR products were selected on –HIS medium and gene replacement at the target sites verified by PCR. The *pho84-URA3* strain was obtained by replacement of the endogenous *PHO84* coding region (codon 1 to stop) by the *K. lactis* URA3 coding region through targeted homologous recombination of a URA3 PCR fragment amplified from pUG72 (Gueldener et al. 2002), followed by selection of transformants on –URA plates.

Media and culture conditions

Yeast strains transformed with plasmids were grown for 3 d on plates before liquid culture for 24 h under exponential conditions (OD₆₀₀ < 0.8) in drop-out minimum medium. The wild-type, Rz, and Rzm strains (Fig. 5B) were grown on YEPD plates at 25°C for 3 d and kept for 2 or 25 d at 4°C followed by cultivation in synthetic complete (SC) minimum medium for 24 h under exponential conditions.

RNA extraction and analysis

Total RNA was extracted with the hot phenol procedure and analyzed by Northern blotting (Camblong et al. 2007) or RT-qPCR (Xu et al. 2009). For Northern blots, all probes were SP6 or T7 (*GAL1*) riboprobes, except for *ACT1*, which was a random primed labeled probe. Quantifications were performed with a Phosphor-Imager. For RT-qPCR gene-specific primers were used for the RT reaction. All primers are described in Supplemental Table 2.

ChIP

ChIPs were performed essentially as described (Camblong et al. 2007). Yeast strains were grown to OD₆₀₀ = 1 in –LEU medium for 15 min at 25°C and cross-linked by the addition of formaldehyde to a final concentration of 1.2%. Cross-linked and sonicated chromatin extracts from 10⁸ cells were immunoprecipitated overnight in the presence of 50 μ L of 50% protein G Sepharose (Amersham Pharmacia) with antibodies against the C-terminal domain of the RNA Pol II (Abcam 8WG16) or the TBP (gift from M. Collart). All immunoprecipitations were performed in triplicate with different chromatin extracts. Immunoprecipitated DNA was quantified by real-time PCR using specific primer pairs (Camblong et al. 2007) and expressed as the fold increase over a nontranscribed intergenic region as in (Zenklusen et al. 2002). Error bars correspond to standard deviations.

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