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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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 Δpho84 strains, each containing a PHO84 ectopic copy replacing either the IVS2 [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.

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 Δpho84 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
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 Δpho84 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 Δpho84 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 Δpho84 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, Ece1, 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 wild-type and Δpho4 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, Δhda1, and Δhda2 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).

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 Δpho84 strain to define their expression in the absence of the endogenous PHO84 gene [Fig. 3B]. While plasmids
Antisense RNA-induced silencing

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 anti-sense expression. Thus, Δpho84 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 Δpho84 nor in Δpho84 Δhda2 strains. Since endogenous antisense RNAs are degraded by Rrp6, we also transformed the GAL-N°1 plasmid into a Δpho84 Δrrp6 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 poll 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 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 +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 Δpho84 (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.
D. hda2 strain transformed with empty vector (V) and plasmid PHO84+HIS5. Transformants were exponentially grown in 2% glucose (GLU), 2% galactose (GAL), 2% glucose and 2% galactose (2% GLU & 2% GAL), and 2% glucose and 2% galactose (2% GLU & 2% GAL) media 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 [PHO84R2] 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 N°1 but containing the Rz or Rzm sequences were constructed [N°1 Rz and N°1 Rzm] and transformed into the Δter strain, which produces a long PHO84 read-through transcript distinguishable from
plasmid-derived PHO84 sense RNAs [Fig. 3D,E]. While transformation of plasmids N°1 and N°1 Rzm triggered silencing of endogenous PHO84 transscripts, 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 Δset1 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 Δset1 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 Δset1 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 Δset1 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 Δset1 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 Δset1 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.
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-N1, as from GAL-N1 (Fig. 4A), are unstable and hard to detect. Moreover Tet-N1 and Tet-N2 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 Δpho84::URA3 was constructed in which the PHO84 coding region [from codon 1 to stop] was replaced by the K. 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 Δrrp6 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 YJR129 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 silencing, depending on the presence of the Tet-O7 and Tet-O2 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-N1, as from GAL-N1 (Fig. 4A), are unstable and hard to detect. Moreover Tet-N1 and Tet-N2 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.

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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

![Figure 7](https://genesdev.cshlp.org/genesdev.cshlp.org)
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), Δset1 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:\textasciitilde \textasciitilde 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 metazoans**

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 naturally occurring mammalian antisense transcripts. In particular, HOTAIR, and Xite noncoding RNAs from, respectively, 3.7- and 5.6-kb-long sequences promote 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 N1–N6 were cloned as Sall–BamH1 PCR fragments [see Supplemental Table 2 for primers] into YCpLac111 and correspond, respectively, to PHO84 positions \(-725\) to \(+165\) [N1, pFS3178], \(-725\) to stop [N2, pFS3181], \(-725\) to 770 [N3, pFS3182], \(-200\) to 165 [N4, pFS3183], \(-200\) to stop [N5, pFS3184], and \(-200\) to 770 [N6, pFS3185]. Plasmid GAL-YCpLac111 [pFS1932] was obtained by transferring the GAL UAS promoter region together with a multiple cloning site as an EcoRI– HindIII fragment from the high-copy galactose-inducible YEp-GAL vector [Lee et al. 1996] into YCpLac111. Construct GAL-N1 [pFS3247] was obtained by subcloning the Sall–BamH1 PHO84 fragment N1 in the inverse orientation behind the GAL plasmid of plasmid GAL-YCpLac111 cut with Sall and BamHI. pCM252-N1 [pFS3312], pCM252-N2 [pFS3333], pCM252-N3 [pFS3478], and pCM252-N5 [pFS3481] were generated by inserting the PHO84 sequence N1–N3 and 5 in the reverse orientation behind the TetO2 promoter of vector pCM252 as BamHI–StuI PCR fragments. pCM252-UA5 [pFS3211] was obtained by cloning the region upstream of PHO84 [position 1 to 723] as a BamH1–StuI fragment behind the TetO2 promoter such that deoxy cycline induces YML122c antisense RNA. To express S. pombe HIS5 antisense transcripts, a BamH1–Stul 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 BamH1 PCR fragments into YCpLac111 to obtain pFS3465, pFS3466, and pFS3469; the same genes were cloned as BamH1 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 by using a two-step PCR strategy and initially cloned into the integrating vector pRS406 cut with XhoI at other genomic locations, as template and cloned into pCM252 to obtain pFS3482. YCpLac111 was obtained by subcloning the region upstream of PHO84 [position 1 to 723] as a BamH1–StuI fragment behind the TetO2 promoter such that deoxy cycline induces YML122c antisense RNA. To express S. pombe HIS5 antisense transcripts, a BamH1–Stul 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 BamH1 PCR fragments into YCpLac111 to obtain pFS3465, pFS3466, and pFS3469; the same genes were cloned as BamH1 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 by 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¢ primer and cloned into pCM252 to obtain pFS31249 and a Rz or Rzm reverse primer (OFS1249 or OFS1299); a 3¢ fragment was generated with a Rz or Rzm forward primer (OFS1247 or OFS1298) and the 3¢ SacI primer (OFS1250). The 5¢ and 3¢ PCR fragments were mixed and reamplified with primers of 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 (pFS3181), pFS3183), pFS3184, and pFS3185 to generate, respectively, pUG27-PHO84 for PHO84Rz and PHO84Rzm genes followed by insertion as a Sall–SacI fragment into p RS406 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 + Smal 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 the forward or the reverse orientation to generate, respectively, pUG27-PHO84 for 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 + Smal 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 the forward or the reverse orientation to generate, respectively, pUG27-PHO84 for 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 + Smal 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 the forward or the reverse orientation to generate, respectively, pUG27-PHO84 for PHO84Rz and PHO84Rzm genes followed by insertion as a Sall–SacI fragment into pRS406 cut with XhoI + SacI to generate pFS3154 and pFS3231.
were linearized with Aval 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-HISS fragments with sequences complementary to the target genes. W303 and Δpho84 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 Rz2 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 (GALI) 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 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 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 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 C and cross-linked by the addition of formaldehyde to a final concentration of 1.2%. 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