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Regulation of anti-sense transcription by Mot1p and NC2 via removal of TATA-binding protein (TBP) from the 3′-end of genes

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ABSTRACT

The activity and dynamic nature of TATA-binding protein (TBP) crucial to RNA polymerase II-mediated transcription is under control of the Mot1p and NC2 complexes. Here we show that both TBP regulatory factors play 'hidden' roles in ensuring transcription fidelity by restricting anti-sense non-coding RNA (ncRNA) synthesis. Production of anti-sense ncRNA transcripts is suppressed by Mot1p- and NC2-mediated release of TBP from binding sites at the 3′-end of genes. In this, Mot1p and NC2 collaborate with the Nrd1p–Nab3p–Sen1p (NNS) complex that terminates the synthesis of anti-sense ncRNAs. In several cases anti-sense ncRNA expression interferes with expression of the cognate sense transcript. Our data reveal a novel regulatory mechanism to suppress anti-sense ncRNA expression and pre-initiation complex (PIC) formation at spurious sites.

INTRODUCTION

The compact genome of *Saccharomyces cerevisiae* encodes ∼6200 protein-coding genes. However its RNA expression profile is far more complex and this has been observed in other organisms too (1). Besides mRNA synthesis by RNA polymerase II (pol II), pervasive transcription of the yeast genome also results in formation of non-coding RNAs (ncRNAs) comprising stable unannotated transcripts (SUTs), cryptic unstable transcripts (CUTs), Nrd1-unterminated transcripts (NUTs) and Xrn1-sensitive unstable transcripts (XUTs) (2–7). Classification of these ncRNAs is based on their transcription termination and degradation pathway, but several ncRNAs belong to more than one class.

Transcription initiation by pol II is restricted to nucleosome-depleted regions (NDRs) of the yeast genome, which are accessible for pre-initiation complex (PIC) components like TATA-binding protein (TBP) and TFIID. The PICs for upstream divergent ncRNAs assemble on 5′-NDRs at promoters of coding genes in an orientation opposite to the mRNA. PIC formation at the end of genes drives synthesis of anti-sense ncRNAs (5,8,9). Typically, divergent and anti-sense ncRNAs are independent from levels of the sense mRNA (8–10). However, a subset of ncRNAs is capable of regulation and fine-tuning mRNA expression level of protein-coding genes, which can be achieved in multiple ways. The ncRNA can influence gene expression by transcription interference in *cis* or in *trans*. Alternatively, ncRNA formation can alter chromatin structure and/or modifications. For example, the *PHO84* anti-sense ncRNA accumulates in mutant yeast deleted for *RRP6* encoding a critical component of the nuclear exosome. This *PHO84* ncRNA represses the sense mRNA by recruitment of a histone deacetylase (HDAC) complex to the mRNA promoter (11,12). Two ncRNAs in mating-type control of sporulation in yeast were discovered, which recruit the SET2 and SET3 chromatin complexes repressive for promoter activity (13).

These recent findings underscore the importance to understand the regulatory mechanisms controlling ncRNA synthesis. One mechanism is the control of chromatin structure by the ISW2 remodeling complex, which restricts the size of the intergenic NDR (14,15). Secondly, the chromatin-assembly factor 1 (CAF1) complex suppresses divergent ncRNA synthesis by histone deposition, which is antagonized by increased nucleosome turnover by the SWI2/SNF2 chromatin remodeler (16). As for the *PHO84* gene, modulation of histone acetylation and methylation is another way to control ncRNA expression (10,17–19). Furthermore, ncRNA levels are restricted at the level of transcription termination and degradation. Most SUTs and XUTs undergo cleavage, poly-adenylation and nuclear export like mRNAs, before they are degraded by the cytoplasmic Xrn1p 5′-3′ exoribonuclease and the nonsense-mediated decay pathway. Similar to small nucleolar/nuclear RNAs (sno/snRNAs) CUT synthesis is terminated by the Nrd1p–Nab3p–Sen1p (NNS) complex, but in this case RNA synthesis is coupled to rapid degradation by the nuclear exosome (20,21). The NNS complex is recruited to 5′-

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regions of genes, which coincides with phosphorylation of the C-terminal domain (CTD) of pol II at serine-5. This modification event results in NNS complex binding via Nrd1p interaction with the CTD (22). Both Nrd1p and Nab3p also contain RNA recognition motifs, which preferentially bind short sequence motifs enriched in ncRNAs and depleted in mRNAs. This provides a termination mechanism selective for ncRNAs (7,23). In conclusion, pervasive transcription of the genome is both controlled at the level of initiation by chromatin-dependent pathways and at the level of transcript stability by selective RNA degradation.

A crucial step in the synthesis of coding and ncRNAs is the formation of a transcription competent PIC, which starts with TBP recruitment to the core promoter (24). Transcriptional output of coding genes is determined by balancing TBP recruitment by the Spt-Ada-Gcn5-acetyltransferase (24) or TFIID complexes (25) with TBP displacement by the Swi2/Snf2-like ATPase Mot1p (human ortholog: BTAF1) and the negative cofactor 2 (NC2) complex consisting of NC2α/Bur6p and NC2β/Ncb2p (24,26–36). Recently, we showed that Mot1p-NC2 suppress intragenic ncRNA transcription in cooperation with the ISWI and the Set2p-Asf1p-Rtt106p chromatin regulators (37). This is consistent with proposals that the Mot1p and NC2 complexes redistribute TBP from the intrinsically preferred TATA-containing to less well-defined ‘TATA-less’ promoters (38).

Here, we extend these studies to investigate the involvement of Mot1p-NC2 in regulation of anti-sense transcription. We employed the anchor-away (AA) technique (39) to deplete the Mot1p or NC2 proteins from the nucleus and we subsequently analyzed anti-sense ncRNA production. Most ncRNAs are rapidly degraded and, therefore, they are difficult to measure. In addition to Mot1p and NC2, we codepleted Nrd1p to detect these ncRNAs and examine collaborative effects of the NNS and Mot1p-NC2 complexes. Interestingly, our findings support a model of collaboration of Mot1p with NC2 in restricting anti-sense ncRNA production by inhibiting TBP binding and PIC formation at the 3′-end of genes. This study provides novel mechanistic insights into the regulatory mechanisms limiting the formation of non-functional and ncRNAs and PIC formation at non-promoter sites of the yeast genome.

MATERIALS AND METHODS

Yeast genetics, media and primers

All *S. cerevisiae* strains used in this study are listed in Supplementary Table S1. They were derived from HHY168 (Euroscarf #Y40343). Cells were grown in yeast extract peptone dextrose (YPD) or synthetic complete (SC) medium supplemented with 2% glucose at 30°C. We created strains with a gene deletion or with C-terminal fusion of the FKBP12-rapamycin-binding (FRB) domain of human mTOR to the gene of interest. Homologous recombination using polymerase chain reaction (PCR) generated DNA fragments was performed and verified by PCR. Details of primers used are listed in Supplementary Table S2.

Plasmids

To measure transcription read-through, the previously described reporter vector pRS415-prADH1-FLC1_FMP40 terminator-SpeI-GFP-CYC (a kind gift from Jeffry Corden (40)) was introduced in an FRB-tagged *NRD1* background strain or the HHY168 control strain. Site-directed mutagenesis of the parental plasmid pRS415-prADH1-FLC1_FMP40 terminator-SpeI-GFP-CYC resulted in the formation of a BamHI site after the *ADH1* promoter. Blunt-end cloning of a BamHI/SpeI-digested pRS415-prADH10-BamHI-FLC1_FMP40 terminator-SpeI-GFP-CYC vector resulted in the control plasmid pRS415-prADH1-BamHI-GFP-CYC, which was also transformed into the *NRD1-FRB* or HHY168 strains. To test the effect of replacing the promoter of *SUT295* with the promoter of *RPS27A* on *SUT295* transcript levels, the *SUT295* promoter or *RPS27A* promoter was cloned together with the downstream *SUT295* transcript in pRS315. To be able to discriminate the *SUT295* transcript arising from the plasmid from the transcript arising from the endogenous locus a shortened version of the *SUT295* transcript that lacked nucleotides 899–1342 was cloned in the pRS315 vector. In short, elongation of two PCR-generated fragments resulted in a DNA fragment consisting of prSUT295_SUT295$\Delta_{899-1342}$ flanked by NotI and SacI restriction sites and with an internal AatII site. This fragment was cloned as a NotI/SacI fragment in NotI/SacI-digested pRS315 resulting in pRS315-NotI-prSUT295-SUT295$\Delta_{899-1342}$-SacI. Elongation of two PCR-generated fragments resulted in a DNA fragment consisting of prRPS27A_SUT295$\Delta_{899-1342}$ with NotI and SacI restriction sites on either site of the fragment and an internal AatII restriction site. This fragment was cloned as a NotI/SacI fragment in NotI/SacI-digested pRS315 resulting in pRS315-NotI-prRPS27A-SUT295$\Delta_{899-1342}$-SacI. Plasmids were verified by sequencing and transformed in the indicated strain backgrounds. Details of plasmids used are listed in Supplementary Table S3.

Culturing of yeast cells

For spot assays overnight cultures from single colonies in YPD at 30°C were diluted to an OD_{600} of 0.15. Five-fold serial dilutions were prepared and spotted on YPD plates containing 1-μg/ml rapamycin as indicated and grown for 3 days at 30°C. For RNA blot and chromatin immunoprecipitation (ChIP) analysis, overnight cultures from single colonies were grown in SC medium unless a plasmid was being maintained in which case SC medium lacking leucine was used. These cultures were diluted to an OD_{600} of 0.15 and grown to OD_{600} of 0.6 at 30°C at 230 rpm. Cultures were then grown for an additional 60 min in the presence or absence of rapamycin (1-μg/ml) and harvested for analysis. When indicated cultures were grown for 90 min at 39°C instead of 60 min at 30°C in the presence or absence of rapamycin. For liquid growth curves, cells were diluted to OD_{600} of 0.15 in YPD in 48-well plates at 30°C in a Tecan Infinite F200 instrument under continuous shaking. OD_{600} was recorded every 10 min. Rapamycin (1-μg/ml) was added at an OD_{600} of 0.6 where indicated.

RNA isolation and blotting

RNA isolation and RNA blotting was carried out as described previously (37). The primers used to generate the strand-specific DNA probes are listed in Supplementary Table S2.

Chromatin immunoprecipitation

ChIP was carried out with minor modifications as described previously (37). In short, formaldehyde cross-linked cells were disrupted using a gene disruptor and sonicated (Bioruptor, Diagenode: seven cycles of 30 s, high settings) to produce an average DNA fragment length of 300 bp. Two hundred microliters of extract was incubated overnight at 4°C with antibody [5-μg affinity purified α-TBP, or 1-μl α-Nrd1 (a gift from F.C.P. Holstege)]. Forty microliters (50% slurry) of protein A+G (for α-TBP) or protein A beads (for α-Nrd1) were added and incubated for 1.5 h at RT. Beads were washed twice with FA-lysis buffer; twice with FA-lysis buffer containing 410-mM NaCl; twice with 10-mM Tris-HCl pH 8.0, 50-mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1-mM ethylenediaminetetraacetic acid and once with TE (pH 8.0). Samples were eluted twice with 50-ml TE-1% sodium dodecyl sulphate for 10 min at 65°C. Cross-linking was reversed overnight at 65°C with 0.1-mg/ml RNAse. Samples were treated with 2.7-mg/ml proteinase K for 2 h at 37°C, and DNA was purified using a PCR purification kit (Qiagen). Samples were analyzed by quantitative PCR, and ChIP signals were normalized relative to HMR (silent mating-type locus) signals (for TBP) or displayed as percent input (for Nrd1p). Experiments were repeated three times to ensure consistency.

PAR-CLIP and 4tU-Seq

A detailed description of experiments and computational analysis of the photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) and 4-thiouracil (4tU)-seq data sets and the ArrayExpress database accession number used for Supplementary Figure S3 can be found in (7).

RESULTS

NNS complex inactivation by Nrd1p depletion does not lead to synthetic growth phenotypes with Mot1p or NC2

We showed previously that two essential proteins can be functionally co-depleted at once using the AA technique (39) by FRB-tagging both proteins in the same strain using different selectable markers (37). To be able to study the effects of Mot1p and NC2 on anti-sense transcription, co-depletion strains were generated by creating *NRD1-FRB* alleles in FRB-tagged *MOT1*, *NC2α/Bur6p* or *NC2β/Ncb2p* background strains as Nrd1p removal allows detection of unstable ncRNAs (7). Firstly, we tested the effectiveness of the FRB-tagging of *NRD1* by assaying growth on plates and in suspension cultures (Supplementary Figure S1). Strains expressing *NRD1-FRB* failed to grow on plates with rapamycin, which is consistent with earlier observations (7). Growth curve comparisons of single AA strains with double AA strains in suspension cultures after rapamycin addition indicated that *Mot1p*, *NC2α* or *NC2β* co-depletion does not exacerbate *NRD1-FRB* growth phenotypes. ChIP analysis showed that nuclear depletion of Nrd1p leads to reduced binding of Nrd1p to target genes (Supplementary Figure S2). Depletion of Mot1p or NC2α on the other hand had little effect on Nrd1p target gene association. Co-depletion of Nrd1p and Mot1p or NC2α seemed to reduce Nrd1p binding further.

Mot1p-NC2 and the NNS complex repress formation of anti-sense ncRNAs

To investigate effects of Mot1p-NC2 depletion on anti-sense transcription we investigated expression changes of three pairs of annotated sense and anti-sense units: *CYB2-SUT295*, *RAD4-CUT119* and *GYP5-CUT402* (Supplementary Figures S3 and S4 (2,4–6)). We refer to the originally annotated transcript as the sense transcript and the CUTs and SUTs on the opposite strand as anti-sense transcripts. Recently, the Cramer lab performed 4tU-seq analyses to monitor global RNA synthesis changes upon nuclear depletion of Nrd1p (7). Their data show increased expression of *SUT295*, *CUT119* and *CUT402* in Nrd1p depletion conditions as a result of termination defect. Nrd1p and Nab3p binding motifs, determined by PAR-CLIP, are enriched in *SUT295*, *CUT119* and *CUT402* and depleted in *CYB2*, *RAD4* and *GYP5* (vertical green and brown lines in Supplementary Figure S3) explaining selective transcription termination of these anti-sense RNAs. We performed RNA blot analysis using single-stranded DNA probes to discriminate between sense and anti-sense transcripts (probe locations in Supplementary Figure S4). Indeed, increased *SUT295*, *CUT119* and *CUT402* levels were detected upon Nrd1p depletion (Figure 1). In contrast, *CUT119* and *CUT402* ncRNAs were undetected and *SUT295* remained low upon Mot1p, NC2α or NC2β depletion. To determine functional connection between the early termination pathway and Mot1p-NC2 on anti-sense ncRNA repression, ncRNA levels were analyzed in co-depletion strains. Interestingly, co-depletion of Mot1p or NC2 with Nrd1p caused a marked increase in all anti-sense RNAs compared to single depletion strains.

Depletion of Mot1p-NC2 increases TBP binding at anti-sense promoters present at the 3′-end of protein-coding genes

We hypothesized that Mot1p-NC2 inhibits anti-sense ncRNA expression by preventing TBP binding and subsequent PIC formation at the 3′-end of genes. To test this ChIP assays for TBP were performed. We analyzed TBP occupancy at the promoters, open-reading frames (ORFs) and 3′-ends of the sense mRNA transcripts (for primer location: Supplementary Figure S4). Depletion of Mot1p or NC2α led to a clear increased occupancy of TBP at the 3′-end of *CYB2*, *RAD4* and *GYP5*, whereas the occupancy of TBP in ORFs remained low (Figure 2). Although we observed increased TBP binding at the *RAD4* promoter after Mot1p or NC2α depletion (Figure 2B), this was not associated with increased *RAD4* transcription (Figure 1B). As

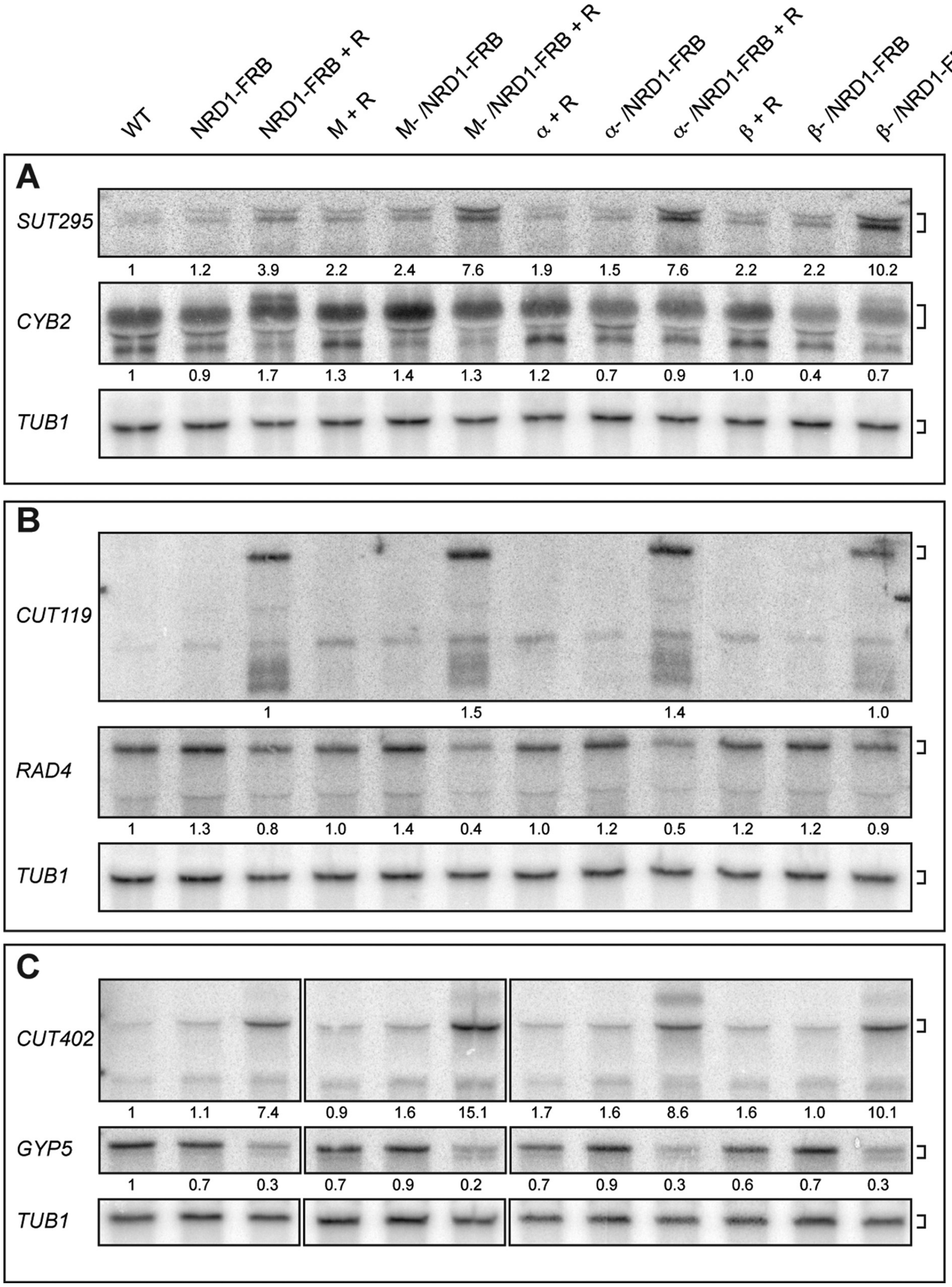
WT
NRD1-FRB
NRD1-FRB + R
M + R
M- /NRD1-FRB
M- /NRD1-FRB + R
α + R
α- /NRD1-FRB
α- /NRD1-FRB + R
β + R
β- /NRD1-FRB
β- /NRD1-FRB + R
A
SUT295
1 1.2 3.9 2.2 2.4 7.6 1.9 1.5 7.6 2.2 2.2 10.2
CYB2
1 0.9 1.7 1.3 1.4 1.3 1.2 0.7 0.9 1.0 0.4 0.7
TUB1
B
CUT119
1 1.5 1.4 1.0
RAD4
1 1.3 0.8 1.0 1.4 0.4 1.0 1.2 0.5 1.2 1.2 0.9
TUB1
C
CUT402
1 1.1 7.4 0.9 1.6 15.1 1.7 1.6 8.6 1.6 1.0 10.1
GYP5
1 0.7 0.3 0.7 0.9 0.2 0.7 0.9 0.3 0.6 0.7 0.3
TUB1

Figure 1. Depletion of Mot1p, NC2α, NC2β and Nrd1p increases anti-sense ncRNA expression levels and decreases mRNA levels in some cases. (**A**) Total RNA was isolated and used for RNA blot analysis of sense and anti-sense RNA species arising from the *CYB2* locus using strand-specific DNA probes. Cells were grown to OD_{600} of 0.6 at 30°C and cultures were then grown for an additional 60 min in the presence or absence of rapamycin (1-μg/ml), as indicated. (**B**) As in (A) except that the analysis was performed for RNA species from the *RAD4* locus. (**C**) As in (A) except that the analysis was performed for RNA species from the *GYP5* locus. Quantitations of the bands are shown below each panel relative to the WT strain or NRD1-FRB + R as indicated, and relative to the loading control *TUB1*. M, *Mot1-FRB*; α, *NC2α-FRB*; β, *NC2β-FRB*; R, rapamycin; WT, wild-type.

expected Nrd1p depletion did not increase TBP binding, since it affects anti-sense ncRNA expression at the level of transcription termination and not of initiation (Figure 2). In short, Mot1p-NC2 inactivation increases TBP binding at 3′-end of genes, which offers an explanation for their effects on anti-sense RNA expression.

SUT295 functions as an independent transcription unit

To obtain further insight into Mot1p and NC2 regulation of anti-sense RNA expression we cloned the *SUT295* promoter in front of a shortened version of the *SUT295* transcript (lacking nucleotides 899–1342) in pRS315. This allows discrimination of ectopic expression from the en-

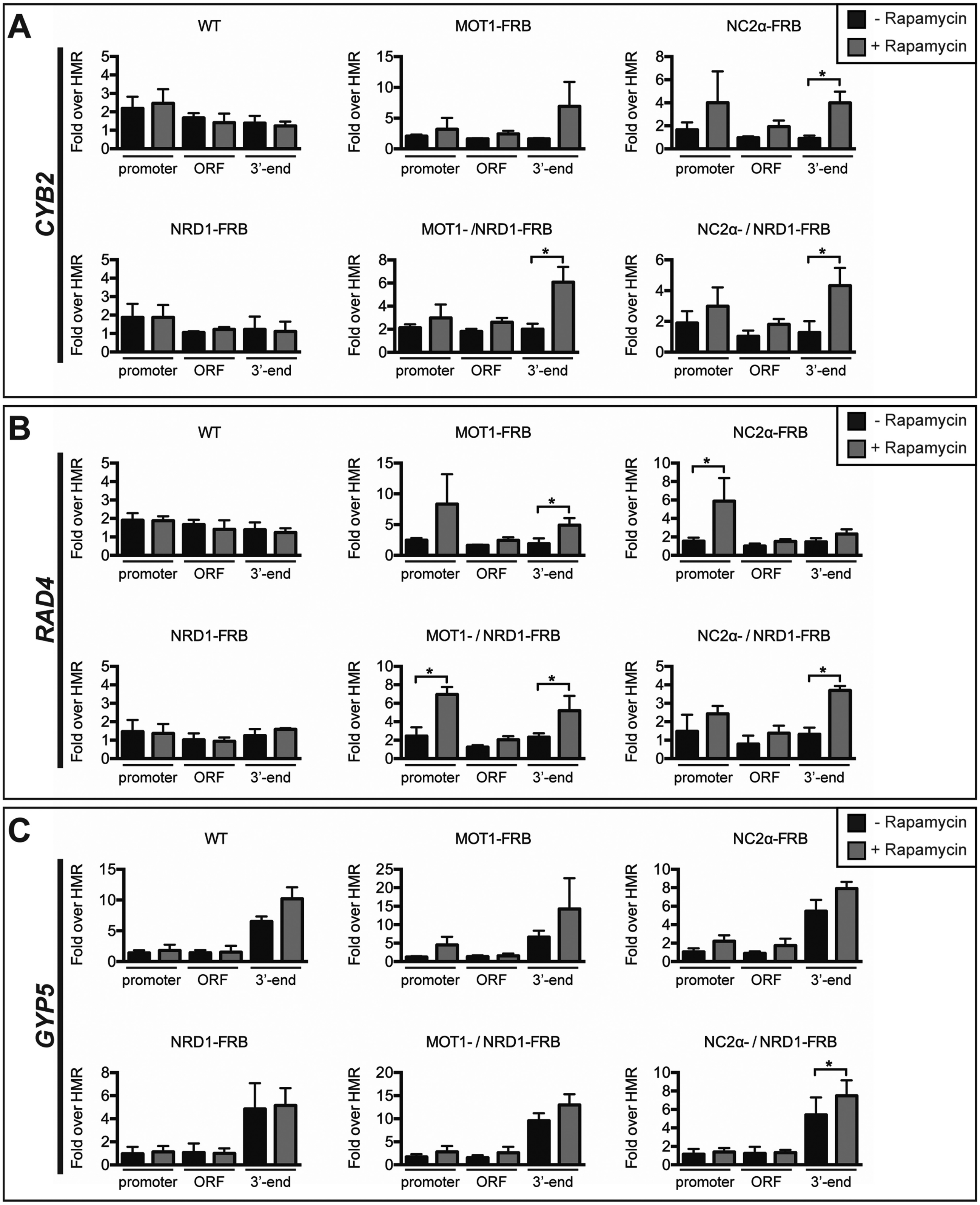

Figure 2. Depletion of Mot1p-NC2 leads to increased TBP binding at anti-sense promoters present downstream of the 3′-end of genes. (**A**) ChIP analyses of the *CYB2* promoter, ORF and 3′-end using TBP antibodies, real-time PCR signals were normalized to the silent *HMR* locus. Cells were grown as in Figure 1, cross-linked and harvested. Each graph displays TBP occupancy at the indicated locations (promoter, ORF and 3′-end) and background strains (WT, Nrd1-FRB, Mot1-FRB, Mot1-FRB Nrd1-FRB, NC2α-FRB and NC2α-FRB Nrd1-FRB). (**B**) As in (A) except that the analysis was performed for the *RAD4* locus. (**C**) As in (A) except that the analysis was performed for the *GYP5* locus. Note that the divergently transcribed *RPL36B* gene is located close to the 3′-end of *GYP5* (Supplementary Figure S4C), which may be responsible for the higher TBP ChIP signals. Significant differences ($P < 0.05$ by Student's *t*-test) are indicated (asterisk), $n = 3$.

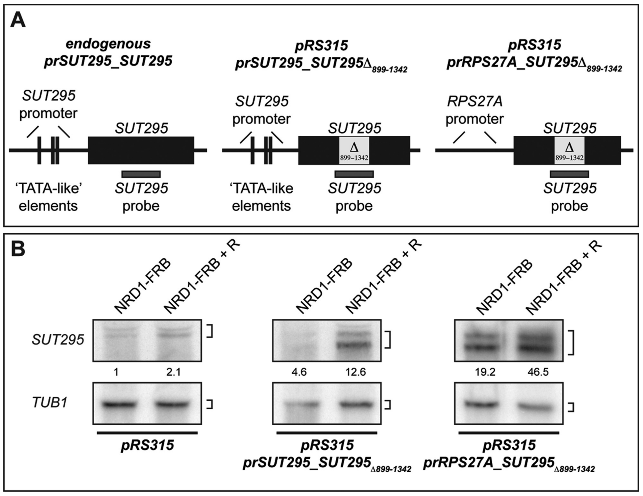
A
endogenous prSUT295_SUT295
pRS315 prSUT295_SUT295Δ899-1342
pRS315 prRPS27A_SUT295Δ899-1342
SUT295 promoter
SUT295
'TATA-like' elements
SUT295 probe
Δ 899–1342
RPS27A promoter
B
NRD1-FRB
NRD1-FRB + R
SUT295
TUB1
1
2.1
4.6
12.6
19.2
46.5
pRS315
pRS315 prSUT295_SUT295Δ899-1342
pRS315 prRPS27A_SUT295Δ899-1342

Figure 3. *SUT295* functions as an independent transcription unit. (**A**) Schematic representation of the endogenous *SUT295* locus (left). Schematic representation of the reporter vector containing the *SUT295* locus with a 443-bp internal deletion (nucleotides 899–1342) (middle) to allow discrimination from the endogenous *SUT295* ncRNA. Schematic representation of the reporter vector containing the *RPS27A* promoter upstream of *SUT295* transcribed region with the 443-bp internal deletion (nucleotides 899–1342) (right). (**B**) RNA blot analyses (as in Figure 1) in the indicated strains, which carried the indicated plasmids. Cells were grown in synthetic complete medium without leucine. 'TATA-like' elements in the *SUT295* promoter are represented as vertical black bars. R, rapamycin.

dogenous *SUT295* transcript by size (Figure 3A, left and middle). The plasmids were introduced in the *NRD1-FRB* conditional depletion strain. The ectopically expressed *SUT295* transcripts migrate slightly faster than the endogenous *SUT295*. Similar to the endogenous transcripts, plasmid-derived *SUT295Δ* transcripts were targeted for termination-coupled degradation by the NNS complex (Figure 3B, middle). This indicates that *SUT295* does not rely on its surrounding genomic context for proper expression and regulation. Additionally, we made a construct where the *SUT295* promoter was replaced with the promoter of the *RPS27A* gene (Figure 3A, right). *SUT295* transcripts were still sensitive to NNS complex after substitution when driven by the *RPS27A* promoter (Figure 3B, right). Thus, the transcript sequence but not the promoter type determines sensitivity toward transcription termination-coupled degradation. Altogether, these results indicate that anti-sense ncRNAs function as independent transcription units. The anti-sense promoter influences the level of transcription, but the fate of the transcript is determined by the transcript body.

SUT295, CUT119 and *CUT402* expression levels are influenced by chromatin state

Modulation of chromatin state forms an important regulatory mechanism that affects anti-sense ncRNA expression levels. Motivated by recent studies that link H3K4 trimethylation levels of anti-sense ncRNAs promoters to their transcriptional activity (17,18) we explored the sensitivities of the ncRNAs toward deletion of *SET1*. Set1p is the catalytic subunit of the sole H3K4 methyltransferase complex, COMPASS (Set1C) in yeast (41). RNA analyses indicated that *SUT295, CUT119* and *CUT402* are dependent on the histone methyltransferase for their expression (Figure 4). Additional depletion of Mot1p resulted in a further increase in *SUT295* and *CUT402* ncRNAs, but had no effect on *CUT119* transcripts. This indicates that the opposing effects of Set1p and NNS-Mot1p on ncRNA levels are not interdependent.

Active repression of anti-sense ncRNA also depends on the chromatin-remodeler complex ISW2 that restricts the size of the 3′-NDR (14,15). Thus we tested the effect of deleting its catalytic *ISW2* subunit on *SUT295*, *CUT119* and *CUT402* levels as they are produced from anti-sense promoters located at 3′-NDRs. As a control ncRNA expression levels were analyzed in a *CHD1* deletion strain. CHD1 is a monomeric chromatin remodeler, which organizes chromatin in coding regions and does not influence NDR size (42–44). The levels of *SUT295, CUT119* and *CUT402* remained low in the *ISW2* (Supplementary Figure S5) and *CHD1* deletion backgrounds (Supplementary Figure S6). It could be that elevated ncRNA transcripts in *Δisw2* strains were not detected, due to rapid degradation by the nuclear exosome.

Mot1p-NC2 and NNS use independent pathways for ncRNA suppression

Next, we used the previously described transcription read-through reporter vector (40) to investigate whether depletion of Nrd1p indeed leads to a transcription termination defect in our strains (Figure 5). A control vector that expresses *GFP* under control of the *ADH1* promoter and a vector that contains a terminator sequence with three Nrd1p and Nab3p binding motifs (*FLC1-FMP40* terminator) in between the *ADH1* promoter and the *GFP* reporter were introduced into wild-type (WT) or *NRD1-FRB* strains. RNA blot analyses (Figure 5B) showed that strains containing the control vector express similar levels of *GFP* independent of Nrd1p depletion. As expected the WT strain did not express *GFP* mRNA from the vector with a terminator sequence, which is due to NNS-mediated transcription termination. Upon Nrd1p depletion read-through was observed as evidenced by *GFP* mRNA formation. The ChIP results of Supplemental Figure S2 could suggest that Mot1p and NC2 affect Nrd1p-binding. However, we did observe any effect of Mot1p or NC2β depletion on Nrd1p-dependent read-through (Figure 5B), which indicates that Mot1p or NC2 are not directly involved in the transcription termination function of Nrd1p. Also, no elevated *GFP* mRNA levels were observed upon co-depletion of Mot1p or NC2β with Nrd1p. This is also in line with previously obtained microarray data, which showed that *ADH1* expression is not effected by depletion of Mot1p or NC2β (24).

Thus, the results from these reporter constructs support the model that the Mot1p-NC2 and NNS pathways suppress ncRNA formation by independent mechanisms. Mot1p and NC2 act on TBP bound at the 3′-ends of genes to suppress PIC formation responsible for anti-sense transcription. The NNS complex in its turn acts to restrict ncRNA levels via transcription termination coupled to ncRNA degradation.

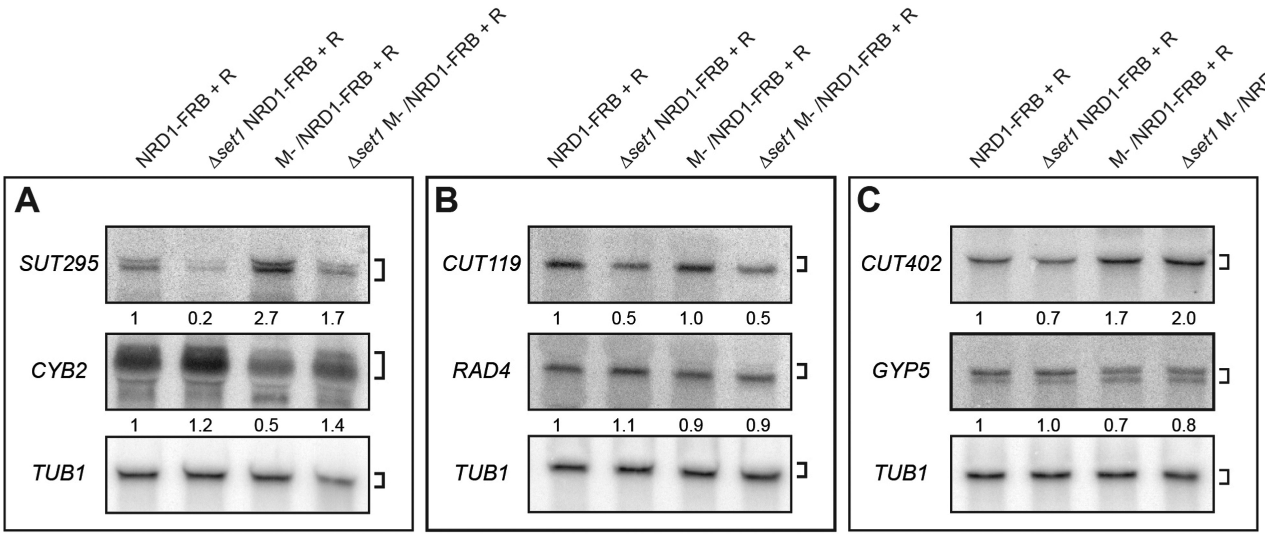
NRD1-FRB + R
Δset1 NRD1-FRB + R
M- /NRD1-FRB + R
Δset1 M- /NRD1-FRB + R
A
SUT295
1 0.2 2.7 1.7
CYB2
1 1.2 0.5 1.4
TUB1
B
CUT119
1 0.5 1.0 0.5
RAD4
1 1.1 0.9 0.9
TUB1
C
CUT402
1 0.7 1.7 2.0
GYP5
1 1.0 0.7 0.8
TUB1

Figure 4. *SUT295, CUT119* and *CUT402* are dependent on the histone methyltransferase Set1p for their expression. (**A–C**) RNA blot analyses (as in Figure 1) performed in different strain backgrounds. M, *Mot1-FRB*; R, rapamycin.

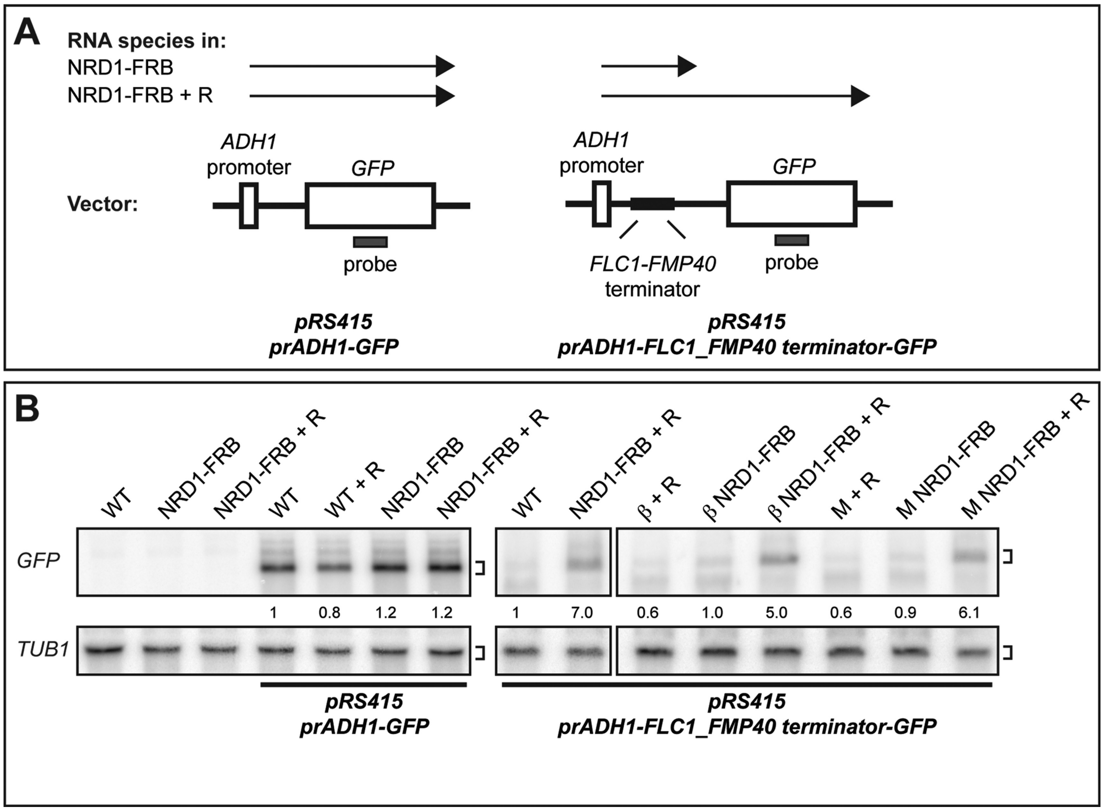
A
RNA species in:
NRD1-FRB
NRD1-FRB + R
ADH1 promoter
GFP
Vector:
probe
FLC1-FMP40 terminator
pRS415 prADH1-GFP
pRS415 prADH1-FLC1_FMP40 terminator-GFP
B
WT
NRD1-FRB
NRD1-FRB + R
WT
WT + R
NRD1-FRB
NRD1-FRB + R
WT
NRD1-FRB + R
β + R
β NRD1-FRB
β NRD1-FRB + R
M + R
M NRD1-FRB
M NRD1-FRB + R
GFP
1 0.8 1.2 1.2
1 7.0 0.6 1.0 5.0 0.6 0.9 6.1
TUB1
pRS415 prADH1-GFP
pRS415 prADH1-FLC1_FMP40 terminator-GFP

Figure 5. Nrd1p depletion leads to read-through transcription from a reporter vector and the read-through is independent of Mot1p-NC2. (**A**) Schematic representation of a control vector containing an *ADH1* promoter upstream of a *GFP* reporter and of the transcription termination read-through reporter vector containing an *ADH1* promoter upstream of a *GFP* reporter with 157 bp of the *FLC1-FMP40* intergenic region, containing three Nrd1p and Nab3p binding motifs in between the *ADH1* promoter and the *GFP* reporter as previously described in (40). (**B**) RNA blot analysis, as in Figure 1 except that the analysis was performed in strain backgrounds that were transformed with the indicated plasmids and the cells were grown in synthetic complete medium without leucine. M, *Mot1-FRB*; β, *NC2β-FRB*; R, rapamycin; WT, wild-type.

DISCUSSION

This study provides novel insights into mechanisms that operate to counteract anti-sense ncRNA formation. Previous studies stressed the importance of chromatin structure and/or modification, and termination-coupled degradation in limiting anti-sense ncRNA levels (2–7,10,14–19). Here, we uncovered novel inhibitory roles for Mot1p and NC2 on anti-sense ncRNA expression. We show that Mot1p and NC2 do not act at the transcription termination level (Figure 5). Instead, Mot1p and NC2 function upstream of the NNS complex and at the level of transcription initiation to prevent anti-sense ncRNA transcription (Figure 1). Anti-sense ncRNAs arise from PICs located at 3′-end of genes (5,8,9). Mellor *et al.* showed that 3′-end of coding genes can function as independent promoters for anti-sense transcription and that PIC levels at 3′-end of coding genes correlate with anti-sense expression level (9). In general, the 3′-end of genes are permissive to assembly of the transcription machinery, which may relate to a reduced nucleosome occupancy (45). This motivated us to test whether TBP occupancy is a regulatory point for anti-sense ncRNA expression control. Our results indicate that Mot1p-NC2 restricts formation of PICs to anti-sense promoters by TBP displacement, which limits anti-sense ncRNA production (Figures 1 and 2). These results support our previous findings with intragenic RNAs (37) and they reveal a widespread role for Mot1p and NC2 in limiting PIC formation responsible for the synthesis of non-functional and ncRNAs.

Furthermore, we showed that anti-sense transcription arises from independent transcription units (Figures 3 and 5). We found that the transcript sequence and not the promoter determined sensitivity toward transcription termination-coupled degradation via the NNS pathway. As expected the output of ncRNA transcription units is dependent by the promoter sequence. A 4-fold increase in *SUT295* expression was detected when it was placed under control of the *RPS27A* promoter instead of its endogenous promoter (Figure 3B). Our results are in agreement with a recent study that performed a 'body-swap' experiment, which determined that the choice of degradation pathway is specified by the transcript body (16).

We identified that the sensitivity toward Mot1p-NC2 regulation varies among anti-sense ncRNAs. We suspect that this depends on the promoter sequence of the ncRNA. Of the three analyzed ncRNAs, *SUT295* expression was the most sensitive toward Mot1p-NC2 inactivation (Figure 1). The *SUT295* promoter contains three 'TATA-like' elements, whereas the *CUT119* promoter contains one and *CUT402* lacks a 'TATA-like' element (Supplementary Figure S4). TATA-containing promoters of ncRNAs could be preferred targets for repression by Mot1p-NC2 as is the case for TATA-containing promoter of protein-coding genes (24,38). It would be interesting to perform genome-wide analysis to see if Mot1p and NC2 display selectivity toward a population of aberrantly localized TBPs. We observed two cases where anti-sense ncRNA expression interfered with formation of the cognate sense transcript (46,47). *CUT119* and *CUT402* expression levels were anti-correlated with mRNA expression levels (Figure 1). Recently, a genome-wide study detected genome-widespread transcript isoform diversity (48). The two *CUT295* transcripts of different lengths detected in this study (Figure 1B) could result from transcription machineries that initiate or terminate at different sites.

Finally, we examined how chromatin state influences anti-sense transcription levels. Our results support previous findings and show that H3K4 methylation correlates with ncRNA expression (Figure 4) (17,18). Inactivation of the catalytic subunit of the chromatin remodeler ISW2 did not result in detectable anti-sense ncRNA levels (Supplementary Figure S5). It is possible that elevated ncRNA transcripts in *Δisw2* strains were not detected, because of rapid degradation by the nuclear exosome.

Multiple potential functions have been ascribed to anti-sense RNAs. They might regulate and fine-tune mRNA expression, act as buffers to prevent cells from responding to weak signals, allow increased expression variability, play roles in condition-specific and environmental stress responses and function as proto-genes that could give rise to protein-coding genes (6,49,50). Some anti-sense transcripts have been identified to be evolutionary conserved across distantly related species (6). This suggests that (some) anti-sense RNAs have important regulatory roles that are selectively maintained during the evolution of species. This motivated studies on the control of ncRNA expression. We have used the yeast *S. cerevisiae* as a model organism to discover regulatory mechanisms that control ncRNAs. Given the universal roles of the Mot1p/BTAF1 protein and the NC2 complex as regulators of TBP dynamics (27–33,35,36) we propose that their orthologs in other eukaryotes play similar roles in the suppression of ncRNA formation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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