

# Roadblock Termination by Reb1p Restricts Cryptic and Readthrough Transcription

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<http://dx.doi.org/10.1016/j.molcel.2014.10.026>

## SUMMARY

Widely transcribed compact genomes must cope with the major challenge of frequent overlapping or concurrent transcription events. Efficient and timely transcription termination is crucial to control pervasive transcription and prevent transcriptional interference. In yeast, transcription termination of RNA polymerase II (RNAPII) occurs via two possible pathways that both require recognition of termination signals on nascent RNA by specific factors. We describe here an additional mechanism of transcription termination for RNAPII and demonstrate its biological significance. We show that the transcriptional activator Reb1p bound to DNA is a roadblock for RNAPII, which pauses and is ubiquitinated, thus triggering termination. Reb1p-dependent termination generates a class of cryptic transcripts that are degraded in the nucleus by the exosome. We also observed transcriptional interference between neighboring genes in the absence of Reb1p. This work demonstrates the importance of roadblock termination for controlling pervasive transcription and preventing transcription through gene regulatory regions.

## INTRODUCTION

In *S. cerevisiae*, two main transcription termination pathways have been described for RNA polymerase II (RNAPII) (for a recent review, see Kuehner et al., 2011). The first pathway involves the cleavage and polyadenylation (CPF/CF) complex and is mainly devoted to the transcription of mRNA coding genes. The CPF/CF complex is recruited at the 3' end of genes by the interaction with the carboxy-terminal domain (CTD) of the largest RNAPII subunit and by the recognition of specific signals within the nascent RNA. After cotranscriptional cleavage of the nascent transcript, the polymerase is released by a mechanism that is still not fully understood and might involve exonucleolytic digestion of the RNAPII-associated RNA. This mode of termination and

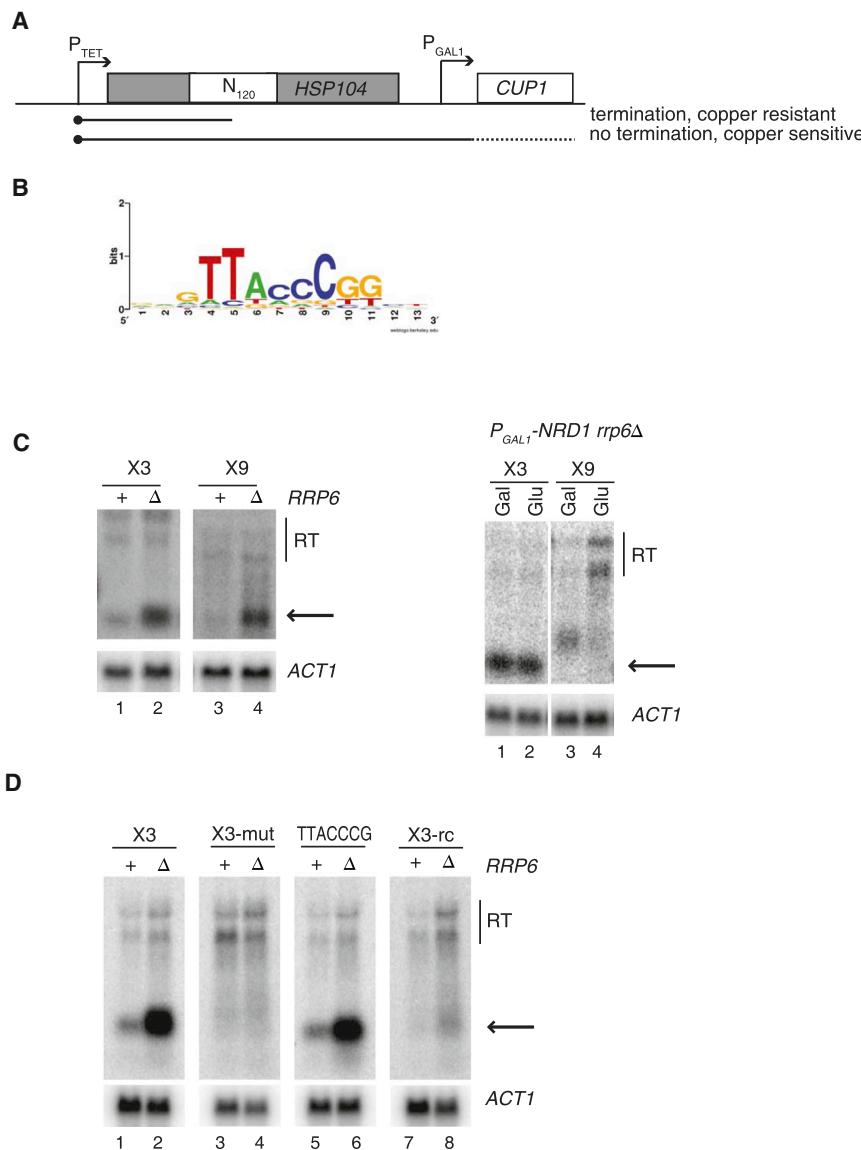
3' end processing produces stable transcripts that are exported to the cytosol for translation.

The second pathway involves the Nrd1p-Nab3p-Sen1p (NNS) complex and was first described for termination of snRNAs/snoRNAs (Steinmetz et al., 2001). This mode of termination is triggered by the recognition of specific motifs on the nascent RNA by the RNA-binding proteins Nrd1p and Nab3p (Creamer et al., 2011; Gudipati et al., 2008; Porrúa et al., 2012; Wlotzka et al., 2011), which precedes release of the polymerase by the Sen1p helicase (Kuehner et al., 2011; Porrúa and Libri, 2013; Hazelbaker et al., 2013). One distinctive feature of NNS-dependent termination is that the released transcript is polyadenylated by a different poly(A) polymerase, Trf4p, a subunit of the TRAMP4 (Trf4p-Air2p-Mtr4p) complex (Wyers et al., 2005). TRAMP4 stimulates degradation by the nuclear exosome, a multimeric enzyme containing two catalytic subunits, Rrp6p and Dis3p.

One important role of the NNS complex is to control pervasive transcription, i.e., the widespread occurrence of spurious transcription events defining noncanonical transcription units (Schulz et al., 2013; Steinmetz et al., 2006). Pervasive transcription is potentially harmful, as it can interfere with transcription of canonical genes and generate toxic noncoding RNA molecules. The NNS complex terminates transcription of cryptic unstable transcripts (CUTs), the major products of pervasive transcription, and targets these RNAs for degradation by the nuclear exosome (Arigo et al., 2006; Thiebaut et al., 2006; Schulz et al., 2013).

Similar to canonical gene transcription, cryptic transcription generally originates from nucleosome-free regions (NFRs; Neil et al., 2009; Xu et al., 2009), and the Reb1p protein plays an important role in the positioning of NFRs. Reb1p contains a bipartite, Myb-like DNA binding domain and was originally described as a transcriptional activator for RNAPII and RNA polymerase I (RNAPI) transcription (Brandl and Struhl, 1990; Kulkens et al., 1992). It has been shown to preferentially bind intergenic regions, and ectopically inserting a Reb1p binding site within an ORF is sufficient to induce formation of an NFR. Reb1p-dependent nucleosome depletion depends on the RSC chromatin remodeling complex and its catalytic subunit, the Sth1p ATPase, which are likely targeted by Reb1p and other factors to sites of NFR formation (Hartley and Madhani, 2009).

Although Reb1p has been reported to be involved in transcription termination of rDNA transcription by RNAPI, recent studies have challenged this notion, showing that a Reb1p-related protein,



**Figure 1. Selection of Terminators from Naive Sequences**

(A) Scheme of the reporter used to select terminators. The random sequence of 120 nt (white box) and the *HSP104* coding sequence (gray boxes) are flanked by the tetracycline repressible ( $P_{TET}$ ) and the *GAL1* promoter ( $P_{GAL1}$ ). Transcripts expected in the event of termination or transcriptional readthrough are indicated as solid lines. Two readthrough species are produced, one ending at a cryptic terminator at the end of  $P_{GAL1}$  and a second at the end of the *CUP1* gene (indicated by a dotted line).

(B) Consensus logo obtained from 83 selected terminators. See also Figure S1A.

(C) Northern blot analysis of transcripts derived from clone X3 or, as a control, from an artificial NNS-dependent terminator (X9; Porrua et al., 2012). Analysis was performed in an *rrp6Δ* background (left panels) or upon metabolic depletion of Nrd1p by growth in glucose of  $P_{GAL1}$ -*NRD1 rrp6Δ* cells (right panels). An arrow indicates the position of the RNAs derived from termination at the selected sequence. RT, readthrough transcripts. See also Figure S1B.

(D) The selected motif is necessary and sufficient for termination. Northern blot analysis of wild-type or *rrp6Δ* strains harboring reporters containing either the clone X3, its mutated version (X3-mut), the motif alone (TTACCCGG), or clone X3 containing the reverse complement of the selected motif (X3-rc).

additional tool adopted by the cell to tame pervasive transcription and ensure the stability of the transcriptome.

## RESULTS

### Selection of Terminators from Naive Sequences

We used an *in vivo* SELEX approach combined with a reporter system in which transcription termination prevents the silencing of a downstream promoter by transcriptional interference to identify sequence motifs inducing RNAPII transcription termination (Figure 1A; Porrua et al., 2012). Briefly, a pool of random sequences was cloned between the Tet-repressible promoter (Garí et al., 1997) and the *GAL1* promoter, driving expression of the *CUP1* gene, which confers copper resistance to yeast. In the presence of termination signals between the two promoters, transcriptional interference is prevented and the  $P_{GAL1}$ -*CUP1* unit is active, which allows selecting terminators from the pool of random sequences based on the copper resistance readout. We isolated several hundred sequences, the majority of which induced transcription termination in an NNS complex-dependent manner (Porrua et al., 2012). However, we also selected a large fraction of sequences (roughly 15%) containing a well-conserved, nonpalindromic sequence motif. Statistical analysis of the nucleotide variations in the selected pool

Nsi1p, is the rDNA termination factor (Kawauchi et al., 2008; Reiter et al., 2012). RNAPII termination is thought to occur by a roadblock mechanism whereby Nsi1p binds its cognate site within the rDNA terminator to induce polymerase release. Although roadblock termination likely occurs for RNAPII, it has not been demonstrated for RNAPII, which predominantly utilizes mechanisms linked to the recognition of signals on the nascent RNA.

Here we show in yeast that Reb1p can terminate RNAPII transcription by roadblock-induced pausing followed by ubiquitination of the stalled polymerase. Reb1p-dependent termination occurs at several sites of cryptic transcription in the genome and produces unstable transcripts that, like CUTs, are degraded by the TRAMP-exosome pathway. We also provide evidence that Reb1p-dependent termination functions as a fail-safe mechanism neutralizing transcriptional leakage from adjacent genes, thereby attenuating transcription interference. These data reveal an important role of Reb1p as an “NFR guard,” thus defining an

generated the highly conserved core motif shown in Figure 1B and a more relaxed flanking nucleotides context (see Figure S1A available online). Copper resistance was indeed due to the occurrence of transcription termination, because short transcripts ending within the selected regions could be detected by northern blot analysis (Figures 1C and S1B, clones X3, X18, and X31). Similar to CUTs, these RNAs are unstable in a wild-type strain and are strongly stabilized in *rrp6Δ* degradation-defective strains (Figures 1C and S1B, left panels). As expected, termination is not dependent on the NNS complex, because metabolic depletion of Nrd1p in a *P<sub>GAL1</sub>-NRD1* strain did not affect termination at these sequences but impaired termination at a control, NNS-dependent clone (X9, Figures 1C and S1B, right panels).

Mutation of the conserved motif led to the loss of copper-resistant growth (data not shown) and the disappearance of the short transcripts, while longer, readthrough RNAs terminating at downstream sites increased (Figure 1D, lanes 3 and 4). Moreover, insertion of this motif alone in a coding region terminated transcription as efficiently as the full-length terminator (Figure 1D, lanes 5 and 6). Interestingly, the termination motif was found in the selected clones preferentially in one orientation, and replacement of this sequence by its reverse complement almost fully abolished termination (Figure 1D, lanes 7 and 8), suggesting directional specificity for termination, at least in the context of our system (see Discussion).

From these experiments we conclude that the selected motif is necessary and sufficient to induce efficient transcription termination.

### The Transcription Factor Reb1p Is Involved in RNAPII Transcription Termination

The motif identified is the putative binding site of two DNA-binding proteins: Reb1p and Nsi1p/Ydr026c (Harbison et al., 2004). These two proteins belong to the family of Myb-like DNA-binding factors. Reb1p functions in transcription activation and is required for the proper positioning of NFRs (Hartley and Madhani, 2009). Nsi1p is required for transcription termination of rDNA genes and is expressed to lower levels than Reb1p (432 versus 7510 molecules per cell; Ghaemmaghami et al., 2003).

To assess if either one of these two factors is required for inducing transcription termination at the selected motif, we constructed yeast strains containing either a deletion of *NS1* or *REB1* under control of the *GAL1* glucose-repressible promoter (*REB1* is essential). Since these two factors could have redundant functions in termination, we also constructed strains carrying both modifications.

As shown in Figure 2A, deletion of *NS1* has no effect on termination (compare lanes 1 and 2 to lanes 5 and 6). However, metabolic depletion of Reb1p induced a clear loss of the short unstable transcript to the profit of a longer readthrough RNA (Figures 2A and S1C), which is diagnostic of a termination defect. Depletion of Reb1p in an *nsi1Δ* genetic background did not further increase readthrough, excluding a possible redundant implication of Nsi1p. From these results we conclude that Reb1p is required for RNAPII transcription termination at these selected terminators.

### Reb1p-Dependent Termination Produces Unstable Transcripts that Are Polyadenylated by Trf4p

Our results indicate that, akin to NNS-dependent termination, Reb1p-dependent termination leads to the production of unstable transcripts. Therefore we investigated whether degradation of these RNAs follows the same pathway as CUTs. Analysis of the poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions as well as RNaseH/oligo dT cleavage indicates that a significant fraction of RNAs stabilized in *rrp6Δ* cells are polyadenylated (Figures 2B and S1D). The polyadenylated fraction is strongly reduced in *rrp6Δtrf4Δ* cells, indicating that Trf4p, presumably within the TRAMP complex, is mainly responsible for adding poly(A) tails to these transcripts (Figure 2B, compare lanes 5 and 6 and lanes 8 and 9; Figure S1D, lanes 3 and 4 and lanes 5 and 6). Similarly to CUTs, Trf4p contributes to efficient degradation, because in *trf4Δrrp6Δ* cells the steady-state level of these transcripts was considerably higher than in single *rrp6Δ* mutant cells (Figure 2B, compare lane 4 to lane 7; Figure S1D, compare lanes 3 and 4 and lanes 5 and 6) and stabilization was also clearly observed in single *trf4Δ* mutant (data not shown). Finally, the core exosome/Dis3p also contributes to degradation, because stabilization of the short RNAs was observed in a catalytic *dis3-exo-* mutant, which was further increased in double *rrp6Δ dis3*-defective cells (data not shown).

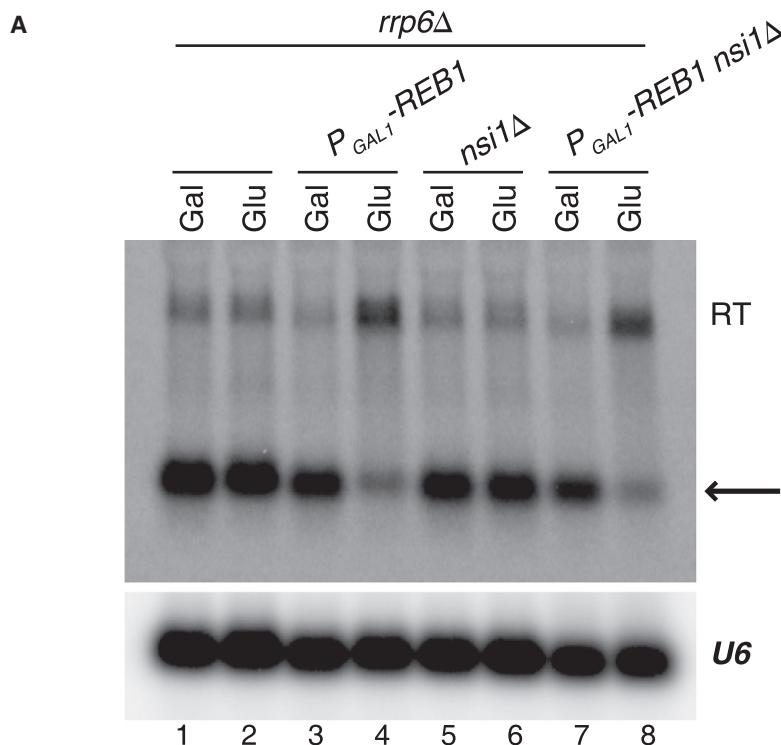
Poly(A)<sup>-</sup> RNAs were also consistently detected (Figure S1D, lanes 1–3), a fraction of which is also present in a wild-type strain, unlike poly(A)<sup>+</sup> species (Figure S1D, lanes 1 and 2). These stable, nonadenylated RNAs most likely represent nascent transcripts that are protected by RNA polymerase from the exosome (see below).

From these experiments, we conclude that transcripts derived from Reb1p-dependent termination events are unstable because they are polyadenylated by Trf4p and degraded by the Rrp6p and Dis3p exosome subunits in wild-type cells. Considering the similar nature of these RNAs and CUTs, we dubbed them Reb1p-dependent unstable transcripts, or RUTs.

### Reb1p Terminates Transcription by DNA Binding Rather Than RNA Binding

The two major pathways of RNAPII transcription termination rely on the essential recognition of the nascent RNA by termination factors (either the CPF/CF or the NNS complex). However, Reb1p is a DNA-binding protein, suggesting that termination might occur via a different mechanism. We first assessed Reb1p binding to the RNA version of its DNA binding site. As shown in Figure 3A, recombinant Reb1p bound efficiently double-stranded DNA with an affinity around 70 nM. However, recognition of the single-stranded DNA or RNA version of the same site was very inefficient, most likely reflecting unspecific binding at the highest concentrations of Reb1p used.

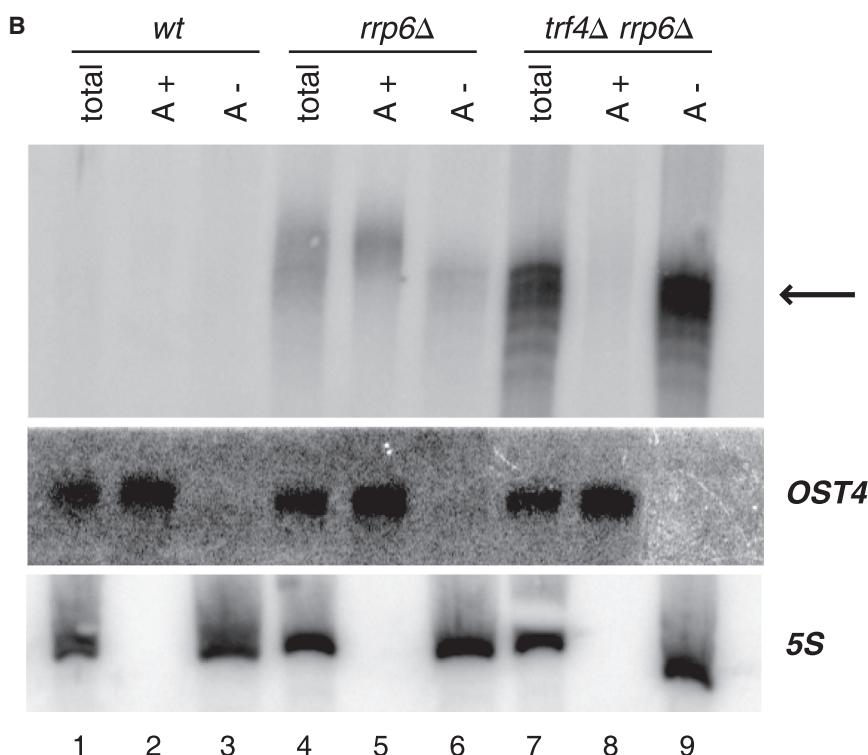
This suggests that binding to the DNA induces transcription termination by a roadblock mechanism. One prediction of such a model is that the Reb1p binding site should not be transcribed. Consistently, mapping by RACE the 3' ends of transcripts produced from different clones selected showed that Reb1p-dependent termination always occur 11–13 nt before the Reb1 site (Figure S2A). This size is compatible with the physical distance between the leading edge of the polymerase (touching

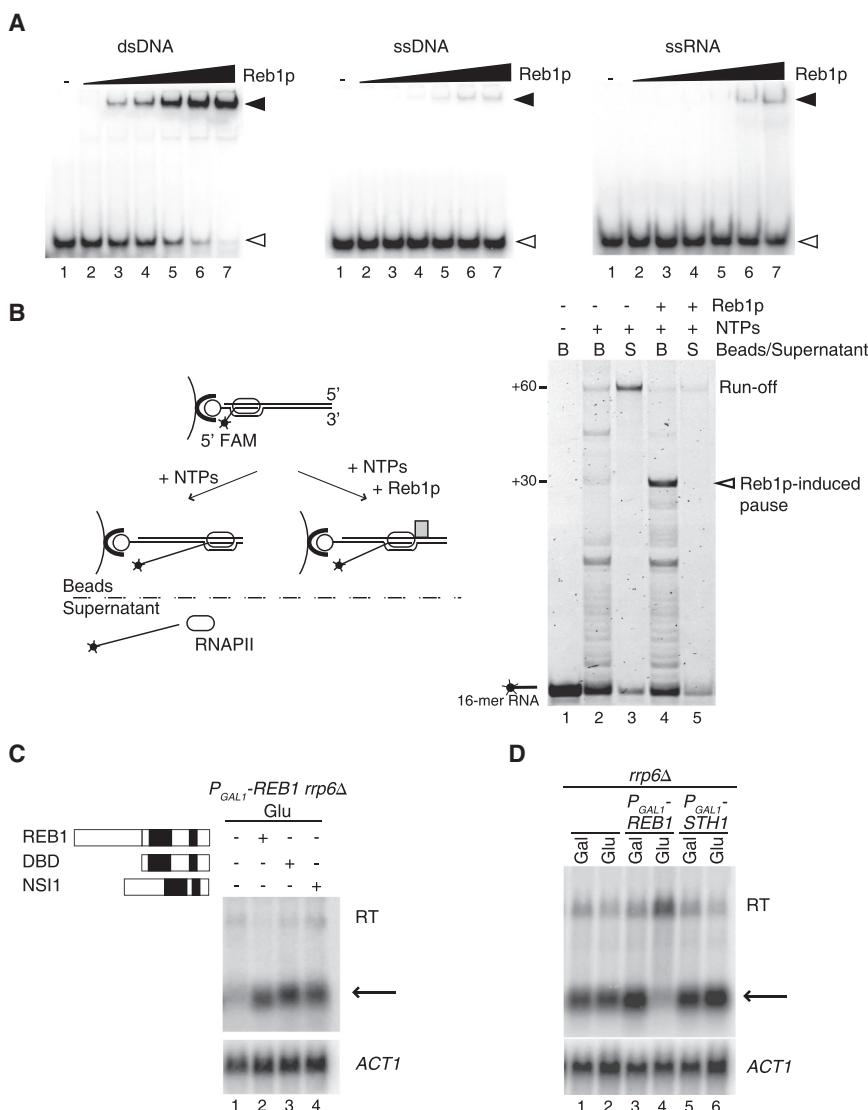


**Figure 2. Reb1p Is Required for Transcription Termination at the Selected Terminators**

(A) Northern blot analysis of transcripts derived from clone X3 upon metabolic depletion of Reb1p, in the presence or absence of Nsi1p as indicated. Labeling is as in Figure 1C.

(B) Reb1p terminated transcripts are polyadenylated by Trf4p. Northern blot analysis of RNAs generated by Reb1p-dependent termination and affinity-selected on oligo-dT magnetic beads. The total poly(A)<sup>-</sup> and poly(A)<sup>+</sup> fractions were analyzed as indicated. Note that poly(A)<sup>-</sup> RNAs are not visible in this experiment in the wild-type strain due to the shorter exposure of the blot. See also Figures S1C and S1D.





**Figure 3. Binding of Reb1p to the DNA Is Sufficient to Elicit Termination**

(A) Recombinant Reb1 (rReb1) binds dsDNA but not ssDNA or RNA. EMSA analysis was carried out using rReb1p and probes of same sequences in a dsDNA, ssDNA, or RNA form containing a Reb1p binding site. The complex formed is indicated by a black arrowhead. Unbound probe is indicated by a white arrowhead. Concentration of rReb1p used was 12.5, 25, 50, 100, 200, and 300 nM (respectively, lanes 2, 3, 4, 5, 6, and 7).

(B) rReb1p induces transcriptional pausing in vitro. In vitro transcription termination assay using immobilized templates and highly purified RNAPII. A scheme of the experiment is shown on the left. Reb1p-dependent pausing is indicated by the accumulation of a transcript that extends up to 12 nt upstream of the Reb1 site but remains associated to the immobilized template (B) and is not released in the supernatant (S). Reb1p is indicated by a gray box.

(C) DNA binding by Reb1p or Nsi1p is sufficient to induce termination in vivo. Northern blot analysis of RNAs extracted from *P<sub>GAL1</sub>-REB1 rrp6Δ* cells containing the X3 reporter. Endogenous Reb1p was depleted by growth in glucose in the presence of wild-type Reb1p, a truncated form of Reb1p containing only the DNA binding domain (DBD) or Nsi1p under control of the *REB1* promoter. Black boxes represent conserved region in Reb1p and Nsi1p.

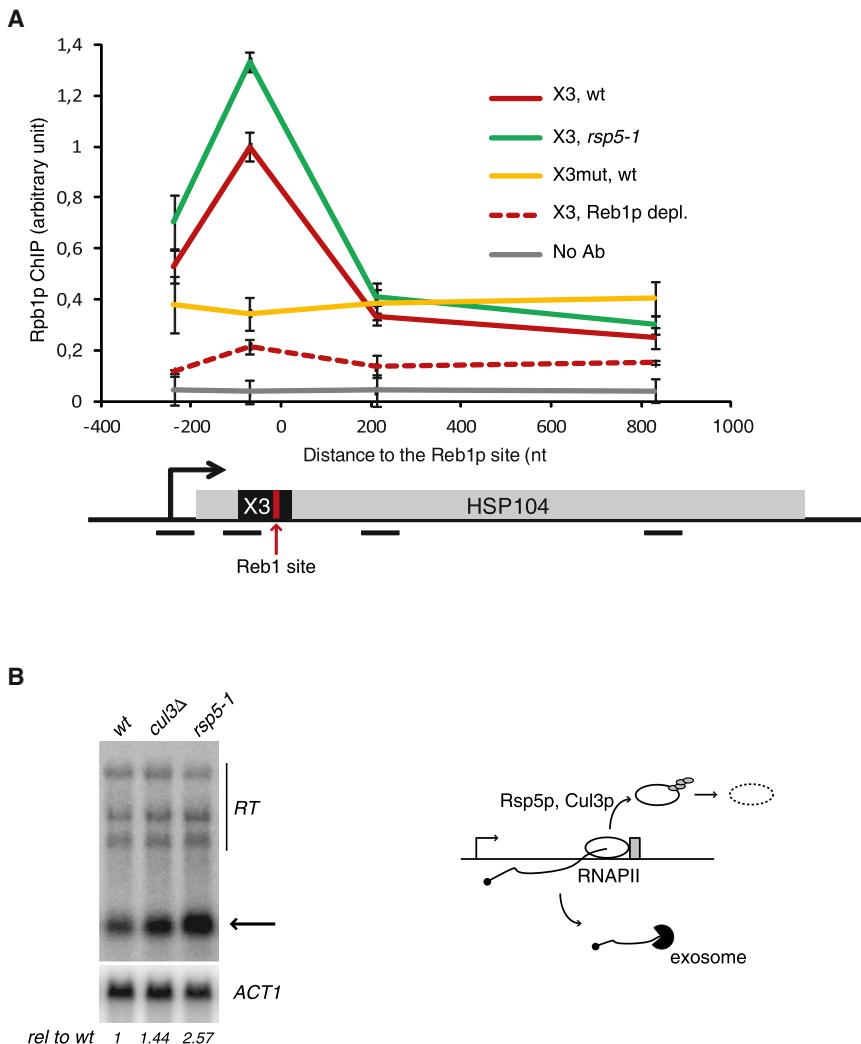
(D) Northern blot analysis of RNAs extracted from cells depleted for Reb1p or Sth1p and containing the X3 reporter. See also Figure S2.

#### Reb1p-Dependent Termination Does Not Require NFR Formation

Reb1p is known to activate transcription by recruiting the RSC chromatin remodeling complex, which, in turn, contributes to the positioning of the NFR (Hartley and Madhani, 2009). Our results from in vitro transcription assays do not rule out the possibility that in vivo DNA binding might be necessary but not sufficient for Reb1p-dependent termination and that formation of an NFR might also be required. To address this question, we generated a version of Reb1p truncated for the first 336 aa (Reb1-DBD), which retains full ability to bind DNA, as verified by EMSA (Figure S2C), but cannot activate transcription (Figure 6C). We also considered that Nsi1p, which binds the same sequence as Reb1p but is only similar in the DNA binding domain, might also terminate transcription when expressed to the same levels as Reb1p. We ectopically expressed Reb1-DBD and Nsi1p under control of the *REB1* promoter, in a strain containing the endogenous *REB1* driven by the glucose-repressible *GAL1* promoter (*P<sub>GAL1</sub>-REB1*). Metabolic depletion of endogenous Reb1p in the presence of Reb1-DBD or Nsi1p indicated that neither protein supports viability (Figure S2B) nor can either activate transcription of Reb1p-dependent genes (Figure 6C; data not shown).

the DNA bound Reb1p) and the catalytic center of the enzyme (where the 3' OH of the nascent RNA is positioned) (Saeki and Svejstrup, 2009). To substantiate these results, we performed in vitro transcription experiments using purified RNAPII and recombinant Reb1p. In this system, an elongation complex was assembled on an immobilized template that contains a Reb1p binding site (Porrua and Libri, 2013) and allowed to transcribe in the presence or absence of rReb1p. Consistent with in vivo observations, in the presence of Reb1p the polymerase pauses roughly 12 nt upstream of the Reb1 site, as indicated by the accumulation of a transcript that remains associated to the template (Figure 3B).

These findings strongly support the notion that the first step in Reb1p-dependent termination is the collision between RNAPII and Reb1p. However, DNA-bound Reb1p alone cannot provoke the release of the polymerase in vitro, suggesting that a mechanism must exist in vivo for dismantling the elongation complex (see below).



**Figure 4. Reb1p-Dependent Termination Involves Transcriptional Pausing In Vivo**

(A) Rpb1p ChIP occupancy on a reporter construct containing the X3 or X3mut sequence, containing the wild-type or the mutated Reb1 site, respectively. The experiment was also performed in *rsp5-1* cells as indicated. A strain metabolically depleted for Reb1p (*P<sub>GAL1</sub>-REB1*, growth in glucose) was included as a control. Average of three experiments; error bars represent standard deviation. A scheme of the construct with the position of the Reb1 site and the positions of the amplified regions is shown on the bottom. See also Figure S3.

(B) Northern blot analysis of RNAs extracted from *rsp5-1* or *cul3Δ* cells containing the X3 reporter. Defects in the clearance of RNAPII paused at the Reb1 site lead to the increased detection of nascent RNAs (indicated by an arrow). Quantification of the nascent RNA levels relative to *ACT1* and to the wild-type for this gel is shown below the panel. From multiple independent analyses, the levels in *rsp5-1* cells relative to wild-type are  $2.68 \pm 0.12$  (average and standard deviation from three experiments). A scheme of the experiment is shown on the right.

### The Mechanism of RNAPII Release in Reb1p-Dependent Termination

The in vitro transcription experiments reported above indicate that Reb1p induces RNAPII pausing but cannot dismantle an elongation complex in this minimal experimental set up. This suggests the existence of a mechanism that ensures the release of RNAPII road-blocked by Reb1p.

We first assessed whether RNAPII pausing could also be detected in vivo around Reb1 sites by chromatin immunoprecipitation (ChIP). To this end we measured RNAPII occupancy at the X3 selected terminator and its mutated version (X3mut). As shown in Figure 4A, the presence of a functional Reb1 site induced a dramatic increase in RNAPII occupancy, which was fully abolished by metabolic depletion of Reb1p. Binding of Reb1p to its site in vivo was verified by ChIP (Figure S3). These findings indicate that Reb1p bound to the DNA induces RNAPII pausing in vivo, which is consistent with the detection of nonadenylated, nascent transcripts associated with paused polymerases (Figures 2B and S1D).

The persistence of paused polymerase is thought to be detrimental for several cellular functions, and it has been shown that polymerases stalled upstream of DNA damage are released by a ubiquitination/degradation mechanism. This implicates mono-ubiquitination by Rsp5p of the largest subunit of RNAPII, Rpb1p, which induces ubiquitin chain extension by the Elc1p-Cul3p complex and subsequent proteasomal degradation (Harreman et al., 2009, and references therein). We surmised that this pathway might also be required for the release of polymerases

Both factors, however, efficiently induced transcription termination at the Reb1p-dependent terminator (Figure 3C, lanes 3 and 4).

To further support the notion that formation of an NFR is not required for termination, we turned to the RSC complex, which is the main effector of Reb1p in NFR formation. Sth1p is the catalytic subunit of the RSC, and its depletion strongly affects Reb1p-dependent NFR formation genome-wide (Hartley and Madhani, 2009). Consistent with this notion, while metabolic depletion of Sth1p using a glucose repressible promoter strongly affected the activation of Reb1p target genes (see below, Figures S5A and 6C), it had no effect on Reb1p-dependent termination (Figure 3D). This strongly suggests that Reb1p-dependent termination does not depend on the ability of this factor to promote nucleosome remodeling.

Together our results indicate that DNA binding is necessary and sufficient, in vivo, to promote Reb1p-mediated transcription termination, and strongly support the notion that termination is triggered by the occurrence of a roadblock to transcription.

paused by a roadblock and assessed Reb1p-dependent termination into an *rsp5-1* or a *cul3Δ* strains, both defective for this ubiquitination pathway. Failure to ubiquitinate Rpb1p is expected to result in increased persistency of the polymerase at the site of Reb1p-dependent pause. This was indeed verified by RNAPII ChIP in *rsp5-1* cells (Figure 4A). This increase was not due to higher Reb1p levels or binding to the DNA, because Reb1p occupancy at the pause site was even slightly lower in *rsp5-1* cells compared to the wild-type (Figure S3).

Increased levels of roadblocked polymerase in *rsp5* or *cul3* mutants are expected to result in increased levels of nonadenylated, nascent RNA. Consistently, we observed a significant increase of short RNAs ending at the site of roadblock in *rsp5-1* and *cul3Δ* cells transformed with the reporter (Figure 4B). These RNAs are stable, because they are detected in cells proficient for nuclear degradation (Figure 4B), and are nonadenylated (data not shown), as expected for nascent transcripts.

From these experiments we conclude that RNAPII roadblocked at sites of Reb1p binding is released by the Rsp5p-Cul3p ubiquitination pathway, akin to the removal of RNAPII paused at sites of DNA damage.

### Reb1p Terminates RNAPII Transcription at Natural Sites

The experiments reported above demonstrate the proof of principle of Reb1p-dependent termination and its mechanism, but not its occurrence in natural cases. To assess the biological relevance of this termination pathway, we analyzed by tiling arrays the transcriptome of cells transiently depleted of Reb1p, using the *P<sub>GAL1</sub>-REB1* strain, with or without the *rrp6Δ* deficiency. These data were crossed to the known genome-wide distribution of Reb1p, as determined by high-resolution ChIP-seq (Rhee and Pugh, 2011) and the position of Reb1p binding sites, and to the distribution of unstable transcripts (Neil et al., 2009; Gudipati et al., 2012). Several cases of Reb1p-dependent termination were indeed identified for natural transcripts, most of which are unstable (Figure S4; data not shown). A characteristic feature of these transcripts, revealed by 3' end SAGE genome-wide analyses (Neil et al., 2009) and our analyses using the artificial terminators (e.g., see Figures S1 and S2), is their well-defined 3' end compared to canonical CUTs, which is due to the different mechanism of termination.

We validated the occurrence of Reb1p-dependent termination for a few model cases (Figures 5 and S5). For instance, an unstable short transcript (*uATP5*) can be detected upstream of the *ATP5* gene, terminating a few nucleotides before a Reb1 site. Transcription termination of *uATP5* was Reb1p dependent, because depletion of the latter strongly affected the levels of the short RNA to the profit of a longer species, terminating at a downstream site. Note that the longer species is itself unstable because it is only visible in an *rrp6Δ* context and at levels that do not fully compensate for the loss of the short transcript. As for other cases reported below, a fraction of the longer RNAs might also be degraded in the cytoplasm.

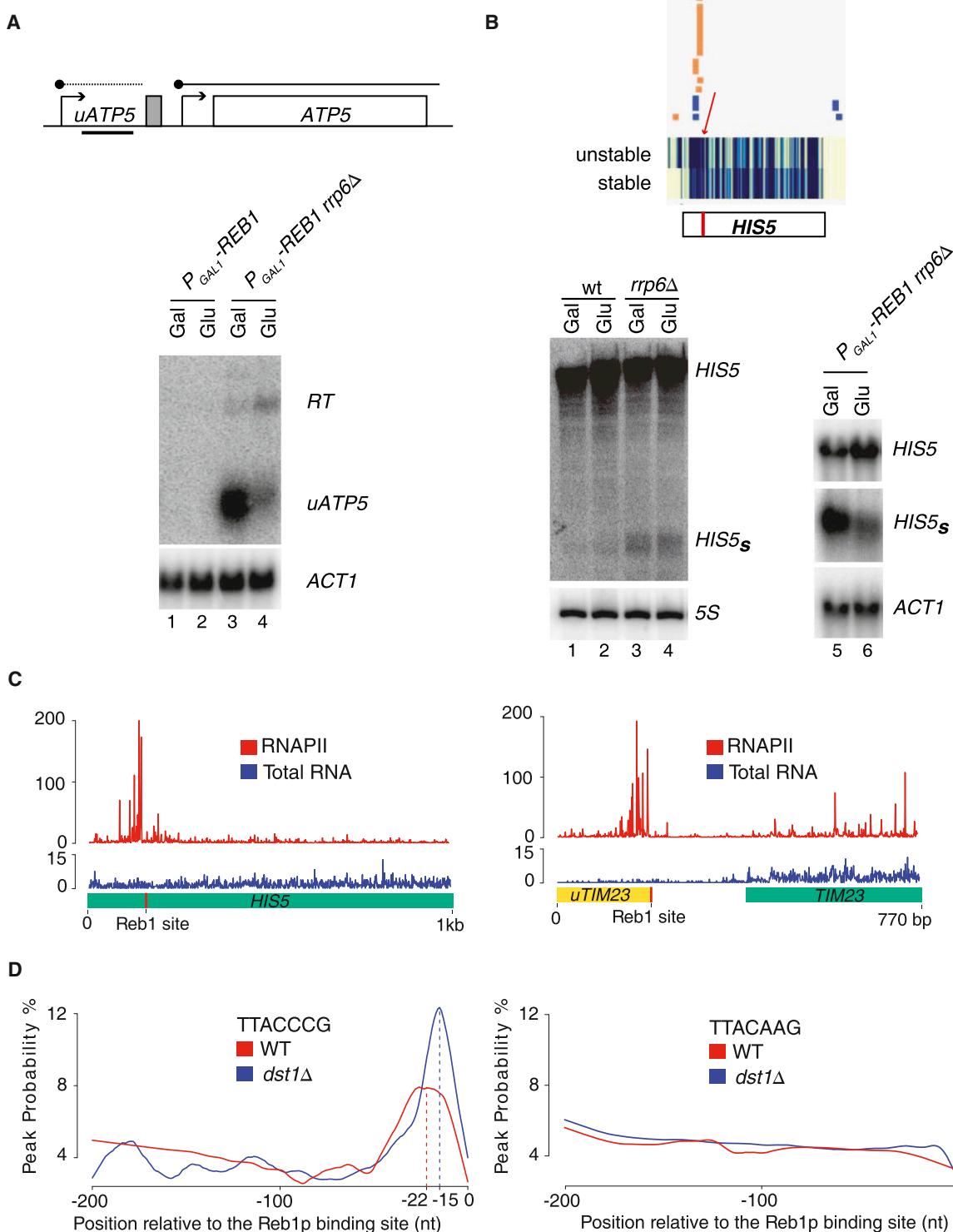
Most Reb1 sites are located in intergenic regions. The case of *HIS5* is peculiar, since this gene contains two Reb1 sites, one upstream and one within the coding region. A short unstable transcript terminating upstream of the internal site can be detected by SAGE and northern blot analysis (Figure 5B; Neil et al.,

2009). Assessing the Reb1p dependency of the short transcript is complicated by the fact that the *HIS5* gene is strongly Reb1p dependent and depletion of Reb1p or the RSC catalytic subunit Sth1p strongly affects *HIS5* expression (Figure S5A). Therefore we ectopically expressed *HIS5* under control of a heterologous promoter and showed that depletion of Reb1p strongly reduced early termination at the Reb1p internal site to the profit of the full-length transcript (Figure 5B, lanes 5 and 6). Whether this unusual organization implies the occurrence of regulated attenuation is unclear so far and is a matter for future studies.

We have shown above that RNAPII pauses in vivo in a Reb1p-dependent manner at an artificial terminator. We explored whether RNAPII pausing could be detected at natural Reb1p termination sites using the high-resolution genome-wide distribution of RNAPII defined by native elongating transcript sequencing (NET-Seq; Churchman and Weissman, 2011). As shown in Figure 5C, prominent RNAPII pausing peaks are precisely located a few nucleotides before the Reb1p binding site at the *HIS5* gene and the RUT upstream of the *TIM23* gene (Figure S5B). Pausing is not expected to be detected at all sites of termination, because accumulation of the signal depends on a balance between transcription levels upstream of the pause site and the rate of clearance of the paused polymerase. For instance, at *uATP5* RUT, increased RNAPII signals can be observed upstream of the Reb1 site, but the overall signal is too low to unambiguously identify a peak as in the case of *HIS5* or *uTIM23* (data not shown). To generalize this finding, we combined data from all Reb1 sites in the genome and plotted the frequency of RNAPII peaks that are at least two standard deviations above the mean at each position in a 200 nt region upstream of the Reb1 site. The distribution of the peak frequency indicates a significantly increased RNAPII pausing 14–25 nt before the average Reb1 site, but not at sites containing single-nucleotide mutations known to strongly affect Reb1p binding (Figure 5D), which is fully compatible with in vitro experiments.

Elongation pausing is known to induce backtracking of RNAPII, during which the 3' end of the nascent RNA is displaced from the catalytic site of the enzyme. The RNA is cleaved by the coordinated action of the polymerase and the TFIIS elongation factor to correctly position the 3' OH of the molecule and resume elongation (Reines et al., 1992; Izban and Luse, 1992). In a wild-type strain, the 3' end of the nascent RNA at the region of pausing is determined by cleavage, which can occur at multiple, closely spaced positions during backtracking; when cleavage is inhibited (as in the absence of TFIIS), the 3' end of the nascent RNA generally coincides with the last position occupied by the polymerase before backtracking. To confirm the occurrence of pausing at the average Reb1 site, we repeated the same analysis in a strain deleted for TFIIS (*dst1Δ*). As shown in Figure 5D, the RNAPII peak was still observed in the mutant, but with a distribution slightly displaced toward the Reb1 site and less dispersed relative to a wild-type strain, consistent with the notion of pausing and backtracking at sites of roadblock.

Together, these results demonstrate the existence of natural sites of Reb1p-dependent transcription termination in the yeast genome, which occurs via a roadblock mechanism.



**Figure 5. Reb1p Terminates Transcription of Natural ncRNAs, “RUTs”**

(A) Northern blot analysis of the unstable transcript located upstream of *ATP5* (*uATP5*). A scheme of the genomic region is shown on the top; the Reb1 site (gray box) is located a few nucleotides downstream of the mapped 3' end of the transcript.

(B) Stable and unstable RNAs species derived from the *HIS5* locus. (Upper panel) Tiling array heatmap and SAGE tags analyses (Neil et al., 2009). The ends of individual RNA species mapped by 3' end SAGE are indicated by orange (unstable fraction) or blue (stable fraction) squares. The position of the Reb1 site in *HIS5* (158 nt downstream of the ATG) is indicated by a red arrow and a red bar in the scheme. (Lower panels) Northern blot analyses revealing a short unstable transcript

(legend continued on next page)

### Functional Cooperation between Termination Pathways to Control Pervasive Transcription

Because Reb1p occupancy is prominent in intergenic regions, in many cases a putative site of Reb1p-dependent termination overlaps with the canonical terminator of a gene, suggesting that different pathways may concur to ensure efficient termination either by acting synergistically or sequentially as reciprocal fail-safe mechanisms.

The *YSY6* locus is one example of an alternative pattern of main and backup termination mechanisms. This gene (Figure S6A) contains a Reb1 site that coincides with the end of the transcript, still transcription termination is mainly dictated by the CPF/CF, as production of *YSY6* RNA is sensitive to mutation of Rna14p (an essential CF subunit; Figure S6B) and insensitive to Reb1p depletion (Figure 6A). However, a low level of transcriptional readthrough naturally occurs that is neutralized by the occurrence of Reb1p-dependent termination. Indeed, upon depletion of Reb1p a longer transcript is produced that is exported and degraded in the cytoplasm by the nonsense-mediated decay (NMD) pathway, as shown by the marked accumulation of this species when NMD is impaired by the *upf1Δ* mutation (Figures 6A and S6C). A similar organization is found at the *YDL233w* locus, where Reb1p-dependent termination acts as a fail-safe mechanism to neutralize leakage from the main CPF/CF termination. In this case, the readthrough transcripts that are not terminated at the Reb1p-dependent site (i.e., when Reb1p is depleted) are degraded both by the exosome and the NMD pathway because they can only be detected in a *dis3-exo<sup>-</sup>* *upf1Δ* context (Figure 6B, lanes 5–8).

In the cases described above, depletion of Reb1p leads to a downregulation of the downstream genes (*DEM1* and *OST4* for *YSY6* and *YDL233w* respectively; Figures 6, S6A, and S6D). One possibility is that increased upstream readthrough due to impairment of the backup termination pathway silences the downstream promoter by transcriptional interference. However, it is also possible that transcriptional activation of the downstream gene depends on Reb1p via the RSC pathway. To distinguish between the two possibilities, we took advantage of the finding that the Reb1-DBD can terminate but is unable to activate transcription (Figure 3C). The expression of *DEM1* and *OST4* was analyzed after depletion of Reb1p in the presence of Reb1-DBD, which should prevent transcriptional interference but should not allow activation. As shown in Figure 6C, the Reb1-DBD could only poorly restore expression of *DEM1*, indicating that the latter critically depends on the activation function of Reb1p. Consistently, expression of *DEM1* was markedly affected by depletion of Sth1p, the catalytic subunit of the RSC (Figure 6C). Therefore, whether transcription interference occurs at the *DEM1* locus in

the absence of Reb1p cannot be reliably determined. In contrast, *OST4* was dependent on Reb1p for activation only to a lesser extent, as shown by the poor effect of Sth1p depletion (Figure 6D). Importantly, expression of the Reb1-DBD alone was sufficient to partially restore *OST4* expression, supporting the notion that silencing of *OST4* upon Reb1p depletion is mainly due to transcriptional interference.

The paradigmatic case of *OST4* suggests that Reb1p-dependent termination could play a general role as a fail-safe mechanism preventing transcriptional interference to occur at contiguous genes. One important prediction of this model is that Reb1p-dependent termination should become essential under conditions where CPF/CF termination is also defective. To test this prediction, we generated a hypomorphic, thermo-sensitive allele of Reb1p containing a mutation in its DNA binding domain, *reb1-ts1*. This mutation only slightly affects growth in an otherwise wild-type context, presumably because DNA binding is only partially affected. However, when combined with the CPF/CF *rna14-3* mutation that is mildly defective at permissive temperature (our unpublished data; Libri et al., 2002), the *reb1-ts1* mutation becomes detrimental for growth (Figure S6E). Importantly, the growth defect of the double mutant can be partially suppressed by expressing the DNA binding domain of Reb1p alone or by Nsi1p, which bind the same sequence and terminate transcription but lack the activation function of Reb1p (Figure S6F). This indicates that the synthetic growth defect is due to loss of fail-safe Reb1p-dependent termination in a context partially defective for CPF/CF termination.

Together these findings support the notion that Reb1p has a dual role in the control of gene expression. On one side, it induces the correct positioning of NFRs, which is essential for transcription activation; on the other side, it plays an important role in “protecting” promoter regions from the deleterious effect of even modest readthrough transcription from neighboring transcription units.

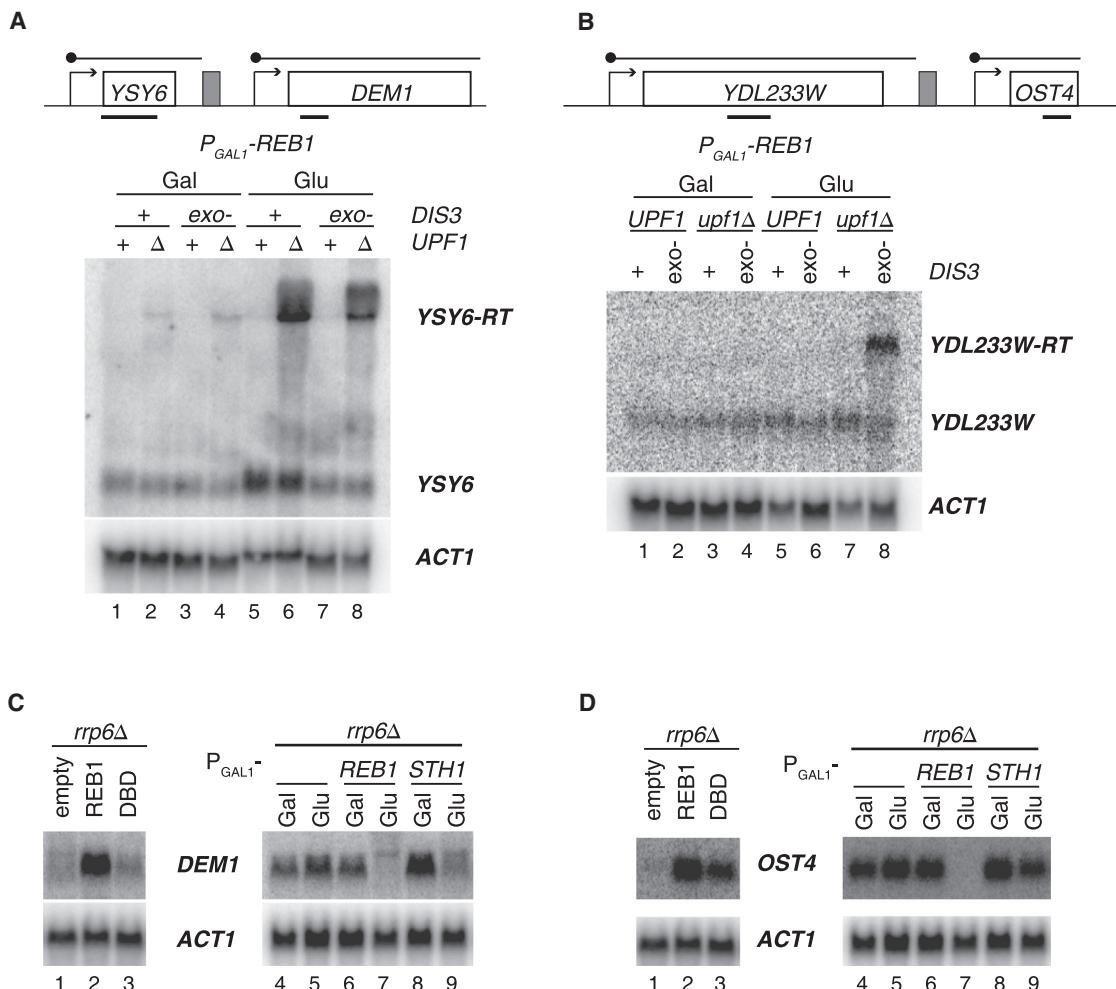
### DISCUSSION

Because the yeast genome is compact and extensively transcribed, efficient transcription termination is very important for robust and stable gene expression. In this study we describe an additional pathway for transcription termination that depends on the DNA binding factor Reb1p, a factor hitherto implicated in the activation of transcription (see Figure 7 for a model). These data open up an additional perspective on the insulation of transcriptional units, the control of pervasive transcription, and the role of DNA binding proteins in these processes.

(HIS5<sub>S</sub>) derived from roadblock termination at the Reb1 site in an *rrp6Δ* strain (left panel, lanes 3 and 4). (Right) RNAs derived from *HIS5* expressed under the control of a heterologous promoter (*P<sub>TET</sub>*) in a *P<sub>GAL1</sub>-REB1 rrp6Δ* strain. See also Figure S5A.

(C) RNAPII occupancy revealed by NET-Seq analysis (red histograms) at *HIS5* and *TIM23* (data from Churchman and Weissman, 2011). Total RNA signal in the same regions is shown in blue as a control. An unstable transcript (*uTIM23*) terminated by Reb1p is located immediately upstream of *TIM23*. The position of the Reb1 site is indicated by a red bar in the scheme. See also Figure S5B.

(D) Metasite analysis of RNAPII pausing revealed by NET-Seq (Churchman and Weissman, 2011) upstream of Reb1 sites in the genome. The plots show the frequency of polymerase pausing peaks in a 200 nt window preceding a generic Reb1 site (TTACCCG) or a mutated sequence that cannot bind Reb1p (TTACAAG), calculated over all the sites in the genome aligned at position 0. The analysis was performed in a wild-type strain (red) or a TFIIS mutant (*dst1Δ*, blue). See also Figure S7.



**Figure 6. Reb1p-Dependent Termination Functions as a Fail-Safe Mechanism**

(A and B). Reb1p-dependent termination functions as a fail-safe mechanism for *YSY6* and *YDL233W* mRNA termination. Northern blot analysis of *YSY6* and *YDL233W* mRNAs under the indicated conditions and strains. Readthrough transcripts are only detected upon Reb1p depletion when NMD is impaired in *upf1Δ* cells (*YSY6*) or when both the exosome and NMD are affected (*YDL233W*).

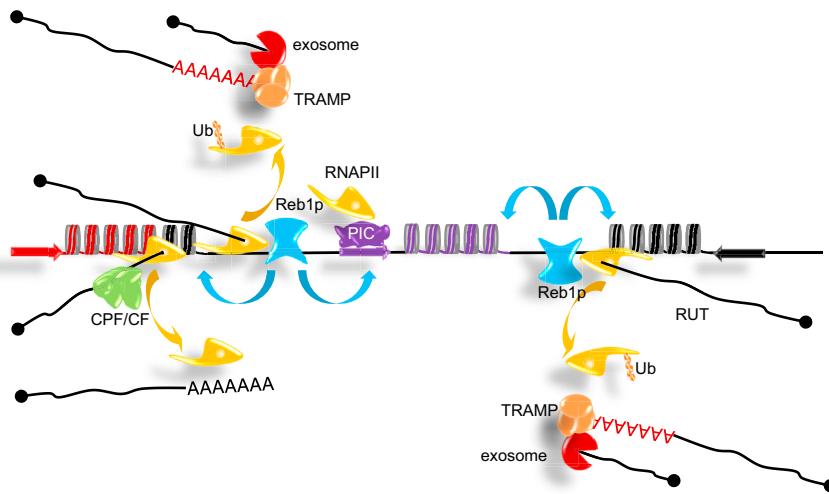
(C) Northern blot analysis of *DEM1* expression. *DEM1* expression depends on Reb1p and Sth1p and is not restored by expression of the Reb1p DNA binding domain.

(D) Northern blot analysis of *OST4* expression. In the absence of Reb1p, expression of *OST4* can be restored with the reestablishment of fail-safe termination upon expression of the Reb1p DNA binding domain alone. The position of the Reb1 site is indicated by a gray box. See also Figure S6.

### Roadblock Termination Is a General and Conserved Mechanism

Roadblock termination has been shown to occur for transcription of rDNA genes by RNAPI in several species, including yeast. Termination is triggered by the collision between the transcribing polymerase and a DNA-bound factor that is TTF1 in the mammalian system and most likely Nsi1p in yeast (Evers and Grummt, 1995; Evers et al., 1995; Reiter et al., 2012). However, the sensitivity to roadblocks is not a specific feature of RNAPI, and the three RNA polymerases can be paused in vitro by the DNA-bound Reb1p (Lang et al., 1994; this study). The inability to overcome a DNA-bound obstacle might therefore be an intrinsic property of RNA polymerases and possibly an ancient mechanism of termination, which is underscored by the occurrence of

roadblock termination in bacteria (Belitsky and Sonenshein, 2011; Pavco and Steege, 1990). We show here that roadblock termination also occurs in vivo for RNAPII. This indicates that in spite of the existence of elongation factors and chromatin-remodeling factors to allow transcription through nucleosomes in vivo, the cell apparently lacks specific mechanisms to efficiently remove nonhistone, DNA-bound proteins in front of transcribing RNA polymerases. Reb1p-dependent roadblock termination is also very likely to be conserved in other species. Reb1 sites are similarly excluded from regions of active elongation in several Hemiascomycetes species (*S. paradoxus*, *K. lactis*, *S. mikatae*, *S. bayanus*, and *C. glabrata*) ( $p < 10^{-4}$ ) to an extent that is not likely to be due only to the role of Reb1p in NFR formation. For comparison, the distribution of Cbf1p,



**Figure 7. A Schematic Model Depicting the Role of Reb1p in the Positioning and Protection of Nucleosome-free Regions and in the Control of Pervasive Transcription**

The role of Reb1p in promoting transcription initiation and NFR formation is indicated by blue arrows. Binding of Reb1p can also induce RNAPII pausing by a roadblock mechanism. Additional details are in the text. Ub, ubiquitin. PIC, pre-initiation complex.

nucleosome repositioning factor (Kent et al., 2004) that cannot路block RNAPII (our unpublished data), is less markedly skewed (the difference is significant at the 98% confidence level; data not shown).

#### How Many Roadblocks?

These considerations beg the question of what restricts roadblock termination for the cell to cope with a wealth of DNA binding activities that could potentially interfere with transcription. It is likely that not all DNA-bound proteins elicit RNAPII pausing and termination. For instance, we observed that binding of Gal4p does not elicit termination of upstream transcription regardless of growth conditions (data not shown). On the other side, binding to the DNA of the *E. coli* Lac repressor was found to prevent expression of SV40 large T antigen in mammals, suggesting RNAPII roadblocking (Deuschle et al., 1990). Similarly, a short sequence motif that is a binding site for a DNA binding factor in the adenovirus major late promoter has been suggested to prevent elongation (Connelly and Manley, 1989). Roadblock termination must depend on a complex interplay of several competing events: first, nucleosomes might occlude binding sites for several factors; second, transcription itself might prevent the interaction of potential roadblockers with the DNA, either by sterically hindering binding sites or by inducing a chromatin structure that limits the exposure of these sites. The ability of a DNA binding factor to counteract these competing events, in terms of abundance and affinity for the DNA, conceivably qualifies a roadblocker, and Reb1p might occupy a pre-eminent position in this respect.

#### The Mechanism of Roadblock Termination

RNAPII pausing and the consequent protection of nonadenylated RNAs are readily observed at sites of roadblock termination using NET-Seq (Figure 5; data from Churchman and Weissman, 2011) and PAR-CLIP (Creamer et al., 2011; data not shown) data. Although pausing has also been proposed to occur as a prerequisite of CPF/CF- and NNS-dependent termination, the same techniques do not detect significant RNAPII accumulation at these sites, suggesting that localized RNAPII pausing (or slow

clearance of the paused polymerase) might more specifically characterize roadblock termination.

An interesting feature of Reb1p-induced roadblock is its directionality. It was originally demonstrated that only when bound to a site in the "G-rich" orientation relative to the direction of transcription can Reb1p路block RNAPII in vitro (Lang and Reeder, 1993; Lang et al., 1994).

Although we did not observe in vitro a similar directionality with RNAPII (data not shown), one orientation of the Reb1 site was markedly preferred in our selection experiment, and its reverse orientation could not terminate transcription in our reporter system (Figure 1D), suggesting directionality. Interestingly enough, the preferred site orientation for roadblocking RNAPII is the "C-rich," i.e., opposite to that for RNAPI. However, natural sites of roadblock termination were observed upstream of the Reb1 site in both orientations (e.g., the site upstream OST4 is "G-rich"), and RNAPII pausing was also detected by "metasite" analysis when the Reb1 site is in the "G-rich" orientation (Figure S7A). This suggests that the geometry of the RNAPII-Reb1p collision affects the efficiency of the roadblock. The "G-rich" orientation would be sufficient to路block the less robust RNAPII elongation in vitro and the generally low frequency transcription in vivo (Zenklusen et al., 2008). Still, it does not suffice to arrest transcription driven by the strong Tet-repressible promoter in our more stringent selection system, because high-frequency firing might efficiently outcompete Reb1p binding.

Differently from RNAPI (Lang et al., 1994; Mason et al., 1997a, 1997b), release of roadblocked RNAPII does not require a release sequence element because the isolated 8 nt Reb1 site is sufficient for termination (Figure 1D). However, we cannot exclude that, in addition to the Rsp5p and elongin-cullin ubiquitination system, other factors might contribute to the release of paused RNAPII, as proposed for the Rat1p exonuclease and the Sen1p helicase in RNAPII termination (Kawauchi et al., 2008). Nevertheless, we have shown that the CPF/CF- and the NNS-dependent termination pathways are dispensable for at least a few tested cases of Reb1p-dependent termination (data not shown; Figures 1C and S1B).

It remains unclear whether ubiquitination of roadblocked RNAPII is followed by degradation of the enzyme, in analogy with what was demonstrated for RNAPII stalled upstream of DNA damage (Somesh et al., 2005; Verma et al., 2011; Wilson et al., 2013). Destruction of one polymerase molecule per termination event might not look economical, but advantages are

probably to be found in the overall low evolutionary cost of the system, which requires very limited sequence information (typically 8–10 nt) and uses enzymatic pathways selected for other purposes. This pathway appears to be well adapted for neutralizing low levels of transcription at “sensitive” locations such as NFRs and in any instances where the benefits of compact termination signals might justify its costs in terms of polymerase losses (e.g., in the case of regions “crowded” with transcription units).

### Functional Significance of Roadblock Termination

Because Reb1p-dependent termination leads to the production of unstable transcripts, it is a reasonable assumption that its significance relates more to the control of transcription than to the generation of functional RNA molecules. The bulk of cryptic transcription originates in NFRs, where Reb1p is preferentially localized, and it is very possible that pervasive transcription events are neutralized early by Reb1p (or other roadblockers) within the NFR from which they originate, and escape detection because of their small size. We suggest that Reb1p and roadblock termination in general might contribute to suppress promoter bidirectionality.

Importantly, Reb1p has also a role in preventing transcriptional interference, sometimes as part of a fail-safe termination pathway (Figure 7). Regions where transcription originates in NFRs are extremely sensitive to “invasions” from neighboring polymerases. Because elongating transcription induces a chromatin state that is repressive for initiation (for a review, see Jensen et al., 2013), even low levels of transcription through promoters suffice to inhibit initiation (Bumgarner et al., 2012; Castellano et al., 2013; van Werven et al., 2012). Thus, isolating regions of transcription initiation from elongating polymerases is a major challenge, and the preferential localization of Reb1p in intergenic regions might be economically exploited to sustain such a double role as a transcriptional activator and an NFR “guard.” The protection of the *OST4* gene from interfering readthrough transcription is paradigmatic in this regard. The importance of enforcing termination is underscored by the existence of fail-safe pathways described for other genes, such as the one triggered by cleavage of the nascent RNA by the endoribonuclease Rnt1p (Ghazal et al., 2009; Rondón et al., 2009).

To estimate the extent of Reb1p-dependent termination in the yeast genome, we can propose a minimal figure based on the occurrence of robust RNAPII pausing at sites of termination (Figures 4, 5, and S7). When we considered all the putative Reb1 sites in the yeast genome based on sequence alone, roughly 12% show an RNAPII pausing signal upstream (Figures 5D and S7A). However, when we restricted the analysis to the subset of sites with the highest actual Reb1p occupancy (MacIsaac et al., 2006), this figure approaches 40% (Figure S7B). This could still be an underestimation, because sites of low transcriptional activity and cases of fail-safe termination are likely overlooked.

Finally, many cases of roadblock pausing/termination might translate into regulation, not only at the level of transcription but also splicing and 3' end processing. Altering the abundance or the binding affinity of DNA binding factors might affect pausing at sensitive locations, repress or generate regulatory transcription, or more directly attenuate the expression of genes. The case of *HIS5* described here might be paradigmatic in this

respect, and investigating the regulatory potential of its unusual architecture is an exciting future perspective.

### EXPERIMENTAL PROCEDURES

#### Yeast and Constructs

Yeast strains and oligonucleotides used in this study are listed in Supplemental Experimental Procedures. Plasmids were generally constructed using homologous recombination in yeast with standard procedures and are listed in Supplemental Experimental Procedures. Standard molecular biology analyses including transcriptome analyses are described in Supplemental Experimental Procedures. In vivo selection of terminators has been extensively described elsewhere (Porrua et al., 2012). The statistical analysis of the selected pool of sequence was performed as described in Supplemental Experimental Procedures.

#### Genome-wide Analyses of Reb1p-Induced Polymerase Pausing

Reb1p-induced RNAPII pausing was analyzed using the NET-seq data sets (Churchman and Weissman, 2011) in wild-type and *dst1Δ* context. A total of 917 occurrences of the Reb1p core binding site (TTACCCG) were identified in the yeast genome (SGD R62).

To analyze RNAPII pausing at each of these sites, we searched for local peaks of polymerase occupancy in an upstream window of 200 nt. A peak was defined as a read value that is higher than the mean plus two times the standard deviation calculated over all the nonzero read values in the 200 nt window. All the windows containing fewer than three nonzero read values were excluded from this calculation, resulting in a total of 555 (wild-type) and 604 (*dst1Δ*) processed sites for the “TTACCCG” sequence. The same analysis was performed for polymerases transcribing toward the reverse complement of the site (GGGGTAA, 534 occurrences for the wild-type and 594 for the *dst1Δ* data set), and at a mutated site (TTACaaG) that does not bind Reb1p as a control. In order to make each metasite analysis comparable, the number of peaks occurring at any given position in the 200 nt window was divided by the total number of nonzero read values at that position, which represents peak frequency. Note that only the occurrence of a peak and not its height was taken into account. To obtain the plots shown in Figures 5 and S7, a smoothing of the data was performed using the “supsmu” R function.

### ACCESSION NUMBERS

The whole tiling array set of data has been deposited into ArrayExpress under accession number E-MTAB-2241.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.molcel.2014.10.026>.

### AUTHOR CONTRIBUTIONS

J.C. designed and performed experiments and wrote the paper; O.P. designed and performed experiments; T.C. designed and performed bioinformatics analysis; O.P. and T.C. contributed equally to this work. J.B., C.Z., and F.L. performed experiments; L.M.S. designed experiments; D.L. designed experiments and wrote the paper.

### ACKNOWLEDGMENTS

We would like to thank J. Svejstrup for the kind gift of strains, and F. Pugh for allowing access to unpublished data; Libri lab members for fruitful discussions; and J. Svejstrup, N. Proudfoot, and F. Feuerbach for critical reading of this manuscript. This work was supported by the CNRS (D.L.), the Danish National Research Foundation (D.L.), the Agence Nationale pour la Recherche (ANR, ANR-08-Blan-0038-01 and ANR-12-BSV8-0014-01 to D.L.), the Fondation pour la Recherche Médicale (FRM, programme Equipes 2013 to D.L.), the Fondation Bettencourt-Schuller (prix Coup d’Elan 2009 to D.L.), the National

Institutes of Health (L.M.S.), and the Deutsche Forschungsgemeinschaft (L.M.S.). This study was technically supported by the EMBL Genomics Core Facility. O.P. was supported by fellowships from the EMBO and the FRM. This research was carried out within the scope of the Associated European Laboratory LEA "Laboratory of Nuclear RNA Metabolism."

Received: January 30, 2014

Revised: June 9, 2014

Accepted: October 29, 2014

Published: December 4, 2014

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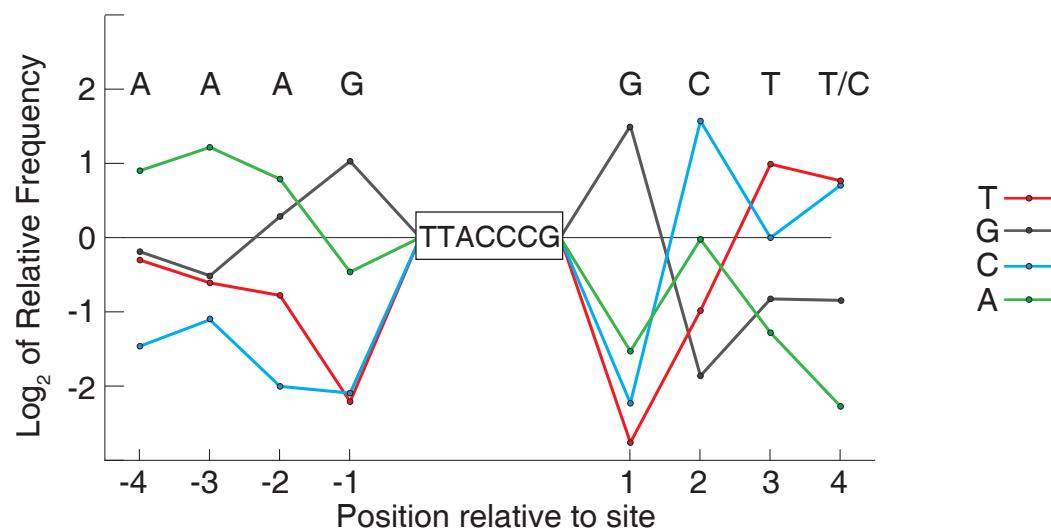
Molecular Cell, Volume 56

## **Supplemental Information**

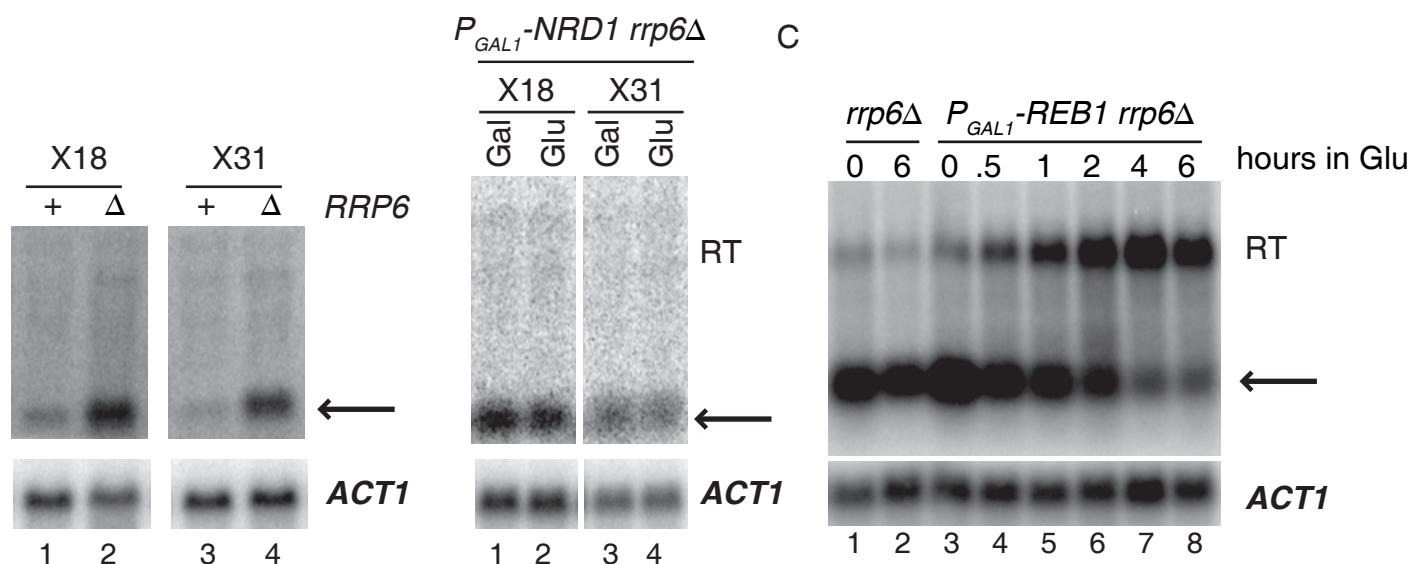
### **Roadblock Termination by Reb1p Restricts Cryptic and Readthrough Transcription**

Jessie Colin, Tito Candelli, Odil Porrua, Jocelyne Boulay, Chenchen Zhu,  
François Lacroute, Lars M. Steinmetz, and Domenico Libri

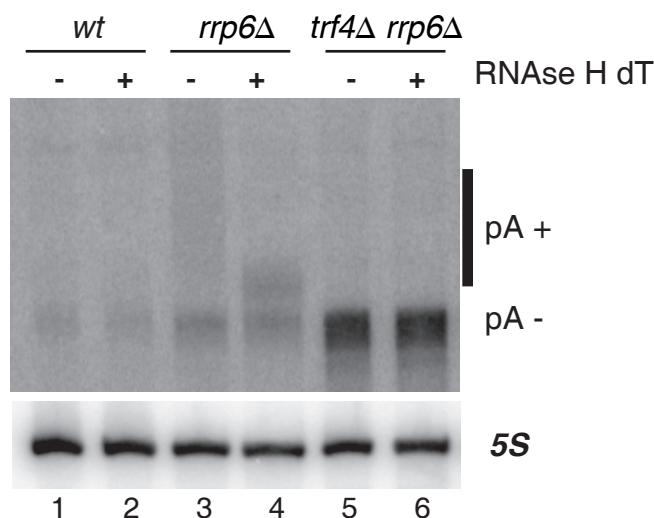
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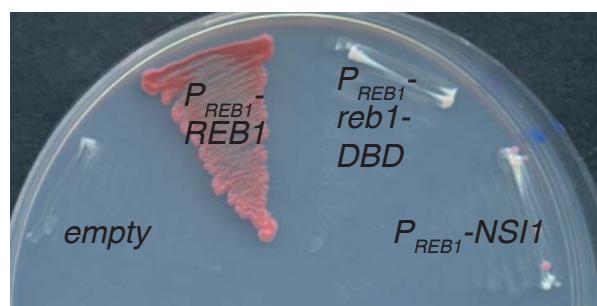
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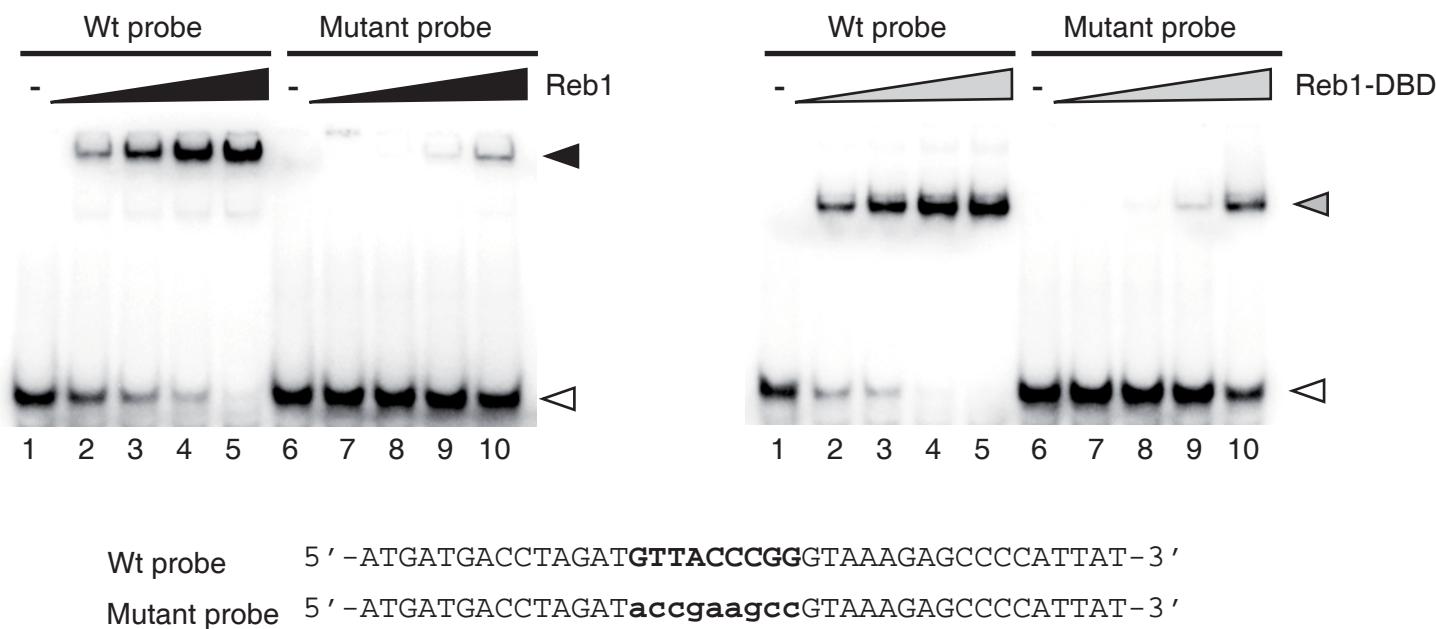
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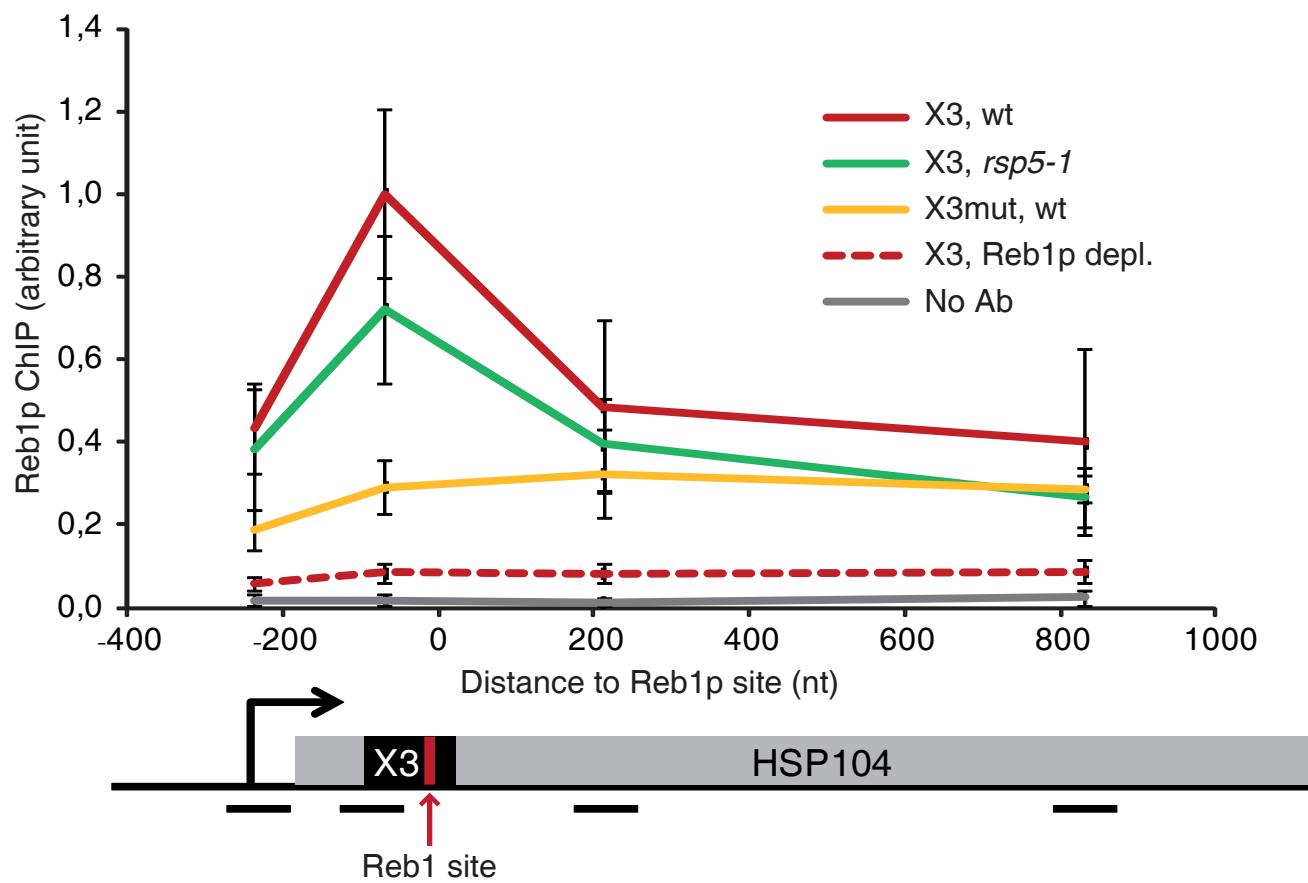
		Distance to Reb1p site (nt)
HSP::Reb1	TATTCTAGCTGC ↓ CTTCATTGAAACGG <b>TTACCCGG</b>	15
X3	ATTCGCAAGGACT ↓ ATAGTTCATTTAG <b>TTACCCGG</b>	14
X18	GATAACCGGTACCG ↓ CATTGAAAAGA <b>TTACCCGG</b>	13
X20	TATGGCTTGGTGAG ↓ AGCAAGGAAGG <b>TTACCCGG</b>	12
X25	ACGATAAACGCTG ↓ CTGGTCGTTACG <b>TTACCCGG</b>	14
X28	ATTCGCAAGGACTAT ↓ AGTTCATTTAG <b>TTACCCGG</b>	12
X31	TCGGTAGGATGAAT ↓ AGGTCAAGAAAG <b>TTACCCGG</b>	13

B

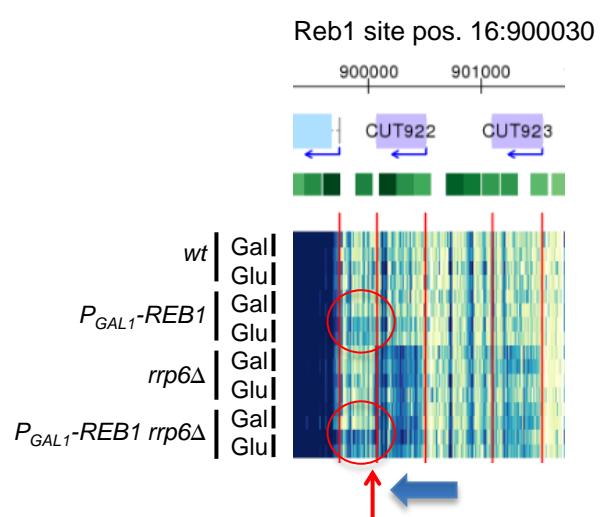
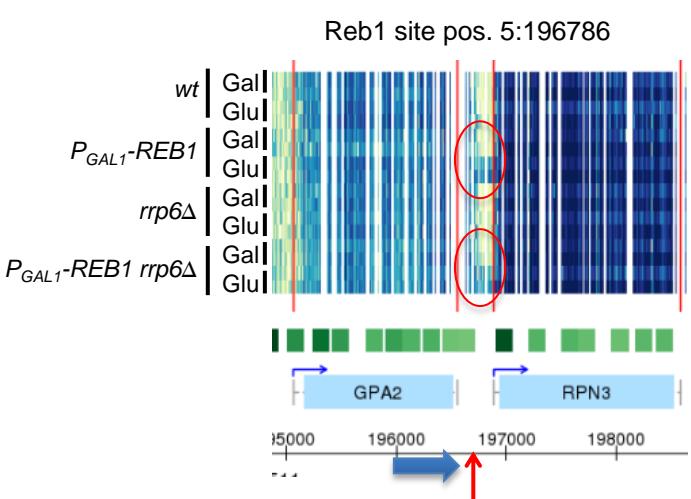
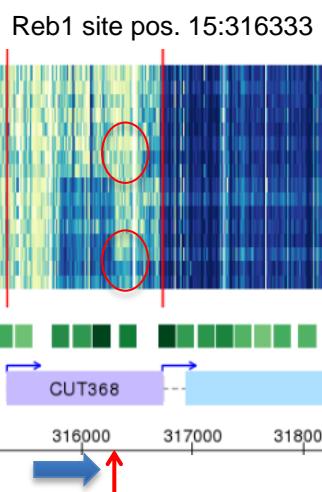
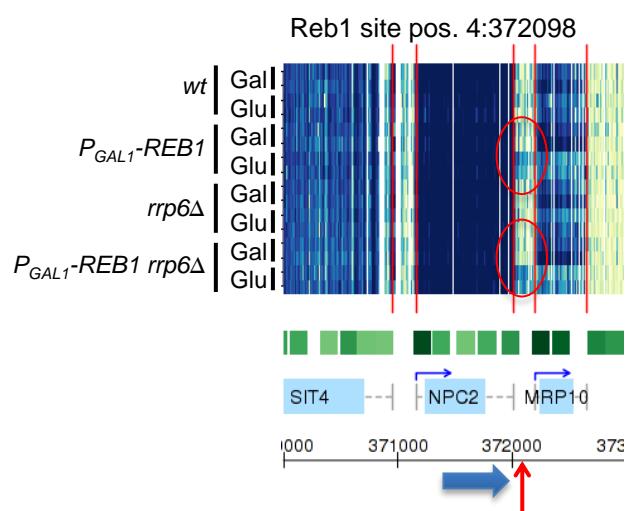
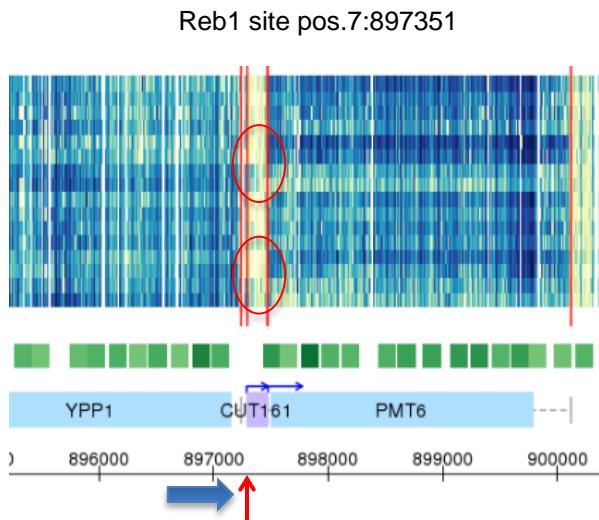
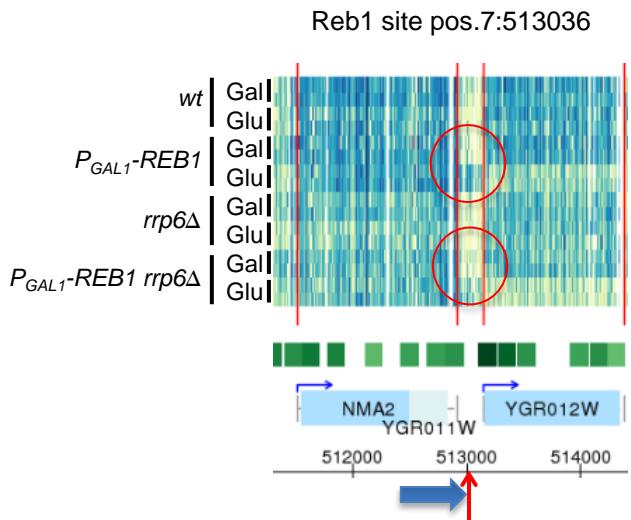


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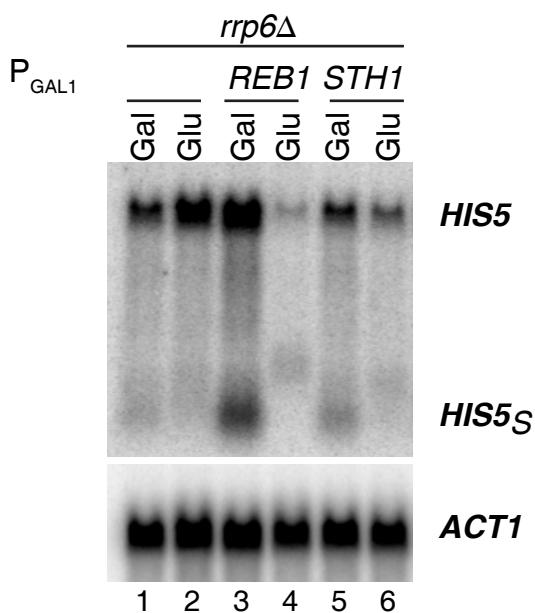




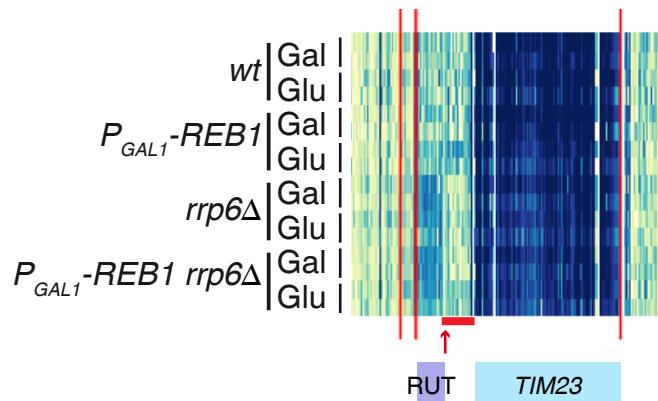
Colin et al., Figure S3, related to figure 4



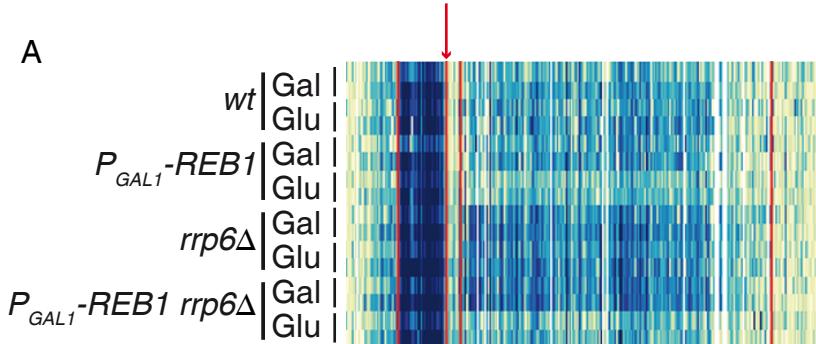
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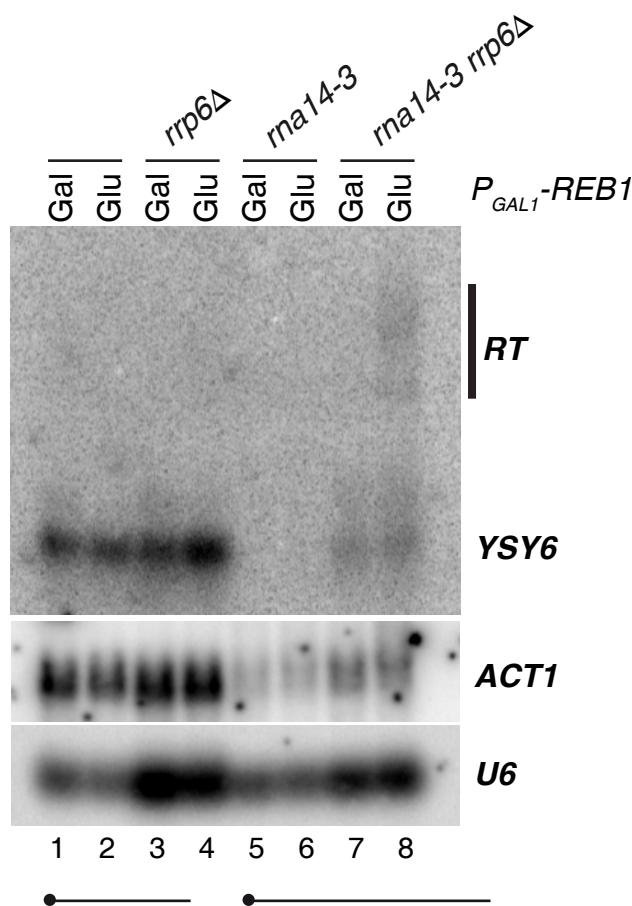
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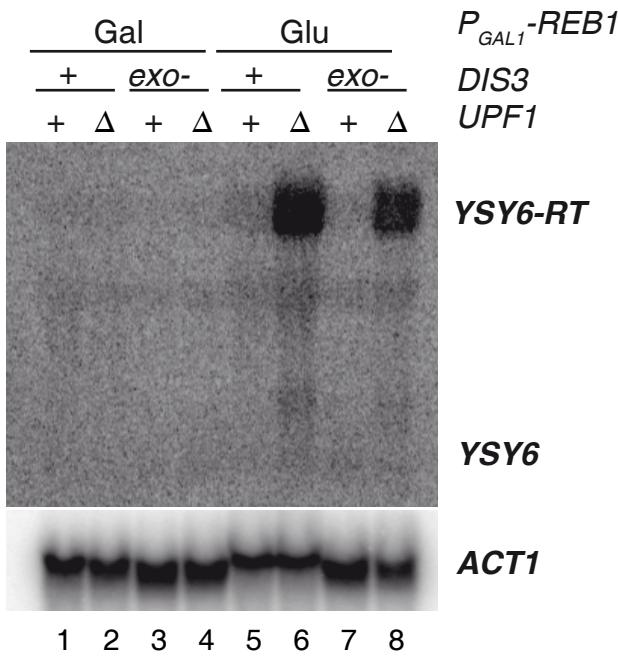
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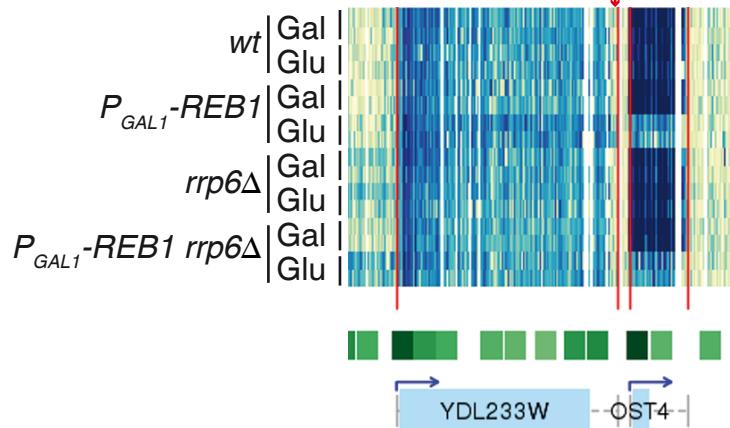
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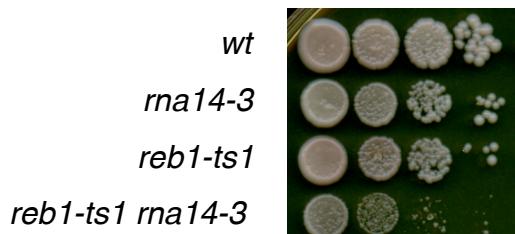
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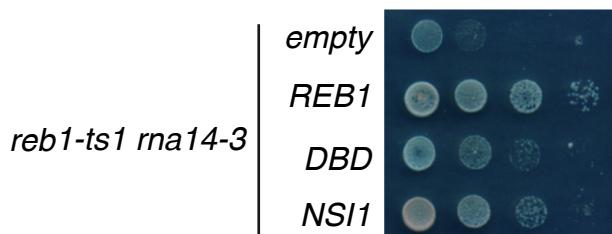
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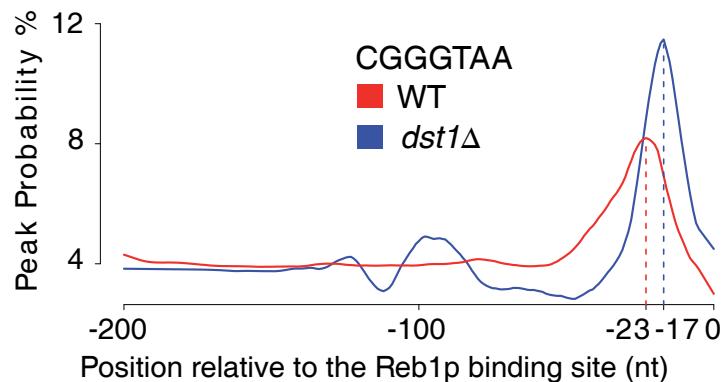
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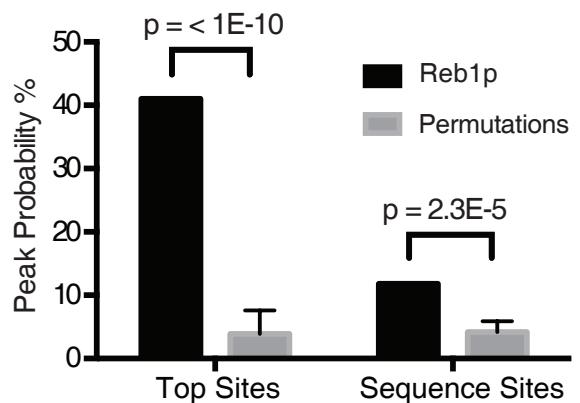
F



A



B



## Supplemental Figure Legends

Figure S1. Reb1p induces RNAPII transcription termination. Related to Figures 1 and 2. **A.** Statistical analysis of the nucleotides flanking the Reb1p site in the selected terminators. For every position flanking the core Reb1p site, the plot indicates the  $\log_2$  of the ratio between the nucleotide frequencies in the selected and the non-selected pools. **B.** Northern-blot analysis of RNAs generated from two additional selected clones (X18 and X31). Similarly to clone X3 (Figure 1C), termination at these sequences is independent of the NNS pathway (right panels) and generates unstable transcripts that are stabilized in *rrp6Δ* cells (indicated by an arrow, left panel). **C.** Time course of Reb1p depletion in cells containing the Reb1p-dependent terminator X3. Labeling as in Figure 1. **D.** Northern-blot analysis of the polyadenylation status of transcripts generated by termination at the X3 sequence. Poly(A) tails were degraded by RNase H/oligo dT treatment as indicated. The smear of polyadenylated species in *rrp6Δ* cells (indicated by a solid bar) disappears when the gene coding for the poly(A) polymerase Trf4p is also deleted (compare lanes 3 and 5) and collapses to species with short poly(A) tails that cannot be further degraded by RNase H/oligo dT treatment (compare lanes 3 and 4). The band present in the wild type strain is nonadenylated because is insensitive to oligo dT/RNaseH digestion and cannot be detected after oligo-dT selection (Figure 2B).

Figure S2. Related to Figure 3 **A.** RACE mapping of 3' ends of transcripts generated by Reb1p-dependent termination within several independent selected sequences. Termination always occurs 12 to 15 nucleotides before the Reb1p-binding site, independently of the sequence. Clone HSP::Reb1 contains only the Reb1p binding site inserted in the *HSP104* coding sequence. The mapped 3' ends are indicated by an arrow and the Reb1p binding site is represented in bold. **B.** The DNA binding domain of Reb1p is not sufficient to support growth. *P<sub>GAL1</sub>-REB1* cells containing Reb1p, the DNA-binding domain of Reb1p or Nsi1p, under control of the *REB1* promoter are grown on glucose plates to deplete endogenous Reb1p. Complementation only occurs with wild type Reb1p. **C.** Electrophoretic mobility shift assay (EMSA) using either rReb1p or its DNA binding domain. Both proteins bind efficiently dsDNA carrying a Reb1p binding site but not its mutated version.

Figure S3. Reb1p occupancy does not increase in *rsp5-1* cells. Related to Figure 4. Reb1p occupancy determined by ChIP on a template carrying the X3 sequence in wild type or *rsp5-1* cells. As in figure 4,

the experiment was also performed with a clone containing a mutated Reb1p site and after Reb1p depletion as a control.

Figure S4. Related to Figures 5 and 6. Heatmap of additional examples of natural transcripts terminated by the Reb1p-dependent pathway. For all these examples, an extended read-through transcript (red oval) is readily detected upon Reb1p depletion. Reb1 site is indicated by a red arrow.

Figure S5. Related to Figure 5C and 6A **A**. Reb1p and the RSC are required for the activation of *HIS5*. Northern-blot analysis of *HIS5* RNAs. Depletion of either Reb1p or Sth1p affects expression of full length *HIS5*. **B**. Heatmap of the RNA signals derived from the *TIM23* locus revealed by tiling arrays. A Reb1p-dependent cryptic unstable transcript (*uTIM23* RUT) is detected upstream of *TIM23* in an *rrp6Δ* strain. Upon Reb1p depletion in glucose-containing medium, an extended transcript can be detected (extension marked by a red bar). The position of the Reb1p binding site is indicated by a red arrow.

Figure S6. Related to Figure 6 **A**. Heatmap of transcripts derived from the *YSY6/DEM1* locus showing that depletion of Reb1p induces a downregulation of *DEM1*. The position of the Reb1 site is indicated by a red arrow. Note that under these conditions the elongated transcripts generated from read-through at the *YSY6* locus (presumably *YSY6-DEM1* chimeric RNAs) cannot be detected because degraded by the NMD pathway in the cytoplasm (see part B and figure 6B). **B**. Reb1p-dependent termination functions as a fail-safe mechanism at the *YSY6* gene. When canonical CPF/CF termination is impaired in an *rna14-3* mutant (compare lanes 1-4 to 5-8), read-through transcripts cannot be observed even upon impairment of nuclear degradation in an *rrp6Δ* background (lanes 5 and 7). However, longer and heterogeneous read-through species (RT, indicated by a black bar) are clearly visible when Reb1p-dependent termination is also impaired by metabolic depletion of Reb1p (lane 8). **C**. Same blot as in figure 6B, hybridized with a probe specific for the extended *YSY6-DEM1* chimeric species (RT). The position of the probe used is shown on the scheme. **D**. Heatmap of transcripts derived from the *YDL233W/OST4* locus. Upon depletion of Reb1p, readthrough occurs at the *YDL233W* gene (not markedly visible in these conditions, see figure 6C) and the downstream gene *OST4* is downregulated. The Reb1p binding site is indicated by a red arrow. **E**. The integrity of Reb1p function is required for normal growth when the CPF/CF termination pathway is also partially impaired. Both the *reb1-ts1* and *rna14-3* alleles are hypomorphic and grow relatively well at the permissive temperature. The double mutant is strongly impaired. **F**. The function of Reb1p in termination is required for growth. *reb1-ts1 rna14-3* cells were transformed with plasmids expressing wild type Reb1p, a truncated form of Reb1p

containing only the DNA binding domain or Nsi1p. The DNA binding domain of Reb1p is sufficient to improve growth of *reb1-ts1 rna14-3* cells.

Figure S7. A. Metasite analysis of RNAPII pausing upstream of Reb1p binding sites in the genome. Related to Figure 5E. The plots show the profile of the frequencies of polymerase pausing peaks in the 200 positions preceding the reverse complement of the Reb1 site used in figure 5E. Analysis and labeling as in figure 5E. B. Statistical analysis of the extent of RNAPII pausing at the best 109 sites showing the highest Reb1p occupancy (top sites) or at all the sites in the genome (sequence sites) irrespective of occupancy. The RNAPII peak frequency at the nucleotide corresponding to the maximum of the profile (position -17 relative to the site) is plotted and compared with the distribution of values obtained from randomly sampling equivalent number of sites from the genome. Error bars in the random distribution correspond to the standard deviation generated by the simulation.

## Supplemental Experimental Procedures

### RNA analysis

Northern blot analyses were performed with standard procedures, using 5% acrylamide/7.5M urea or 1.2% agarose/0.67%formaldehyde gels. RNAs were transferred to Hybond N+ membrane (GE Healthcare) and probed with 5' end-labeled oligonucleotides or PCR fragments labeled by random priming (Megaprime kit, GE Healthcare). Hybridizations were performed in UltraHyb or UltraHyb-Oligo (Ambion) commercial buffers at 42°C. Analysis of the polyadenylation status of transcripts was performed either by cleaving the poly(A) tail with RNaseH (Invitrogen) and oligo(dT) or by affinity selection of poly(A)<sup>+</sup> species with oligo(dT)-Dynabeads (Invitrogen) according to the manufacturer instructions. 3' RACE were performed using a commercial kit (Ambion) according to the manufacturer instructions.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (Rougemaille et al., 2008). Immunoprecipitations were performed with anti-HA (F-7) or anti-Rpb1(Y-80) antibodies from Santa Cruz. Data presented are the average of at least three biological replicates and error bars represent standard deviations.

### Analyses of the selected pool of sequences

Sequences containing Reb1p-dependent terminators were first identified by visual detection of the Reb1p binding site in a low number of clones generated by manual sequencing. A larger pool was subsequently assembled from large scale sequencing data. The sequence logo was generated with Weblogo (<http://weblogo.berkeley.edu/logo.cgi>) using 83 sequences previously aligned with Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

Nucleotides flanking the core Reb1p binding sites were statistically analyzed using a subset of the selected sequences containing only the most highly represented clones (i.e. each clone representing at least 2% of the selected pool) and the total pool of naive sequences (1,431,308 unique sequences) to estimate background frequencies. Selected sequences were purged to prevent biases due to single nucleotide variants of the same clone (Thomas-Chollier et al., 2011). A total of 1025 sequences were present in the Reb1p binding sites enriched pool. The analysis was performed by extracting all the occurrences of the consensus core Reb1p binding site "TTACCCG", plus 4 nt on each side, from both the naive and the enriched pool (1828 and 45 sequences respectively). For each flanking position, we

plotted in figure S1A the  $\log_2$  ratio of the frequency of each nucleotide in the enriched versus the naive pool.

### ***In vitro* analysis of Reb1p nucleic acid binding and function in termination**

rReb1 and rReb1-DBD were produced and purified as previously described (Porrua and Libri, 2013) Electrophoretic mobility shift assays (EMSA) were performed using recombinant proteins and 5'-labelled probes of same sequence (5'-ATGATGACCTAGAT**GTTACCCGGGTAAAGAGCCCCATTAT** -3') in double stranded DNA, single stranded DNA, or RNA forms. The mutant probe has the sequence (5'-ATGATGACCTAGAT**accgaagcc**GTAAAGAGCCCCATTAT -3'). *In vitro* transcription termination assays were performed essentially as previously described (Porrua and Libri, 2013).

### **Transcriptome analyses by tiling microarrays**

RNAs for tiling arrays hybridizations were prepared from strains containing the *P<sub>GAL1</sub>-REB1* construct (or the endogenous *REB1* gene as a control) after 2 hours of growth in glucose to minimize indirect effects. At this time point Reb1p depletion induces a clear termination defect (Figure S1C), but no marked effects on growth can be observed. Hybridizations and analyses were performed as previously described (Xu et al., 2011) Briefly, total RNA was treated with RNase-free DNaseI using Turbo DNA-free kit (Ambion). For first-strand cDNA synthesis, 20 µg of total RNA was mixed with 1.72 µg of random hexamers, 0.034 µg of oligo(dT) primer and incubated at 70°C for 10 min followed by 10 min at 25°C, then transferred on ice. The synthesis included 2,000 units of SuperScript II Reverse Transcriptase, 50 mM TrisHCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 0.01 M DTT, dNTP + dUTP mix (0.5 mM for dCTP, dATP and dGTP; 0.4 mM for dTTP and 0.1 mM for dUTP, Invitrogen), 20 µg/mL actinomycin D in a total volume of 105 µL. The reaction was carried out in 0.2 mL tubes in a thermal cycler with the following thermal profile: 25°C for 10 min, 37°C for 30 min, 42°C for 30 min followed by 10 min at 70° for heat inactivation and 4°C on hold. Samples were then subjected to RNase treatment of 20 min at 37°C (30 units RNase H, Epicentre, 60 units of RNase Cocktail, Ambion). First-strand cDNA was purified using the MinElute PCR purification kit (Qiagen) and 5 µg were fragmented and labeled using the GeneChip WT Terminal labeling kit (Affymetrix) according to manufacturer's protocol. The labeled cDNA samples were denatured in a volume of 300 µl containing 50 pM control oligonucleotide B2 (Affymetrix) and Hybridization mix (GeneChip Hybridization, Wash and Stain kit, Affymetrix) of which 250 µl were hybridized per array (*S. cerevisiae* yeast tiling array, Affymetrix, PN 520055). Hybridizations were carried out at 45°C for 16 h with 60 rpm rotation. The staining was carried out using the GeneChip Hybridization, Wash and Stain kit with fluidics protocol FS450\_0001 in an Affymetrix Fluidics station.

Table of strains used in this study

strain	genotype	Source/reference
W303	<i>ura3-1, ade2-1, his3-11,5, trp1-1, leu2-3,112, can1-100</i>	(Thomas and Rothstein, 1989)
DL671 BMA64	<i>ura3-1, ade2-1, his3-11,5, trp1Δ, leu2-3,112, can1-100</i>	(Baudin et al., 1993)
DLY678	as BMA64, MAT $\alpha$ , <i>trf4::KAN<sup>R</sup>, rrp6::URA3</i>	
DLY815	as BMA64, MAT $\alpha$ , <i>rrp6::KAN<sup>R</sup></i>	
DLY885	as W303, MAT $\alpha$ , <i>HIS::P<sub>GAL1</sub>-NRD1, rrp6::KAN<sup>R</sup></i>	(Thiebaut et al., 2006)
DLY1582	as W303, MAT $\alpha$ , <i>hsp104::LEU CUP1::KAN<sup>R</sup></i>	
DLY1583	as W303, <i>hsp104::LEU cup1::KAN<sup>R</sup> rrp6::KAN<sup>R</sup></i>	
DLY1650	as W303, <i>HIS::P<sub>GAL1</sub>-NRD1, rrp6::KAN<sup>R</sup></i>	
DLY1905	as BMA64, <i>reb1-ts1</i>	This study
DLY1912	as BMA64, MAT $\alpha$ , <i>HIS::P<sub>GAL1</sub>-HA-REB1</i>	This study
DLY1914	as BMA64, MAT $\alpha$ , <i>HIS::P<sub>GAL1</sub>-HA-REB1 rrp6::KAN<sup>R</sup></i>	This study
DLY1968	as BMA64, <i>nsi1::URA3kl rrp6::KAN<sup>R</sup></i>	This study
DLY1970	as BMA64, <i>HIS::P<sub>GAL1</sub>-HA-REB1 nsi1::URA3kl, rrp6::KAN<sup>R</sup></i>	This study
DLY1986	<i>reb1-ts1 rna14-3</i>	This study
DLY2109	<i>HIS::P<sub>GAL1</sub>-HA-REB1 rna14-3</i>	This study
DLY2110	<i>HIS::P<sub>GAL1</sub>-HA-REB1, rna14-3, rrp6::KAN<sup>R</sup></i>	This study
DLY2210	as BMA64, MAT $\alpha$ , <i>HIS::P<sub>GAL1</sub>-HA-REB1, dis3-D551N-protA::TRP1kl</i>	This study
DLY2213	as BMA64, <i>HIS::P<sub>GAL1</sub>-HA-REB1, his5::LEU2cg, rrp6::URA3</i>	This study
DLY2240	as W303, <i>cul3::TRP</i>	
DLY2241	W303	
DLY2242	as W303, <i>rsp5-1::HIS</i>	(Harreman et al., 2009)
DLY2275	<i>HIS::P<sub>GAL1</sub>-HA-REB1, rsp5-1::HIS</i>	This study
DLY2304	<i>HIS::P<sub>GAL1</sub>-HA-REB1, upf1::TAP::TRP1kl</i>	This study
DLY2305	<i>HIS::P<sub>GAL1</sub>-HA-REB1, upf1::TAP::TRP1kl, dis3-D551N-protA::TRP1kl</i>	This study
DLY2351	<i>HIS::P<sub>GAL1</sub>-HA-STH1, rrp6::KAN<sup>R</sup></i>	This study

Table of plasmids used in this study

pDL431	pCM190(TRP1)- <i>P<sub>TET</sub></i> -HSP104- <b>X3</b> -HSP104- <i>P<sub>GAL1</sub></i> -LACZ
pDL438	pCM190(TRP1)- <i>P<sub>TET</sub></i> -HSP104-“ <b>GTTACCCGG</b> ”-HSP104- <i>P<sub>GAL1</sub></i> -LACZ
pDL457	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X9</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL459	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X18</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL460	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X20</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL641	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X25</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL642	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X28</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL643	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X31</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL513	pCM188
pDL537	pCM188- <b><i>P<sub>REB1</sub></i>-REB1</b>
pDL538	pCM188- <b><i>P<sub>REB1</sub></i>-reb1-DBD</b>
pDL539	pCM188- <b><i>P<sub>REB1</sub></i>-NSI1</b>
pDL550	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104-“ <b>GTTACCCGG</b> ”-HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL551	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X3mut</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL552	pCM190(URA3)- <i>P<sub>TET</sub></i> -HSP104- <b>X3rc</b> -HSP104- <i>P<sub>GAL1</sub></i> -CUP1
pDL665	pCM190- <b>HIS5</b>
pDL572	pCM188- <i>P<sub>TET</sub></i> -HSP104- <b>X3</b> -HSP104- <i>T<sub>CYC1</sub></i>
pDL573	pCM188- <i>P<sub>TET</sub></i> -HSP104- <b>X3mut</b> -HSP104- <i>T<sub>CYC1</sub></i>

Table of oligonucleotides used in this study

name	Sequence 5'-3'	Gene	use
DL190	TTGAGCCAACGTCAAAATCGTTAGAGCCCTTCTGTAAATT GCCTTGGTCGTTCAT	HSP104	probe PNK
DL275	ATCTCTTGTAAAACGGTTCATCC	U6	probe PNK
DL377	ATGTTCCCAGGTATTGCCGA	ACT1	PCR (Klenow probe)
DL378	ACACTTGTGGTGAACGATAG	ACT1	
DL751	TTTCCCAGAGCAGCATGACT	CUP1	probe PNK
DL1198	GCGGAGATAACTCCAAGTTAT	HSP104	qPCR
DL1199	TAGAGCAAACAATATATGGTC	HSP104	qPCR
DL1202	AAGGACGACGCTGCTAACATC	HSP104	qPCR
DL1203	AGATCTATATTCTGTTATTGGT	HSP104	qPCR
DL1359	CCTTATACATTAGGTCCCTT	P <sub>TET</sub>	qPCR
DL1360	ATCCCCCGAATTGATCCGG	P <sub>TET</sub>	qPCR & RNase H
DL2812	GAATGCGGTGACCGGTATCG	HSP104-Reb1-BS	qPCR
DL2813	CCTATACATATTCTAGCTGCC	HSP104-Reb1-BS	qPCR
DL1566	AGTTGATCGGACGGGAAAC	5S	probe PNK
DL2321	TGCTAACACCAGCAGTACGTGTGCCG	HIS5	PCR (Klenow probe)
DL2322	GGTCTTGCTCAATTCACTGGAG	HIS5	
DL2323	ATAGCAGGGTGCAGAGGTG	YSY6	PCR (Klenow probe)
DL2324	ACACCACCCACTACGAGAAG	YSY6	
DL2325	TGCAAAGGACCAAAGACAGC	uATP5	PCR (Klenow probe)
DL2326	CCAGCACCGTCTCTCGAAAG	uATP5	
DL2370	CGGCACACACGTACTGCTGG	HIS5	RNase H
DL2668	CGTCGTGGTGCAGGGAGAGATACCGCTGTTGCGTCGCCT CTTTCGCGTCAAGGGC	YDL233W	probe PNK
DL2669	GGAGACATGGTGGAGTCAACAGCATGG	OST4	probe PNK
DL2840	TGGGCCGGGCACTAACAAAC	DEM1	PCR (Klenow probe)
DL2841	TCGCTCAACAATGCTCCCTCC	DEM1	
DL2910	CGCATCCATAAATGACAACGCGGGTAATACTGCC	YSY6-RT	probe PNK
DL2503	GGCTAACCGCGTGGTGCAGTACCAATGAACTATAATCTAGGT TTGATGATGACCTAGATTATAGTTCATTGT <b>TTACCCGGAC</b> TCAGAGCC	Non-template strand of IVT substrate containing a Reb1 binding site (bold)	
DL2504	GGCTCTGAGT <b>CCGGTAAC</b> ACAATGAACTATAATCTAGGT CATCATCAATTCCATACTGGCCTGGTCATTGCACCACCG CGT	Template strand of IVT substrate containing a Reb1 binding site (bold)	
DL2492	UGCAUUUCGACCAGGC	Fluorescently 5' labeled RNA oligonucleotide for promoter- independent assembly of elongation complex (IVT)	

## Supplemental References

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