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The Reb1-homologue Ydr026c/Nsi1 is required for efficient RNA polymerase I termination in yeast

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Several DNA *cis*-elements and *trans*-acting factors were described to be involved in transcription termination and to release the elongating RNA polymerases from their templates. Different models for the molecular mechanism of transcription termination have been suggested for eukaryotic RNA polymerase I (Pol I) from results of *in vitro* and *in vivo* experiments. To analyse the molecular requirements for yeast RNA Pol I termination, an *in vivo* approach was used in which efficient termination resulted in growth inhibition. This led to the identification of a Myb-like protein, Ydr026c, as *bona fide* termination factor, now designated Nsi1 (NTS1 silencing protein 1), since it was very recently described as silencing factor of ribosomal DNA. Possible Nsi1 functions in regard to the mechanism of transcription termination are discussed.

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Introduction

Transcription termination requires specific DNA and/or RNA elements that directly or indirectly interfere with the elongating RNA polymerase machinery. Properly terminated transcription results in a length restricted transcript, which is disposed for posttranscriptional modifications and the release of the RNA polymerase from the DNA template. Similarities in the structure of bacterial, eukaryotic and archaeal ternary transcription complexes indicate that termination mechanisms must overcome related energetic and structural constraints (Peters *et al*, 2011).

In bacteria, two different classes of signals are sufficient to terminate transcription. First, a stem loop in the nascent RNA in which a series of U-residues follow a G + C-rich element leads to pausing and destabilization of transcription complexes (see Larson *et al*, 2008 and reviewed in Peters *et al*, 2011). Only interactions between DNA, RNA and the RNA polymerase, but no auxiliary transcription regulator are required to trigger this intrinsic termination. The second class of signal includes binding of Rho, a homohexameric ring protein, to the nascent RNA transcript, which triggers the dissociation of the RNA polymerase from RNA and the template in an ATP-dependent manner (see Richardson, 2003 and reviewed in Peters *et al*, 2011).

Termination of many eukaryotic RNA polymerase II (Pol II) transcripts involves pausing of the enzyme and processing of the synthesized transcript (reviewed in Richard and Manley, 2009). Pol II often generates transcripts that are subsequently polyadenylated, which was reported to play an important role in transcription termination of protein-coding genes (Whitelaw and Proudfoot, 1986; Logan *et al*, 1987; Connelly and Manley, 1988) and it has been speculated that transcription through the polyA-signal induces structural changes of the elongation complex leading to its release from the DNA template (Orozco *et al*, 2002; Park *et al*, 2004; Nag *et al*, 2006). The involvement of additional termination factors was not excluded. In the last few years, a collision-based model has been developed, in which endoribonucleolytic cleavage and subsequent rapid 5′–3′ degradation of the remaining Pol II-associated transcript leads to a clash between the exonuclease and elongating Pol II releasing the polymerase from the template (see as reviews Tollervey, 2004; Kuehner *et al*, 2011). Nevertheless, no evidence for such a model was found when it was tested in a purified *in vitro* system (Dengl and Cramer, 2009). Termination of Pol II-transcripts that are not polyadenylated depends primarily on factors that contain RNA/DNA helicase activity, possibly to destabilize the ternary elongation complex (see as review Richard and Manley, 2009), thus resembling possibly Rho-dependent termination in prokaryotes.

Like many Pol II-transcripts, Pol I-synthesized ribosomal RNA (rRNA) transcripts from yeast and mammals are processed at their 3′-end during termination or shortly before transcription is terminated (Kempers Veenstra *et al*, 1986; Kuhn and Grummt, 1989; van der Sande *et al*, 1989). However, in apparent contrast to many other transcription systems, efficient Pol I termination depends on specific DNA

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elements to which protein factors are recruited. The Sal box in mammals and a 61-bp long DNA stretch in yeast are sufficient for termination *in vitro* when recognized by the DNA-binding proteins TTF-I (mammals) and Reb1 (yeast), respectively (Grummt *et al*, 1986; Bartsch *et al*, 1988; Kuhn *et al*, 1988, 1990; Ju *et al*, 1990; Lang and Reeder, 1993; Lang *et al*, 1994). Termination occurs 13 or 16 nucleotides upstream of the binding site, respectively. The binding sites for both proteins have to be correctly oriented to cause termination *in vitro* (Grummt *et al*, 1985; Lang and Reeder, 1993). Interestingly, TTF-I and Reb1 can terminate both murine and yeast Pol I transcription *in vitro* (Kuhn *et al*, 1990; Mason *et al*, 1997b). In yeast, the minimal DNA element supporting efficient Pol I termination *in vitro* contains the 11-bp long Reb1-binding site on its 3′-end and a 10–15 bp long T-rich DNA stretch 12–20 bp upstream of the Reb1-binding site (Lang *et al*, 1994; Lang and Reeder, 1995; see Figure 1A for a schematic representation). Reconstitution of termination *in vitro* from purified yeast Pol I and recombinant Reb1 suggested that DNA-bound Reb1 pauses Pol I and cooperates with the T-rich DNA stretch to release the transcript.

Despite the rather conclusive data about Reb1 function for Pol I termination *in vitro*, its role for Pol I termination *in vivo* remained ambiguous. (i) Although a 53-bp long DNA stretch containing the Reb1-binding site at the 3′-end of the rRNA gene and upstream sequences were shown to be required for efficient *in vivo* termination (Reeder *et al*, 1999), only background levels of Reb1 binding to this site *in vivo* could be detected (Kawauchi *et al*, 2008). In contrast, mammalian

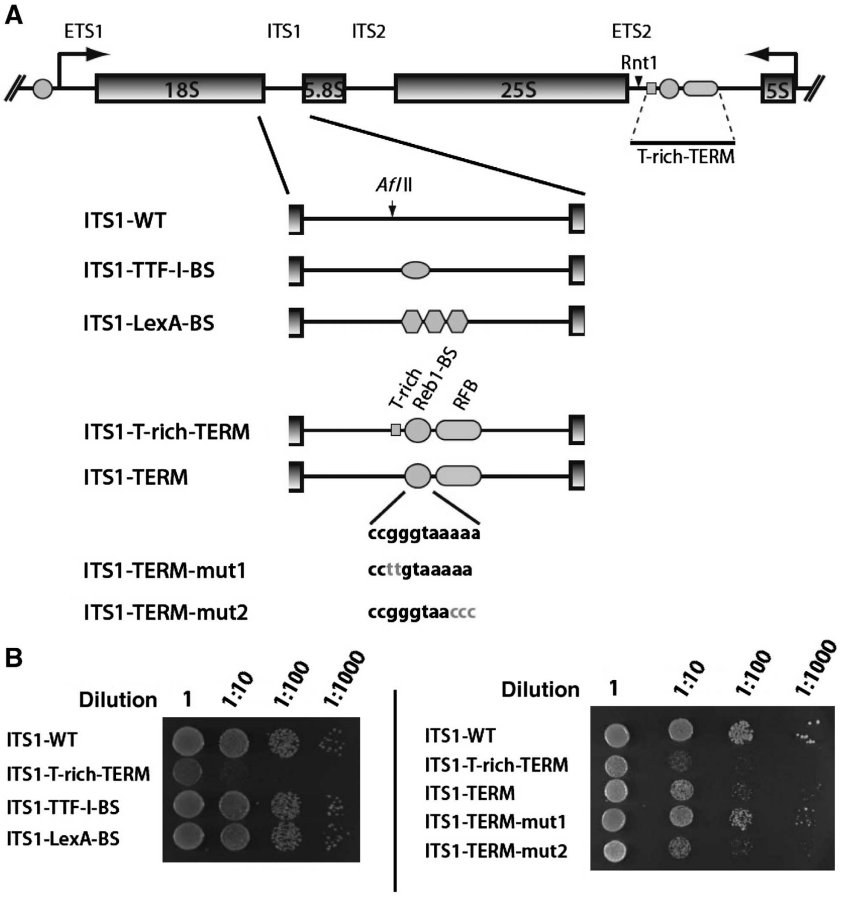


Figure 1 Establishment of an *in vivo* system to study Pol I termination. (**A**) Schematic representation of one repeat unit of the yeast rDNA locus and of the genetic modifications performed by insertion of DNA elements into ITS1. The direction of transcription at the 35S rRNA gene and 5S rRNA gene is marked with arrows. The coding sequences for mature 18S, 5.8S, 25S and 5S rRNAs (rectangles), external transcribed spacers (ETS1, ETS2), internal transcribed spacers (ITS1, ITS2), as well as a T-rich element (filled square), two predicted Reb1-binding sites (Reb1-BS; filled circle), a sequence coding for a Rnt1-cleavage site (Rnt1, arrowhead) and a RFB (filled rounded rectangle) are indicated. Additional DNA elements were inserted in the ITS1 region of every rDNA repeat at the *Afl*II restriction site: WT refers to a WT-ITS1 without insertion. ITS1-TTF-I-BS refers to an insertion of a single DNA-binding site of the mouse Pol I transcription termination factor TTF-I (filled oval). ITS1-LexA-BS refers to an insertion of a DNA-binding cluster for the bacterial LexA protein (filled hexagons). ITS1-T-rich-TERM refers to the DNA element(s) at the 3′-end of the yeast 35S rRNA gene, which has been implicated in Pol I termination, starting with a T-rich element and ending after the RFB (for description of the symbols see above). ITS1-TERM lacks the T-rich element and is referred to as the terminator element in this study. ITS1-TERM-mut1 and ITS1-TERM-mut2, respectively, are derivatives that carry mutations in the putative Reb1 DNA-binding site, which either abolish or enhance Reb1 binding *in vitro* (Lang and Reeder, 1993). The respective sequences of the WT Reb1-binding site as well as the two mutated sequences are indicated, with the altered bases labelled in grey. (**B**) Integration of the yeast terminator element into ITS1 leads to growth reduction, whereas the integration of DNA-binding sites of heterologous proteins shows no growth phenotype. Yeast strains (y1598, y1599, y2038, y2042, y2273, y2274, y2339) carrying rDNA loci with a ITS1-WT, or ITS1 DNA element insertions as described in Figure 1A (ITS1-T-rich-TERM, ITS1-TTF-I-BS, ITS1-LexA-BS, ITS1-TERM, ITS1-TERM-mut1, ITS1-TERM-mut2) were grown to an OD_{600} of 0.2 at 30°C. The same amount of cells was spotted on YPD plates in a 1:10 serial dilution series, as indicated above the panels and incubated for 2 days at 30°C.

TTF-I binds to the terminator *in vivo* (Nemeth *et al*, 2008; Lessard *et al*, 2010). (ii) A four-fold reduction of the cellular Reb1 amount *in vivo* did not significantly enrich non-terminated transcription-run-on fragments at the physiological termination site (Kawauchi *et al*, 2008). On the other hand, Reb1-depletion moderately increased non-terminated transcripts from minigene reporter constructs from which pre-rRNA processing sites have been deleted (Braglia *et al*, 2010b). (iii) Reb1 binds to many control regions of Pol II transcribed genes and is essential in yeast, which makes it difficult to analyse primary effects on Pol I termination *in vivo* (Ju *et al*, 1990; Morrow *et al*, 1993; Pinskaya *et al*, 2009). (iv) In reporter constructs, ~70% of all transcripts terminate at the T-stretch immediately before the Reb1-binding site *in vivo* (Reeder *et al*, 1999), whereas in the presence of purified recombinant Reb1 only about 20% of Pol I generated transcripts are terminated and released *in vitro*. This suggests that additional factors are required to support efficient Pol I termination *in vivo*.

In addition to the minimal stretch of DNA (61 bp) which is sufficient for termination *in vitro* when the appropriate factor is bound, further *cis*-elements appear to be involved in Pol I termination *in vivo*. Since inactivation of the endonuclease Rnt1 stabilizes non-terminated transcripts, it was suggested that cleavage of the nascent RNA at the Rnt1 site 32–65 bp upstream of the T-rich DNA stretch (59–92 bp upstream of the Reb1-binding site, see Figure 1A for a schematic representation) supports termination (Reeder *et al*, 1999; Prescott *et al*, 2004). *In vivo* non-terminated transcripts are extended either to a second—'failsafe'—terminator, which resides about 250 nt downstream of the 25S rRNA 3′-end (van der Sande *et al*, 1989; Reeder *et al*, 1999; Prescott *et al*, 2004) or to the replication fork barrier (RFB; see Figure 1A for a schematic representation), which is located about 300 bp downstreams (El Hage *et al*, 2008). Fob1, a protein binding to RFB and blocking DNA replication forks (Takeuchi *et al*, 2003; Huang *et al*, 2006), was suggested to be involved in efficient termination (El Hage *et al*, 2008). Based on these and other findings and similar to the mechanism proposed for Pol II transcription termination, a model for Pol I termination has been put forward (El Hage *et al*, 2008; Kawauchi *et al*, 2008; Braglia *et al*, 2010b). Elongating Pol I is paused in the vicinity of the Reb1-binding site. Rnt1 cleaves co-transcriptionally the nascent pre-rRNA. The 5′-end of the cleaved transcript serves then as substrate for the exonuclease Rat1, which progressively degrades the Pol I-bound transcripts with the help of the helicase Sen1 and releases Pol I from the template by an eventually collision-based mechanism. Such a *torpedo*-like termination mechanism was suggested to function even in the absence of the Rnt1-dependent cleavage. In cells carrying Pol I minigenes, which lack the Rnt1-cleavage site, a second—'failsafe'—cleavage site at the T-rich DNA stretch by a yet not identified endonuclease was proposed. Cleavage at this site then allows co-transcriptional recruitment of the exonuclease Rat1 and subsequent termination by the above mechanism (Braglia *et al*, 2010b). Recently, another factor, the polynucleotide kinase Grc3 was reported to be involved in Pol I termination (Braglia *et al*, 2010a).

Here, we established a strategy to study Pol I transcription termination *in vivo* using growth reduction of yeast as an easy read-out to determine efficient transcription termination. *Saccharomyces cerevisiae* strains can be constructed in which all of the 150–200 ribosomal DNA (rDNA) copies on chromosome XII have been genetically modified (Wai *et al*, 2000). Thus, we integrated different DNA elements within the internal transcribed spacer 1 (ITS1; see Figure 1A for a schematic representation) between the DNA sequences coding for the 18S and 5.8S rRNAs of all ~150 chromosomal rDNA repeats. If an integrated DNA element was potent in Pol I termination, rRNA synthesis was impaired leading to growth defects. This enabled us to (re-)investigate the requirements of *cis*-elements for Pol I termination. In agreement with previous reports, we observed that integration of a DNA element containing the termination site proximal of the putative Reb1p-binding site into the ITS1 leads to premature transcription termination *in vivo*. As previously reported (Lang *et al*, 1994; Lang and Reeder, 1995), the T-rich DNA stretch further increased termination efficiency also in this system. Importantly, a Myb-related protein (Ydr026c/Nsi1), which was recently described as silencing factor of ribosomal DNA (Ha *et al*, 2012) functions as efficient terminator protein *in vivo*. Our data are consistent with the results of previous *in vitro* studies and emphasize a model in which the combination of a T-rich DNA stretch and a specific termination factor (Nsi1) bound to its recognition site supports efficient Pol I transcription termination *in vivo*.

Results

A system to study termination of Pol I transcription in vivo

The budding yeast *S. cerevisiae* contains a single rDNA cluster consisting of 150–200 tandemly repeated copies of 35S rRNA genes on chromosome XII, which are transcribed by Pol I. Recently, an approach was developed allowing to genetically manipulate the yeast rDNA locus such that each single repeat unit carries the respective genetic modification (Chernoff *et al*, 1994; Wai *et al*, 2000). Using this methodology, we constructed yeast strains carrying modified rDNA loci with insertions of different DNA sequences at the ITS1 spacer element upstream of the DNA sequence coding for the A_2 processing site (we refer to this region as the ITS1 region in the remainder of the manuscript). Manipulation of the 35S rRNA gene sequence is delicate and may lead to aberrant processing of the 35S rRNA precursor affecting ribosome production and cell growth. Therefore, the ITS1 region was chosen, since previous reports showed that depletion of parts within the ITS1 do not necessarily affect ribosome biogenesis and cellular growth (Lindahl *et al*, 1994; Liang and Fournier, 1997). In fact, pilot experiments were carried out, showing that integration of random DNA-elements had none of the above consequences (data not shown). Figure 1A shows a schematic representation of one rDNA repeat with the integration site and the various inserted DNA-fragments. Correct integration of the respective DNA sequences was confirmed by PCR from genomic DNA and subsequent sequencing (data not shown). Since premature transcription termination at the integration site will lead to the production of incomplete rRNA precursors, impaired ribosome biogenesis and thus result in growth defects, this set-up provides a straightforward and sensitive read-out system to identify DNA elements with the potential to terminate Pol I transcription.

Yeast strains were established bearing either rDNA repeats with a wild-type (WT) ITS1 region (Figure 1A, ITS1-WT), or

an ITS1 region with integrated binding sites for the bacterial LexA protein (Figure 1A, ITS1-LexA-BS) or the murine transcription termination factor TTF-I (Figure 1A, ITS1-TTF-I-BS). Furthermore, a 322-bp long DNA stretch from the 3′-end of the rRNA gene containing the T-rich DNA stretch, the Reb1-binding site, and the RFB (Figure 1A, ITS1-T-rich-TERM) and the same fragment without the T-rich sequence were inserted into the ITS1 region. These fragments will be referred to as 'T-rich- terminator' and 'terminator' elements, respectively in this manuscript, since—although their DNA sequence was originally described as rRNA enhancer (Elion and Warner, 1986)—it was also shown to be involved in transcription termination (Kempers Veenstra *et al*, 1986; Lang and Reeder, 1993; Reeder *et al*, 1999). The integrated element lacked the sequence coding for the Rnt1-cleavage site. Incorporation of binding sites for the bacterial LexA protein, or the murine transcription termination factor TTF-I into the rDNA ITS1 region led to no detectable growth defects, when compared with a control strain with a rDNA locus carrying no insertion in ITS1 (Figure 1B, compare ITS1-LexA-BS, and ITS1-TTF-I-BS with ITS1-WT; Supplementary Table 1). In contrast, integration of the yeast T-rich-terminator element resulted in significantly reduced growth (Figure 1B, compare ITS1-T-rich-TERM with ITS1-WT; Supplementary Table 1).

Next, we asked if the T-rich DNA stretch and the Reb1-binding site contribute to growth reduction in this *in vivo* system. To this end, we analysed yeast strains with truncated or mutated versions of the terminator element inserted in ITS1 (Figure 1A; Supplementary Table 1, ITS1-TERM, ITS1-TERM-mut1, ITS1-TERM-mut2). Deletion of the T-rich DNA stretch attenuated the growth defect observed in the strain carrying the full terminator element (Figure 1B; Supplementary Table 1, compare ITS1-TERM with ITS1-T-rich-TERM). A mutation of the Reb1-binding site, which was shown to abolish Reb1 binding *in vitro* (Lang and Reeder, 1993), resulted in almost no growth reduction (Figure 1B; Supplementary Table 1, ITS1-TERM-mut1). In contrast, a mutation in the Reb1-binding site increasing the binding affinity to Reb1 (Lang and Reeder, 1993) caused a stronger growth defect even in the absence of the T-rich DNA stretch similar to that observed after insertion of the full terminator element (Figure 1B; Supplementary Table 1, compare ITS1-TERM-mut2 with ITS1-T-rich-TERM). Taken together, these findings are consistent with previous studies, indicating that a functional Reb1-binding site is required for efficient Pol I transcription termination and that the presence of the T-rich DNA stretch increases the efficiency of transcription termination both *in vitro* (Lang *et al*, 1994; Lang and Reeder, 1995) and *in vivo* (Reeder *et al*, 1999; Braglia *et al*, 2010b).

Ydr026c is recruited in vivo to the rDNA terminator element

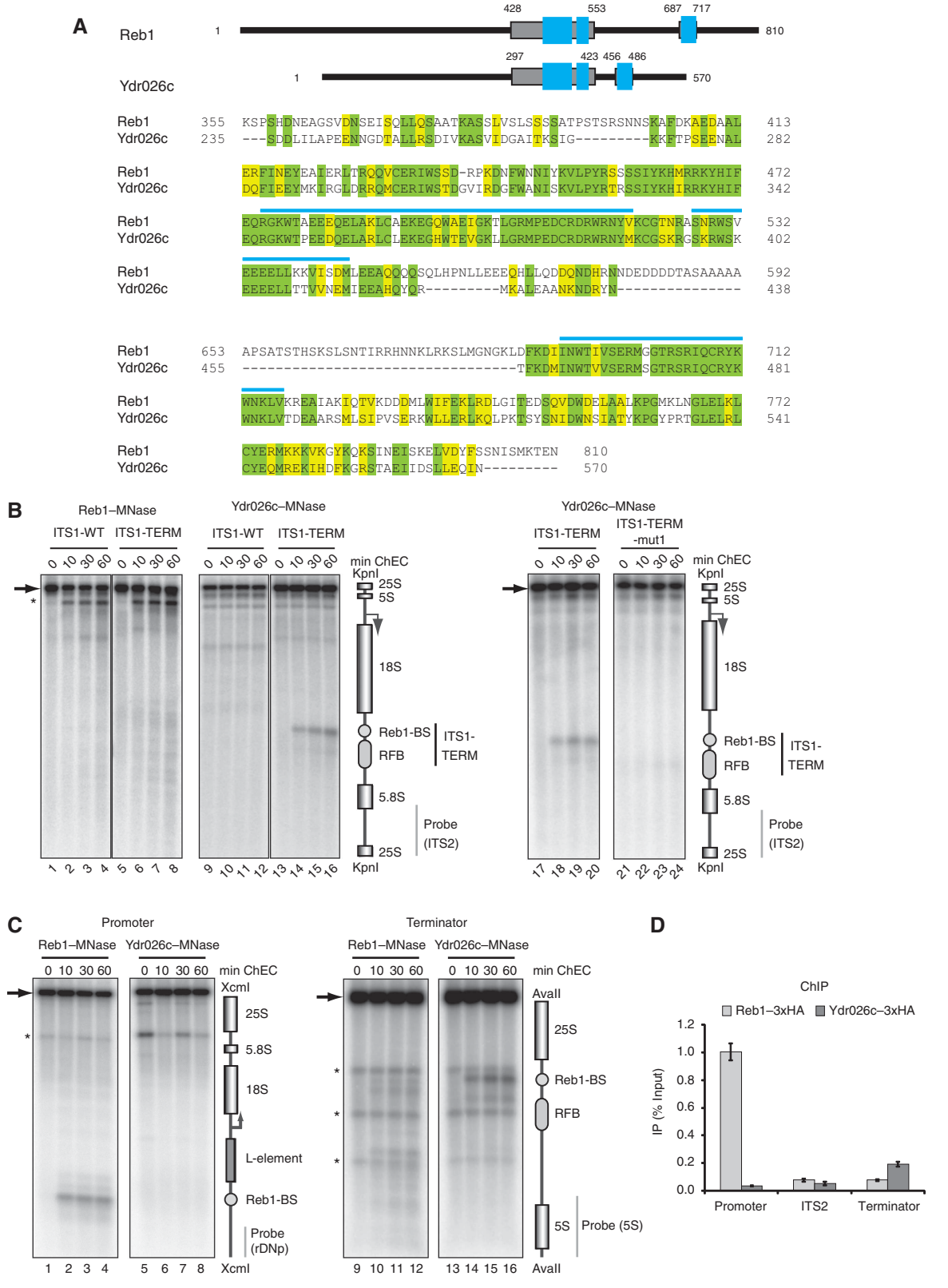
Transcription termination of Pol I *in vitro* can be induced by DNA-associated proteins like the Myb-like protein Reb1 or its mammalian counterpart TTF-I, which bind to the respective *cis*-elements in yeast or in mammals. A possible explanation for the growth defect after integration of the terminator element into ITS1 is that formation of DNA–protein complexes interferes with transcription elongation, resulting in pausing or premature termination. Therefore, we searched for proteins bound to the integrated terminator *in vivo*. Among the candidates we analysed were the yeast RNA Pol I enhancer binding protein Reb1 (Ju *et al*, 1990) and the replication fork binding protein Fob1 (Kobayashi and Horiuchi, 1996). Furthermore, we included the gene product of the yeast ORF YDR026c, which interacts with Fob1 in two-hybrid assays (Mohanty and Bastia, 2004) and shows sequence homology to Reb1, TTF-I and other Myb-related proteins (Figure 2A and data not shown). To monitor recruitment of the candidate proteins to the integrated terminator element, we employed chromatin endogenous cleavage (ChEC) (Schmid *et al*, 2004; Merz *et al*, 2008). This method allows to precisely map association of proteins with DNA within large genomic areas. A factor of interest is expressed as a MNase fusion protein from its endogenous chromosomal locus. Cells are treated with formaldehyde to crosslink the respective proteins to the DNA. After nuclei isolation calcium is added, thus activating the MNase that cleaves in close proximity of the specific binding site of the fusion protein. After DNA isolation cleavage events can be monitored at any genomic locus of interest by Southern blot using the indirect endlabelling technique. ChEC analyses revealed cleavage by Ydr026c–MNase (Figure 2B) and Fob1–MNase (data not shown) in strains carrying the terminator element integrated within ITS1 but not in strains with a WT-ITS1 region (Figure 2B, Ydr026c–MNase, compare ITS1-TERM with ITS1-WT). Cleavage by Ydr026c–MNase was strongly reduced when the Reb1-binding site was mutated (Figure 2B, ITS1-TERM-mut1). Thus, binding of Ydr026c at the integrated terminator element correlates with the observed growth defect (see Figure 1B).

In contrast, cleavage by the Reb1–MNase fusion protein was (if at all) barely detectable at the integrated terminator element (Figure 2B, Reb1–MNase), whereas robust cleavage of the fusion protein was observed at the Pol I promoter-proximal Reb1-binding site in both, the strain carrying a terminator element or no insertion in ITS1 (Figure 2B, Reb1–MNase, asterisk on the left).

We also compared binding of Reb1 and Ydr026c at the natural terminator element and the Reb1-binding site in close proximity of the Pol I promoter of the rRNA gene in strains carrying rDNA loci with a WT-ITS1. ChEC and chromatin immunoprecipitation (ChIP) experiments indicated that Reb1 preferentially associates with its binding site upstream of the rDNA promoter but only poorly with its binding site within the termination element (Figure 2C and D, see also Supplementary Figure S1). This is in good agreement with other studies investigating Reb1 interaction with the rDNA (Kawauchi *et al*, 2008; Goetze *et al*, 2010), and with the fact that depletion of the cellular Reb1 pool does not impair Pol I termination *in vivo* in a WT background (Kawauchi *et al*, 2008; Braglia *et al*, 2010b). It is also consistent with earlier findings, demonstrating that Reb1 has a higher affinity for the promoter-proximal binding site than for the binding site within the terminator region in *vitro* (Morrow *et al*, 1989; Chasman *et al*, 1990). Conversely, Ydr026c was detected at the Reb1-binding site within the termination element (see also Ha *et al*, 2012), but not at the Reb1-binding site near the Pol I promoter (Figure 2C and D, see also Supplementary Figure S1). Thus, the above analyses suggest that the Reb1-homologue Ydr026c is mainly associated with the rDNA terminator element whereas Reb1 binds preferentially to the promoter-proximal DNA-element *in vivo*.

Ydr026c is required for growth reduction in strains carrying the integrated terminator element

We observed a good correlation between Ydr026c binding to the terminator element integrated in ITS1 and the observed growth reduction in this strain (Figures 1B and 2B, ITS1-TERM; Supplementary Table 1). If Ydr026c is required for the observed growth defect, the defect should be rescued by YDR026C deletion. YDR026C was deleted in yeast strains carrying either an rDNA locus with a WT-ITS1 (ITS1-WT *ydr026c*Δ) or an rDNA locus with the terminator element lacking the T-rich DNA stretch integrated in ITS1 (ITS1-TERM-*ydr026c*Δ). Both strains and the corresponding strains carrying a WT YDR026C locus were transformed with vector pGAL-YDR026C bearing the YDR026C coding sequence under



the control of the galactose inducible GAL1 promoter. Growth was tested on selective agar plates in the presence of glucose or galactose as carbon source. Disruption of YDR026C suppressed the reduced growth in the strain with the terminator element integrated in ITS1 when ectopic expression of YDR026c was repressed in the presence of glucose (Figure 3, panel GLC; Supplementary Table 1). Induction of YDR026c expression on galactose containing plates restored a strong growth defect in the strain carrying the integrated terminator element when compared with the strain carrying a reconstituted rDNA locus with no insertion in ITS1 (Figure 3, panel GAL; Supplementary Table 1). Strikingly, the growth defect caused by the terminator element in ITS1 was significantly enhanced when YDR026c was overexpressed (Figure 3, compare ITS1-TERM in panels GLC and GAL, see also Supplementary Table 1). Altogether these analyses strongly suggested that recruitment of Ydr026c to the terminator element integrated at the ITS1 of the rDNA is required to evoke the observed growth inhibition.

Specific recruitment of heterologous DNA-binding factors to the rDNA ITS1 region may affect yeast cell growth

Several reports demonstrated that the association of a DNA-binding protein with DNA *in vitro* is sufficient to pause and terminate Pol I-transcription (Kuhn *et al*, 1990; Jeong *et al*, 1995). Therefore, we investigated whether recruitment of heterologous DNA-binding proteins to the yeast ITS1 region of the rDNA locus *in vivo* leads to growth inhibition. The proteins selected were the *Escherichia coli* transcriptional repressor LexA and the myb-like murine Pol I transcription termination factor TTF-I, which were both expressed as MNase fusion proteins to allow detection of their binding at the rDNA locus by ChEC. Expression of these fusion proteins from plasmids under the control of the galactose inducible GAL1 promoter in yeast strains carrying a WT-ITS1 within rDNA locus did not affect growth (Figure 4A, panel GLC, compare ITS1-WT pGAL (empty vector) with pGAL-TTF-I–MNase, and pGAL-LexA–MNase, see also Supplementary Table 1). However, galactose-dependent expression of TTF-I, but not of LexA–MNase fusion proteins led to (slight but) detectable growth inhibition in strains carrying the respective protein-binding DNA elements inserted in ITS1 (Figure 4A, panel GAL, compare ITS1-TTF-I-BS and ITS1-LexA-BS; Supplementary Table 1). Interestingly, ChEC analyses demon-

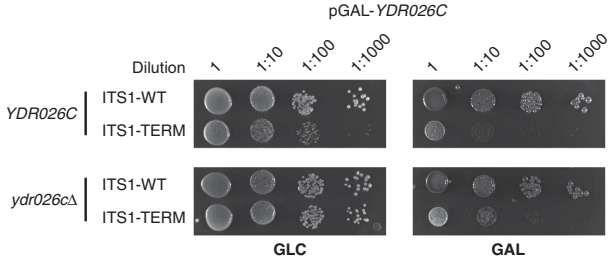


Figure 3 Growth defects observed in yeast strains carrying the terminator element integrated at the rDNA ITS1 region depend on expression of Ydr026c. Yeast strains (y1599, y2042, y2276, y2281) carrying rDNA loci with a ITS1-WT, or an ITS1 DNA element insertion as described in Figure 1A (ITS1-TERM), and being WT in YDR026C or being deleted in the gene (*ydr026c*Δ) were transformed with plasmid 1803 (pGAL-*YDR026C*) for galactose inducible expression of the protein. Cells were grown in selective complete media lacking leucine (SC-LEU) and containing raffinose to an OD_{600} of 0.2 at 30°C. The same amount of cells was spotted on SC-Leu plates containing either glucose (GLC) or galactose (GAL) as carbon source in 1:10 serial dilution series, as indicated above the panels and incubated for 2–3 days at 30°C.

Figure 2 The Reb1-homologue Ydr026c associates with the yeast terminator element *in vivo*. (**A**) Schematic representation and amino-acid sequence alignment of Ydr026c and Reb1. Regions sharing homology with the Myb-DNA-binding domain are depicted as blue boxes in the schematic representation or as blue lines on top of the alignment. Yellow or green rectangles denote similar or identical amino-acid residues, respectively. (**B**) ChEC analyses reveal Ydr026c binding at the terminator element integrated at ITS1 *in vivo*. Yeast strains (y2050, y2054, y2090, y2093, y2241) carrying rDNA loci with a ITS1-WT, or ITS1 DNA element insertions as described in Figure 1A (ITS1-TERM, ITS1-TERM-mut1), and expressing either Reb1–MNase, or Ydr026c–MNase fusion proteins from the respective chromosomal gene locus, were grown to exponential phase. After formaldehyde crosslinking, cells were harvested and crude nuclei were isolated. The nuclei suspension was incubated in the absence (0) or presence of calcium activating DNA cleavage by MNase fusion proteins for the times indicated on top of the panels (min ChEC). DNA was isolated, linearized with the indicated restriction enzyme (*Kpn*I), separated in an agarose gel and analysed in a Southern blot by indirect endlabelling using the indicated probe (*Kpn*I (ITS2)) (Supplementary Table 4). The cartoon on the right shows a map of the corresponding rDNA-fragment of around 10 kb. The positions of the 18S, 5.8S and 25S rRNA coding sequences, of the integrated terminator element or derivative (ITS1-TERM, ITS1-TERM-mut1) and of the target sequence of the radioactive probe are depicted. An arrow on the left marks the position of the full-length *Kpn*I fragment. An asterisk on the left labels an Reb1–MNase mediated cleavage event at the promoter-proximal Reb1-binding site. (**C**) ChEC analysis reveals specific Ydr026c–MNase mediated cleavage at the terminator element located at the 3′-end of the rRNA gene. (y2050, y2090) carrying rDNA loci with a ITS1-WT and expressing either Reb1–MNase, or Ydr026c–MNase fusion proteins from the respective chromosomal gene locus, were grown to exponential phase. ChEC analysis were performed as in (**B**); however different restriction enzymes and probes (Supplementary Table 4) were used to analyse the 35S rRNA gene 5′-end and upstream region (panels labelled 'Promoter'), and the 35S rRNA gene 3′-end and downstream region (panels labelled 'Terminator'), respectively. The cartoon on the right shows a map of the corresponding rDNA-fragments of around 5.6 and 2.1 kb, respectively. The positions of the 5S, 5.8S, 18S and 25S rRNA coding sequences, Reb1-binding sites, the RFB and the transcription start sites (arrows) and of the target sequence of the radioactive probe are depicted. The L-element refers to the left flanking region of the yeast rDNA locus, which is part of the IGS in yeast strains carrying the reconstituted rDNA loci. An arrow on the left marks the position of the full-length *Xcm*I and *Ava*II fragments. Asterisks on the left label DNA-fragments of unknown origin, potentially cross-hybridizing with probes rDNp or 5S. We note an additional band between RFB and 5S, which appears after Calcium addition to nuclei from cells expressing an Reb1–MNase fusion protein. This fragment might be a product of non-specific MNase cleavage at a hypersensitive site. (**D**) ChIP reveals a reciprocal relationship of Reb1 and Ydr026c binding at the promoter and at the 3′-end of the 35S rRNA gene. Yeast strains (y2050, y2090) expressing Ydr026c–, and Reb1–MNase fusion proteins with a C-terminal triple HA-tag were grown to exponential phase. Cells were treated with formaldehyde and ChIP experiments were performed as described in Materials and methods. The amounts of specific DNA-fragments present in the input and retained on the beads were determined by quantitative PCR with primer pairs amplifying the promoter region (969/970, see region 1 in the schematic representation in Figure 5), the ITS2 region (2864/2865, see region 4 in the schematic representation in Figure 5), or the terminator element (2884/2885, see region 7 in the schematic representation in Figure 5). The bar graphs depict percent of total input DNA retained after ChIP of the respective triple HA-tagged protein (see legend of the graph). Error bars represent the standard deviation of three independent ChIP experiments, each of which was analysed in triplicate quantitative PCR reactions.

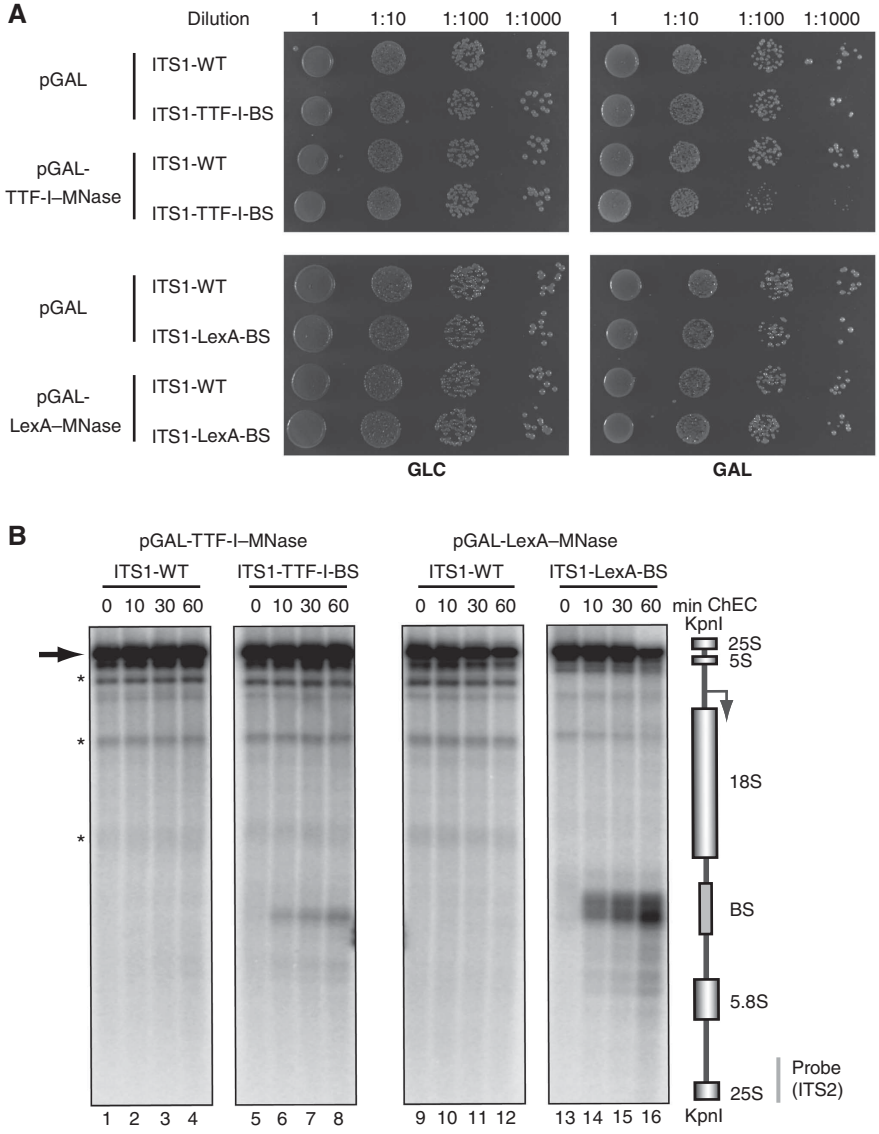


Figure 4 Binding of mouse TTF-I, but not of bacterial LexA to their respective binding sites integrated at ITS1 of yeast rDNA leads to growth defects. (**A**) Yeast strains carrying TTF-I-binding sites integrated at ITS1 of the rDNA are impaired in growth upon heterologous inducible expression of mouse TTF-I protein. Yeast strains (y1598, y1599, y2038) carrying rDNA loci with a ITS1-WT, or ITS1 DNA element insertions as described in Figure 1A (ITS1-TTF-I-BS, ITS1-LexA-BS), were transformed with plasmids 230, 1806, 1808 (pGAL, pGAL-LexA–MNase, pGAL-TTF-I–MNase) as a control, or for galactose inducible expression of the respective DNA-binding protein. Cells were grown in selective complete media lacking leucine (SC-LEU) and containing raffinose to an OD600 of 0.2 at 30°C. The same amount of cells was spotted on SC-Leu plates containing either glucose (GLC) or galactose (GAL) as carbon source in 1:10 serial dilution series, as indicated above the panels and incubated for 2 and 3 days at 30°C, respectively. (**B**) Heterologous expression of TTF-I–MNase and LexA–MNase leads to specific cleavage events at their binding sites integrated at ITS1 of the rDNA. Yeast strains (y1598, y1599, y2038) carrying rDNA loci with a ITS1-WT, or ITS1 DNA element insertions as described in Figure 1A (ITS1-TTF-I-BS, ITS1-LexA-BS), were transformed with plasmids 1806, 1808 (pGAL-LexA–MNase, pGAL-TTF-I–MNase) for galactose inducible expression of the respective DNA-binding protein. Cells were grown in selective complete media lacking leucine (SC-LEU) and containing raffinose to an OD600 0.3, before galactose was added to a final concentration of 2% and incubation was continued for 4 h at 30°C. Cells were subjected to ChEC analysis as described in the legend to Figure 2B. An arrow on the left marks the position of the full-length *Kpn*I fragment. Asterisks on the left label DNA-fragments of unknown origin, potentially cross-hybridizing with probe ITS2.

strated that both MNase fusion proteins bind to their recognition sequence within ITS1 (Figure 4B). The observation that binding of mammalian TTF-I within the yeast ITS1 correlates with growth inhibition supports the conclusion drawn from previous *in vitro* studies that binding of the mammalian TTF-I to the template interferes with transcription elongation of yeast Pol I (Kuhn *et al*, 1990; Mason *et al*, 1997b). The DNA element containing the TTF-I-binding site in our study included neither a yeast-specific termination sequence, nor the mammalian T-rich-sequence upstream the TTF-I-binding site, which has been shown to support termination *in vitro* (Mason *et al*, 1997a). Our results suggest that the growth defect observed after insertion of certain sequences into ITS1 depends on the nature of the bound protein. Thus, association of the bacterial LexA protein with its binding sites inserted into ITS1 does not lead to growth reduction and therefore seems not to efficiently interfere with Pol I transcription elongation.

Ydr026c is required for efficient termination of Pol I transcription in vivo

Next, we wanted to analyse more directly whether Ydr026c is involved in termination of yeast Pol I transcription at the natural terminator element at the 3′-end of the rRNA gene. Using ChIP analysis followed by quantitative PCR, we investigated co-precipitation of rDNA-fragments with Pol I in yeast strains carrying a WT allele or a complete deletion of YDR026C (ITS1-WT *ydr026c*Δ and ITS1-WT). In the presence of Ydr026c, co-precipitation of DNA-fragments immediate downstream of the Reb1-binding site with Pol I was close to background levels (5S gene), whereas they efficiently co-precipitated with Pol I in the absence of Ydr026c (Figure 5A, ITS1-WT *ydr026c*Δ and ITS1-WT; compare amplicons 9 and 10 immediate downstream of the Reb1-binding site, with amplicon 8 upstream of the Reb1-binding site and amplicons 13 in the 5S gene and 14 in the PDC1 gene (background)). This indicates that Pol I reads through the terminator element in the YDR026c deletion strain. Very similar results were obtained when the terminator element was integrated in the ITS1 region. Efficient co-precipitation of rDNA-fragments with Pol I downstream of the integration site was only observed in the YDR026C deletion background (Figure 5A, ITS1-TERM and ITS1-TERM-*ydr026c*Δ; compare amplicon 4/5 with 6). Consistent with the previous results, Pol I read through was also observed at the termination element integrated in ITS1 in the YDR026C deletion background (Figure 5A, ITS1-TERM-*ydr026c*Δ; compare amplicons 8 and 9 with amplicon 10).

To investigate whether Pol I associated with rDNA regions downstream of the physiological terminator in the YDR026C deletion strain is still transcriptional active, transcriptional run on (TRO) experiments were performed (Figure 5B). Nuclei were prepared from cells carrying an YDR026C WT locus or a complete deletion of the gene. After addition of radiolabelled nucleotides elongating Pol I molecules stalled on the rRNA genes upon nuclei preparation are allowed to continue RNA chain elongation for a limited time. Thus, the synthesis of radiolabelled RNA during TRO should be proportional to the number of active Pol I complexes still associated with the template DNA. After RNA extraction radioactive transcripts were hybridized to a membrane with immobilized single-stranded DNA-probes corresponding to the PCR-fragments analysed in the ChIP analysis depicted in Figure 5 (for details, see Supplementary data). Radioactive signals on the blot were determined for each DNA probe. Since Pol I occupancy at the 5′-region of the 35S rRNA gene was comparable in both strains (Figure 5A, amplicon 3), we used the radioactive signal obtained with amplicon 3 for normalization (Figure 5B, see bar graph for quantitative analysis). For RNAs isolated from both stains, the hybridization signals were very similar for DNA probes 1–8 spanning a region upstream of the Pol I promoter to the end of the 25S rRNA coding sequence. In contrast, signals obtained for hybridization with amplicons 9–12 covering a sequence downstream of the termination element were significantly higher in the case of RNAs isolated from nuclei of the YDR026C deletion strain (Figure 5B, see bar graph for quantitative analysis).

Altogether, these data suggest that Ydr026c binding to the rDNA terminator element is required to efficiently terminate transcript elongation and Pol I–rDNA association.

Previously, Pol I association with single rRNA genes was investigated by chromatin spreading and subsequent electron microscopy (McKnight and Miller, 1976; French *et al*, 2003). When applying this approach to a yeast strain carrying the terminator element integrated in the ITS1 region, an increased population of rRNA genes with atypical appearance was evident. In these analyses rRNA genes appear as characteristic Christmas-tree-like structures, with RNA Pol I molecules forming the electron-dense stem of the tree, whereas nascent rRNA transcript extend as the branches of the tree from the central Pol I molecules. Two representative Christmas-tree-like structures for WT and mutant yeast cells are depicted in Figure 5C. In comparison to Christmas-tree-like structures in a yeast strain carrying a WT-ITS1, the average tree length in the strain carrying the terminator region integrated in ITS1 was almost two-fold shorter (Figure 5C, see also Supplementary Figure S2 for the total data set analysed and statistical evaluation). Shortening of these tree-like structures in nucleolar regions of chromatin spreads can be explained by premature termination of transcription elongation at terminator elements integrated in the rDNA ITS1 region. Consistent with this, Northern blot analyses indicated that the ratio of 25S rRNA to 18S rRNA was reduced in strains in which the terminator element was integrated in the ITS1 region (Figure 5D). A decrease in the 25S to 18S ratio was observed in the strains containing the terminator element with and without T-rich DNA stretch (Figure 5D, compare ITS1-WT, with ITS1-TERM and ITS1-T-rich-TERM). In contrast, imbalance of 18S rRNA and 25S rRNA was partially abrogated in a yeast strain carrying a terminator element mutated in the Reb1-binding site integrated in the ITS1 region (ITS1-TERM-mut1).

Together the above analyses all support the idea that integration of the 322-bp long terminator element within ITS1 is sufficient to trigger efficient premature termination and to reduce Pol I occupancy. To execute transcription termination, it requires a functional Reb1-binding site. The presence of the T-rich DNA stretch supports efficiency of termination but is not absolutely required. Our data further indicate that recruitment of Ydr026c to the rDNA terminator element is an important prerequisite to ensure efficient termination of Pol I transcription *in vivo*.

Discussion

Analysing Pol I termination in vivo

Several approaches exist to study Pol I termination *in vivo*. Many of them depend on analysis of RNA synthesized from the chromosomal rDNA locus or from episomal reporter constructs. Others, as for example ChIP or Miller chromatin spread analyses, investigate termination by mapping Pol I molecules on the rDNA sequence on a population average or single molecule basis, respectively. Here, we developed an *in vivo* system in which Pol I termination efficiency can be directly correlated with growth phenotypes. This enabled us both to determine the requirement of *cis*-elements for *in vivo* termination and to test for possible termination factors since their inactivation suppresses growth defects. Thus, we identified Nsi1 as Pol I-transcription termination factor.

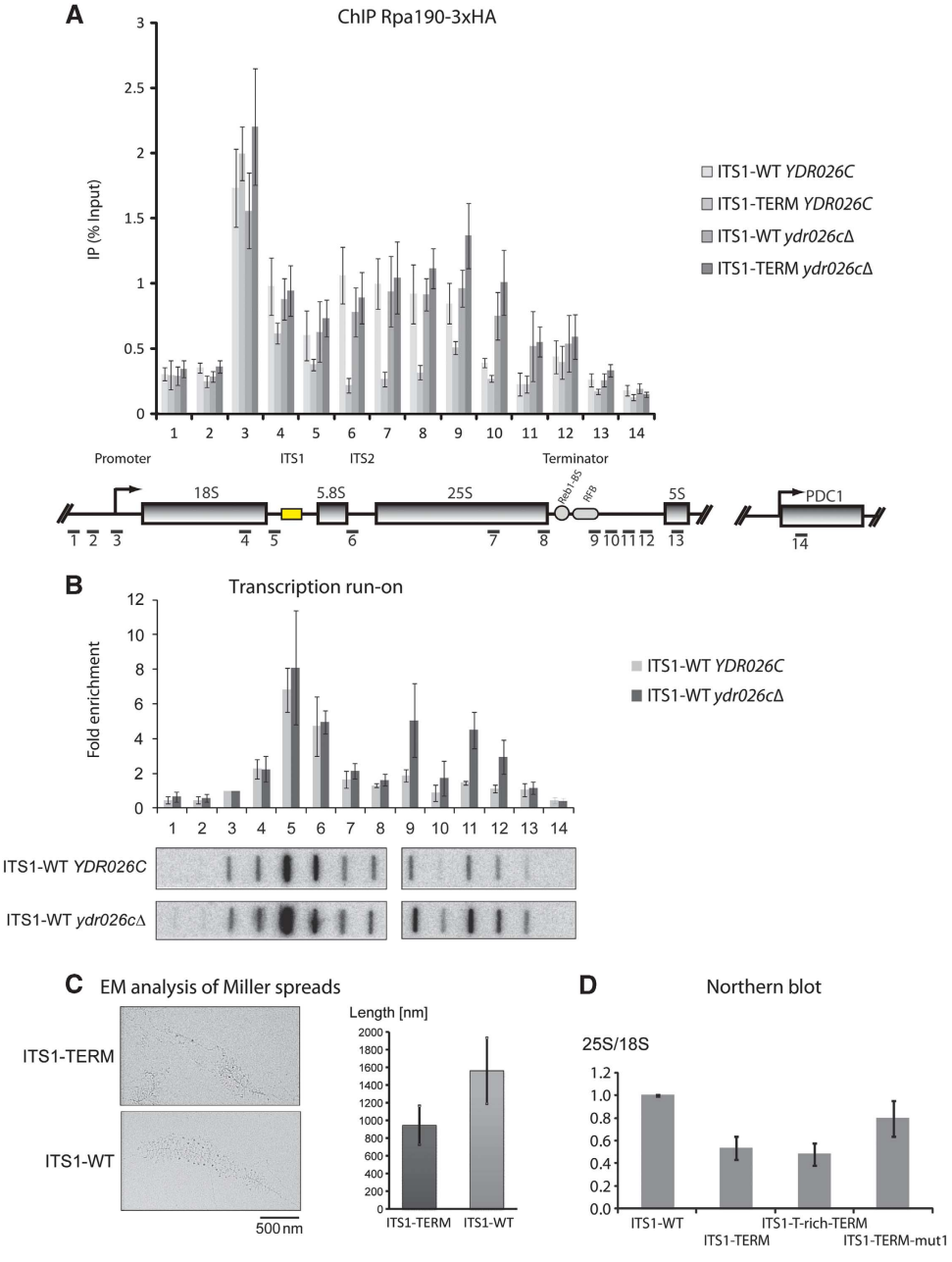
The 322-bp long 3′-region of the 35S rRNA gene inserted within ITS1 was found to be very potent in Pol I transcription termination. The inserted DNA sequence contains several *cis*-elements, which were suggested to play a role in termination. The Reb1-binding site and the T-rich DNA stretch were both

shown to support termination *in vitro* (Lang *et al*, 1994; Lang and Reeder, 1995) and *in vivo* (Reeder *et al*, 1999; Braglia *et al*, 2010b; this study). Using the above *in vivo* system to test termination efficiency, we could show that a two basepair substitution in the Reb1-binding site is sufficient to restore near WT growth (Figure 1B, ITS1-TERM-mut1), confirming the essential function of this *cis*-element for Pol I termination (Lang and Reeder, 1993; Reeder *et al*, 1999). The 3′-region of the 35S rRNA gene further contains a 'failsafe'—terminator (van der Sande *et al*, 1989; Reeder *et al*, 1999; Prescott *et al*, 2004), and a RFB (El Hage *et al*, 2008). Our *in vivo* system will now help to closer characterize how the different *cis*-elements and proteins binding to these sites as for example the RFB-binding protein Fob1 (Takeuchi *et al*, 2003; Huang *et al*, 2006) terminate Pol I transcription. ChEC analyses of MNase-tagged Nsi1 in a Δfob1 strain (data not shown) and ChIP analyses of TAP-tagged Nsi1 in a Δfob1 strain (Figure 2C in Ha *et al*, 2012) suggest a significant but no complete reduction of Nsi1 association with the termination element, indicating that Fob1 may be involved in Nsi1 recruitment. On the other hand, preliminary experiments suggested that Nsi1-dependent Pol I transcription termination could occur at least to some extent even in the absence of the Fob1-binding sites (data not shown). The molecular basis of a possible Fob1–Nsi1 cooperativity is currently under investigation.

It appears, that the 322-bp long termination region neither completely abrogates transcription when inserted in ITS1 (our data) nor at its physiological position (Reeder *et al*, 1999; El Hage *et al*, 2008). It is possible that transcription has to proceed at a very reduced level to assure other functions like the genomic stability of the rDNA locus.

Nsi1 is a Reb1- and Myb1-related protein

Our findings that Nsi1 binds the rRNA gene terminator element and is required for Pol I transcription termination in yeast resolves a hitherto existing puzzle. The designation



of Reb1 as *bona fide* termination factor for yeast Pol I transcription was well accepted. This was based on *in vitro* and *in vivo* transcription analyses, the specific binding of purified Reb1 to DNA elements important for Pol I transcription termination, and its homology to the mammalian Pol I transcription termination factor TTF-I (Morrow *et al*, 1989, 1990; Lang and Reeder, 1993; Lang *et al*, 1994; Mason *et al*, 1997b; Reeder *et al*, 1999; Braglia *et al*, 2010b). However, this view was challenged because Reb1 binding to the terminator *in vivo* was—if at all—barely detectable (Kawauchi *et al*, 2008; Goetze *et al*, 2010) (Figure 2C and D; Supplementary Figure S1), partial depletion of Reb1 did not significantly affect Pol I termination *in vivo* (Kawauchi *et al*, 2008; Braglia *et al*, 2010b), and efficiency of Pol I termination *in vitro* using the Reb1-binding site from the yeast terminator element was rather low (about 20% (Lang *et al*, 1994)). It is thus likely that the affinity of Reb1 for this binding site is not high enough to support efficient binding *in vivo*. Along these lines, the promoter-proximal Reb1-binding site in which three adenines are exchanged against cytosines strongly increases complex formation with Reb1 *in vitro* and the potential of this element to terminate Pol I transcription *in vitro* and *in vivo* (Lang *et al*, 1994; Reeder *et al*, 1999). These results explain the different binding behaviour of Reb1 to either the promoter-proximal Reb1-binding site or the binding site within the terminator (see Figure 2D). In good agreement, the above mutation leads to efficient recruitment of endogenous Reb1 (Supplementary Figure S3) and slightly enhances the growth defect in the *in vivo* system (Figure 1B, ITS1-TERM-mut2). Interestingly, Nsi1 does not interact with the promoter-proximal Reb1-binding site but binds efficiently when this sequence replaces the Reb1-binding site in the terminator element (Figure 2C and D; Supplementary Figure S3). This indicates that additional features are required to recruit Nsi1 to the terminator element.

Our data show that Reb1 binds only weakly to the yeast rRNA gene terminator element whereas Nsi1 efficiently binds to this site *in vivo* (Figure 2C and D; Supplementary Figure S1). Furthermore, Nsi1 is required for the observed growth defect when the terminator element is inserted into ITS 1 of the yeast rRNA genes and the growth defect can be restored upon conditional expression of the protein (Figure 4). However, it cannot be excluded that Reb1 still contributes to Pol I transcription termination. Since neither direct physical interaction studies nor functional *in vitro* analysis of Nsi1 have been performed to date, it is possible that other proteins like Reb1 or Fob1 (see above) participate in Nsi1-recruitment and/or in Nsi1-dependent termination of Pol I transcription. Along these lines, it has recently been reported that partial depletion of Reb1 causes a two-fold increase in read-through transcripts if the Rnt1-cleavage site at the 3′-end of the 35S transcript is mutated (Braglia *et al*, 2010b).

Reb1 and Nsi1 can bind to similar DNA-sequences, albeit with different preferences (Figure 2B–D; Supplementary Figure S1) (Harbison *et al*, 2004). We consider it as unlikely that both proteins are functional paralogs, which solely compete for binding at distinct DNA sites. First, when ChEC analyses were performed with nuclei from strains of NSI1 WT strains, or with nuclei from strains carrying a complete deletion of the gene, no significant difference in Reb1–MNase mediated cleavage within the termination element was observed (Supplementary Figure S4). Second, compared

Figure 5 Pol I association with rRNA gene sequences with or without DNA-binding sites for Pol I termination factors inserted into ITS1 and correlation with steady-state rRNA levels. (**A**) Effects of YDR026C deletion on Pol I occupancy in proximity of the rDNA terminator element as analysed by ChIP. Yeast strains (y1620, y2094, y2229, y2234) carrying rDNA loci with a ITS1-WT, or an ITS1 DNA element insertion as described in Figure 1A (ITS1-TERM), being WT in YDR026C or being deleted in the gene (*ydr026c*Δ), and expressing the Pol I subunit Rpa190 as a MNase fusion protein with a C-terminal triple HA-tag were grown to exponential phase. ChIP experiments were performed as described in the legend to Figure 2D. The amounts of specific DNA-fragments present in the input and retained on the beads were determined by quantitative PCR with primer pairs amplifying the regions 1–9 of the rDNA depicted in the schematic representation on the bottom of the figure. Primer pairs used were 3249/3250 (1), 3022/3023 (2), 969/970 (3), 712/713 (4), 1049/2863 (5) 2864/2865 (6), 710/711 (7), 2882/2883 (8), 2884/2885 (9), 2886/2887 (10), 2421/2422 (11), 2419/2420 (12), 920/921 (13), 613/614 (14). The bar graphs depict percent of total input DNA retained after ChIP of the triple HA-tagged Rpa190. Error bars represent the standard deviation of three independent ChIP experiments, each of which was analysed in triplicate quantitative PCR reactions. Note: the slight enrichment of fragments upstream of the Pol I promoter and the 5S rRNA gene co-purifying with Pol I, compared with the PDC1 fragment could be due to some residual Pol I association with the 'non-transcribed' rDNA regions. Alternatively, the 5S rRNA gene and upstream sequences could be enriched by being part of larger DNA-fragments produced during the sonication process for ChIP sample preparation, which contain the 35S rRNA coding sequence and can thus be co-precipitated with Pol I. (**B**) Deletion of YDR026c leads to an increased production of radiolabelled RNAs hybridizing to DNA sequences downstream of the terminator region in transcription run on experiments. After transcription run-on conditions, radiolabelled RNA from isolated nuclei of either WT cells (y1599) or the YDR026c deletion strain (y2276) were hybridized to single-stranded DNA immobilized on a Nylon membrane. The single-stranded DNA probes were derived by PCR from genomic DNA using the same primer pairs as for the ChIP experiment described in Figure 5A. After hybridization with RNAs from YDR026C, WT and YDR026c deletion strains membranes were exposed to a imaging plate (Fujifilm) and radioactive signals were quantified using the FLA-3000 imaging system (Fujifilm). The hybridization signal obtained with DNA probe 3 spanning the Pol I promoter was used for normalization. Mean values and standard deviations of the fold enrichment over amplicon 3 of three hybridization experiments (one biological replicate and one technical replicate) are depicted in form of a bar graph. Note: the quantitative data were not corrected for content of incorporated ^{32}P-UMP. (**C**) Insertion of the terminator element into ITS1 leads to a decreased density of Pol I molecules downstream of the Reb1-binding site as visualized in EM analysis. Miller chromatin spreading analyses were performed with yeast strains (y1599, y2042) carrying rDNA loci with a ITS1-WT, or an ITS1 DNA element insertion as described in Figure 1A (ITS1-TERM). Two representative electron micrographs from typical 'Christmas-tree'-like structures likely corresponding to actively transcribed rRNA genes in the two different strains are shown on the left. A bar on the bottom indicates the scale of the images. The length of the regions with high polymerase density (electron-dense stem of the 'Christmas trees') with extending nascent rRNA transcripts (branches of the 'Christmas trees') was determined for a total number of 20 and 26 molecules for strains y2042 and y1599, respectively (see Supplementary Figure S2 for details). The average length and standard deviation are depicted in the graph on the right. (**D**) Insertion of the terminator element into ITS1 leads to an imbalance in the steady-state level of 18S and 25S rRNAs. Yeast strains (y1599, y2042, y2273, y2274) carrying rDNA loci with a ITS1-WT, or ITS1 DNA element insertions as described in Figure 1A (ITS1-T-rich-TERM, ITS1-TERM, ITS1-TERM-mut1) were grown to exponential phase. RNA was extracted, separated by gel electrophoresis and Northern blot analysis was performed as described in Materials and methods. Membranes were hybridized with radioactively labelled oligonucleotides 205 and 212 specific for 18S, and 25S rRNA, respectively. Radioactive signals on the blot were quantified by phospho imager analysis as described in Materials and methods. The graph depicts the 25S/18S rRNA ratio, which was normalized to the value obtained with strain y1599 (ITS1-WT), which was arbitrarily set to 1.

with Reb1, which is a rather abundant protein (about 7000 molecules per cell) distributed over the entire nucleus, Nsi1-expression is >10-fold lower and the protein shows a strong nucleolar accumulation (Ghaemmaghami *et al*, 2003; Huh *et al*, 2003; Ha *et al*, 2012). Third, in a genome-wide location study of 203 transcriptional regulators, including Reb1 and Nsi1 (Harbison *et al*, 2004), enrichment for both, Reb1 and Nsi1 has only been shown for two intergenic sequences (the promoter regions of the genes coding for uridine kinase URK1 and for the RNA-dependent ATPase PRP2) (http://jura.wi.mit.edu/young_public/regulatory_code/GWLD.html). However, no further significant overlap of other genomic Nsi1 and Reb1-binding sites has been reported, indicating that the two proteins may have different nuclear functions.

The C-terminal half of Nsi1 shares the c-Myb-related DNA-binding domain not only with Reb1, but also with the mammalian transcription termination factor TTF-I (Reeder and Lang, 1997). Mammalian TTF-I is characterized as a multifunctional factor binding at three different regions within the murine rDNA locus and appears to be important for several different processes: it plays a role in Pol I transcription initiation, activation and silencing (Nemeth *et al*, 2004; Schmitz *et al*, 2010), pre-rRNA processing (Lessard *et al*, 2010), transcription termination (Kuhn and Grummt, 1989) and replication fork stalling (Gerber *et al*, 1997). A multifunctional Reb1-homologue, which is involved in both termination of transcription and replication fork blocking, was also described in *Schizosaccharomyces pombe* (Zhao *et al*, 1997). In *S. cerevisiae*, some of these functions seem to be carried out by different DNA-binding proteins: Reb1 which binds upstream of the rDNA promoter and might play a role in the regulation of rRNA gene transcription (Morrow *et al*, 1989), the Reb1-homologue Nsi1 which is required for termination (this work) and the replication fork blocking protein Fob1 (Kobayashi and Horiuchi, 1996). On the other hand, a few not further characterized genes coding for Myb-related proteins are present in mammals, which all share the same or a highly similar DNA-binding domain. One of it (NP_808373) shows an even higher sequence similarity with Nsi1 than TTF-I. Thus, it will be interesting to see whether and how these mammalian TTF-I homologues interfere with rRNA gene transcription.

Nsi1 and transcription termination

Efficient cellular rRNA production is the result of many interconnected processes. rRNA processing and proper 3′-end formation of 25S rRNA requires the coordinated activity of endonucleases (Rnt1) (Elela *et al*, 1996; Kufel *et al*, 1999) and exonucleases (Rat1) (Petfalski *et al*, 1998) as well as of the helicase Sen1 (Ursic *et al*, 1997). All three factors were recently suggested to play an important role in Pol I transcription termination *in vivo* favouring a release of the transcribing Pol I machinery by a torpedo-like mechanism. In this model, Rat1-dependent digestion of Rnt1-cleaved, accessible pre-rRNA 5′-ends releases the paused Pol I transcription machinery from the rDNA by collision (El Hage *et al*, 2008; Kawauchi *et al*, 2008). Efficient termination requires the Reb1-binding site, and possibly a protein that binds to this site and delays RNA chain elongation (Reeder *et al*, 1999; Braglia *et al*, 2010b). Interestingly, termination occurs also in the absence of the endonuclease Rnt1 (Braglia *et al*, 2010b), which cleaves the pre-rRNA downstream of the 25S 3′-end thereby generating an entry site for Rat1 (El Hage *et al*, 2008; Kawauchi *et al*, 2008). It was postulated that in such a scenario, co-transcriptional RNA cleavage by an unknown endonuclease at a site in close proximity of the T-rich DNA stretch allows recruitment of the 5′–3′exonuclease Rat1, which digests the nascent rRNA chain, eventually leading to a destabilization of the transcription machinery (Braglia *et al*, 2010b). Therefore both, the Reb1-binding site, and co-transcriptional cleavage either by Rnt1 or by the putative endonuclease at the T-rich DNA stretch are important elements to support termination. However, earlier *in vitro* analysis demonstrated that binding of a specific DNA-binding protein (i.e., Reb1) in combination with an adjacent T-rich DNA stretch is sufficient to terminate transcription and to dissociate Pol I from the template (reviewed in Reeder and Lang, 1997).

Our *in vivo* data confirm that termination can be induced in the absence of the known Rnt1-cleavage site and its efficiency is further improved in the presence of the T-rich DNA stretch. Binding of Nsi1 to the terminator element represents a necessary prerequisite, which then might pause the elongating Pol I in proximity of the T-rich DNA stretch (which is located only about 16 bp upstream of the Reb1-binding site) to which finally an unknown nuclease has to get access for cleavage. Since >25 nucleotides of the DNA template are always covered by yeast Pol II (Gnatt *et al*, 2001) it is difficult to explain how a structurally related RNA polymerase like Pol I allows cleavage by an accessory nuclease within this region when it is stalled at the terminator region. Alternatively, cleavage might be executed by an intrinsic Pol I-cleavage activity. In fact, the Pol I subunit A12.2 was previously reported to be involved in both RNA cleavage and transcription termination (Prescott *et al*, 2004; Kuhn *et al*, 2007). Interestingly, association of murine TTF-I to a DNA stretch, which was integrated within the ITS1 region and contained only its cognate binding site led to growth reduction in our *in vivo* system (Figure 4; Supplementary Table 1). It will be interesting to see whether TTF-I binding leads to the release of Pol I from the template (perhaps including co-transcriptional cleavage) and liberation of the nascent RNA, or whether TTF-I association simply stalls the elongating polymerase.

Nsi1 binding to the natural termination element at the 3′-end of the rRNA gene as well as to the termination element integrated in ITS1 leads to a detectable drop in Pol I density downstream of the Nsi1-binding site (Figure 5A). Since Pol I termination occurs also in the absence of the T-rich DNA stretch (Figure 5A) Nsi1 or an Nsi1-associated factor probably triggers the dissociation of Pol I from the rDNA template. The existence of an additional releasing factor was suggested for both mammalian and yeast Pol I transcription systems (Mason *et al*, 1997a; Tschochner and Milkereit, 1997; Jansa *et al*, 1998). Future genetic analysis using the here presented approach to analyse Pol I termination efficiency by yeast growth may help to identify further possible *trans*-acting factors.

Nsi1 activity might link transcription termination to rRNA gene silencing and life span regulation

When this work was prepared for publication a manuscript by Ha *et al* (2012) was submitted, in which Ydr026c (Nsi1) is described as a nucleolar rDNA-binding factor involved in rDNA silencing of RNA Pol II transcription. Importantly,

Nsi1 contributes to rDNA stability and life span extension and interacts with Sir2, a protein involved in silencing of Pol II transcription at the rDNA locus (Bryk *et al*, 1997; Smith and Boeke, 1997), rDNA recombination (Gottlieb and Esposito, 1989; Kobayashi *et al*, 2004) and ageing (Sinclair and Guarente, 1997; Kaeberlein *et al*, 1999). Since Nsi1 is both required for efficient Pol I transcription termination and involved in rDNA silencing and rDNA-stabilization (Ha *et al*, 2012), it is possible that properly terminated Pol I transcription contributes to preserve genomic integrity of the rDNA, which in turn may affect ageing. Future analyses will show whether Ydr026c has independent roles in these different processes or whether its effects on rDNA-stabilization, lifespan extension and/or silencing can be explained by its function as transcription termination factor.

Materials and methods

Tables of the used strains, oligos and plasmids as well as most methods are described in the Supplementary data.

Chromatin endogenous cleavage analyses

For ChEC experiments, yeast strains were grown in yeast peptone dextrose (YPD) at 30°C until they reached an OD_{600} of 0.5. Strains carrying expression plasmids for TTF-I–MNase or LexA–MNase were grown in selective complete media with raffinose lacking leucine at 30°C. When the cultures reached an OD_{600} of 0.3, galactose was added to a final concentration of 2% and incubation was continued for 4 h at 30°C. Formaldehyde fixation and isolation of crude nuclei, and ChEC were performed as previously described (Merz *et al*, 2008).

EM analysis

Chromatin spreading was performed as described in Osheim *et al* (2009) with minor modifications. Each solution was buffered with di-Sodium Tetraborate. Carbon-coated grids were glow discharged to make them hydrophilic. Grids were examined with a transmission electron microscope (Jeol 1200 EX) at 80 kV. The images were obtained using a digital camera (AMT-USA). The length of the Miller's spread were measured using Image J (National Institutes of Health).

Southern blot and Northern blot analyses

Southern blot analysis was carried out as described previously (Merz *et al*, 2008). RNA extractions, Northern blotting and probe preparation were performed as described in (Ferreira-Cerca *et al*, 2005). The FLA-3000 imaging system (FUJI) and the MultiGauge software (FUJI) were used for data collection and quantification, respectively. A list of probes used for blot hybridization is presented in Supplementary Table 5.

Chromatin immunoprecipitation analyses

ChIP was essentially performed as described elsewhere (Goetze *et al*, 2010). Primer pairs used for amplification are listed in Supplementary Table 2. Data was analysed with the comparative quantitation module of the RotorGene analysis software. Retention of specific DNA-fragments was calculated as the percentage of total input DNA. The mean values and error bars are derived from at least three independent ChIP experiments analysed in triplicate quantitative PCR reactions.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: AR developed the *in vivo* assay. AR, JP-F, JG and PM designed and constructed plasmids and strains. AR and HS performed and interpreted ChEC analysis. SH designed, performed and evaluated termination assays. SH and JP-F performed and evaluated run-on transcription. LW and AN analysed yeast mutants and generated expression strains. PM devised the strategy. PM, JG and HT designed the experiments, analysed the data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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