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Intrinsic Properties Of Rna Polymerase I And Trans-Acting Factors Control Transcription Elongation Efficiency

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INTRINSIC PROPERTIES OF RNA POLYMERASE I AND TRANS-ACTING FACTORS CONTROL TRANSCRIPTION ELONGATION EFFICIENCY

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BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Transcription of ribosomal (r) DNA by RNA polymerase I (Pol I) is the initial step of ribosome synthesis. Pol I transcription is unique in its high rate of initiation, specific organization within the nucleolus and tight connection to cell growth and proliferation. Moreover, transcription elongation by Pol I is functionally coupled with rRNA processing and assembly of the ribosomes. Regulatory insights into transcription elongation by Pol I and its interface with rRNA processing are limited, despite decades of research. To fill that gap, we asked several important questions: Do the obvious functional divergences between Pol I and other eukaryotic polymerases extend to the catalytic core? What are the components of Pol I transcription elongation complex and how do these trans-acting factors control rRNA synthesis? Is there a functional link between Pol I transcription elongation rate and ribosome biogenesis? To begin to address these broad questions, we investigated the roles of Spt5 and the RNA polymerase trigger loop in yeast rRNA synthesis and ribosome assembly.

The Spt4/5 complex is involved in Pol I and Pol II transcription and nascent transcript maturation. In this study we demonstrated that Spt5 directly associates with Pol I and binds to the two catalytic subunits of Pol I and to the subunits A49/A34.5 that act in elongation. Interactions of Spt5 with Pol I and other components of the rDNA transcription apparatus support a direct role for Spt5 in Pol I transcription elongation.

To study the degree of functional divergence between Pols I and II we examined the role of the trigger loop, a flexible domain directly involved in nucleotide addition. Analysis of Pol I trigger loop mutants suggested that analogous mutations in Pol I resulted in different phenotypes than in Pol II. The experiments using chimeric Pol II enzymes carrying the trigger loop regions of Pol I suggest that functional consequences of mutations that alter trigger loop dynamics are determined not solely by the trigger loop sequence but rather by the protein context of the enzyme. Altogether, our data suggest that Pol I and Pol II have different rate-limiting steps.

In vivo characterization of the Pol I trigger loop mutants revealed ribosome assembly defects consistent with the model that the rate of elongation by Pol I is coupled to ribosome biogenesis. Based on our data we propose that optimal elongation rates are required for proper rRNA processing and that slowing Pol I elongation complexes beyond a certain rate leads to impaired ribosome biogenesis. This novel hypothesis explains the tight link between Pol I transcription elongation and rRNA maturation and highlights significance of co-transcriptional events during ribosome production.

Together, these findings enhance our understanding of the mechanisms of Pol I transcription elongation and its potential roles in orchestration of rRNA processing and ribosome biogenesis.

Keywords: RNA polymerase I, transcription, ribosomal RNA

DEDICATION

For my dearest family that lives so far away and for my new family here, in Birmingham, Alabama.

Thank you for your love and support.

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THE UNIQUE PROPERTIES OF RNA POLYMERASE I REFLECT ITS SPECIFIC ROLES IN EUKARIOTIC TRANSCRIPTION

"Introduction" has been adapted for journal style requirements and submitted for publication.

Transcription by Multisubunit RNA Polymerases

Transcription is the universal process by which organisms transcribe their genes. Enzymes that convert genetic information encoded by DNA into RNA are called DNA dependent RNA polymerases (RNAPs). All cells utilize multi-subunit RNAPs for transcription of their genomes. While prokaryotic cells have a single RNA polymerase for the transcription of their genomes, eukaryotic cells utilize at least three specialized nuclear RNAPs: RNA polymerase I (Pol I), RNA polymerase II (Pol II) and RNA polymerase III (Pol III). Pol I transcribes ribosomal DNA (rDNA); Pol II synthesizes all messenger RNAs (mRNAs) and some small non-coding RNAs; and Pol III primarily produces transfer RNAs (tRNAs) and 5S rRNA. The diversity of multi-subunit RNAPs, their conservation, and subunit composition are summarized in Table 1.

All multi-subunit RNAPs synthesize the RNA chain from the DNA template *de novo* using nucleoside triphosphate (NTP) substrates via the two metal ion mechanism of catalysis (Steitz, 1998). The active site contains invariable acidic residues within the catalytic loop that coordinate the magnesium ions responsible for nucleotidyl transfer [reviewed in (Nudler, 2009)]. The active center of the enzymes includes the binding sites for the 3'-hydroxyl of the nascent RNA and the NTP insertion site, thus, bringing together and activating the reactive groups of the substrates. The formation of the phoshodiester bond is followed by translocation of the newly formed RNA 3'-end by one nucleotide with respect to polymerase. Translocation is coupled to the release of the pyrophosphate product [reviewed in (Brueckner et al., 2009)].

Bacteria	Archaea	Eukarya[1]		
		Pol I	Pol II	Pol III
β'	A'/A''	A190	Rpb1	C160
β	B'/B''	A135	Rpb2	C128
α	D	AC40	Rpb3	AC40
α	L	AC19	Rpb11	AC19
ω	K	Rpb6	Rpb6	Rpb6
	H	Rpb5	Rpb5	Rpb5
	G	Rbp8	Rbp8	Rbp8
	N	Rpb10	Rpb10	Rpb10
	P	Rpb12	Rpb12	Rpb12
		A12.2 (N-ribbon)	Rpb9	C11 (N-ribbon)
	E	A43	Rpb7	C25
	F	A14	Rpb4	C17
		A49 (N-terminal)	TFIIFα*	C53
		A34.5	TFIIFβ*	C37
	TFEα		TFIIEα*	C82
	TFEβ	A49 (C-terminal)	TFIIEβ*	C34
				C31
	TFS	A12.2 (C-ribbon)	TFIIS (C-ribbon)	C11 (C-ribbon)
б				

Table 1. Diversity of the Multisubunit RNA Polymerases. Subunit composition and homology of the multisubunit RNA polymerases from the three domains of life are presented. The universally conserved polymerase subunits are shaded in green; the archaeo-eukaryote-specific functional core subunits are in yellow, the polymerase stalk components are in tan. All of the conserved subunits or the Pol I/III subunits (in grey) homologous to Pol II trans-acting factors (in blue) are organized in the same rows. *Asterisk* represents partial structural homology.
[1] Eukaryotic RNA polymerase subunits are according to *S. cerevisiae* nomenclature.

Transcription involves a number of steps, beginning with initiation. During transcription initiation, gene promoters are recognized by the general transcription factors which assist in polymerase recruitment [reviewed in (Vannini and Cramer, 2012)]. Promoter recognition and preinitiation complex (PIC) formation are followed by the so-called “open” complex formation that involves multiple events including conformational changes within the nucleic acid template and the polymerase. The polymerase enters productive transcription elongation after a series of abortive initiation cycles and promoter escape. In principle, once the stable elongation complex is formed, polymerase alone is capable of RNA chain elongation and of proofreading, though the presence of

specific *cis*- and *trans*- factors help control the elongation process, affecting both transcription efficiency and processivity [reviewed in (Landick, 2009; Liu et al., 2013; Martinez-Rucobo and Cramer, 2013)]. After the transcript synthesis is complete, the polymerase terminates transcription and releases the RNA product. Transcription termination occurs using several different mechanisms depending on the polymerase system and/or on the *cis*-/*trans*- acting factors [reviewed in (Peters et al., 2011; Richard and Manley, 2009)].

Even though all multi-subunit RNA polymerases are highly conserved, there are important structural and functional differences between the related RNAP complexes [reviewed in (Cramer, 2002)]. This work will focus on unique features of Pol I transcription system and its comparison to Pol II as the best-studied enzyme among eukaryotic polymerases.

The RNA Polymerase I Complex

The Pol I complex consists of 14 subunits (Table 1), seven of which are shared with Pols II and/or III. Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12 are common for all three enzymes, whereas AC40 and AC19 are found in Pols I and III. The remaining seven subunits (A190, A135, A49, A43, A34.5, A14 and A12.2) are specific for Pol I though they share sequence or structural homology with Pol II's or Pol III's subunits or trans-acting factors (Cramer et al., 2008; Vannini and Cramer, 2012).

The structural insights in Pol I architecture were gained based on the electron microscopy (EM) studies, homology map comparisons to Pol II and X-ray crystallographic analysis of Pol I-specific peripheral subunits (Bischler et al., 1998;

Bischler et al., 2002; De Carlo et al., 2003; Geiger et al., 2008; Geiger et al., 2010; Klinger et al., 1996; Kuhn et al., 2007; Schultz et al., 1990; Schultz et al., 1993). Most of the biochemical distinctions between Pol I and Pol II have been attributed to peripheral surfaces. However, some important functional differences between the paralogs also involve the polymerase core and the catalytic center (Carter and Drouin, 2009; Jennebach et al., 2012; Kuhn et al., 2007; Viktorovskaya et al., 2013). In this chapter, the major structural differences identified between Pols I and II are highlighted.

The A190 and the A135 subunits of the functional core.

The two largest Pol I-specific subunits, A190 and A135 form opposite sides of the central "cleft" (Kuhn et al., 2007). The active center of the enzyme is located deep inside the "cleft" limited by the "wall" domain. Mutations in the largest subunits often affect the basic functions of the polymerase such as the complex assembly and the catalytic properties (Archambault and Friesen, 1993; Schneider et al., 2007; Viktorovskaya et al., 2013). A190 and A135 associate with 8 more subunits (AC40, AC19, Rpb5, Rpb6, Rpb8, Rpb10, Rpb12, and A12.2) to form the functional core of the eukaryotic polymerase. The functional core contains a minimal subset of subunits proposed to be sufficient for nonspecific transcription (Bischler et al., 2002; Edwards et al., 1991; Lanzendorfer et al., 1997).

The overall morphology was investigated by various EM techniques confirming that the Pol I core is similar to that of Pol II, especially at the active center (Bischler et al., 1998; Bischler et al., 2002; De Carlo et al., 2003; Klinger et al., 1996; Kuhn et al., 2007). Despite the general resemblance of Pol I and Pol II, the homology model and the EM

studies identified Pol I-specific surfaces created by the A190 and A135 subunits. The most significant differences were observed for the Pol I "clamp" domain, a flexible region involved in stabilization of the elongation complex (Kuhn et al., 2007). The clamp was positioned to entirely close off the cleft in the EM structure. In agreement with structural data, the three major insertions in the region corresponding to the clamp were detected when compared with the Pol II sequence. It is not known how the described distinctions in the clamp affect Pol I activity. Additional surfaces unique to Pol I are generated by the extended "dock", the truncated "foot" domains as well as a polymerase "jaw" region with a substantial insert (Kuhn et al., 2007). These distinct regions might have evolved for the interactions with specific transcription factors at the rDNA promoter. Indeed, the dock domain is a site of interaction with the PIC component, core factor (Knutson and Hahn, 2011; Vannini and Cramer, 2012).

The first sequence comparison of the largest subunits of Pols I and II revealed an overall conservation (Allison et al., 1985). However, even early studies observed differences between the catalytic subunits, such as the presence of the well-known C-terminal repeat of Rpb1 in Pol II that is absent in Pols I and III. A recent sequence-based *in silico* analysis of the two largest subunits from the three eukaryotic RNAPs suggests that functional differences exist even between the most conserved regions of the paralogs such as the cleft loops (Carter and Drouin, 2009). The identified variations cluster near the active center and involve regions responsible for the interactions with the DNA-RNA hybrid. These results suggest RNAPs might differ in their ability to maintain a stable RNA-DNA hybrid.

Thus, the structural studies and the *in silico* approach aimed to compare the catalytic core of Pols I and II proposes that important differences in the functional core might exist despite high conservation (Carter and Drouin, 2009; Kuhn et al., 2007). Consistently, recent analysis using Pol I mutants as well as chimeric Pol II enzymes hosting conserved active center regions of Pol I demonstrated that the functional differences between Pols I and II extend to the polymerase active center (Viktorovskaya et al., 2013). The results of this study suggest that Pols I and II might have different rate-limiting steps of the nucleotide addition cycle. Further analyses of the structural and functional divergences between the active centers are required to understand unique catalytic properties of the three RNAPs.

The A12.2 core subunit and Pol I's cleavage activity.

In addition to the catalytic subunits, the core includes another Pol I-specific factor-A12.2. A12.2 is the only non-essential component of the functional core. The absence of A12.2 results in a slow growth and heat-sensitive phenotype (Nogi et al., 1993). Interestingly, A12.2 has two functional domains: the N-terminus of A12.2 is homologous to Pol II's Rpb9 subunit whereas the C-terminal ribbon domain of A12.2 shows sequence similarity to the Pol II transcription factor TFIIS [Table 1; (Van Mullem et al., 2002)]. The N-terminal zinc binding domain of A12.2 anchors the subunit to the Pol I complex and plays a role in Pol I complex integrity. It was shown that Pol I purified from either *rpa12* deletion or N-terminal truncation mutants had sub-stoichiometric amounts of A49 and, possibly, A43 and A14. Consistent with its homology to Rpb9, A12.2 occupies a

location within the Pol I complex similar to the known position of Rpb9 in Pol II (Kuhn et al., 2007).

Functional analysis of the second domain of A12.2 containing the C-terminal zinc ribbon did not detect any significant role for this region *in vivo* (Van Mullem et al., 2002). The domain homologous to A12.2's C-terminal motif is a part of TFIIS which stimulates RNA cleavage activity of Pol II. This cleavage activity has been shown to be important for reactivation of an arrested complex and for Pol II fidelity (Cheung and Cramer, 2011; Koyama et al., 2007). In agreement with sequence homology, *in vitro* studies confirmed that the C-terminus of A12.2 is required for RNA hydrolysis by Pol I (Kuhn et al., 2007). The role of A12.2 in RNA hydrolysis explains why the intrinsic RNA cleavage activity of Pol I is strong, in contrast to the weak cleavage activity of Pol II. Crosslinking mass spectrometry analysis of the Pol I complex suggested that the C-terminus of A12.2 transiently interacts with the active site via the polymerase pore in a TFIIS-like manner (Jennebach et al., 2012; Ruan et al., 2011). In addition, decreased Pol I cleavage activity in the *rpa12*Δ mutant might explain the observed read-through beyond the transcription termination site on rDNA (Braglia et al., 2011; Prescott et al., 2004; Ruan et al., 2011). Thus, A12.2 as a "built-in" cleavage factor may provide higher accuracy and efficiency of rRNA synthesis by Pol I.

The peripheral subunits of Pol I.

The A49, A43, A34.5, and A14 peripheral subunits are required for promoter-dependent transcription of rDNA. The A43 and A14 subunits form the stalk of the

polymerase responsible for transcription initiation, whereas the A49 and A34.5 sub-complex primarily functions as an intrinsic elongation factor (Kuhn et al., 2007).

The A14/43 sub-complex. The three eukaryotic as well as archaeal RNA polymerases share a homologous structure that protrudes from the core enzyme named the stalk [Table 1; reviewed in (Grohmann and Werner, 2011)]. Pol I's stalk is formed by the A14/A43 heterodimer and structurally is very similar to its counterparts in other polymerases (Bischler et al., 2002; Geiger et al., 2008; Hu et al., 2002; Kuhn et al., 2007; Meka et al., 2003; Peyroche et al., 2002; Todone et al., 2001).

Early studies revealed that A43 is essential for cell viability, whereas A14 is dispensable (Smid et al., 1995; Thuriaux et al., 1995). Either the lack of A14 or a C-terminal truncation of A43 results in temperature – sensitivity. Purification of Pol I from these strains generates transcriptionally inactive enzymes deprived of A14, A43 and ABC23 subunits suggesting decreased Pol I complex stability of the mutants (Peyroche et al., 2002; Smid et al., 1995). The Pol I complex lacking A14/A43 is active in non-specific but not in promoter-dependent transcription *in vitro* (Lanzendorfer et al., 1997; Peyroche et al., 2002; Peyroche et al., 2000). Functional studies demonstrated that the A14/A43 sub-complex is crucial for recruitment of Pol I to the rDNA promoter via direct association with the transcription initiation factor Rrn3 (Peyroche et al., 2000). Interaction of Pol I and Rrn3 via the A43 subunit is essential for the formation of a complex competent for transcription initiation and is regulated for proper control of rRNA synthesis.

The A34.5/A49 sub-complex. The A34.5 and A49 subunits make a stable sub-complex within Pol I that shares similarity with Pol III's heterodimer C37/53 but does not have a

counterpart among Pol II's subunits [Table 1; (Kuhn et al., 2007)]. Instead, the A49/A34.5 heterodimer structurally resembles the Pol II transcription factors TFIIF and TFIIE (Geiger et al., 2010). X-ray crystallographic studies suggest that the dimerization module of the A49/A34.5 sub-complex is similar to TFIIF whereas the C-terminal domain of A49 may be related to the parts of TFIIE. The location of the A34.5/A49 heterodimer on the polymerase complex also resembles the location of respective regions of TFIIF and TFIIE on Pol II as shown by the crosslinking mass spectrometry analysis (Jennebach et al., 2012). The positioning of the A34.5/A49 sub-complex in proximity to the cleft and the clamp is in agreement with the earliest EM data (Bischler et al., 2002).

Both A34.5 and A49 are non-essential: deletions of either of the genes encoding these two polypeptides as well as the double mutant are viable but defective in rRNA production (Gadal et al., 1997; Liljelund et al., 1992). The lack of A34.5 subunit does not generate noticeable growth defect or conditional lethality; however, it manifests a moderate caffeine-sensitivity. In the absence of A34.5, there is a slight decrease in A49 stoichiometry within the polymerase *in vivo* suggesting that A34.5 stabilizes association of the heterodimer with Pol I (Beckouet et al., 2008; Gadal et al., 1997).

In contrast to A34.5, deletion of the A49 subunit dramatically impairs cell growth and leads to inviability at lower temperatures (Liljelund et al., 1992) suggesting that a major contribution to the A34.5/A49 sub-complex's activity is provided by A49. Functional studies of the *rpa49*Δ strain indicate the roles of A49 in Pol I transcription initiation and elongation (Albert et al., 2011; Beckouet et al., 2008; Beckouet et al., 2011). Structural insight and biochemical analysis of the A34.5 and A49 subunits suggest that they primarily function in transcription elongation and Pol I processivity (Gadal et al., 1997;

Geiger et al., 2010; Huet et al., 1975; Kuhn et al., 2007), however, roles in promoter opening and transcription initiation were proposed as well (Geiger et al., 2010; Jennebach et al., 2012). Interesting, the crosslinking analysis yielded an important discovery that the domains of A34.5/A49 may be mobile on the surface of Pol I with some regions potentially reaching the funnel and even the active center of the enzyme (Jennebach et al., 2012). The proposed functions of A34.5/A49 in Pol I transcription initiation and elongation are in agreement with its similarity to TFIIF and TFIIE.

Altogether, structural analysis suggests that architecture of the whole Pol I complex is structurally, functionally and evolutionarily related to Pol II complex in association with TFIIS, TFIIF and TFIIE (Jennebach et al., 2012; Vannini and Cramer, 2012).

RNA Polymerase I Transcription Initiation

Initiation is the best characterized step in Pol I transcription in yeast and mammals. Transcription starts when the initiation complex assembles at the gene promoter. Genetic and biochemical approaches have identified the major rDNA promoter elements and general transcription factors required for Pol I initiation. Unlike Pol II or Pol III initiating from multiple types of promoters, Pol I utilizes only a single rDNA promoter. That makes yeast Pol I initiation relatively simple with very few factors essential for robust specific transcription initiation – Rrn3, TATA-binding protein (TBP), core factor (CF) and upstream activation factor (UAF). Most of the initiation factors are highly conserved between lower and higher eukaryotes (Figure 1); however, some important differences exist, described below in this section.

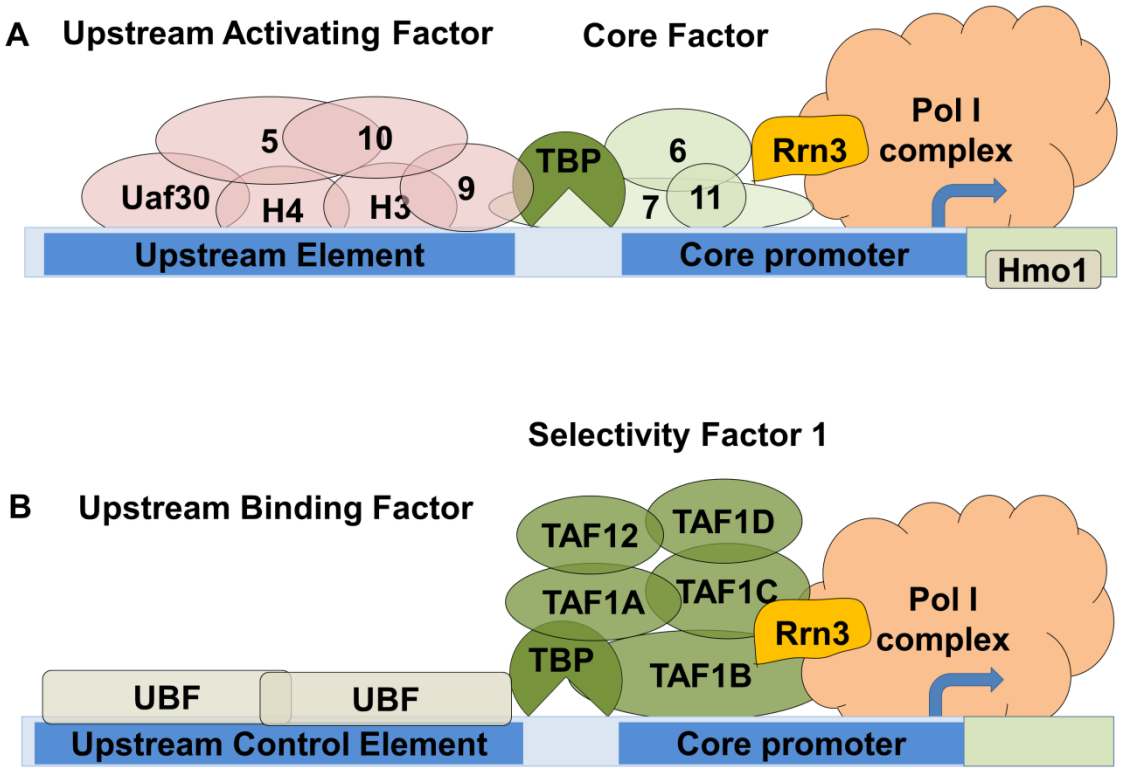

Figure 1. Pol I transcription initiation. Cartoon diagram of factors required for transcription initiation by Pol I in yeast (A) and mammals (B). The yeast factors indicated by numbers represent certain Rrn factors (i.e. the number 6 represents the Rrn6 protein). Subunits of individual factors are colored the same and reflect their functional analogy or shared conservation between the yeast and mammalian factors.

Rrn3 is a key target for control of Pol I transcription initiation.

Rrn3 is an essential factor that directly binds Pol I in the absence of DNA template (Blattner et al., 2011; Milkereit and Tschochner, 1998; Peyroche et al., 2000; Yamamoto et al., 1996). Rrn3 associates with Pol I between its sub-complexes, AC40/19 and A14/43, making a critical contact with the A43 subunit to generate initiation competent complex. Inactivation of Rrn3 by the point mutations compromises rRNA synthesis and decreases Pol I occupancy at the rDNA promoter (Blattner et al., 2011; Claypool et al., 2004). Structural study of Rrn3 identified a unique HEAT repeat fold and a surface exposed "serine patch" region (Blattner et al., 2011). This "serine patch" is required for association with Pol I and for efficient transcription initiation *in vivo*.

When bound to Pol I, Rrn3 interacts with the promoter-associated core factor (CF) in yeast or the selectivity 1 (SL1) factor in mammals (Miller et al., 2001; Peyroche et al., 2000; Yuan et al., 2002). Thus, the generally accepted model is that the initiation competent Pol I – Rrn3 complex is recruited to the rDNA promoter via protein-protein interactions with the transcription factors bound to the template. However, some evidence presented by the Rothblum lab suggests that human Rrn3 (called TIF-IA) might have a DNA binding activity required for rDNA transcription (Stepanchick et al., 2013). The detailed mechanism by which initiation competent RNA polymerase I recognizes the committed template is an area of active study.

After initiation Rrn3 is released from the elongation complex, and Rrn3 dissociation depends on A43 and A49 Pol I subunits (Beckouet et al., 2008; Bier et al., 2004; Claypool et al., 2004; Philippi et al., 2010). Interestingly, it was shown that human Rrn3 not only dissociates from Pol I, but cannot activate transcription in a subsequent reaction

(Hirschler-Laszkiewicz et al., 2003). This does not seem to be true for yeast, though yeast Rrn3 requires hours of incubation with Pol I to form initiation competent complex *in vitro* (Keener et al., 1998b). The mechanisms by which Rrn3 is loaded on Pol I to ensure rapid and efficient initiation *in vivo* remain to be elucidated.

It was shown that just a minor portion of both Rrn3 and Pol I are in a stable complex with each other in the cell, and only this small fraction is active in transcription initiation (Milkereit and Tschochner, 1998). Thus, availability of the initiation competent complex signifies a limiting step for Pol I transcription. Indeed, the interaction of Rrn3 and Pol I is highly controlled responding to the conserved TOR (target of rapamycin) pathway (Claypool et al., 2004; Milkereit and Tschochner, 1998; Philippi et al., 2010), a key system that integrates signals sensing changes in growth factors, nutrient availability, energy status and various physiological stresses in cell [reviewed in (Loewith and Hall, 2011)].

It has been shown that yeast Rrn3 expression and activity are regulated on transcriptional and post-translational levels to match ribosome production to nutrient availability and cellular growth requirements. In stationary phase or under conditions similar to nutritional deprivation, the amount of Rrn3-bound Pol I complex decreases dramatically resulting in reduced transcribing polymerases and a drop in rRNA synthesis (Claypool et al., 2004; Milkereit and Tschochner, 1998; Philippi et al., 2010). The levels of the initiation-competent complexes were dependent on the total Rrn3 content (Philippi et al., 2010). Rrn3 is constitutively ubiquitylated and rapidly degraded by the proteasome. The cellular pool of Rrn3 is restored by *de novo* synthesis under optimal conditions, but not during starvation. Thus, under nutrient deficiency [or TOR signaling inhibition by

rapamycin treatment], the Rrn3 abundance and the Pol I – associated form reduce since *RRN3* expression is blocked. The involvement of the proteasome degradation system to Rrn3-Pol I activity seems to be conserved between yeast and mammals (Fatyol and Grummt, 2008).

Another example of post-translational control of Rrn3 activity involves phosphorylation. Yeast Rrn3 can be phosphorylated *in vivo* and *in vitro* [(Fath et al., 2001) Schneider lab, unpublished data] which was proposed to be regulatory (Blattner et al., 2011; Claypool et al., 2004; Fath et al., 2001). However, to date, no essential sites of modification or protein kinases have been established for yeast Rrn3 growth-dependent control (Blattner et al., 2011; Gerber et al., 2008). Instead, it has been suggested that phosphorylation of Pol I is necessary for formation of the active initiation complex (Fath et al., 2001). In comparison with yeast studies, covalent modifications of mammalian Rrn3 are critical for rRNA synthesis and its regulation (Bierhoff et al., 2008; Cavanaugh et al., 2002; Mayer et al., 2005; Mayer et al., 2004; Zhao et al., 2003).

Altogether, Rrn3 abundance and binding to Pol I is a well-documented, conserved mechanism of ribosome biogenesis control in response to nutrient availability.

The yeast core factor and the TATA-binding protein (TBP).

The yeast core factor (CF) is a heterotrimer consisting of essential Rrn6, Rrn7 and Rrn11 subunits (Keys et al., 1994; Lalo et al., 1996; Lin et al., 1996). CF functions in the recruitment of Pol I to the rDNA. Several lines of evidence suggest that CF physically associates with all of the components of PIC: Rrn3, TBP, UAF and Pol I (Blattner et al., 2011; Knutson and Hahn, 2011; Lalo et al., 1996; Peyroche et al., 2000). It is proposed that the Rrn6 subunit of CF directly binds TBP and Rrn3, and the Rrn7 subunit contacts

UAF, TBP and the polymerase "dock" domain. In addition, CF associates with the core element of the rDNA promoter in the presence of functional Pol I, suggesting that PIC assembly affects rDNA affinity of the CF (Bordi et al., 2001). However, recent studies using chromatin endogenous cleavage (ChEC) assays indicate that the promoter binding by CF and partial PIC assembly can occur in the absence of Pol I or Rrn3 *in vivo* (Goetze et al., 2010).

Pol I, Rrn3 and the CF are sufficient for minimal promoter-specific transcription *in vitro*. However, TBP is required for efficient specific transcription (Bedwell et al., 2012). TBP is a component of all three eukaryotic polymerase initiation machineries (Cormack and Struhl, 1992; Schultz et al., 1992b). TBP associates with the rDNA promoter likely via interactions with CF and/or UAF. Initially, TBP was discovered to bind both CF [via Rrn6 and Rrn7 subunits] and the UAF [through the Rrn9 subunit] suggesting that it acts as an intercessor in PIC assembly (Steffan et al., 1996; Steffan et al., 1998). TBP is clearly required for Pol I activity to mediate the UAF-dependent recruitment of CF to the template *in vivo* (Aprikian et al., 2000; Cormack and Struhl, 1992; Schultz et al., 1992b). These data indicate a dual role of TBP in the Pol I transcription serving as an essential component of the core promoter complex as well as a mediator for the interaction with upstream activators.

While Pol I, Rrn3 and TBP are highly conserved, CF does not have any obvious sequence identity to mammalian Pol I initiation factors. Yeast CF is functionally analogous to the human Pol I factor SL1, composed of TBP and associated factors (TAFs): TAF1A, TAF1B, TAF1C, and TAF1D (Gorski et al., 2007; Zomerdijk et al., 1994). Despite insignificant sequence conservation, it has been proposed that TAF1A, B

and C are orthologous to the CF subunits Rrn6, 7, and 11, respectively (Boukhgalter et al., 2002). Indeed, homology was later confirmed for Rrn7 and TAF1B by functional complementation of the structurally conserved domains (Knutson and Hahn, 2011). In contrast to SL1, TBP is not considered a CF subunit, since the CF and TBP do not form a stable stoichiometric association during purification (Keys et al., 1994). Nevertheless, both CF and SL1 perform similar functions recruiting Pol I to the rDNA promoter via association with Rrn3 (Friedrich et al., 2005; Miller et al., 2001; Peyroche et al., 2000).

It has been recently shown that Rrn7 and TAF1B also share homology with Pol II general factor TFIIB and TFIIB-like Pol III PIC component Brf1 (Blattner et al., 2011; Knutson and Hahn, 2011; Naidu et al., 2011). Rrn7 and TAF1B resemble TFIIB-like domain architecture, especially in the zinc ribbon and cyclin-like fold motifs. Like all TFIIB-like factors, Rrn7 and TAF1B share common domain organization, associate with the polymerase dock domain, bind TBP and recruit polymerase to the promoter [reviewed in (Vannini and Cramer, 2012)]. Furthermore, like other TFIIB-like factors, SL1 has essential post-recruitment roles in transcription independent of Pol I loading on the promoter (Naidu et al., 2011). The exact mechanisms of by which SL1 or CF initiate rDNA transcription remain to be defined.

The Upstream Activation Factor (UAF).

The fourth of Pol I's general initiation factors required for robust specific transcription is UAF (Keener et al., 1998a; Keys et al., 1996). UAF consists of 6 subunits: two histone components - H3 and H4, as well as four specific proteins - Rrn5, Rrn9, Rrn10 and Uaf30 (Keener et al., 1997; Keys et al., 1996; Siddiqi et al., 2001b). UAF is essential for rDNA

transcription by Pol I *in vivo* with Uaf30 being the only dispensable subunit, the absence of which results in a severe growth defect.

UAF is known to bind to the upstream element of the rDNA promoter irrespective of Pol I transcription and defines the location where the PIC can be formed (Bordi et al., 2001; Claypool et al., 2004; Hontz et al., 2008; Keys et al., 1996). The strong DNA binding activity of UAF is supposedly mediated by the histone components, whereas the non-histone subunits (specifically, Uaf30) provide specificity (Hontz et al., 2008). Furthermore, it was demonstrated that the Rrn9 subunit of UAF contacts both TBP and CF (Steffan et al., 1996; Steffan et al., 1998). These protein-protein interactions are required for promoter loading of CF by UAF with assistance of TBP, and thus for functional PIC assembly (Bordi et al., 2001; Steffan et al., 1996; Steffan et al., 1998). Despite the fact that UAF is not required for basal levels of transcription *in vitro*, the presence of UAF is necessary for the template commitment and boosts specific transcription by Pol I in a TBP-dependent manner (Bedwell et al., 2012; Keener et al., 1998b; Keys et al., 1996). Thus, UAF is necessary for activation of ribosome production.

Apart from its role in stimulating Pol I transcriptional activity, UAF prevents Pol II transcription of the ribosomal RNA genes (Oakes et al., 1999; Vu et al., 1999). It has been shown that strains deleted for either *RRN5, RRN9* or *RRN10* genes do not survive or grow extremely slowly, but at a very low frequency give rise to variants showing moderately improved growth rates (Oakes et al., 1999; Siddiqi et al., 2001a; Vu et al., 1999). In these strains, Pol I transcription is completely abolished and rRNA synthesis is achieved by a several fold expansion of the rDNA array transcribed using Pol II from a cryptic promoter upstream from the Pol I initiation site. For that reason, these cells were

called “polymerase switched” (PSW). Furthermore, deletion of the gene encoding another UAF subunit, *UAF30* has similar effects permitting Pol II transcription of the 35S gene (Hontz et al., 2008; Siddiqi et al., 2001b). In *uaf30*Δ cells, UAF occupancy of the rDNA promoter is decreased, consistent with the role of this subunit in specific targeting of the UAF complex, which results in a massive inactivation of the rDNA repeats and ~70% decrease in rRNA production. Remarkably, the *uaf30* mutants use both Pol I and Pol II for rDNA transcription (Siddiqi et al., 2001b). Thus, mutations in genes coding either of the non-histone subunits of UAF result in Pol II invasion of the rDNA. Substitutions in Pol I itself or in CF subunits do not allow Pol II transcription of rDNA suggesting a unique role of UAF in polymerase selectivity at the 35S rRNA genes (Oakes et al., 1999). Recently, it has been demonstrated that absence of the UAF subunits resulted in altered chromatin structure of the rDNA repeats (Goetze et al., 2010). As a result, Pol II and Pol III gained access to the rDNA promoter region. Thus, UAF establishes chromatin structures responsible for polymerase specificity at the rDNA locus.

Unlike the rest of the basal factors, the non-histone subunits of UAF do not have mammalian homologues. The complex seems to be specific for fungi (Al-Khouri and Paule, 2002; Liu et al., 2002; Nakazawa et al., 2008). Mammalian Pol I utilizes an additional factor, called UBF for efficient transcription initiation, which does not resemble yeast UAF. Remarkably, several subunits of UAF share partial homology with the components of SWI/SNF chromatin remodeling complex, including the catalytic protein Snf2 (Liu et al., 2002). Whether the detected sequence homology between UAF and chromatin remodelers reflects functional similarity remains to be tested.

RNA Polymerase I Transcription Elongation

To complete synthesis of the 35S rRNA precursor, Pol I transcribes rDNA with the rate of elongation approximately 60 nucleotides per second (French et al., 2003). In yeast the full size of the pre-rRNA is 6744 nt, which is considerably longer than the average yeast Pol II and III transcripts. Thus, Pol I should be necessarily efficient and processive to ensure successful rRNA biosynthesis.

For decades research was focused on the mechanisms of Pol I transcription initiation, while the significance of the elongation step was overlooked. However, in the past several years interest in Pol I transcription elongation has emerged primarily due to the two major discoveries: first, Pol I transcription elongation is functionally coupled with ribosome biogenesis; and second, Pol I transcription elongation is regulated (Philippi et al., 2010; Schneider et al., 2007; Stefanovsky et al., 2006a; Zhang et al., 2010). Ongoing studies continue to identify factors that affect transcription elongation by Pol I and rRNA biosynthesis as well as mechanisms by which Pol I effectively accomplishes rDNA transcription [reviewed in (Schneider, 2012)].

The role of "co-transcriptionality" in ribosome production.

Ribosome biogenesis commences in a special sub-nuclear compartment – the nucleolus, continues in the nucleoplasm and completes in the cytosol. This complicated process involves multiple steps, starting with the rRNA production by Pol I. The Pol I – derived 35S pre-rRNA is processed via cleavages, folding and modifications. Co- and post-transcriptional association of the rRNA molecules with the ribosomal and non-ribosomal proteins promotes formation of the small and large subunit precursors. The

precursors of ribosomal subunits are then exported from the nucleus into the cytoplasm where they undergo final steps of maturation to form functional 40S and 60S particles. The process of ribosome assembly is highly complex, organized and controlled [reviewed in (Fatica and Tollervey, 2002)].

For a while it was believed that yeast rRNA maturation occurs post-transcriptionally, however, recent studies showed that early steps of rRNA processing and ribosome subunit formation happen on the nascent pre-rRNA transcript (Kos and Tollervey, 2010; Osheim et al., 2004). The fact that the first steps in ribosome assembly occur while Pol I is still transcribing rDNA lead to a model that Pol I transcription elongation is coupled to ribosome assembly (Schneider et al., 2007). Indeed, co-transcriptionality is known to facilitate the assembly of RNP complexes for Pol II or bacterial RNAP [reviewed in (Perales and Bentley, 2009)]. The rate of transcription elongation, especially pausing, is proposed to affect the folding pathway of the nascent transcript and co-transcriptional recruitment of the trans-acting factors to RNA [reviewed in (Pan and Sosnick, 2006)].

A screen designed to isolate Pol I mutants defective in transcription elongation resulted in identification of the Pol I mutant bearing a substitution of the aspartate to glycine in position 784 of the A135 subunit, the *rpa135-D784G* allele (Schneider et al., 2007). Based on sequence homology with Pol II, the D784 residue participates in the catalytic magnesium coordination. Dramatically reduced transcription elongation rate of this Pol I mutant was accompanied by decreased rRNA synthesis, enhanced rRNA degradation and faulty processing. Careful analysis of the *rpa135-D784G* mutant was the first direct support of the model that Pol I transcription elongation rate is linked to rRNA maturation. Moreover, analysis of the ribosome pools in the *rpa135-D784G* mutant

suggested an imbalance in the 60S to 40S particles ratio and resulted in the significant ribosome assembly defects. To date, several more elongation defective Pol I mutants were shown to have impaired rRNA processing and ribosome assembly [(Albert et al., 2011; Viktorovskaya et al., 2013); Chapters 2 and 3]. Altogether, the speed of Pol I transcription affects rRNA processing and assembly of the ribosomes serving as an evidence that multiple stages of ribosome biogenesis are functionally coupled.

Pol I transcription elongation factors.

The properties of transcription elongation by Pol I discussed above reveal the significance of this step in the transcription cycle. However, only a few of the trans-acting factors that influence transcription elongation have been described for Pol I. Some of them are unique to Pol I whereas others are shared with other polymerases.

The conserved Spt4/5 complex. Spt4/5 is a universally conserved transcription factor. Its homologs are known to affect polymerase processivity and transcription elongation in all three domains of life [reviewed in (Grohmann et al., 2011; Hartzog and Fu, 2013; Werner, 2012)]. The eukaryotic Spt4/5 complex has known roles in Pol II transcription elongation and mRNA biogenesis [reviewed in (Saunders et al., 2006)]. Spt4/5 is a heterodimer composed of the essential Spt5 protein, which is believed to be a major functional subunit, and a dispensable Spt4 peptide, primarily involved in stabilization of the complex (Liu et al., 2009). A novel role for yeast Spt4/5 complex in Pol I transcription has been identified (Schneider *et al.,* 2006). Deletion of *SPT4* leads to impairment of rRNA processing/ribosome assembly and a small increase in Pol I transcription elongation rate relative to wild type. Thus, these data suggested that Spt4/5

inhibits Pol I transcription elongation (Schneider et al., 2006). Further studies using point mutations in *SPT5* support the previous model that Spt4/5 can inhibit Pol I transcription *in vivo* (Anderson et al., 2011). Interestingly, characterization of these mutants also identified a positive role for Spt4/5 in Pol I transcription elongation that was not evident from characterization of *spt4Δ* strains. In addition, mutation of *SPT5* resulted in an altered Pol I distribution over the rDNA suggesting transcriptional pausing or local changes in the polymerase velocity as a potential mechanism of Spt4/5-mediated effects on rRNA synthesis. It has been shown that Spt4 and Spt5 associate with Pol I and the rDNA *in vivo*, and Spt5's interaction with Pol I is direct (Beckouet et al., 2011; Lepore and Lafontaine, 2011; Schneider et al., 2006; Viktorovskaya et al., 2011). Moreover, Spt5 interacts with an exosome-associated components, bringing together Pol I transcription elongation, rRNA processing and surveillance machinery (Lepore and Lafontaine, 2011). Altogether, it has been proposed that direct association of Spt4/5 to Pol I and to the processing machinery modulates transcription elongation rates, rRNA maturation and ribosome biogenesis.

The Paf1 complex. Similar to Spt4/5, Paf1 complex (Paf1C) participates in transcription by Pol II, affects chromatin structure and transcript maturation (Mayekar et al., 2013). Paf1C consists of five subunits: Paf1, Ctr9, Rtf1, Cdc73 and Leo1 (Krogan et al., 2002; Mueller and Jaehning, 2002). The Schneider lab has recently discovered that Paf1C is required for efficient transcription by Pol I *in vivo* (Zhang et al., 2009). Since pure Paf1C increased the transcription elongation rate of Pol I in a fully reconstituted *in vitro* system, it definitively showed that the Paf1C complex could influence Pol I transcription directly (Zhang et al., 2010).

The mammalian elongation factors. Unlike yeast, more factors affecting Pol I transcription elongation were identified in mammalian cells. Some of those factors are largely specific for Pol I (such as the Upstream Binding Factor (UBF) and the histone chaperon nucleolin) while others have previously established role in Pol II transcription (like TFIIH and FACT (FAcilitates Chromatin Transcription)) (Assfalg et al., 2012; Birch et al., 2009; Mongelard and Bouvet, 2007; Rickards et al., 2007; Stefanovsky et al., 2006a; Stefanovsky et al., 2006b). These and several other factors were shown to primarily affect transcription elongation by Pol I via the rDNA chromatin organization [reviewed in (Birch and Zomerdijk, 2008; Hannan et al., 1998)]. Consistent with the elongation-linked model of ribosome biogenesis, at least TFIIH and nucleolin were shown to affect Pol I transcription elongation rate and rRNA processing (Ginisty et al., 1998; Nonnekens et al., 2013).

Pol I transcription elongation is regulated.

Though transcription initiation by Pol I is a major step for control of rRNA synthesis, several studies suggested that post-initiation events are also regulated in response to cellular signaling and nutrient availability (Claypool et al., 2004; Philippi et al., 2010; Stefanovsky et al., 2006a; Zhang et al., 2010). On the one hand, the role of the abundance of the Rrn3-Pol I in the growth-dependent control of rRNA synthesis is well-established (Claypool et al., 2004; Laferte et al., 2006; Philippi et al., 2010). On the other hand, a non-degradable *rrn3* mutant was still able to slightly down-regulate initiation competent Pol I levels in response to rapamycin treatment (Philippi et al., 2010). Moreover, under-expression of yeast *RRN3* correlated with reduced Pol I transcription initiation and rRNA

synthesis even under optimal conditions, though to a lesser extent than TOR-dependent shut-down of ribosome production. Therefore, the simple reduction of Rrn3 levels and the resulting decrease in Pol I transcription initiation do not fully explain the observed effects of TOR inhibition on rRNA biogenesis suggesting that additional post-initiation mechanisms of regulation exist.

There are two possible mechanisms by which the abundance of rRNA can be modulated after initiation: first, rRNA is synthesized and rapidly degraded; and second, the elongation by the already engaged polymerases is inhibited. Indeed, it is proposed that the rRNA turnover in yeast is fast (Poll et al., 2009; Reiter et al., 2011). Furthermore, two factors (mammalian UBF and yeast Paf1C) which affect Pol I elongation are shown to be required for growth-dependent control rRNA synthesis (Stefanovsky et al., 2006a; Zhang et al., 2010). Thus, rRNA degradation and transcription elongation are likely to be regulated, establishing an opportunity for the fast rRNA synthesis control in response to changing environmental conditions.

RNA Polymerase I Transcription Termination

Pol I has an extremely high polymerase density of approximately 50 - 60 elongation complexes per 35S rRNA gene (French et al., 2003), which implies very efficient transcription initiation and termination. According to calculations, Pol I termination must occur every 5 seconds in growing yeast under standard conditions (Reeder and Lang, 1997). Inability of Pol I to stop would potentially impede with expression of the proximal 5S rRNA gene as well as misdirect resources away from the production of functional ribosomes. Thus, Pol I transcription termination, although poorly understood, may be

another critical step in the transcription cycle. Careful analysis of the Pol I terminator sequences and identification of the major protein components allowed to generate a series of models describing the mechanisms of termination and transcript release.

An early Reb1-dependent "road block" model.

Reb1 is an essential DNA-binding protein involved in termination by Pol I in addition to other cellular roles (Lang et al., 1994; Lang and Reeder, 1993; Reeder and Lang, 1997). Reb1 has known orthologs in mouse and human (called TTF1), with the highest conservation found within their DNA-binding domain (Evers and Grummt, 1995; Evers et al., 1995). A conserved DNA-binding motif of Reb1 specifically associates with the sequences at the rDNA *in vitro*, especially with the 11-bp region of Pol I terminator (Lang and Reeder, 1993). Association of Reb1 with rDNA terminator was demonstrated to be required and sufficient for the two major steps: polymerase pausing and subsequent transcript release *in vitro*.

The termination site and thus 3'end of the transcript were mapped to the T-rich element right upstream from the Reb1-binding site called the main terminator T1. Mutant forms of Reb1 as well as the altered terminator sequences used in the all purified transcription termination reactions suggested a simple road-block model. According to that model Reb1 bound to the terminator impedes with Pol I elongation promoting pausing which occurs at the upstream T1 element. The T1 site is enriched in T-bases which destabilize interactions of the paused elongation complex with the weak RNA-DNA hybrid, promoting transcript release and removal of the polymerase from the template.

The Torpedo model.

Despite the requirement of the Reb1 binding site, Reb1 does not seem to be crucial for termination *in vivo*, in contrast to *in vitro* data (Kawauchi et al., 2008; Reeder et al., 1999). Because of the increasing discrepancies concerning the role of Reb1 *in vivo* as well as novel data describing various Pol I termination factors, a novel "torpedo-like" model was proposed (El Hage et al., 2008; Kawauchi et al., 2008).

The torpedo model resembles the mechanism proposed for Pol II transcription termination. According to this model, co-transcriptional cleavage of the nascent 35S transcript performed by Rnt1 is required for Pol I termination *in vivo* (El Hage et al., 2008; Kawauchi et al., 2008; Reeder et al., 1999). The RNase III-like endonuclease Rnt1 hydrolyses the nascent transcript across a stem–loop structure within the 3′-ETS (external transcribed spacer), at positions downstream to the 3' end of the 25S rRNA and upstream to the T1 site (Elela et al., 1996; Kufel et al., 1999). The 5'end of the cleaved transcript is modified by the polynucleotide kinase Grc3 (and/or other processing factors) which generates a loading site for the exonuclease complex Rat1/Rai1 and a helicase Sen1 (Braglia et al., 2010; El Hage et al., 2008; Kawauchi et al., 2008). Rat1 promotes 5' to 3' degradation of the transcript approaching to the Pol I elongation complex. Transcription is terminated, when Rat1 reaches Pol I at the T1 element destabilizing the elongation complex.

Indeed, as shown by transcription run-on and ChIP analyses defects in Rnt1 as well as Rat1, Rai1 and Sen1, but not Reb1, compromise termination and lead to transcriptional read-through *in vivo*. When Rat1or Rnt1 activities are inhibited and termination at the T1 site fails, Pol I stops downstream at the "fail-safe" stop site (called T2) (El Hage et al.,

2008; Elela et al., 1996; Reeder et al., 1999). Investigation of the rDNA terminator *in vivo* even in the wild type cells suggests that about 90% of the transcripts are terminated at the T1 element, whereas the rest of the polymerases elongate up to the T2 terminator or the replication fork barrier (RFB) (El Hage et al., 2008).

The combined termination model.

The torpedo model does not exclude involvement of Reb1 in termination, though it devotes a critical role in termination to the Rnt1 endonuclease. However, Rnt1 is not essential for survival, and the extent to which Pol I transcription termination at the T1 site is reduced upon deletion of *RNT1* is marginal (Prescott et al., 2004). These data suggest that additional factors are required to support efficient termination in cells. Two recent studies devoted to the mechanisms of Pol I termination seem to resolve the potential discrepancy in the roles of Rnt1 and Reb1 (Braglia et al., 2011; Reiter et al., 2012).

In the first study a second co-transcriptional cleavage event independent from the Rnt1 nuclease was identified (Braglia et al., 2011). This cleavage occurs at the T1 element serving as an alternative pathway to the primary Rnt1-mediated mechanism. The existence of the alternative pathway explains the moderate phenotype of the *rnt1*Δ mutants.

In the second work a novel role for the Nsi1 protein in Pol I termination was identified (Reiter et al., 2012). Nsi1 shares homology with Reb1 and mammalian TTF1 and likely functions in polymerase pausing and termination. Nsi1 efficiently binds to the terminator region *in vivo* and is required for proper termination. Thus, Nsi1 potentially plays a role previously designated to Reb1 in the "road-block" model.

According to the current model [(Figure 2); reviewed in (Nemeth et al., 2013)], Pol I transcription is primarily terminated at the T1site using the Rnt1-induced transcript cleavage followed by the Rat1 recruitment as described in the "torpedo" model. However, if the polymerase escapes termination at the T1 site, a "fail-safe" mechanism prevents elongation much further. An alternative co-transcriptional cleavage activity at the T1 site provides substrate for the "torpedo" loading on the nascent pre-RNA. In that case, termination occurs at the second T2 terminator and is additionally insured by the RFB sequence (El Hage et al., 2008). This secondary pathway requires the intact Reb1 binding site, might involve Nsi1- (and/or Reb1-) mediated polymerase pausing and is independent from Rnt1.

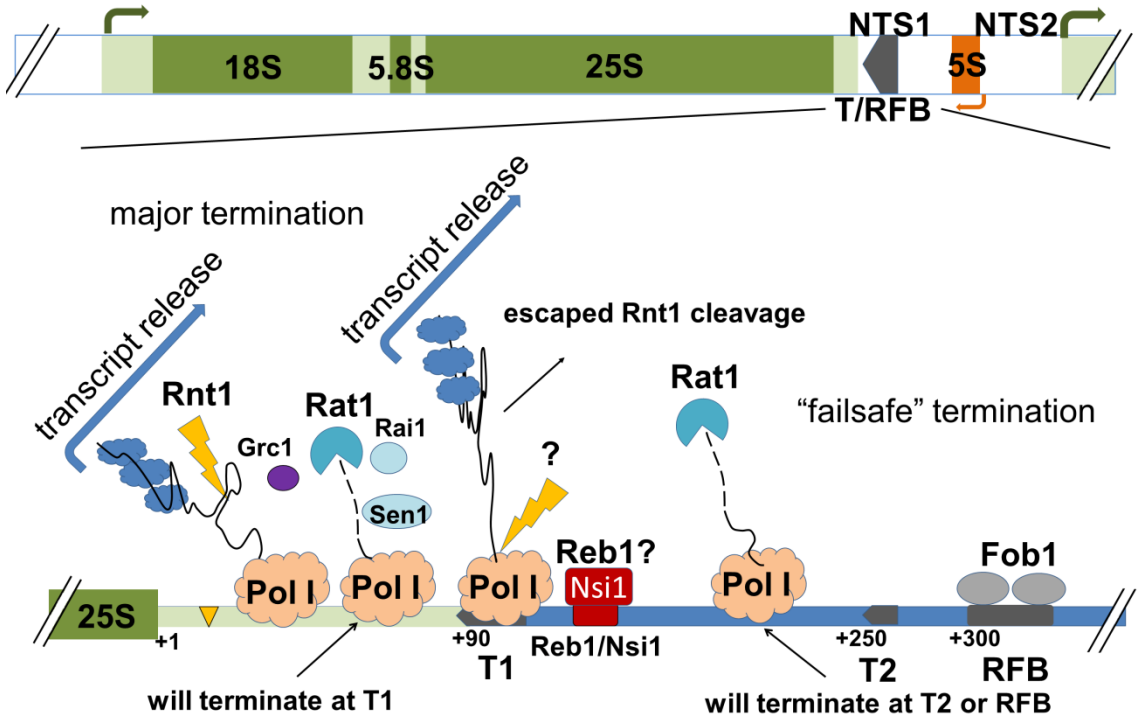

Figure 2. Pol I transcription termination. Cartoon diagram of the yeast Pol I transcription termination factors. The major rDNA sequence elements are indicated at the top. The positions of 25S, 5.8S, 18S and 5S rRNA regions, nontranscribed (NTS) sequences, the 35S terminator (T) and the replication fork barrier (RFB) site are shown. The directions of transcription from the 5S and 35S promoters are specified by the arrows. The Reb/Nsi1 binding site, the T1 and T2 elements are pointed. The positions of the T1, T2 and RFB sites are specified in respect to the 3'end of the 25S rRNA region. The yellow triangle represents the DNA-encoded Rnt1-recognition site. The nascent rRNA transcript emerging from the Pol I elongation complex (in tan) bound by the ribosomal proteins and ribosome biogenesis factors (in blue) is cleaved by Rnt1 (the yellow lightning) and modified by Grc1 (in purple) followed by activities of Rat1/Rai1/Sen1 (in light blue) and Nsi1 or Reb1 (in red) which promote termination at T1. If the elongation complex escapes Rnt1-cleavage, the transcript is cleaved at T1 by unknown endonuclease (yellow lightning with the question mark), recruits the Rat1 "torpedo" and the polymerase terminates at the T2 terminator (or at the RFB site).

The Organization of the rDNA Locus

The rDNA locus has several unique characteristics that significantly contribute to rRNA synthesis control. The local DNA topology, the size of the rDNA array and the chromatin structure were shown to affect primarily Pol I transcription initiation and elongation. However, the ability of Pol I to efficiently terminate also depends on epigenetic state of rDNA (Jones et al., 2007). The epigenetic features specific for the rRNA gene expression in yeast are discussed in this section.

The effects of size of the rDNA array and polymerase clustering.

The rDNA loci of eukaryotes are organized as head-to-tail tandem arrays. In yeast the 35S and 5S pre-rRNA genes are located beside one another in a 9,081-bp unit that is repeated approximately 150-200 times to form one large array (Figure 3). This array localizes on chromosome XII and is estimated to be approximately 1.4 Mb long, accounting for about 10% of the genome (Kobayashi et al., 1998; Schweizer et al., 1969). The organization of rDNA in most other eukaryotes is similar to that in yeast except that the 5S genes (transcribed by Pol III) in most species are found in distinct arrays apart from the Pol I-transcribed rDNA repeats.

In wild type yeast, about half of the rDNA repeats is not transcribed and this characteristic is conserved in eukaryotes. EM analysis of the rDNA chromatin spreads (Miller spreads) can visualize actively transcribed rDNA units, known as the “Christmas trees”, and inactive repeats as the gaps interspacing the trees (French et al., 2003). Although actively transcribed rRNA genes are required for ribosome biogenesis, the presence of the inactive repeats functions in genome stability (Ide et al., 2010).

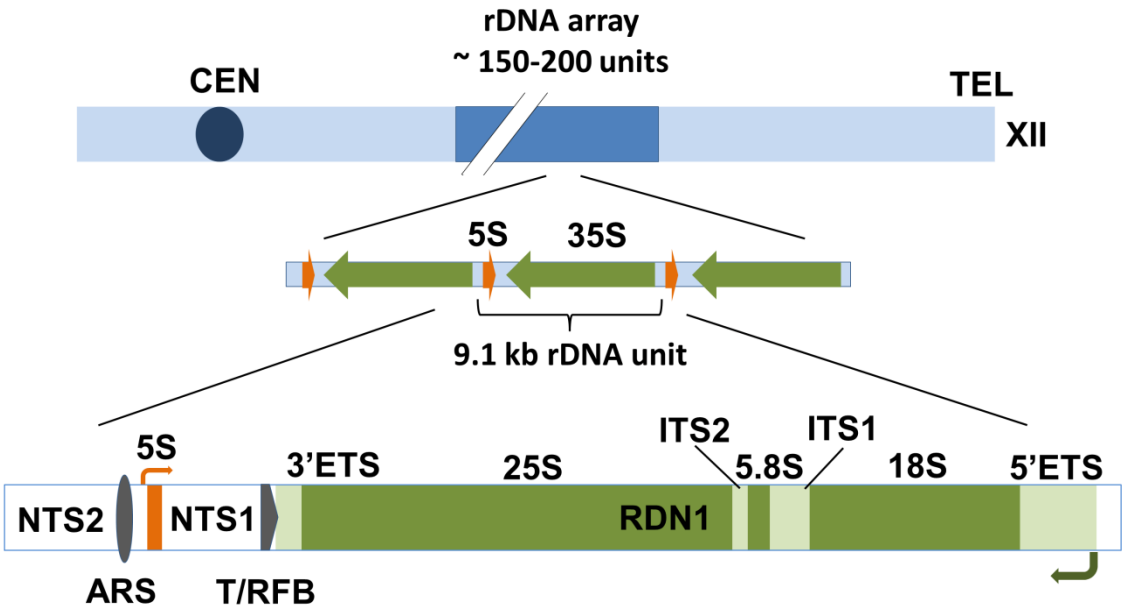

Figure 3. Organization of the ribosomal DNA locus in yeast. Cartoon diagram of the rDNA array. The major rDNA sequence elements are indicated as on Figure 2. The positions of 25S, 5.8S, 18S and 5S rRNA regions, an autonomously replicating sequence (ARS), the 35S terminator (T) and the replication fork barrier (RFB) site, the external and internal transcribed (ETS and ITS) and nontranscribed (NTS) sequences are shown.

Due to its highly repetitive nature, the size of rDNA is not stable; changes in the number of repeats are governed by either homologous recombination or DNA replication (Houseley and Tollervey, 2011; Kobayashi et al., 1998). The effects of the rDNA array size on Pol I transcription and cell function can be monitored using the *fob1*Δ mutation. A deletion of *FOB1* gene generates a collection of strains with the fixed rDNA copy numbers at various levels (Kobayashi et al., 1998). Among these mutants, there are strains with dramatically reduced rDNA copy number (containing ~25 and ~42 repeats per haploid genomes compared to ~150-200 copies in WT cells). Despite that, the cell growth or rRNA synthesis rates are not decreased in the low-copy-number strains due to a number of epigenetic changes of the rDNA not observed in the WT cells (French et al.,

2003; Kobayashi et al., 1998). EM analysis of the 42-copy-number strain revealed that all of the repeats were active. Furthermore, the number of transcribing polymerases per gene was significantly elevated to compensate for the decrease of the rRNA genes. On some genes in the reduced copy strain, there was a polymerase every 41 nucleotides, which is near the limit possible for polymerase packing.

A high density of elongation complexes on the rDNA is rare or absent in Pol II- or Pol III-transcribed genes. Such polymerase clustering might have interesting consequences on the rRNA synthesis. Gadal and colleagues proposes that high Pol I density might help efficient transcription elongation (Albert et al., 2011). Molecular modeling, Miller spreads analysis and co-immunoprecipitation assays suggest that A43 subunit of the leading polymerase contacts A49 subunit of the trailing elongation complex, so that this interaction "pushes" the leading elongation complex forwards (Albert et al., 2011; Beckouet et al., 2008). Genetic support for that model comes from the studies of the *rpa49*Δ strain on the high- and low-copy number backgrounds. It has been shown that defects caused by the absence of the A49 subunit are partially suppressed by the high polymerase density in the reduced rDNA copy number strains.

Another potential outcome from the polymerase clustering is potentially increased sensitivity towards intrinsic pausing/arrests. The stalled Pol I complex would generate "traffic jams". For that reason Pol I might have evolved more efficient catalytic properties compared to Pol II (Viktorovskaya et al., 2013).

Altogether, the size of the rDNA array affects the proportion of the active repeats as well as polymerase loading and density over the active genes. This characteristics unique

for Pol I might have important consequences on the rRNA synthesis in some genetic backgrounds.

Active *versus* inactive rDNA repeats.

Each of the rRNA genes contains an identical subset of *cis*-acting elements and can be transcribed by Pol I. However, only approximately half of the rDNA units are transcribed in the growing yeast cells. Active and inactive rRNA genes can be separated by gel-electrophoresis after cross-linking (Dammann et al., 1993). Alternatively, both states of the rDNA can be visualized using EM of the Miller chromatin spreads (French et al., 2003). Studies in yeast and mammals demonstrated that alteration in the ratio of active versus inactive units is not critical for regulation of rRNA synthesis. However, accessibility of the rDNA to transcription factors affects rRNA production under some developmental or growth conditions (Claypool et al., 2004; Conconi et al., 1989; French et al., 2003; Oakes et al., 2006a; Oakes et al., 2006b; Sanij et al., 2008; Stefanovsky and Moss, 2006).

Inactive rDNA repeats have nucleosomes packed in a beads-on-a-string structure with density of approximately 35-40 nucleosomes per gene as estimated by EM [Figure 4; (Dammann et al., 1993; Johnson et al., 2013; Merz et al., 2008)]. Such "closed" heterochromatin structure would potentially prevent Pol I from transcription of the unit since Pol I was shown to be sensitive to the presence of nucleosome-containing template *in vitro* (Birch et al., 2009; Rickards et al., 2007).

There is more controversy concerning the chromatin structure of the actively transcribed units. The active repeats are heavily loaded with closely spaced Pol I

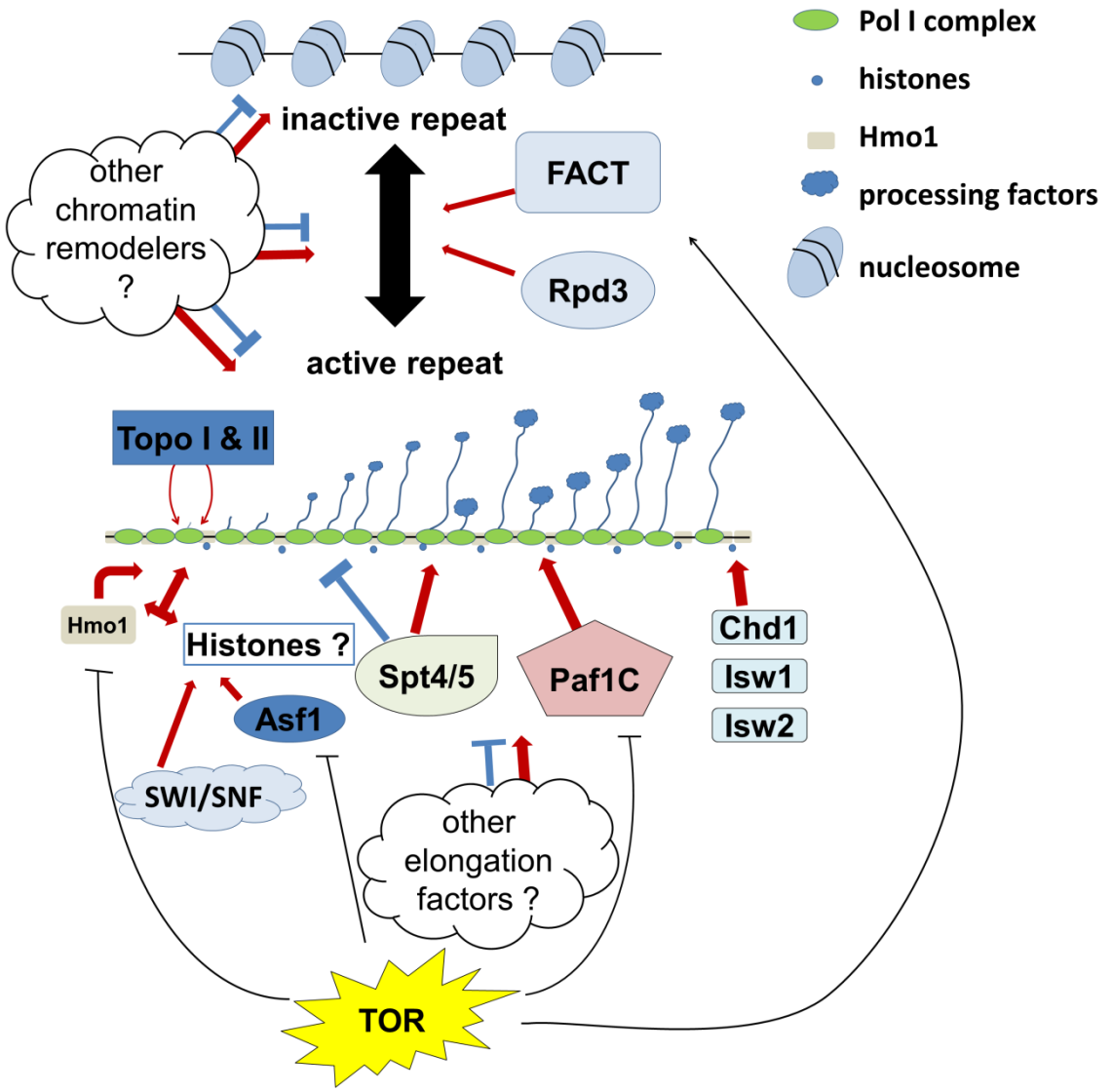

Figure 4. Pol I transcription elongation and the rDNA chromatin. Cartoon diagram of the yeast Pol I transcription elongation and chromatin remodeling factors. A dynamic nature of the active (at the bottom) and inactive (on the top) rDNA repeats is specified by the two-headed arrow. The positive (red arrows) and negative (blue block signs) effects (direct or indirect) of the certain factors on Pol I transcription or the rDNA chromatin state are indicated. The known targets of the TOR-signaling pathway (the yellow multi-pointed star) are showed by the arrows or the block signs.

elongation complexes (French et al., 2003). Using the psoralen-crosslinking or the chromatin endogenous cleavage (ChEC) assays, it has been shown that actively transcribed repeats are devoid of nucleosomes at their coding region (Dammann et al., 1993; Merz et al., 2008). Instead, is has been proposed that such "open" chromatin is organized in a specialized structure containing Hmo1 [see below; (Merz et al., 2008)]. EM analysis further confirms that the portions of the coding region free from polymerases appear "smooth" and nucleosome-free (Johnson et al., 2013).

On the other hand, the ChIP assay of the low-copy-number strains containing only active repeats strongly suggests the presence of histones over the rDNA (Jones et al., 2007). For that reason, the "open" rRNA genes were proposed to have "unphased" or dynamic nucleosome structure. Though it is clear, that canonical nucleosomes are absent on the active rDNA (Johnson et al., 2013), histones might still be present forming alternative structures or sub-nucleosomal particles in the "open" chromatin [as suggested in (Albert et al., 2012; Chen et al., 2012; Jones et al., 2007)].

The role of Hmo1 protein in ribosome synthesis.

Hmo1 is a component of the rDNA chromatin specific for the actively transcribed rRNA genes (Merz et al., 2008). Hmo1 is a non-essential protein that associates with the rDNA promoter and coding region (Gadal et al., 2002; Hall et al., 2006; Kasahara et al., 2007). It belongs to the high mobility group (HMG) family and shares partial structural and functional similarity with mammalian UBF. Deletion of *HMO1* has a very mild phenotype, though becomes limiting in certain genetic backgrounds (Gadal et al., 2002).

A mild decrease in rRNA synthesis rate and delay in rRNA processing were detected in the *hmo1*Δ strains (Gadal et al., 2002; Hall et al., 2006).

Ribosome biogenesis requires coordinated equimolar amounts of the four rRNAs and ribosomal proteins. In addition to its positive role in the rDNA transcription, Hmo1 was also shown to associate with the promoters of several ribosomal genes and affect their expression (Berger et al., 2007; Hall et al., 2006). In the absence of Hmo1, the expression of the rRNA and ribosomal proteins in response to TOR-signaling is uncoupled. Thus, it has been proposed that Hmo1 plays a role in coordinated synthesis of rRNA and ribosomal proteins. Altogether, Hmo1 is an important component of rDNA chromatin involved in Pol I transcription and ribosome biogenesis.

Chromatin modification and remodeling factors in rDNA.

Even though the ratio of active to inactive rDNA units does not regulate rRNA synthesis in growing yeast (French et al., 2003), establishment and maintenance of these two states are important for Pol I transcription (Johnson et al., 2013; Sandmeier et al., 2002).

During stationary phase or starvation the percentage of open rDNA genes is greatly reduced (Dammann et al., 1993). The rDNA units that used to be actively transcribed become inactivated and get "heterochomatinized" until cells are provided with the necessary nutrients (Oakes et al., 2006b; Sandmeier et al., 2002). This conversion of active to inactive units is dependent on a histone deacetylase Rpd3 and chromatin remodeling factor Spt16 (Johnson et al., 2013; Oakes et al., 2006b). Rpd3 and Spt16 (a component of the FACT) are responsible for the histones deposition on the rDNA genes

during diauxic shift. In either *rpd3*Δ or *spt16* cells, rDNA repeats stay in an "open" conformation upon diauxic shift even though the rRNA synthesis was still repressed. The defects in nucleosome disruption in the *spt16* mutant were accompanied by an inability to fully activate Pol I transcription when cells exit stationary phase and re-transition to active growth.

Several other chromatin remodeling factors or histone chaperones are suggested to affect Pol I transcription in yeast: SWI/SNF complex, Asf1/Rtt109, Spt6, Chd1, Isw1 and Isw2 [Figure 4; (Beckouet et al., 2011; Chen et al., 2012; Jones et al., 2007; Zhang et al., 2013)]. Moreover, histone modifications were shown to regulate Pol I activity, Hmo1 occupancy over rDNA and rRNA processome recruitment in a TOR-dependent manner (Chen et al., 2012). Involvement of these and other factors in rDNA chromatin and Pol I transcription is a subject for future studies.

rDNA "silencing" of Pol II transcription.

In wild type cells, a specific chromatin structure represses recombination and prevents transcription from Pol II promoters within the rDNA array, the telomeres and the mating type loci. Such "silencing" is established by the histone deacetylase Sir2 [reviewed in (Blander and Guarente, 2004; Guarente, 1999)].

At the rDNA locus, Sir2 is a part of the RENT complex (regulator of nucleolar silencing and telophase exit) together with Net1 and phosphatase Cdc14 (Shou et al., 2001; Straight et al., 1999). Directly and via recruitment of chromatin modification factors, RENT establishes a heterochromatic state at the rDNA (Bryk et al., 2002; Fritze et al., 1997; Oakes et al., 1999). Deletion of *SIR2* results in hyper-recombination and

permits transcription of the Pol II-reporter genes inserted in the rDNA. Association of Sir2 with the rDNA is affected by multiple proteins, including the Pol I complex (Goetze et al., 2010; Huang and Moazed, 2003; Straight et al., 1999). Interestingly, both Net1 and Cdc14 subunits of RENT directly affect Pol I transcription (Clemente-Blanco et al., 2009; Shou et al., 2001). The functional interplay between Pol I transcription and the rDNA silencing factors is an area of active research.

DNA topology effects.

Unwinding of the DNA in the course of transcription generates localized negative supercoiling behind and positive torsion ahead of the elongation complexes [reviewed in (Vos et al., 2011)]. Topoisomerase I acting together with RNase H and topoisomerase II are required to relieve either type of topological stresses induced by the moving polymerases. Since Pol I transcription is highly efficient, accumulation of the local DNA torsion around the elongation complex might impair polymerase movement. Indeed, recently it has been shown that topoisomerase activity is required for Pol I initiation, processivity and proper elongation rate (El Hage et al., 2010; French et al., 2011; Ray et al., 2013). Involvement of topoisomerases in rDNA transcription is conserved between yeast and mammals [as suggested in (Drygin et al., 2010; Ray et al., 2013)].

For a long time it has been known that Top1 and Top2 (yeast topoisomerases I and II) localize to the nucleolus and affect rRNA synthesis at Pol I transcription elongation step (Brill et al., 1987; Brill and Sternglanz, 1988; Schultz et al., 1992a). The mechanisms by which topoisomerases function in Pol I transcription were uncovered when the *top1*Δ and *top2* mutants were studied using EM analysis and biochemical assays (El Hage et al.,

2010; French et al., 2011). It has been shown that deletion of the non-essential *TOP1* gene affects Pol I processivity, though with no signs of slowed elongation rates. EM analysis of the *top1*Δ strain suggests that absence of topoisomerase I activity generates "melted" DNA bubbles behind the elongating polymerases and site-specific polymerase pileups at the active rDNA repeats. Furthermore, when Top1 function was further compromised by inhibition of the RNase H activity, decreased Pol I transcription elongation rate was readily detected in the double mutant.

Unlike Top1, mutations in *TOP2* were shown to slow Pol I transcription elongation consistent with the model that positive topological stress in front of the moving polymerase impedes its forward translocation (French et al., 2011). In agreement, the *top1 top2* double mutant resulted in a total loss of rDNA transcription due to severe processivity defects. Pol I transcription was completely blocked about 1/3 of the way through the rDNA coding region (within the 18S rRNA sequence). Moreover, human topoisomerase IIα was shown to directly affect Pol I PIC assembly and stability (Ray et al., 2013). These data suggest that topoisomerases are critical for the ability of Pol I to initiate and elongate throughout the rDNA and, thus, for efficient ribosome production.

Aims of the Study

The aim of the studies presented in the following chapters is to expand our understanding on the mechanisms of Pol I transcription elongation, especially in comparison to the well-studied "model" eukaryotic polymerase – Pol II.

The first chapter is devoted to the Spt4/5 complex, a conserved transcription elongation and RNA biogenesis factor for Pol I and Pol II [reviewed in (Hartzog and Fu,

2013)]. The role of the Spt4/5 in Pol II transcription is clear; its function in rRNA synthesis remained largely unknown. By defining the interactions of Spt5 subunit with the components of Pol I machinery in comparison to Pol II we aimed to understand the mechanism by which Spt4/5 complex affects rDNA transcription (Viktorovskaya et al., 2011).

In the second chapter we investigated the roles of conserved residues within the active site of Pol I in comparison to Pol II. Construction of a collection of Pol I mutants and Pol II/Pol I chimeric enzymes allowed us to directly compare the function of the conserved domain of the active center called the trigger loop, in catalysis of the two related polymerases. The results presented in the second chapter are significant for our understanding of Pol I transcription and RNA polymerase evolution (Viktorovskaya et al., 2013).

The third chapter aimed to establish the relationship between Pol I transcription elongation rates and ribosome biogenesis by studying the consequences of the various perturbations in Pol I activity. Previously, we proposed that the rate of Pol I transcription elongation affects ribosome biogenesis (Schneider et al., 2007). Using a collection of Pol I mutants, we further supported this model and expanded our understanding of functional coupling between rDNA transcription and ribosome assembly.

Together, these findings will provide a better understanding of the mechanisms of the rDNA transcription by Pol I and its link to one of the most essential cell components – the ribosome.

YEAST TRANSCRIPTION ELONGATION FACTOR SPT5 ASSOCIATES WITH RNA POLYMERASE I AND RNA POLYMERASE II DIRECTLY.

by

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Format adapted and errata corrected for dissertation

ABSTRACT

Spt5 is a transcription factor conserved in all three kingdoms of life. Spt5 homologues from bacteria and archaea bind the largest subunit of their respective RNA polymerases. Here we demonstrate that Spt5 directly associates with RNA polymerase I (Pol I) and RNA polymerase II (Pol II) in yeast through its central region containing conserved NGN and KOW domains. Deletion analysis of *SPT5* supports our biochemical data, demonstrating the importance of the KOW domains in Spt5 function. Far Western blot analysis implicates A190 of Pol I as well as Rpb1 of Pol II in binding Spt5. Three additional subunits of Pol I may also participate in this interaction. One of these subunits, A49, has known roles in transcription elongation by Pol I. Interestingly, *spt5* truncation mutations suppress the cold-sensitive phenotype of *rpa49*Δ strain which lacks the A49 subunit in the Pol I complex. Finally, we observed that Spt5 directly binds to an essential Pol I transcription initiation factor, Rrn3, and to the ribosomal RNA (rRNA). Based on these data we propose a model in which Spt5 is recruited to the rDNA early in transcription, and that it plays an important role in rRNA synthesis through direct binding to the Pol I complex.

INTRODUCTION

The study of ribosome biogenesis is fundamentally important. Multiple studies have connected dysregulation of the factors that affect ribosome biogenesis with cell transformation and cancer (1, 2). Transcription of ribosomal DNA (rDNA) by RNA polymerase I (Pol I) is the initial step of ribosome synthesis. In *Saccharomyces cerevisiae* (for simplicity referred to as “yeast”) Pol I produces the 35S rRNA precursor, which is

co-transcriptionally and post-transcriptionally processed into the mature 18S, 5.8S and 25S rRNA species that form the bulk of the small and large ribosomal subunits.

Pol I consists of 14 subunits; seven of which are Pol I-specific while the other seven are shared with RNA polymerase II (Pol II) and/or RNA polymerase III (Pol III). Pol I transcription initiation *in vivo* requires four additional factors: TATA-binding protein (TBP), Rrn3, core factor (CF), and the upstream activating factor (UAF). Formation of the Rrn3-Pol I complex is a crucial step in the recruitment of Pol I to the promoter. As such, this step in transcription is tightly regulated. However, the initiation step alone cannot account for the magnitude of regulation of Pol I transcription observed *in vivo* (3, 4). Recent data demonstrate that transcription elongation by Pol I is also regulated, and that transcription elongation by Pol I is functionally coupled to rRNA processing (5-7). Thus, factors that influence the elongation step in transcription may play one or more important roles in overall ribosome synthesis.

Although Pol I transcription elongation remains understudied, significantly more is known about factors that affect Pol II transcription elongation. One of these factors is the conserved Spt4/5 complex. In mammalian and *Drosophila* cells, Spt4/5 induces pausing of the Pol II elongation complex in close proximity to the promoter (reviewed in 8). This pause is thought to facilitate pre-mRNA 5'-capping and can be a target for the regulation of gene expression. After release from the pause Spt4/5 remains associated with the elongation complex and stimulates downstream transcription elongation (reviewed in 8). In yeast cells a similar positive role for Spt4/5 in Pol II transcription has been described (9-11). However, to date no negative role for Spt4/5 in Pol II elongation has been observed in yeast.

In addition to its role in Pol II transcription, the Spt4/5 complex acts as a Pol I transcription elongation factor (12). We showed that Spt4 and Spt5 associate with Pol I and rDNA *in vivo*. Deletion of *SPT4* leads to impairment of rRNA processing and a small increase in Pol I transcription elongation rate relative to wild-type (WT). We proposed that Spt4/5 inhibits Pol I transcription elongation (12). Further studies using point mutations in *SPT5* support the previous model that Spt4/5 can inhibit Pol I transcription *in vivo* (13). Interestingly, characterization of these mutants also identified a positive role for Spt5 in Pol I transcription elongation that was not evident from characterization of *spt4Δ* strains (13).

SPT5 is an essential gene conserved throughout eukarya (14, 15), and Spt5 homologoues share similar domain organization (Figure 1A). The NGN (NusG N-terminal homology) domain and the KOW (Kyrpides, Ouzounis, Woese) domains of Spt5 display sequence homology and structural similarity to the N-terminal domain (NTD) and the C-terminal KOW domain of the bacterial transcription factor NusG (16-19). Unlike bacterial NusG with its single KOW motif, eukaryotic Spt5 has multiple KOW domains. For example, there are four KOW motifs in yeast Spt5 (9, http://www.ebi.ac.uk/interpro), whereas human Spt5 (hSpt5) has six (http://www.ebi.ac.uk/interpro).

Spt5 homologues from eukaryotes and prokaryotes associate with the RNA polymerase elongation complexes (9, 20-23). NusG was shown to bind directly to bacterial RNA polymerase (RNAP) through its N-terminal domain but not through its KOW domain (21). In contrast, the region containing the KOW motifs of hSpt5 was shown to bind to Pol II *in vitro* (22, 23).

In this study we characterized the interaction of Spt5 with Pol I and Pol II in yeast. We showed that Spt5 associates with Pol I directly *in vitro* and that the central region of Spt5 (including the NGN and the KOW domains of Spt5) mediates this interaction. Identical regions associate with yeast Pol II. Far Western analysis identified four subunits of Pol I that can interact with Spt5 – A190, A135, A49 and A34.5. *In vivo* analyses support our biochemical data, demonstrating the importance of the KOW domains in Spt5 function. We also observed that the cold-sensitive phenotype of *rpa49*Δ strains is suppressed by *spt5* mutations. Finally, we identified an unexpected direct association of Spt5 with the Pol I transcription initiation factor Rrn3. Together, these observations support a model in which Spt4/5 plays direct roles early in transcription elongation by Pol I.

Figure 1

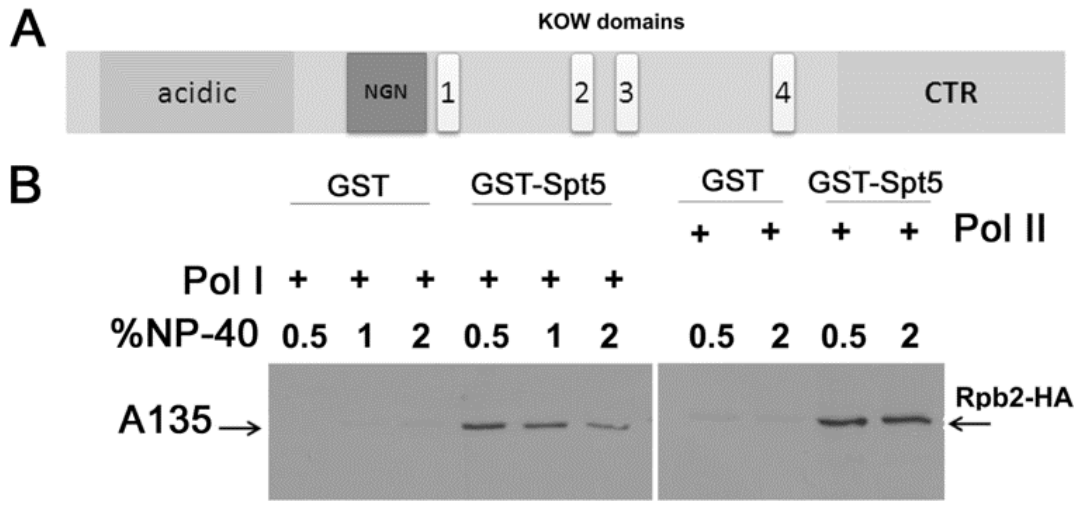

Fig. 1. **Spt5 associates with Pol I directly.** (**A**) Yeast Spt5 domain organization. (**B**) GST-only or recombinant Spt5 were tested for binding to the pure Pol I or Pol II complexes (as indicated by the plus signs). Western Blot analysis was performed using anti-HA antibody (12CA5) to detect $(HA)_3$ – $(his)_7$-C-terminal tags on the A135 subunit of Pol I or Rpb2 subunit of Pol II.

Note: From "Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly" by O. V. Viktorovskaya, F. D. Appling and D. A. Schneider, 2011, *The Journal of Biological Chemistry, 286(21)*, p. 18827

EXPERIMENTAL PROCEDURES

***Yeast strains, media and growth conditions*.** Strains used in this study are listed in Supplementary Table 1. Cells were grown in YEPD [for recipes see (7)] at 30°C unless otherwise indicated in the text or figure legends.

Diploid strains were sporulated in liquid sporulation medium (10 g/L potassium acetate and 5mg/L zinc chloride plus required nutrients) for 5 days with mild agitation at room temperature. Tetrads were dissected using a Zeiss Axioskop 40 Tetrad micromanipulator.

Chromosomal deletions, leading to C-terminal truncations of Spt5 were constructed using standard PCR-based strategies (24). The nomenclature of the mutant alleles indicates the amino-acid length of the corresponding proteins [e.g. *spt5(1-930)* expresses a $(His)_7$-hemagglutinin $(HA)_3$-tagged protein that includes the first 930 amino acid residues of Spt5]. Spt5 protein abundance was measured by Western blot analysis using an anti-HA antibody (12CA5) and compared to epitope-tagged WT Spt5 (data not shown).

The *spt5*Δ*KOW2* mutant allele carries a deletion of a region of *SPT5* that corresponds to amino-acids 515-578. The *spt5*Δ*KOW2* strain bears a chromosomal deletion of *SPT5* complemented by expression of the mutant allele on a single copy CEN plasmid (Supplementary Table 2) and was obtained by tetrad dissection of a diploid strain containing a heterozygous deletion of *SPT5* (*spt5*Δ::*HIS3Mx6*/*SPT5*).

Cloning of SPT4 and SPT5 into bacterial expression vectors. We cloned regions of *SPT5* corresponding to the indicated domains into pET41a (Novagen). We used the predicted Spt5 domain structure from European Bioinformatics Institute Database

(EMBL-EBI; http://www.ebi.ac.uk/interpro/) and from (9) to select our domain endpoints. The plasmids expressing Spt5 and Spt5 domains are described in Supplementary Table 2.

For the expression of the Spt4/5 complex *SPT4* and *SPT5* were cloned into pRSFDuet-1 and pETDuet-1 vectors (Novagen), respectively. We introduced a FLAG-$(His)_6$-TEV-hemagglutinin $(HA)_3$–$(His)_6$ epitope tag to the C-terminus of Spt5 as described in Supplementary Table 2.

Sequences of the primers used for the cloning of *SPT5* or *SPT4* and detailed description of cloning strategy are listed in Supplementary Table 3 and Supplemental Methods.

Protein Purification. Pol I and Pol II were purified using a $(His)_7$-$(HA)_3$ C-terminal epitope tag on the A135 and Rpb2 subunits, respectively, using the same strategy described previously (25). His_6-Rrn3 was expressed in *E. coli* BL21(DE3) from pNOY3162 plasmid and purified as described previously (25).

Full-length Spt5 and Spt5 truncation constructs were expressed in *E.coli* BL21(DE3) as N-terminal $(His)_6$-GST-fusion proteins in the presence of 0.5mM IPTG. The cells were lysed using a French Press (Thermo IEC) in TIB buffer [20mM HEPES (pH 7.5), 400mM NaCl, 0.1mM EDTA, 10% glycerol, 0.05% NP-40, 1mM DTT, 2mM PMSF and protease inhibitor cocktail (Roche)]. The lysates were cleared by centrifugation at 20000 x g for 15 min at 4°C. The proteins were purified from the soluble fraction using Ni-affinity chromatography followed by glutathione affinity purification and a second Ni affinity-purification step. Equal concentrations of the proteins were used when performing GST-pull down assays or Far-Western Blot Analysis.

His_6-Spt4 and His_6-Spt5 were co-expressed in *E.coli* BL21(DE3) in the presence of 0.5mM IPTG. The Spt4/5 complex was purified from the soluble fraction of the cell extract using Ni-affinity chromatography followed by anion-exchange chromatography (HiTrap Q, GE Healthscience), a second Ni-affinity-purification step and a final anion-exchange step (monoQ, GE Healthscience).

GST pull-down assay. To test for interactions between Spt5 and Pol I or Pol II, HA-tagged Pol I and Pol II were incubated in binding buffer [20mM Tris-HCl pH 8.0, 250mM NaCl, 1mM EDTA, 1mM DTT, 2mM PMSF, a protease inhibitor cocktail (Roche), 1% NP-40 (or an indicated percentage of NP-40)] with GST-tagged Spt5 (full-length or mutant constructs) immobilized on glutathione resin (GE Healthcare). To test for an interaction between Spt5 and Rrn3 or the Pol I-Rrn3 complex, we immobilized GST-Spt5 on glutathione resin in binding buffer and added either Pol I, Rrn3, or pre-bound Pol I-Rrn3 complex. The Rrn3-Pol I complex was formed as described previously (25) using an equimolar ratio of Pol I : Rrn3 or with excess of Rrn3 (ratios 1 : 3.5 and 1 : 5). The resin was then washed three times with binding buffer and analyzed by Western Blot probed with an anti-HA antibody (12CA5) or with a polyclonal anti-Rrn3 antibody.

Far-Western Blot Analysis. Far-Western Blot Analysis was performed as described previously (26) with the following modifications. Pure Pol I and Pol II subunits were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, the blot was incubated with equal concentration of the GST-fused probes in the probe dilution buffer (1X PBS, 0.05% Tween, 2% milk, 1mM DTT), followed by a series of washings. We then proceeded with the standard Western Blot analysis protocol using a polyclonal anti-GST antibody (Z-5, Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assay (EMSA). The rRNA oligonucleotide (corresponding to the first 40 nucleotides of 35S rRNA) was labelled with ^{32}P at the 5'-end using T4 polynucleotide kinase (New England BioLabs). EMSA was performed as described previously (27) with modifications described in detail in Supplementary Methods.

RESULTS

Spt5 associates with Pol I directly *in vitro*. Spt4 and Spt5 form a heterodimer that influences transcription elongation by Pol I (12). Previously, we observed a physical interaction (co-purification and co-immunoprecipitation) of these proteins with Pol I. To determine whether this association is direct or mediated through a network of other proteins we tested for binding of Spt5 to Pol I *in vitro*.

We expressed yeast Spt5 with an N-terminal GST tag in *E.coli* and purified the protein using three affinity steps. Endogenously expressed Pol I bearing an HA-tag on the A135 subunit was purified from yeast. These proteins were used to perform a GST pull-down assay in which Spt5 was immobilized on glutathione sepharose resin and incubated with Pol I. After multiple washes, bound Pol I was detected by Western blot analysis using an anti-HA antibody (12CA5). As a positive control, we included HA-tagged Pol II purified from yeast. Human Pol II has been shown previously to directly bind hSpt5 (22, 23), and as expected (9), yeast-derived Pol II directly associated with yeast Spt5 (Figure 1B). Consistent with our results, the Cramer lab has recently shown that recombinant yeast-derived Spt4/5 binds directly to pure Pol II (28). As for Pol II, we observed specific

association of Pol I with GST-Spt5 but not GST only (Figure 1B). Thus, Spt5 directly and stably binds to Pol I.

The central region of Spt5 containing the NGN and the KOW domains binds to Pol I *in vitro*. To identify the domain(s) of Spt5 that bind to Pol I and/or Pol II, we constructed a series of Spt5 variants fused to GST and purified these constructs from *E. coli* (Figure 2A, B). After three affinity-purification steps, these proteins were used in GST-pull down assays with either yeast-derived Pol I or Pol II. After multiple washing steps the bound polymerases were detected by Western blot analysis (Figure 2C, 2E).

The same regions of Spt5 are required for binding to either Pol I or Pol II (Figure 2C, 2E). The N-terminal region containing the NGN domain (construct B) binds to Pol I, though the amount of polymerase retained on the beads is less than in the presence of the full-length Spt5 (Figure 2F). Since the first 244 amino acid residues do not bind to polymerases (construct A, figure 2C, 2D, 2E), we conclude that the NGN domain mediates the detected association with Pol I and Pol II. This observation supports the model that the interaction of the NGN domains with RNA polymerases is conserved in all three kingdoms of life (20, 21, 28, 29).

The mutant proteins that contained the central region of Spt5 including the KOW domains and related sequences (e.g. the linkers between the KOW motifs) but that lacked the NGN domain (constructs D and E) also associated with both polymerases (Figure 2C, 2D). However, the binding of these constructs to Pol I was not as robust as for the full-length protein (Figure 2F). Indeed, binding of both polymerases to Spt5 was more robust when the immobilized construct contained a larger region including the NGN domain and at least two KOW domains (constructs C, G, H, J, K). These constructs retained as much

Figure 2

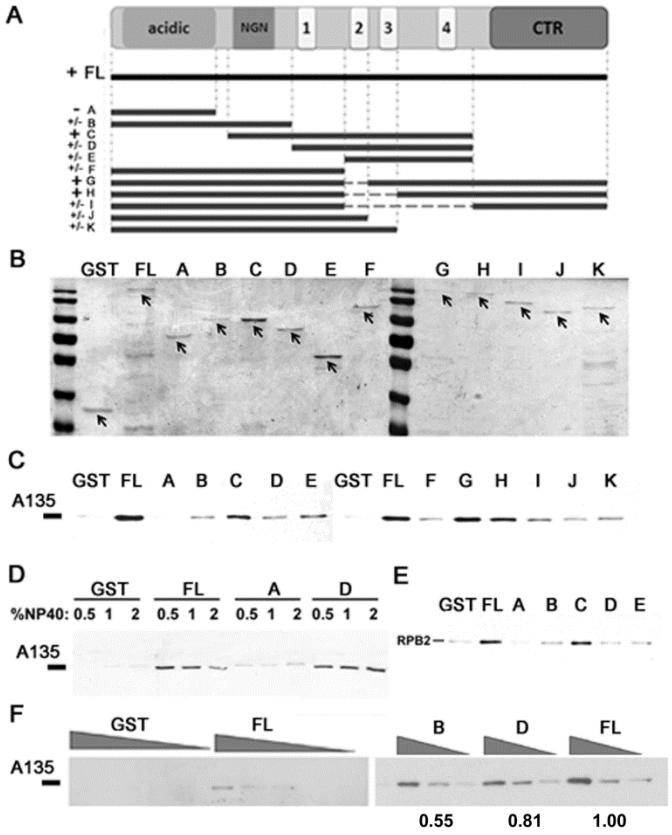

Fig. 2. **The central region of Spt5 (including the NGN and KOW domains) binds to Pol I *in vitro.*** (**A**) A schematic representation of Spt5 constructs used in the study. Dashed lines correspond to end points of individual constructs. Plus signs indicate binding to Pol I. (**B**) The Spt5 constructs were expressed in *E.coli* and purified using a three-step purification. The proteins were visualized with Coomassie Blue. (**C**) The protein constructs A-K were tested for the association with Pol I. Bound Pol I was detected using Western Blot analysis with anti-HA antibody. (**D**) Pol I bound to the construct D, but not to the construct A or GST-only control in variable concentrations of NP-40. (**E**) The Spt5 constructs A-E were tested for the association with Pol II. Western Blot analysis with anti-HA antibody was used to detect bound Pol II. Association of constructs F-K with Pol II was similar to that with Pol I (data not shown). (**F**) Semi-quantitative analysis of Pol I association with Spt5 constructs B and D in comparison to the full-length Spt5. We used 1 μM of either GST-Spt5 (FL and constructs B or D) or GST-only and incubated with 3-fold serial dilutions of Pol I (starting from 0.5 μM). Bound Pol I was detected as in C and quantified using Quantity One software (BioRad Laboratories).

Note: From "Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly" by O. V. Viktorovskaya, F. D. Appling and D. A. Schneider, 2011, *The Journal of Biological Chemistry, 286(21)*, p. 18828 Copyright 2011 by the American Society of Biochemistry and Molecular Biology, Inc. Reprinted with permission.

Pol I or Pol II as full-length Spt5. Therefore, we conclude that unlike bacteria and archaea, in eukaryotes the NGN domain of Spt5 is not sufficient for robust association with RNA polymerases. Instead, the additional sequences, most likely the KOW domains, appear to enhance the association of Spt5 with both Pol I and Pol II. These data demonstrate that the association of Spt5 with RNA polymerases is conserved but apparently more complex in eukaryotes compared to prokaryotes; consistent with the more complex domain structure of eukaryotic Spt5.

KOW domains are important for survival. We have shown that the central region including the NGN and at least two KOW domains is important for robust association of Spt5 with both polymerases *in vitro*. Recent data demonstrated that the NGN domain and the KOW domains of Spt5 are essential for survival (30). However, since the presence of all four of the KOW domains is not required for binding to Pol I and Pol II *in vitro*, we predicted that deletion of one or more KOW motifs would not be lethal in yeast.

To test our hypothesis and to verify our biochemical data *in vivo*, we constructed a variety of *spt5* mutant strains carrying either C-terminal truncations or an internal deletion of KOW2 (Figure 3A). These strains were tested for viability under different conditions. We confirmed previously published data that the CTR region of Spt5 is not essential in yeast (31). The mutants *spt5(1-930)* and *spt5(1-886)* have mild growth defects at permissive temperatures (30°C and 25°C) and cold-sensitive phenotypes (slow growth at 16°C) (Figure 3B and data not shown). Interestingly, deletion of KOW4 together with the CTR deletion [allele *spt5(1-797)*] is viable. This strain also exhibits mild growth defects at 30°C (Figure 3B) and more severe cold sensitivity than *spt5(1-930)* and *spt5(1-886)*. Spt5 abundance in all three mutant strains was not altered

Figure 3

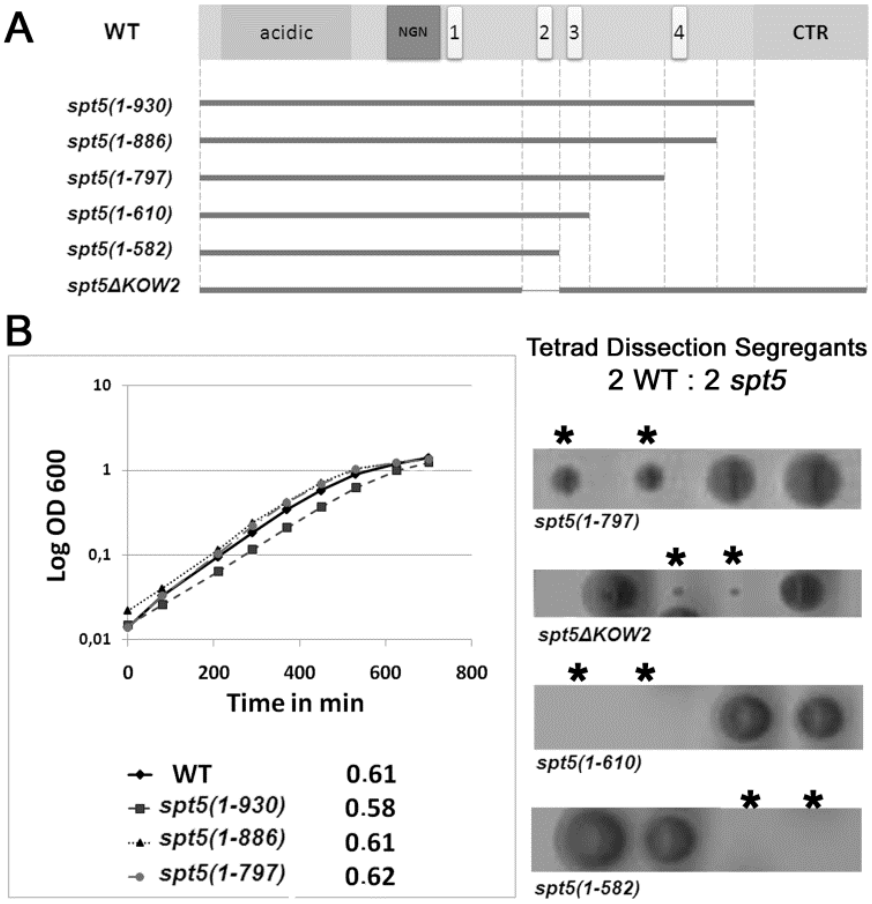

Fig. 3. **KOW domains are important for survival.** (**A**) Schematic representation of the *spt5* mutant alleles constructed for *in vivo* studies. (**B**) Growth curves for WT, *spt5(1-930)*, *spt5(1-886)* and *spt5(1-797)* strains at 30°C are shown with the doubling time indicated (below plot). Individual haploid segregants from heterozygous *spt5/SPT5* diploid strains are shown on the right. Each tetrad yielded two wild type (WT) spores and two *spt5* mutants (indicated by an asterisk). The *spt5ΔKOW2* strain was incubated at 25°C, three other strains were grown at 30°C. The pictures were taken on the fourth day for the *spt5(1-797)* strain and on the 7th day for the rest of the strains.

Note: From "Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly" by O. V. Viktorovskaya, F. D. Appling and D. A. Schneider, 2011, *The Journal of Biological Chemistry*, *286(21)*, p. 18829 Copyright 2011 by the American Society of Biochemistry and Molecular Biology, Inc. Reprinted with permission.

compared to WT (measured by Western blot, data not shown). These results are consistent with our biochemical data showing that the region containing KOW4 and/or CTR is not required for binding of Spt5 to Pol I or Pol II *in vitro* (Figure 2).

A mutant allele of *SPT5* that contains an internal deletion of the KOW2 region (*spt5*Δ*KOW2*) is also viable. However, this strain has the most severe phenotype among the described non-lethal *spt5* mutants exhibiting very slow growth at 25°C (Figure 3B) and lethality at 30°C. Both *spt5*Δ*KOW2* and *spt5(1-797),* have one of the KOW domains deleted, however, the phenotype of the *spt5*Δ*KOW2* allele is significantly more severe than that of the *spt5(1-797)* mutant. We conclude that KOW2 is more critical for Spt5 function than KOW4 and the CTR combined. The observation that *spt5(1-797)* and *spt5*Δ*KOW2* strains are viable supports our biochemical data indicating that fewer than four KOW domains is sufficient for interaction with RNA polymerases (constructs J, H, K from Figure 2).

We were not able to isolate cells carrying deletions of two or more KOW domains. Spores carrying *spt5(1-582),* which lacks the C-terminal region including CTR, KOW4 and KOW3 do not grow at 30°C or 25°C (Figure 3B). Even expansion of the C-terminal deletion to include the 188 amino acid residue linker between KOW3 and KOW4 [allele *spt5(1-610)*] is lethal (Figure 3B). These data are consistent with previously observed lethality of an *spt5(1-640)* allele (30). To determine if these lethal *spt5* constructs could still associate with Pol I we examined their expression and co-immunoprecipitation with the polymerase in heterozygous diploid cells. We did not detect any significant difference in the abundance of these truncated Spt5 proteins compared to the full-length protein when expressed in heterozygous diploids [*spt5(1-582)/SPT5* or *spt5(1-640)/SPT5*;

measured by Western blot analysis (data not shown)]. Moreover, truncated Spt5 expressed in the *spt5(1-640)/SPT5* diploid strain was still able to associate with Pol I in co-immunoprecipitation experiments (data not shown), consistent with our *in vitro* pull-down assays (Figure 2). These data demonstrate that the lethality of the *spt5(1-582)* and *spt5(1-610*) alleles is most likely due to defects in Spt5 function rather than impaired protein folding or defects in the binding of Spt5 to the polymerases.

Our analysis of *spt5* deletion mutants shows that cells with minor disruption of the region containing KOW domains can survive. However, larger deletions within the KOW domains are lethal. These results support the functional importance of the KOW domains *in vivo* and are consistent with our *in vitro* data.

Multiple subunits of Pol I associate with Spt5 *in vitro*. Since we have shown that Spt5 physically interacts with Pol I, we wished to determine which subunits of Pol I are involved in the interaction. To do this we used Far-Western blot analysis. Blots carrying purified Pol I and Pol II were probed with three different Spt5 constructs fused to GST (Figure 4A) and with purified GST as a negative control. Binding of the probes was detected using an anti-GST antibody (Figure 4B, 4C). A duplicate lane from the original SDS-PAGE gel was stained with silver nitrate to determine the identity of the polymerase subunits. We detected binding of all three Spt5-probes to the largest subunits of both Pol I and Pol II – A190 and Rpb1 (Figure 4B, 4C). The A190 subunit of Pol I is homologous to Rpb1, and to the β’ subunit of prokaryotic RNA polymerases (32). The NGN-like domains in bacteria and archaea are known to bind to the β’ subunit or its homologues (20, 28, 29, 33). Rpb1 of human Pol II was also shown to bind to human Spt5 (22) and

we confirmed that interaction for yeast Pol II (Figure 4B). Thus, the observed interaction between Spt5 and the largest subunit of Pol I is conserved.

Two out of three Spt5-probes (probes C and D) also associated with three additional Pol I subunits – A135, A49 and A34.5. The third probe (probe F) associated with A135 and A34.5 in addition to A190. Binding of the probe F to the A49 subunit was not detected. Thus the NGN domain is not sufficient for association with A49.

A135 is the second largest subunit of Pol I and, together with A190 forms the catalytic core of the polymerase. The A34.5 and A49 subunits associate with one another and act as an intrinsic elongation factor (34). Though all three probes associated with the A34.5 subunit, the binding to A34.5 might be a false positive. A34.5 bears a lysine-rich region at its C-terminus, which can lead to robust non-specific interactions *in vitro*.

Our data suggest that Spt5 is capable of binding to the largest subunits of Pol I as well as subunits that specifically participate in transcription elongation. Thus, it is likely that Spt5 associates with Pol I through multiple contacts, mediating its direct effect on transcription elongation.

Mutations in *SPT5* suppress the *rpa49*Δ phenotype. Mutations in *SPT5* have been shown previously to interact with mutations in genes encoding Pol II subunits or Pol II transcription factors (9, 11, 31). We therefore tested for genetic interactions of *spt5* alleles with mutations in Pol I. *RPA49* encodes the A49 subunit of Pol I and is not an essential gene (35). Pol I lacking the A49 subunit is defective in transcription initiation and elongation (34, 36). We observed that Spt5 has affinity for A49 in Far Western blot analysis (Figure 4). Since Spt4/5 can affect Pol I transcription elongation (12, 13) we tested for genetic interactions between *rpa49*Δ and multiple *spt5* alleles.

Figure 4

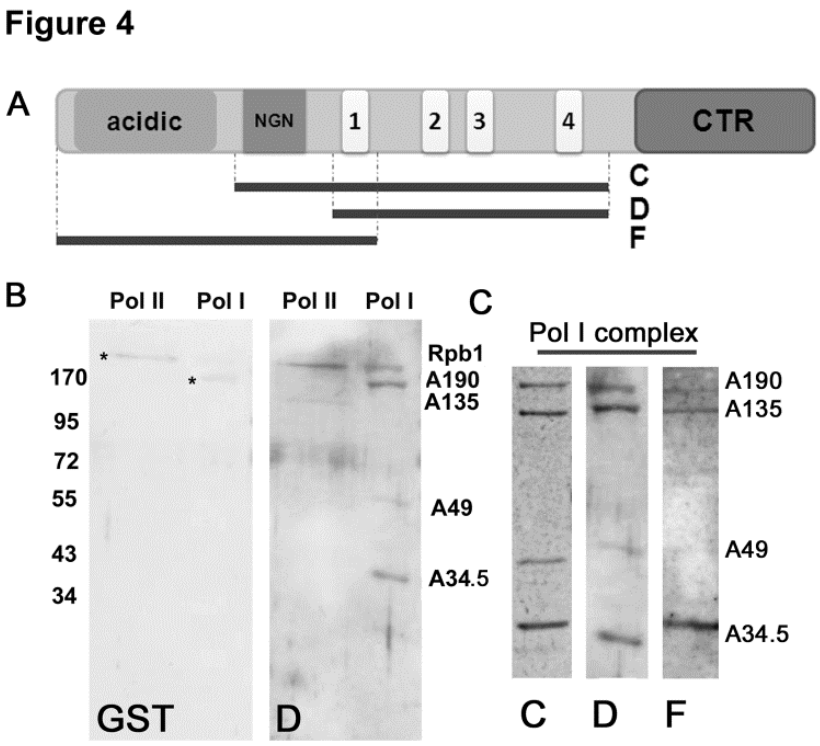

Fig. 4. **Spt5 associates with four Pol I subunits *in vitro***. (**A**) Schematic representation of the Spt5 constructs (C, D, F – the same as on figure 2A) used as probes in Far-Western Blot analysis. (**B**) Binding of construct C or the GST-only probe (GST) to Pol I and Pol II subunits was tested by Far-Western Blot analysis. The bands detected after the incubation with the probe C correspond to the Rpb1 subunit of Pol II and to the A190, A135, A49 and A34 subunits of Pol I. Asterisks show weak non-specific binding of the GST-only probe to Rpb1 and A135. (**C**) Association of the probes C, D and F with Pol I subunits was tested by Far-Western Blot analysis as in figure 4B.

Note: From "Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly" by O. V. Viktorovskaya, F. D. Appling and D. A. Schneider, 2011, *The Journal of Biological Chemistry*, *286(21)*, p. 18830 Copyright 2011 by the American Society of Biochemistry and Molecular Biology, Inc. Reprinted with permission.

We created double mutants bearing *rpa49*Δ combined with either *spt5(1-930)*, *spt5(1-886)* or *spt5(1-797)* alleles. Deletion of *RPA49* results in cold-sensitivity with limited or no growth at 25°C (37). However, when *spt5* mutations were combined with *rpa49*Δ, the double mutants supported growth at 25°C (Figure 5A and data not shown). We found that all of the *spt5* alleles partially suppress the cold-sensitive phenotype of *rpa49*Δ. Furthermore, other recently isolated *spt5* alleles and *spt4*Δ suppressed the cold-sensitivity of *rpa49*Δ (13). Thus, genetic interactions between *RPA49* and *SPT5* or *SPT4* support the model that Spt4/5 influences Pol I transcription elongation. Suppression of the *rpa49*Δ phenotype by *spt5* alleles is consistent with previously characterized negative roles for the Spt4/5 complex in Pol I transcription (12, 13). Though the molecular mechanisms of the observed suppression remain to be discovered, they are likely to be direct and may involve physical interactions of Spt5 with A49.

Spt5 directly associates with Rrn3 and rRNA. Spt5 is known to associate with both the 35S rRNA coding region and its promoter region (12). We hypothesize that Spt5 is recruited to the Pol I complex soon after transcription initiation occurs. Although Spt5 directly binds to Pol I, the Pol I initiation factors or the RNA transcript could also participate in the early recruitment of Spt5 to the rDNA. To test this hypothesis we examined the interaction of Spt5 with the Pol I initiation factor Rrn3 and with rRNA.

Rrn3 associates with Pol I and escorts the polymerase to the promoter. After initiation of transcription, Rrn3 is released from the complex (3, 38, 39). Co-immunoprecipitation experiments showed that Spt5 and Rrn3 interact with each other *in vivo* (data not shown). Since both Spt5 and Rrn3 are known to bind to the Pol I complex, we tested if there is a direct association between recombinant Rrn3 and Spt5 *in vitro*. We observed direct

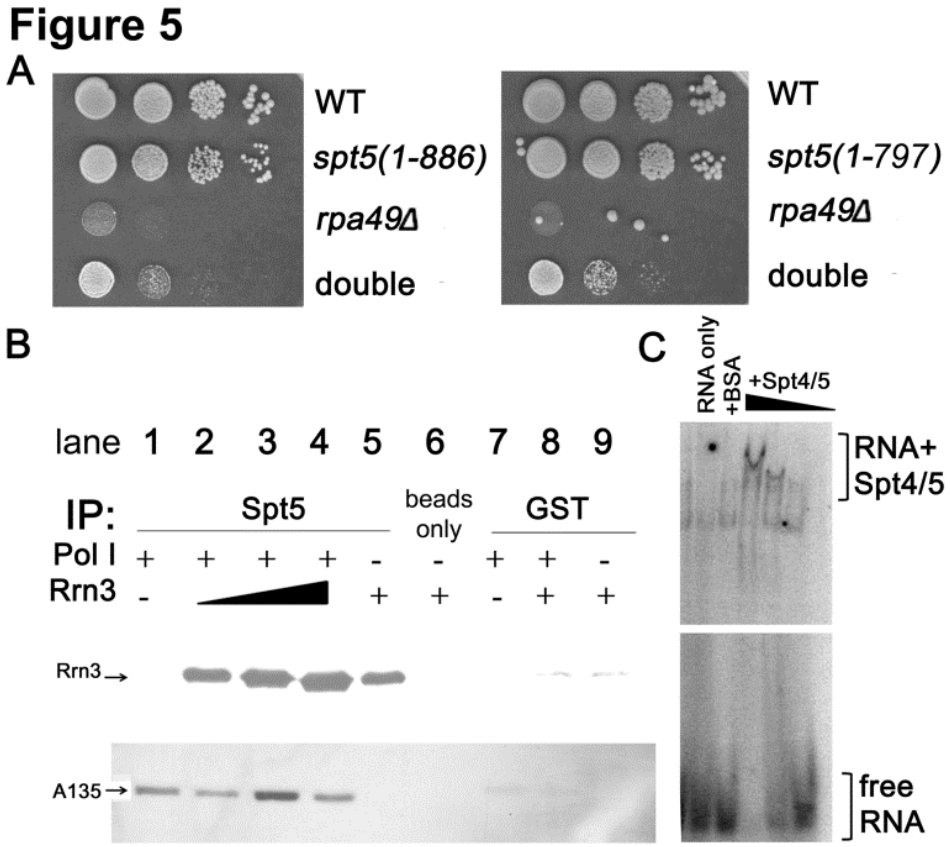

Fig. 5. **Spt5 interacts physically and genetically with Pol I transcription machinery**. (**A**) *spt5(1-886)* [on the left] and *spt5(1-797)* [on the right] alleles partially suppress *rpa49*Δ cold-sensitive phenotype. The strains were plated on YEPD media as serial 10-fold dilutions and incubated at 25°C. The pictures were taken on the sixth day. Several spontaneous suppressors can also be observed as bigger sized colonies for *rpa49*Δ but not for the double mutants. (**B**) Spt5 (lanes 1-5) or GST-only (lanes 7-9) immobilized on glutathione resin were tested for interaction with either Pol I (lane 1), Rrn3 (lane 5) or Pol I – Rrn3 (lanes 2-4) complex [as indicated by the plus signs above the lanes]. Rrn3 was incubated with the glutathione resin without GST or Spt5 as an additional negative control (lane 9). Bound Rrn3 and Pol I were detected by Western Blot analysis using anti-Rrn3 or anti-HA antibody, respectively. (**C**) Spt4/5 binding to an rRNA oligonucleotide was tested using EMSA. Free RNA and RNA-Spt4/5 complexes visualized by autoradiography are indicated.

Note: From "Yeast Transcription Elongation Factor Spt5 Associates with RNA Polymerase I and RNA Polymerase II Directly" by O. V. Viktorovskaya, F. D. Appling and D. A. Schneider, 2011, *The Journal of Biological Chemistry, 286(21)*, p. 18830

binding of recombinant Rrn3 to recombinant Spt5 in a GST pull-down assay (Figure 5B, lane 5).

Since we detected a physical interaction of Spt5 with Rrn3, we tested for genetic interactions between *spt5* and *rrn3* mutations. We created double mutants carrying either *spt5(1-886)* or *spt5(1-797)* allele with *rrn3(S213P)*. The *rrn3(S213P)* mutation reduces the Pol I transcription initiation rate under non-permissive conditions (37°C) (3). The double mutants showed an additive phenotype under all conditions tested (30°C, 25°C, 33°C, 37°C and media with addition of 6-azauracil), indicating an absence of epistatic interactions between the alleles tested (data not shown).

Thus, Spt5 directly associates with Rrn3, however, we do not detect a significant effect of Spt5 on Pol I transcription initiation (no genetic interactions between *RRN3* and *SPT5* and data from 12, 13). We hypothesize that the observed physical interaction of Spt5 with Rrn3 contributes to the recruitment of Spt5 to the Pol I complex at the rDNA promoter rather than to the participation of Spt5 in Pol I transcription initiation.

The Spt4/5 complex was shown to require a nascent transcript longer than 18 nt for efficient recruitment to the reconstituted *Drosophila* Pol II elongation complex *in vitro* (40). We hypothesized that the synthesis of the nascent transcript by Pol I might also contribute to Spt5 recruitment. We tested for binding of recombinant Spt4/5 complex to an rRNA oligonucleotide using an electrophoretic mobility shift assay (EMSA). An oligonucleotide corresponding to the first 40 bp of 35S rRNA labeled with ^{32}P at the 5'-end was used in the assay. To separate the bound RNA complexes from free RNA we performed native PAGE. We detected a slow migrating RNA band in the presence of the Spt4/5 but not in the presence of Bovine Serum Albumin (BSA) control. The association

of the rRNA oligonucleotide with the Spt4/5 complex was concentration dependent (Figure 5C). These data confirm that Spt4/5 is an RNA-binding complex, and the rRNA transcript could aid recruitment of Spt4/5 to the early elongation complex.

Taken together, we have identified three factors present in the early Pol I elongation complex that directly bind to Spt5 and might contribute to recruitment of Spt5 to the rDNA - Pol I, Rrn3 and rRNA.

DISCUSSION

We have shown that Spt5 interacts with Pol I directly *in vitro* through its central region containing conserved NGN and KOW domains and can associate with four subunits of Pol I – A190, A135, A49 and A34. Moreover, we confirmed our biochemical data using *spt5* mutations and genetic interactions of *SPT5* with *RPA49*. We also detected direct binding of Spt5 to Rrn3 and to rRNA, leading to the hypothesis that these factors together with Pol I might participate in recruitment of Spt5 to the rDNA. All of these data support the model that Spt5 is recruited to the rDNA early in transcription and that it plays important direct roles in rRNA synthesis.

The direct association of Spt5 with Pol I. The first evidence for a physical interaction between Spt5 and Pol I was demonstrated in a mass-spectrometry screen for proteins that co-purified with Spt5 (11). Later we confirmed that Spt4 and Spt5 co-purified with the Pol I complex from growing yeast cells (12). Whether this interaction was direct or mediated by a network of other proteins remained undefined.

In this study we have shown that Spt5 directly binds to Pol I *in vitro*. This is the first demonstration of a direct interaction of Spt5 with Pol I. Based on our previous data and

the results obtained here, we propose that direct association of Spt4/5 with Pol I is important for transcription of rDNA *in vivo*.

Interestingly, the prokaryotic homologue of Spt5, NusG, is known to participate in the control of rRNA operon transcription elongation and anti-termination in *E.coli* through direct binding to the RNAP elongation complex (41). Though the overall mechanisms by which NusG and Spt5 control synthesis of rRNA are quite different, they certainly share some common biochemical properties.

The roles of the NGN and the KOW domains of Spt5 in polymerase binding. The binding of hSpt5 to Pol II was shown to be mediated by the central region of hSpt5 containing the KOW motifs (22, 23). In our study, we also observed that the region containing KOW domains and related sequences (e.g. the linker regions between the KOW motifs) of yeast Spt5 is sufficient for its interaction with Pol I and Pol II. The most likely interpretation of these data is that the conserved KOW domains are necessary for direct interaction with the polymerases, however, we cannot exclude the possibility that other motifs from the central region are crucial for this binding.

We also concluded that both polymerases associate with the NGN domain of Spt5 in yeast. These data are consistent with the known association of the homologous NTD of NusG and of the NGN domain of archaeal Spt5 with their respective RNA polymerases (20, 21, 28, 29). We propose that the association of the NGN domain of hSpt5 with Pol II is also conserved, though it was not defined in previous studies (22, 23). The most likely explanation for this potential discrepancy is that the homology of NusG and Spt5 through the KOW motifs was clear upon initial characterization of Spt5 (9, 22, 23), whereas the homology of the NTD of NusG and Spt5 through the NGN domain was defined later

(16). Thus, the constructs containing the N-terminal region of hSpt5 with the full-sized NGN domain were absent in the pull down assays with human Pol II in both previous studies (22, 23).

The most robust association of Spt5 with Pol I or Pol II *in vitro* was observed with constructs containing the central region of Spt5 including the NGN domain and at least two KOW domains. In addition, these regions (the NGN domain, the KOW domains as well as the intervening amino acids) are important for cell viability (Figure 3; and 30). We propose that the interaction of the NGN domain with RNA polymerases is conserved between prokaryotes and eukaryotes. Based on the known roles of the NTD of NusG in RNAP elongation, we speculate that the homologous NGN domain of Spt5 participates in the direct modulation of transcription elongation by Pol I and Pol II. However, unlike prokaryotes, the binding of the eukaryotic Spt5 proteins to RNA polymerases is additionally stabilized by interactions with the multiple KOW domains. These domains may also play critical roles in recruitment of multiple other eukaryotic transcription elongation and RNA biogenesis factors.

Our model for Spt5-Pol I interaction is consistent with the model for Spt5-Pol II association recently published by the Cramer lab (28). They predicted that the NGN domain of eukaryotic Spt5 contacts Pol II above the active center cleft, and the flexible KOW1 domain is located between the top of the clamp and the wall. The details of association between full-length Spt5 and Pol II are still not known but the extended eukaryote-specific region including the rest of the KOW domains and the CTR could reach any position on the Pol II surface (28).

Interaction of Spt5 with RNA polymerase subunits. NusG homologues in bacteria and archea were shown to interact with the largest subunits of their cognate RNA polymerases (20, 28, 29, 33). The homologues of the largest subunit of prokaryotic RNAP in eukaryotic RNA polymerases are A190, Rpb1 and Rpc1 [for Pol I, Pol II and Pol III, respectively (32)]. Rpb1 was shown to associate with hSpt5 in Far-Western Blot analysis (22). Using the same approach we confirmed these data for yeast Spt5 and Rpb1. We further observed that Spt5 associates with the A190 subunit of Pol I. Surprisingly, we also detected association of Spt5 with three additional Pol I – specific subunits – A135, A49 and A34.5. In agreement with these data, three of the four Pol I subunits identified here [A190, A135 and A49] were also previously identified in the mass-spectrometric analysis of the Spt5-associated proteins (11). As mentioned, the A49 and A34.5 subunits, which form a sub-complex within the Pol I complex, have intrinsic elongation activity (34). If the observed interaction of Spt5 with these two subunits occurs *in vivo*, Spt5 could directly modulate the effect of A49/A34.5 on Pol I transcription elongation. In support of this model, we detected genetic interactions between *RPA49* and *SPT5*.

A proposed model for the roles of Spt5 in transcription by Pol I. We have previously shown that Spt4/5 plays a role in Pol I transcription elongation and rRNA processing (12). We observed negative effects of Spt4/5 on Pol I transcription previously (12), however, recent studies revealed dual (negative and positive) roles for Spt4/5 in rRNA synthesis (13).

Based on the multiple functions of Spt4/5 in cells it is hard to differentiate direct versus indirect effects of Spt4/5 on Pol I transcription. This study demonstrates that Spt5

binds to Pol I *in vitro*, supporting a direct role for the Spt4/5 complex in rRNA synthesis *in vivo*.

Spt4 and Spt5 are associated with the rDNA coding sequence as well as the promoter (12). In this study we have shown that Spt5 binds the initiation factor Rrn3. However, none of the previous studies identified significant Spt4/5-mediated effects on Pol I transcription initiation *in vivo* (12, 13). In support of these data, we also did not detect any epistatic interactions between *SPT5* and *RRN3*. Thus, we speculate that the interaction of Spt5 with Rrn3 facilitates the recruitment of Spt5 to the rDNA near the promoter.

Based on our previous data and the results of this study we propose a model for the interactions of Spt4/5 with the rRNA synthesis apparatus. Pol I transcription initiation occurs when a Pol I – Rrn3 complex is recruited to the rDNA promoter; soon after initiation, Rrn3 is released from the Pol I complex. During the transition from initiation to elongation (while Rrn3 is still bound to Pol I) Spt4/5 is recruited to the rDNA by virtue of its interactions with Rrn3, the nascent rRNA transcript and Pol I. Thus, it appears Spt5 is engaged by the transcription machinery during the initiation or early elongation step. This model is supported *in vivo* by co-immunoprecipitation and ChIP data showing Spt4/5-Rrn3 interaction and co-localization of these factors at the rDNA promoter (3, 12, 36). After the release of Rrn3, Spt4/5 stays associated with the elongation complex to modulate rRNA synthesis.

Spt5 can inhibit as well as promote Pol I transcription elongation. Our studies of *spt5* mutants and observed genetic interactions confirm the importance of Spt5 functions for efficient rRNA synthesis and proper rRNA processing *in vivo* (12, 13). Based on the

recent structural data for archeal Spt5-RNAP complex (28, 29), Spt5 is thought to lock the RNA-DNA hybrid of the transcription bubble in the cleft of the polymerases increasing the elongation complex stability. Taking the homology between the multisubunit polymerases as well as between various Spt5/NusG homologues into account, this function of Spt5 is very likely to be true in the Pol I system as well. The exact mechanisms by which Spt4/5 affects of Pol I elongation remain to be elucidated.

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FOOTNOTES

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Supplementary Table 1. Strains used in this study

Strain	Description	Reference
NOY396 (WT)	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100*	Yano and Nomura, 1991
NOY397 (WT diploid)	*MAT***a**/*α ade2-1/ ade2-1 ura3-1/ ura3-1 trp1-1/trp1-1 leu2-3 112/ leu2-3 112 his3-11,15/his3-11,15 can1-100/can1-100*	Yano and Nomura, 1991
NOY2166	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:HIS3Mx6***	Schneider *et al*., 2006
NOY2164	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***SPT5-(HA)3-(His)7:HIS3Mx6***	Schneider *et al*., 2006
DAS50	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa49Δ::LEU2***	Zhang *et al*., 2010
DAS576	Same as DAS50, except *MATα*	Anderson *et al*., 2010
NOY1075	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100* ***rrn3 (S213P)***	Claypool *et al*., 2004
DAS605	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-930)- $(HA)_3$ -$(His)_7$:HIS3Mx6***	this study
DAS606	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-886) -$(HA)_3$-$(His)_7$:HIS3Mx6***	this study
DAS607	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-797) -$(HA)_3$-$(His)_7$:HIS3Mx6***	this study
DAS608	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5Δ::HIS3*** **[pRS316-*spt5ΔKOW2*]**	this study
DAS609	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-930)- $(HA)_3$ -$(His)_7$:HIS3Mx6 rpa49Δ::LEU2***	this study
DAS610	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-886) -$(HA)_3$-$(His)_7$:HIS3Mx6 rpa49Δ::LEU2***	this study
DAS611	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-797) -$(HA)_3$-$(His)_7$:HIS3Mx6 rpa49Δ::LEU2***	this study
DAS121	*MAT***a**/*α* diploid ***SPT5/spt5Δ::HIS3*** derived from NOY397	this study
DAS612	*MAT***a**/*α* diploid ***SPT5/spt5(1-797) -$(HA)_3$-$(His)_7$:HIS3Mx6*** derived from NOY397	this study
DAS613	*MAT***a**/*α* diploid ***SPT5/spt5(1-610) -$(HA)_3$-$(His)_7$:HIS3Mx6*** derived from NOY397	this study
DAS614	*MAT***a**/*α* diploid ***SPT5/spt5(1-582) -$(HA)_3$-$(His)_7$:HIS3Mx6*** derived from NOY397	this study
DAS615	*MAT***a**/*α* diploid resulted from cross DAS50 x DAS605	this study
DAS616	*MAT***a**/*α* diploid resulted from cross DAS50 x DAS606	this study
DAS617	*MAT***a**/*α* diploid resulted from cross DAS50 x DAS607	this study
DAS655	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-886) -$(HA)_3$-$(His)_7$:HIS3Mx6 rrn3 (S213P)***	this study
DAS654	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-797) -$(HA)_3$-$(His)_7$:HIS3Mx6 rrn3 (S213P)***	this study

Supplementary Table 2. Plasmids used in this study

Yeast Plasmids			
pRS316	pBluescript, *CEN6, ARSH4, URA3*		Sikorski and Hieter, 1998
pRS316-*spt5*Δ*KOW2*	pRS316 derivative carrying *spt5*Δ*KOW2*		this study
pRS316-*SPT5*	pRS316 derivative carrying *SPT5*		this study
plasmids for protein expression in bacteria			
pET41a	expression of N-terminal $(His)_6$-GST	GST (Figure 2)	Novagen
pDAS619	expression of $(His)_6$-GST- fusion with full-length yeast Spt5	GST-Spt5 (Figure 2)	this study
pDAS620	expression of the $(His)_6$-GST-fusion of the N-terminal region of Spt5 (amino-acid residues 3-244)	construct A (Figure 2)	this study
pDAS621	expression of the $(His)_6$-GST-fusion of the N-terminal region of Spt5 (amino-acid residues 3-380)	construct B (Figure 2)	this study
pDAS622	expression of the $(His)_6$-GST-fusion of the central region of Spt5 (amino-acid residues 272-830)	construct C (Figure 2)	this study
pDAS623	expression of the $(His)_6$-GST-fusion of the central region of Spt5 (amino-acid residues 379-830)	construct D (Figure 2)	this study
pDAS624	expression of the $(His)_6$-GST-fusion of the central region of Spt5 (amino-acid residues 519-830)	construct E (Figure 2)	this study
pDAS625	expression of the $(His)_6$-GST-fusion of the N-terminal region of Spt5 (amino-acid residues 3-515)	construct F (Figure 2)	this study
pDAS626	expression of the $(His)_6$-GST-fusion of Spt5 containing an internal deletion (amino-acid residues deleted 515-578)	construct G (Figure 2)	this study
pDAS627	expression of the $(His)_6$-GST-fusion of Spt5 containing an internal deletion (amino-acid residues deleted 515-618)	construct H (Figure 2)	this study
pDAS628	expression of the $(His)_6$-GST-fusion of Spt5 containing an internal deletion (amino-acid residues deleted 515-832)	construct I (Figure 2)	this study
pDAS629	expression of the $(His)_6$-GST-fusion of the N-terminal region of Spt5 (amino-acid residues 3-563)	construct J (Figure 2)	this study
pDAS630	expression of the $(His)_6$-GST-fusion of the N-terminal region of Spt5 (amino-acid residues 3-614)	construct K (Figure 2)	this study
pNOY3162	expression of $(His)_6$-Rrn3	Rrn3	Keener *et al*., 1998
pDAS633	expression of $(His)_6$-Spt4 from pRSFDuet-1 (Novagen)	Spt4	this study
pDAS634	expression of $(His)_6$-Spt5 with C-terminal FLAG-$(His)_6$-TEV-$(HA)_3$-$(His)_6$ tag from pETDuet-1 (Novagen)	Spt5	this study

Supplementary Table 3. Oligonucleotides used in the study and the cloning strategy

primer	nucleotide sequence
F	atta**ccgcgg**acaactcggacacaaacgtgag
272_F	attt**ccgcgg**tgccccagaggtttctcctacc
379_F	aata**ccgctg**atgatgttgctttggaagaag
519_F	ataa**ccgcgg**tttcacaatcaatcaagaaggc
578_F	aaat**agatct**tcctatctctactctgaggaaa
618_F	tata**agatct**tcaaacaagcagagaagttacc
832_F	ttaa**agatct**acactcgaagaacaaacacatt
R1	attt**ggatcc**ttaatgacctccccatgtactgtt
R2	attt**aagctt**ttaatgacctccccatgtactgtt
244_R	atta**ggatcc**ttagtcttcttctgaagtcttgttcaa
380_R	taat**ggatcc**ttaaacatcatcagatttgtttgg
563_R	taaa**ggatcc**ttaagtagcaatatctttcgtggttct
614_R	aatt**ggatcc**ttaaaatgtcacttgaccttgctctac
830_R	aatt**ggatcc**ttattcgacagtagctttatcaccatt
SPT5_F	atgc**aagctt**atgagtgacaactcggacacaaacgtgagc
SPT5_R	atgc**cccggg**atgacctccccatgtactgttaccaccatag
SPT4_F	atgc**ggatcc**gatgtctagtgaaagagcctgtatgctgtgtgg
SPT4_R	atgc**aagctt**ttactcaacttgactgccatccctcggtttg
SacII, *BglII*, *BamHI*, *SmaI* and *HindIII* restriction sites used for the cloning are shown in **bold**	
plasmid	**strategy of the cloning of various *SPT5* regions into pET41a**
pDAS619	full-length *SPT5* PCR amplified using F and R1 primers cloned into pET41a
pDAS620	*SPT5* region PCR amplified using F and 244_R primers cloned into pET41a
pDAS621	*SPT5* region PCR amplified using F and 380_R primers cloned into pET41a
pDAS622	*SPT5* region PCR amplified using 272_F and 830_R primers cloned into pET41a
pDAS623	*SPT5* region PCR amplified using 379_F and 830_R primers cloned into pET41a
pDAS624	*SPT5* region PCR amplified using 519_F and 830_R primers cloned into pET41a
pDAS625	pDAS619 restriction digestion with *BglII* and subsequent re-ligation
pDAS626	PCR fragment of *SPT5* produced using 519_F and R2 cloned into pDAS619
pDAS627	PCR fragment of *SPT5* produced using 618_F and R2 cloned into pDAS619
pDAS628	PCR fragment of *SPT5* produced using 832_F and R2 cloned into pDAS619
pDAS629	*SPT5* region PCR amplified using F and 563_R primers cloned into pET41a
pDAS630	*SPT5* region PCR amplified using F and 614_R primers cloned into pET41a

Supplementary Methods.

Cloning of *SPT5* and *SPT4*. We used the predicted Spt5 domain structure from European Bioinformatics Institude Database (EMBL-EBI; http://www.ebi.ac.uk/interpro) and from Hartzog *et al*., 1998. The full-length Spt5 (pDAS619) and the Spt5 constructs A, B, C, D, E, J and K (pDAS620-624, pDAS629 and pDAS630) were cloned into pET41a expression vector (Novagen) using *SacII* and *BamHI* restriction sites introduced into the flanks of the PCR-amplified *SPT5* sequences. The construct F (pDAS625) was derived from pDAS619 using digestion with *BglII* and *BamHI* endonucleases and subsequent re-ligation of the plasmid. The internal deletion Spt5 mutants G, H and I (pDAS626-628) were derived from pDAS625 using *BglII* and *HindIII* restriction digestion and followed by the cloning in 3'-end *SPT5* sequences flanked by the *BglII* and *HindIII* restriction sites.

For the expression of the Spt4/5 complex we used pDAS633 and pDAS634 vectors. pDAS633 was obtained by cloning *SPT4* flanked by the *BamHI* and *HindIII* restriction sites (amplified from genomic DNA using PCR with DAS988 and DAS989 primers). pDAS634 was obtained by cloning *SPT5* flanked by the *HindIII* and *XmaI* restriction sites (amplified from genomic DNA using PCR with DAS986 and DAS987 primers).

The plasmids are listed in the Supplemental Table2. The sequences of the primers used for the cloning and the description of the constructs are listed in the Supplemental Table 3.

EMSA protocol. The rRNA probe was incubated for 20 min on ice with 10 μg of Bovine Serum Albumin (BSA), recombinant Spt4/5 complex (12 μg, 2.4 μg and 0.6 μg) or without protein addition in the binding buffer (10mM Hepes pH 7.6, 3mM $MgCl_2$,

20mM KCl, 1mMDTT, 5% glycerol) in 20 µl total volume. Then 2X loading buffer (40% glycerol, 0.1% Bromphenol Blue) was added; free RNA and RNA-protein complexes were resolved by electrophoresis on an 8% native poly-acrylamide gel in 1X TBE running buffer. Gel was dried down onto Whatman paper and visualized using a phosphoimager (GE Health Sciences).

DIVERGENT CONTRIBUTIONS OF CONSERVED ACTIVE SITE RESIDUES TO TRANSCRIPTION BY EUKARYOTIC RNA POLYMERASES I AND II.

by

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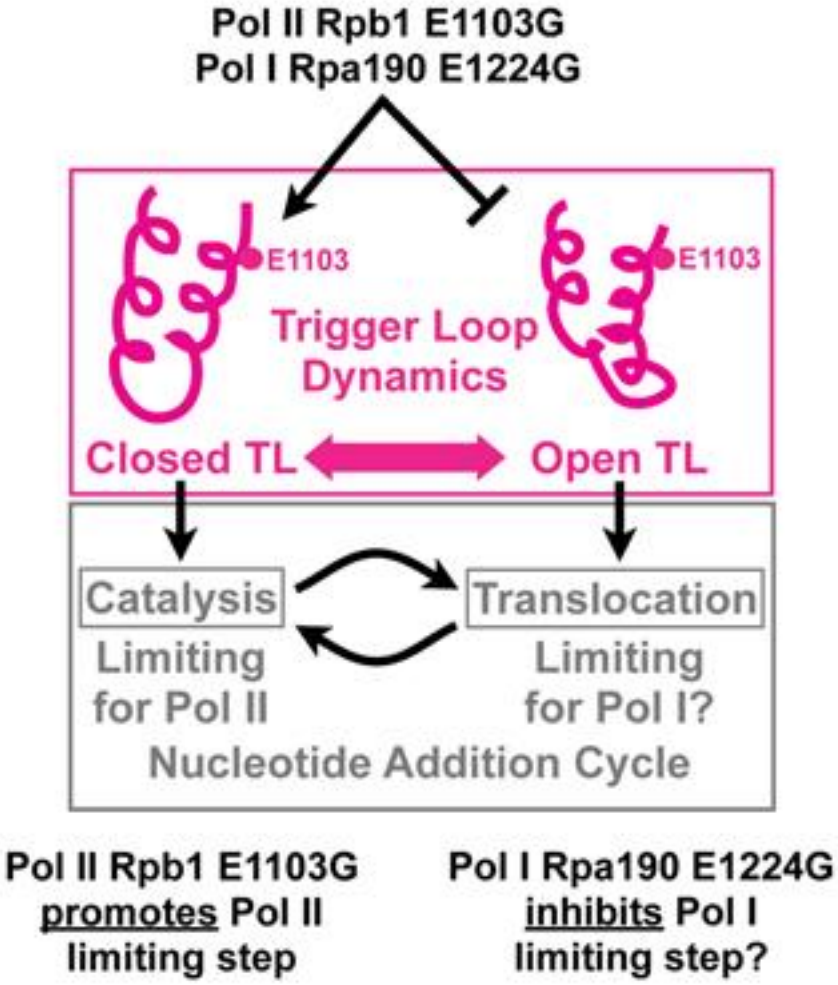
SUMMARY

Multisubunit RNA polymerases (msRNAPs) exhibit high sequence and structural homology, especially within their active sites, which is generally thought to result in msRNAP functional conservation. However, we show that mutations in the trigger loop (TL) in the largest subunit of RNA polymerase I (Pol I) yield phenotypes unexpected from studies of Pol II. For example, a well-characterized gain-of-function mutation in Pol II results in loss-of-function in Pol I [Pol II: *rpb1- E1103G;* Pol I: *rpa190-E1224G*]. Studies of chimeric Pol II enzymes hosting Pol I or Pol III TLs suggest that consequences of mutations that alter TL dynamics are dictated by the greater enzymatic context and not solely the TL sequence. Although the *rpa190-E1224G* mutation diminishes polymerase function, when combined with mutations that perturb Pol I catalysis, it enhances polymerase function, similar to the analogous Pol II mutation. These results suggest that Pol I and Pol II have different rate-limiting steps.

GRAPHICAL ABSTRACT



INTRODUCTION

All cells use one or more DNA-dependent RNA polymerases to transcribe their genomes. Although the subunit composition varies between polymerases, obvious sequence and structural homology is preserved in all domains of life (Cramer, 2002). In each enzyme, the catalytic center is formed between the two largest subunits, and these subunits exhibit the highest degree of sequence homology between even highly divergent species.

Prokaryotic cells utilize a single RNA polymerase for synthesis of all RNAs; however, eukaryotes have evolved three specialized nuclear RNA polymerases: RNA polymerase I (Pol I) transcribes the ribosomal DNA, RNA polymerase II (Pol II) transcribes all protein-coding genes and most loci that encode regulatory RNAs, whereas RNA polymerase III (Pol III) primarily synthesizes transfer RNA (tRNA). Of the three nuclear polymerases, Pol II has been studied most extensively, largely due to its diverse portfolio of target genes and its intimate connection to cell differentiation and development. Importantly, the other nuclear polymerases (Pols I and III) account for the vast majority of cellular transcription (Warner, 1999) though they have many fewer transcriptional targets.

Transcription of the rDNA by Pol I accounts for more than 60% of total transcription in growing cells (Warner, 1999). The pre-rRNA is co- and post-transcriptionally processed into mature rRNA species (18S, 5.8S and 25S rRNA in budding yeast) and incorporated into ribosomes. Thus, Pol I transcription is necessarily robust, processive and tightly regulated. Cryo-EM analysis of the Pol I structure and its comparison to Pol II supports high conservation of the active center as predicted from sequence conservation

(Cramer et al., 2008; Kuhn et al., 2007). Although ribosome biogenesis and thus Pol I transcription are critical to all cells, little is known about the details of Pol I catalysis.

The catalytic mechanism of transcription is thought to be very similar or identical among msRNAPs stemming from abundant sequence conservation. Structural comparisons between bacterial and archaeal RNA polymerases and Pol II from *Saccharomyces cerevisiae* (referred to as "yeast" herein) have identified a very high degree of structural homology, especially within the active centers. Coordinated conformational changes in two flexible domains near the active center, the bridge helix and the trigger loop (TL), are proposed to drive each round of nucleotide addition [reviewed in (Brueckner et al., 2009; Kaplan and Kornberg, 2008; Martinez-Rucobo and Cramer, 2013)]. Structural, biochemical and functional studies using both prokaryotic RNA polymerases and eukaryotic Pol II have demonstrated that the involvement of these features in catalysis is conserved across all domains of life (Tan et al., 2008; Vassylyev et al., 2007; Wang et al., 2006).

The TL is a small flexible domain in the largest subunit of msRNAPs that plays a critical role in nucleotide addition. The TL has been observed in a number of conformations from unfolded ("open") to folded ("closed") that are proposed to promote incorporation of the matched NTP and govern translocation [Figure 1A and (Bar-Nahum et al., 2005; Kaplan, 2010; Kaplan et al., 2008; Larson et al., 2012; Martinez-Rucobo and Cramer, 2013; Yuzenkova et al., 2010)]. Additional studies have shown that alternative intermediate conformations of the TL may contribute to pausing or arrest of elongation complexes (Nayak et al., 2013; Toulokhonov et al., 2007; Zhang et al., 2010a). The dynamic interaction between the TL and other domains within the active center is an area

of intense study due to its direct implications for gene expression and regulation thereof [reviewed in (Landick, 2009)]. Although structural and biochemical studies have revealed important roles for the TL in transcription elongation, there is much to learn about the precise mechanism by which this conserved domain of the polymerase functions.

Point mutations in the TL lead to a wide range of phenotypes including increased or decreased RNA polymerization rates, suppressed or enhanced pausing, enhanced or decreased forward translocation, increased backtracking and altered transcriptional fidelity (Bar-Nahum et al., 2005; Kaplan et al., 2008; Kireeva et al., 2008; Larson et al., 2012; Tan et al., 2008; Yuzenkova et al., 2010; Zhang et al., 2010a). In yeast Pol II, a large collection of mutations in and around the TL have been described (Kaplan et al., 2012; Kireeva et al., 2012). Characterization of the effects of these mutations on Pol II enzymatic properties has enhanced our understanding of Pol II transcription and models for its mechanism.

One of the best-characterized mutations affecting Pol II TL function is *rpb1-E1103G*. The *rpb1-E1103G* allele results in a "hyperactive", or gain-of-function, phenotype (Kaplan et al., 2012; Kaplan et al., 2008; Kireeva et al., 2008; Malagon et al., 2006). The highly conserved E1103 residue is located within base helix C of the TL but distal to the active site and does not make direct contact with substrates (Wang et al., 2006). E1103G mutant polymerases have increased polymerization activity and elevated misincorporation rates. The E1103G mutation promotes active site closure by stabilization of the substrate-interacting "closed" state of the TL (Kireeva et al., 2008). Stabilization of this state is presumed to lead to the observed enhancement of catalysis and misincorporation. The TL must open to release pyrophosphate subsequent to catalysis and it is thought that TL

opening may also be required for translocation (Brueckner et al., 2009; Da et al., 2012; Erie and Kennedy, 2009; Larson et al., 2012). Consistent with such a model, single molecule analyses of *rpb1-E1103G* Pol II show increased rate of nucleotide addition but impaired translocation (Larson et al., 2012). Therefore, E1103 substitution affects multiple steps during transcription, but since catalysis is normally limiting for Pol II, the net effect of E1103G is an increased elongation rate. Examination of the structural consequences of E1103G suggests that it disrupts an important interdomain contact leading to increased flexibility of the TL or destabilization of the open state (Kaplan et al., 2012; Kaplan et al., 2008; Kireeva et al., 2008; Kireeva et al., 2012). Molecular dynamics simulations, using the *Thermus thermophilus* RNAP structure, support the interpretation that mutation of this conserved acidic residue affects TL mobility (Kireeva et al., 2012). In agreement, substitutions in amino acids nearby the conserved glutamate also result in hyperactive enzymes in bacteria and archaea (Bar-Nahum et al., 2005; Tan et al., 2008; Zhang et al., 2010a).

Based on the TL's structural and functional conservation, we anticipated functional identity (or at least similarity) between the TLs of yeast Pols I and II. To test this hypothesis we made a series of mutations in the Pol I TL and compared the phenotypes to analogous mutations in *RPB1* of Pol II. From these mutations, it was clear that Pol I is less tolerant to mutation of the TL than Pol II. Of the viable mutant strains, we chose to further characterize two alleles analogous to those that resulted in gain-of-function phenotypes for Pol II. To our surprise, both mutations (*rpa190-E1224G* in base helix C and *rpa190-F1205H* in the nucleotide interacting region of the TL) reduced the elongation rate of Pol I. Using a set of chimeric alleles of *rpb1* (Pol II), fusing TLs of either *rpa190*

(Pol I) or *rpo31* (Pol III) into *rpb1*, we demonstrate that the divergent functions of the TL residues are not an obligatory feature of the loop sequence, but rather a consequence of the protein context of the RNA polymerase. Furthermore, we show that *rpa190-E1224G* suppresses phenotypic defects of other *rpa190* alleles predicted to alter catalysis, suggesting that impaired transcription elongation by *rpa190-E1224G* is also context dependent. These data are consistent with a model in which different steps in transcription elongation are rate-limiting for Pol I compared to Pol II. These results demonstrate that assumption of strict conservation of function between msRNAPs can be problematic and that each RNA polymerase system must be carefully scrutinized in its own right.

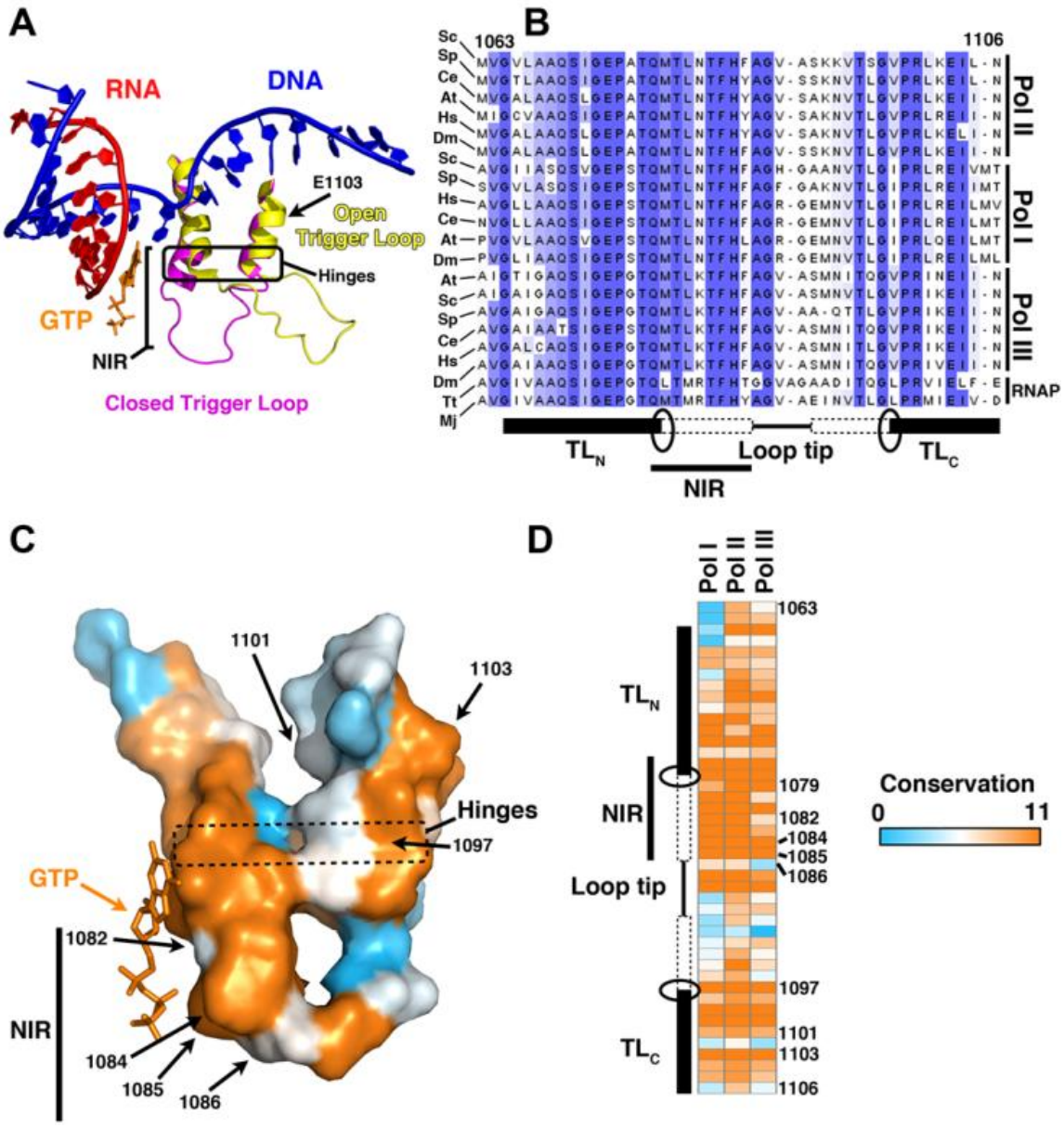


Figure 1. **Overview of Pol II trigger loop and conservation among multisubunit RNA polymerases**. **A)** Schematic of Pol II active site showing TL in open (yellow, PDB 1Y1V (Kettenberger et al., 2004)) or closed conformation (magenta). Closed TL structure is from PDB 2E2H (Wang et al., 2006) and this structure also contained the GTP substrate (orange) shown. NIR = nucleotide interacting region. **B)** Multiple sequence alignment of RNA polymerase large subunits from three domains of life. Eukaryotic Pol I-III polymerase large subunits are from *S. cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Caenorhabditis elegans* (Ce), *Arabidopsis thaliana* (At), *Homo sapiens* (Hs), and *Drosophila melanogaster* (Dm). Bacterial polymerase TL is from the β′ subunit of *Thermus thermophilus* (Tt) and archaeal TL is from the A″ from subunit of *Methanocaldococcus jannaschii* (Mj). Identical and similar residues are shaded.

Alignment generated in MUSCLE (Edgar, 2004) and displayed in Jalview 1.0 (Waterhouse et al., 2009). Schematic below alignment indicates the positions of the N- and C-terminal TL helices (TL_N and TL_C), ovals represent the positions of the hinges, dashed rectangles indicate the extent of TL helices folding or stabilization upon reaching the closed conformation, and NIR is the TL region directly implicated in substrate interaction. **C)** Surface representation of isolated TL from closed conformation [PDB 2E2H (Wang et al., 2006)] showing structural arrangement of residues mutated in various constructs and their positions within the folded TL. Conserved residues based on model organism alignment from Figure 1B (eukaryotic subunits only) are color-coded based on conservation as determined by Jalview using a MUSCLE alignment. Most-conserved residues are orange, least-conserved are light blue (scale as in Figure 1D). **D)** Conservation of TL residues within Pol I, Pol II, Pol III large subunits. Large subunit alignments were generated from eukaryotic Pol large subunit CDD entries (Marchler-Bauer et al., 2013) (cd02584 - Rpb1, cd02735 - Rpa190 and cdk02736 - Rpo31) using MUSCLE and Jalview to generate conservation scores, and scores were plotted as a heat map using GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/). Most conserved residues are in orange, least conserved are in light blue. Structural figures made using Pymol v. 0.99 (The PyMOL Molecular Graphics System, Schrödinger, LLC).

RESULTS

Pol I is less tolerant to TL mutations than Pol II *in vivo*. The TL is conserved across all three domains of life (Figure 1B-1D). To characterize the degree to which TL function is conserved between RNA polymerases, we conducted a mutational analysis of the Pol I TL. We constructed eight substitutions in the TL-encoding region of *RPA190*. These substitutions were located in the substrate proximal, nucleotide interacting region (NIR) as well as in the hinge region of base helix C (TL_C) (Figure 1C). Each mutation was analogous to a previously characterized, viable allele of *RPB1*. Unlike in Pol II, three of these mutations in *RPA190* were lethal (superscript indicates position in Rpb1: $H1206Y^{H1085Y}$, $N1203S^{N1082S}$ and $G1218D^{G1097D}$), as assessed by plasmid shuffle and tetrad analysis (Table 1 and data not shown).

Table 1. Phenotypic comparison of point mutations in *RPA190* and *RPB1* TLs.

Pol II		Pol I	
mutation	**Genetic/biochemical classification**	**mutation**	**relative growth rate (% of WT)**
N1082S	LOF	N1203S	lethal
F1084H	GOF	F1205H	63 ± 7
H1085Y	LOF	H1206Y	lethal
H1085Q	LOF	H1206Q	64 ± 13
F1086S	LOF	F1207S	100 ± 6; *cs*
G1097D	GOF	G1218D	lethal
L1101S	GOF	L1222S	59 ± 2
E1103G	GOF	E1224G	89 ± 6; *cs*

Table comparing phenotypes of the corresponding TL mutants in Pol I (this study) and Pol II (Kaplan et al., 2012). Mean relative growth rates measured in liquid YEPD media at 30°C for the Pol I mutants are averaged from at least three independent experiments each performed in duplicate, with ± SD value indicated. "*cs*" stands for cold-sensitivity observed at 23°C and 18°C. GOF and LOF are abbreviation for gain- and loss-of-function phenotypes of Pol II mutants as established in (Kaplan et al., 2012).

Of the five mutations that supported growth, three mutant strains exhibited severe growth defects relative to WT (F1205H^{F1084H}, H1206Q^{H1085Q} and L1222S^{L1101S}; Table 1). Both F1207S^{F1086S} and E1224G^{E1103G} exhibited minor growth defects compared to the WT strain under optimal conditions and manifested mild cold-sensitivity. To determine if mutations in the Pol I TL resulted in effects mechanistically similar to those observed for Pol II, we focused subsequent analyses on two mutations that were gain-of-function for Pol II: F1205H and E1224G.

F1205H and E1224G mutations in the Pol I TL result in reduced elongation rates. If TL functions in catalysis and translocation by Pol I were similar or identical to that of the Pol II TL, we would predict that mutations in identical residues of the TL would result in similar effects on transcription. To test this hypothesis, we expressed and purified WT, A190-F1205H or A190-E1224G Pol I enzymes from yeast (see Experimental Procedures). Initially we measured enzyme activity using an *in vitro* multiround transcription assay (Bedwell et al., 2012; Keener et al., 1998). In principle, this assay can detect mutation-dependent effects on a variety of steps in the transcription cycle (initiation, promoter escape or elongation). We observed only minor differences in the activity of the mutant enzymes compared to WT Pol I (Figure S1). Thus, the mutant enzymes were active but not obviously hyperactive relative to WT.

Since the *in vitro* multiround assay is not sensitive to modest changes in transcription elongation rate, we performed a single-round transcription elongation assay (Schneider, 2012). We determined that at 200 µM NTPs the WT elongation rate was ~18-20 nucleotides per second, whereas the elongation rates of both mutant enzymes were slower (F1205H = ~8 nt/sec and E1224G = ~12 nt/sec; Figure 2A and 2B). Neither mutation in

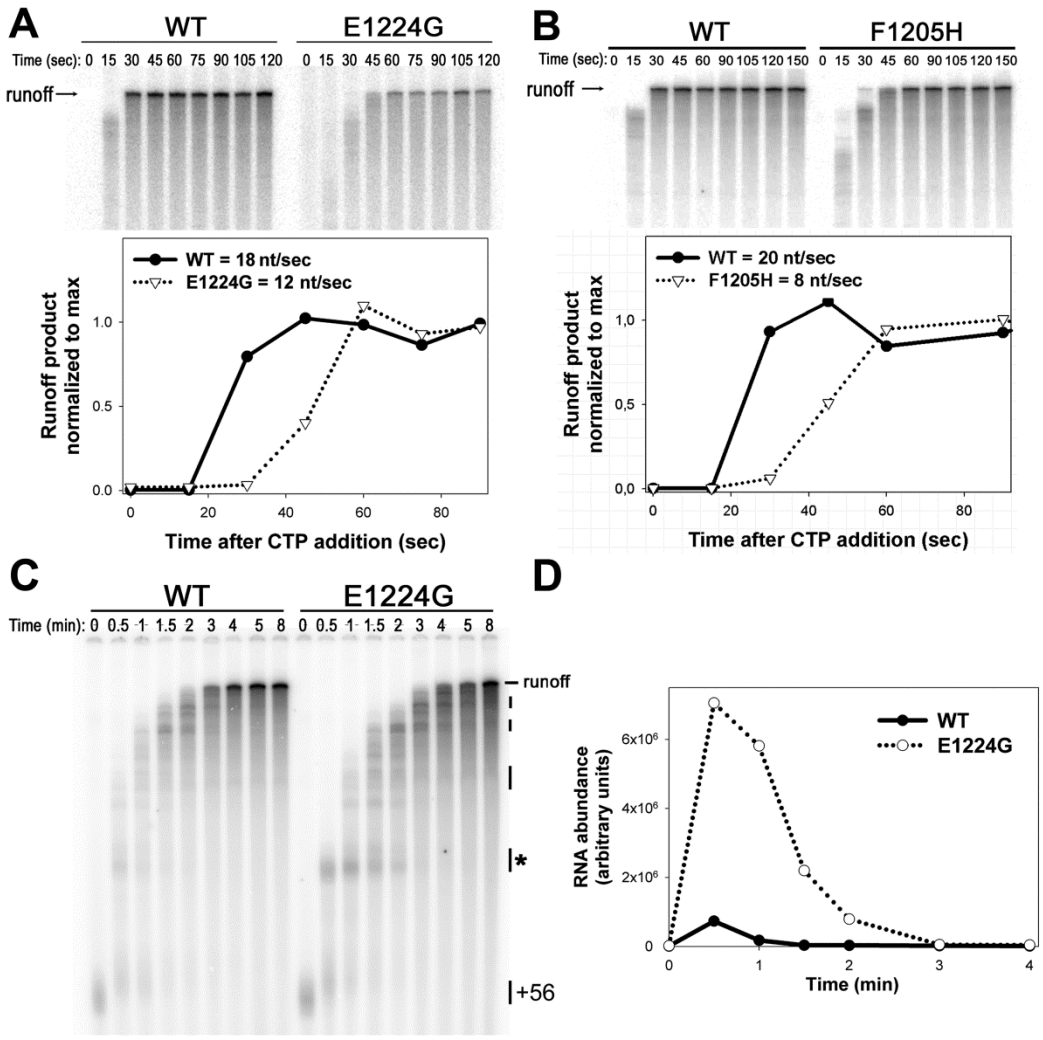


Figure 2. **The F1205H and E1224G polymerases have decreased transcription elongation rates *in vitro*. A)** Transcription elongation assays were performed for WT and E1224G Pol I using 200 μM ATP, UTP, CTP and 20 μM GTP (and 10 μCi α-^{32}P GTP). The ^{32}P-labeled transcripts were separated by gel-electrophoresis and visualized using phosphorimaging. Runoff product accumulation was quantified using ImageQuant software and plotted versus time. The transcription elongation rates for the mutant and WT polymerases were approximated from the lag time required for maximal runoff product accumulation and the length of the runoff product (745 nt). **B)** Transcription elongation assay for WT and F1205H Pol I enzymes performed as in panel A. **C)** Transcription elongation assays for WT and E1224G Pol I were performed as for panel A, but with low NTP concentrations (20 μM ATP, UTP, CTP, 2 μM GTP). The positions of the synchronized complexes at +56 (prior to CTP addition), the runoff product and the sequence-specific pauses are indicated. **D)** Signal intensity for one of the major pause sites (labeled with *asterisk*, panel C) was quantified using Image Quant software and plotted versus time. The data shown are representative experiments. Each assay was performed at least three independent times. Quantification of pause intensity from C is presented in Figure S2.

Pol I resulted in hyperactivity, rather both mutant enzymes were impaired for transcription elongation *in vitro*, contrary to observations for analogous Pol II mutations.

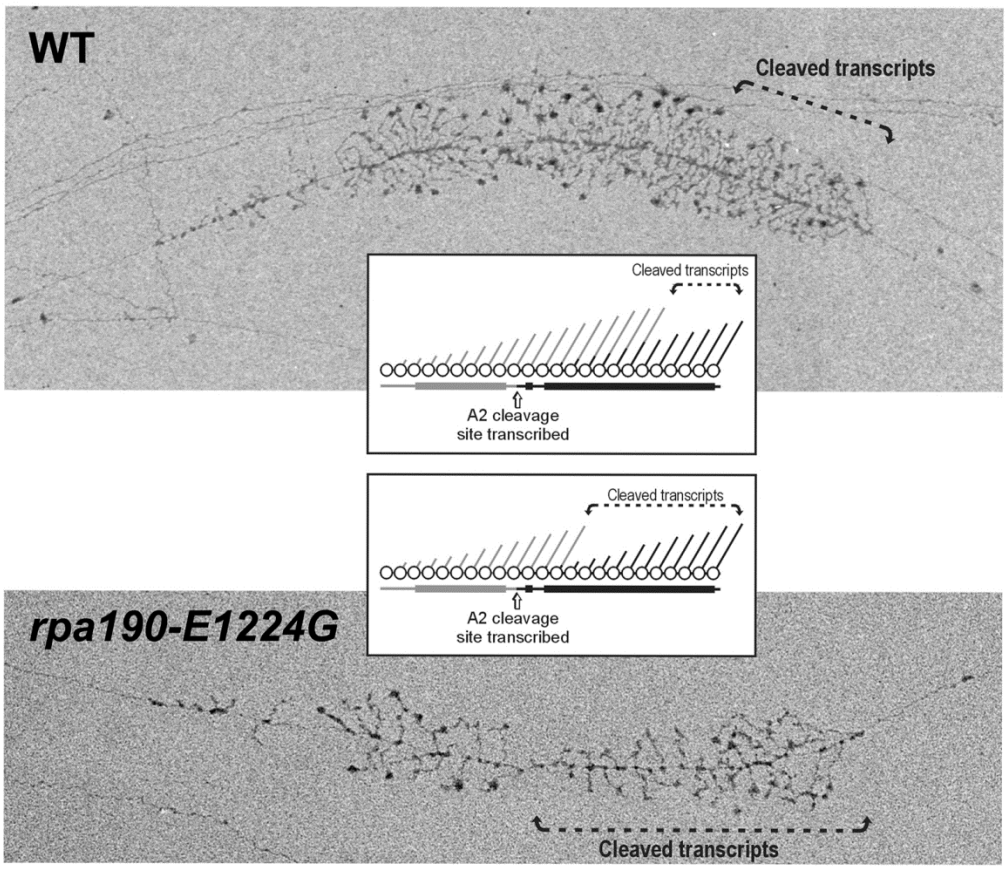
A190-E1224G polymerase demonstrates enhanced pausing *in vitro*. Since the *rpb1-E1103G* mutation is the best-characterized mutation of the Pol II TL, we extended our comparison, focusing on the analogous mutation in Pol I, *rpa190-E1224G*. In addition to increased overall elongation rate, the Rpb1-E1103G Pol II mutant enzyme showed decreased pausing *in vitro* (Kireeva et al., 2008; Malagon et al., 2006). To measure pause-tendency for the WT and mutant Pol I, we used lower NTP concentrations in order to favor pausing. We observed that both WT and mutant Pol I paused at the same sites on the rDNA template. Remarkably, the magnitude and duration of these pauses were significantly increased in the A190-E1224G mutant compared to WT (Figure 2C and 2D and Figure S2). Consistent with assays performed in high NTP concentrations, the net elongation rate of the mutant was also slower than WT when NTP concentration was limiting. Thus, unlike E1103G Pol II, the analogous mutation in Pol I increased pausing rather than decreasing it. The observation of enhanced pausing, though in contrast to Pol II data for the same mutation (Malagon et al., 2006), supports a model that the TL plays a role in pause entry and escape (Nayak et al., 2013; Toulokhonov et al., 2007; Weixlbaumer et al., 2013).

Pol I is not hyperactive in the *rpa190-E1224G* strain. To test whether our observations *in vitro* were valid *in vivo*, we performed a series of analyses. We observed no increase in rRNA synthesis in the E1224G strain relative to WT (Table S1), and we confirmed that any potential overexpression of rRNA was not masked by alteration of the rDNA copy number, decreased transcription initiation by Pol I or exosome-dependent

decay of rRNA (Figure S3). Thus, the mutant allele does not result in hyperactive Pol I *in vivo*.

To directly assess the effects of the E1224G mutation on Pol I activity *in vivo*, we used single-molecule analysis of transcription by electron microscopy of Miller chromatin spreads. From the Miller spreads, we observed no large defects in polymerase density per gene or any change in the percentage of actively transcribed genes (Figure 3). These data suggest that transcription initiation is not defective for the mutant enzyme. Since this approach results in static images, one cannot directly assess changes in transcription elongation rate; however, analysis of the individual genes and nascent transcripts yielded insights into the enzymes' elongation properties *in vivo*. Multiple previous studies using Miller chromatin spreading have identified features of active rRNA genes that are characteristic of moderately impaired elongation rate [*e.g.* after mycophenolic acid (MPA) treatment or in topoisomerase mutants; (French et al., 2011)]. When compared to rDNA repeats in WT cells, co-transcriptional processing of the rRNA occurs on a greater percentage of nascent rRNA transcripts in the mutant and at a position in which the polymerase is located closer to the 5′-end of the gene, resulting in a distinctive 'double gradient' of transcript length. This appearance is interpreted to reflect RNA processing continuing at its normal rate while polymerase elongation rate is reduced. These characteristics were readily observed in the great majority of genes in *rpa190-E1224G* Miller spreads but were rarely observed in control genes (Figure 3). Thus, *rpa190-E1224G* has *in vivo* consequences for Pol I transcription consistent with reduced elongation rate.

A



B

	pols/gene	st. dev.	n	% active	n % active	% double gradient	n % double gradient
WT	51	17.6	65	65.1	43	<1	197
rpa190-E1224G	50.3	14.2	189	75.7	148	92	132

Figure 3. EM analysis of Miller chromatin spreads confirms an elongation defect in *rpa190-E1224G* Pol I. A) Electron micrographs showing examples of single, active rDNA genes from WT and *rpa190-E1224G* strains. Gene regions displaying cleaved transcripts are marked on the micrographs with arrowed brackets. The inset schematic illustrates the characteristic co-transcriptional cleavage patterns that correlate with either normal (top) or reduced transcription elongation rate by Pol I. The "double gradient" pattern (bottom schematic) was seen in 92% of 132 rDNA genes in the mutant strain, but in less than 1% of 197 WT genes. **B)** Multiple rDNA repeats from WT and mutant cells were analyzed and from these counts the average number of polymerases per gene and the percentage of actively transcribed genes were determined. The value "n" reports the number of genes analyzed for each analysis (and within each strain). The standard deviation observed for "pols/gene" is indicated.

Genetic interactions between *rpa190-E1224G* and Pol I elongation factors. As shown in Table 2, we tested the *rpa190-E1224G* allele for genetic interactions with mutations in several genes encoding Pol I subunits (*rpa49*Δ, *rpa135-D784G*, *rpa12*Δ), as well as factors involved in Pol I transcription initiation (*uaf30*Δ, *rrn3-S213P*), elongation (*spt4*Δ, *spt5(1-797)*, *paf1*Δ) and rRNA quality control (*trf4*Δ, *rrp6*Δ). We observed synthetic lethality between *rpa190-E1224G* and *rpa12*Δ mutations. This result is particularly interesting, since neither *rpa190-E1224G* nor *rpa12*Δ single mutations affect the growth rate dramatically under permissive conditions [Table 1; (Nogi et al., 1993)]. The *RPA12* gene encodes the A12.2 subunit of Pol I and is involved in polymerase assembly and intrinsic transcript cleavage (Kuhn et al., 2007; Nogi et al., 1993). This result is analogous to synthetic lethality between *rpb1-E1103G* and the Pol II cleavage factor deletion *dst1*Δ (Malagon et al., 2006). Furthermore, we observed that the *rpa190-E1224G* mutation genetically interacted with three additional alleles: *rpa49*Δ, *rpa135-D784G* and *paf1*Δ. All three of these mutations have been shown to impair Pol I transcription elongation (Kuhn et al., 2007; Schneider et al., 2007; Zhang et al., 2010b). If *rpa190-E1224G* were hyperactive, one might expect suppression of these elongation defective alleles based on behavior of *rpb1-E1103G* (Kaplan et al., 2012). However, we observed synergistic growth defects in the double mutants. These data support a model that E1224G substitution impairs Pol I transcription elongation and sensitizes cells to defects in other factors that promote Pol I elongation.

Table 2. Genetic interactions between *rpa190-E1224G* and mutations that influence Pol I transcription suggest a role for E1224 in transcription elongation.

Function in rRNA synthesis	allele tested	genetic interaction with *rpa190-E1224G*
Pol I subunits involved in transcription elongation	***rpa12*Δ**	**lethal**
	***rpa49*Δ**	**synergistic slow, ts**
	rpa135-D784G	**synergistic slow**
Pol I transcription elongation factors	***paf1*Δ**	**synergistic slow**
	*spt4*Δ	additive slow
	spt5(1-797)	additive slow
Pol I transcription initiation factors	*uaf30*Δ	additive slow
	rrn3-S213P	additive slow
rRNA quality control	*trf4*Δ	additive slow
	*rrp6*Δ	additive slow

Context-dependent functions of Pol I TL residues revealed by chimeric *RPB1-RPA190* alleles. To explain how identical mutations in related polymerases yield divergent outcomes, we can envision several models. These models fall into two general categories. First, subtle substitutions *within* the TL sequence may alter the responsiveness of the domain to mutations in conserved residues. In this model, the role of Pol II E1103 or analogous residues in the Pol I or Pol III TLs would depend on the TL sequence in which it were placed. Second, amino acids *outside* of the TL differentially interact with the TL, altering the functional consequence of mutations in conserved residues, such as E1103G.

To distinguish between these models we constructed a series of *rpb1* chimeric alleles, in which the TL domain from Pol II was substituted with the corresponding sequences from either Pol I or Pol III. We also included E1103G-substituted versions to measure its effect on different TL sequences in the Pol II context (Figure 4A). Expression levels of chimeric Pol II enzymes were measured for additional control (see Methods and Figure S4A-S4D). Almost all of the chimeric *rpb1* alleles supported growth, confirming functional conservation of the TL among the three RNA polymerases.

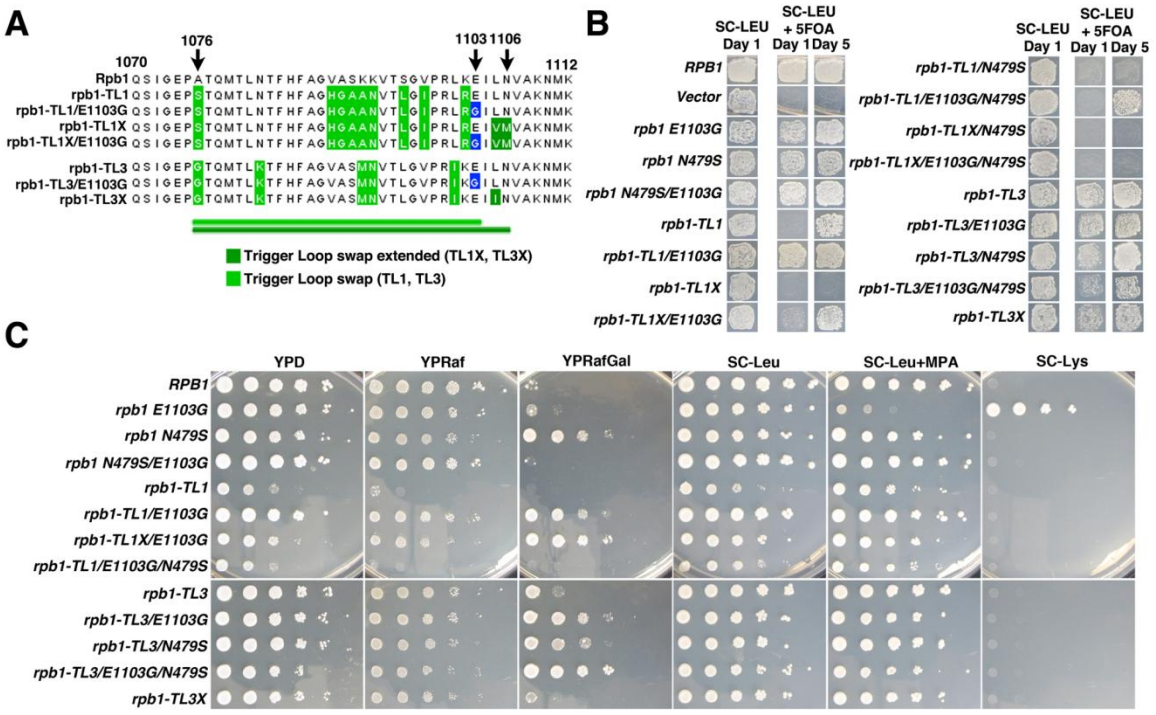


Figure 4. Chimeric alleles of *RPB1* support the model that sequences in the Pol I trigger loop impair Pol II function. A) Summary of chimeric *RPB1* alleles used in this study showing amino acid sequence of TL region for each. **B)** Plasmid shuffle results measuring complementation ability of individual *rpb1* alleles. Growth in the presence of 5-FOA indicates proficiency for loss of the *RPB1 URA3* plasmid and the resulting growth of chimeric alleles as the sole source of Rpb1. Cells were grown 30°C and images for 1 or 5 days growth are shown. **C)** 10-fold dilutions of cultured viable strains were plated on indicated growth media. Phenotypes were assessed as follows: growth on YEP with raffinose and galactose as carbon sources (YPRafGal), compared to YEP with raffinose alone (YPRaf), was scored as suppression of galactose toxicity conferred by *gal10Δ56*. Mycophenolic acid (MPA) sensitivity was scored by comparison of growth in SC-Leu to that on SC-Leu +MPA (20 μg/ml). To assess the Spt phenotype, cells expressing the chimeric alleles of *RPB1*, all of which also contained the Spt-reporter allele *lys2-128δ*, were plated on SC-Lys medium. Cells with the Spt$^-$ phenotype will grow in the absence of lysine, whereas Spt$^+$ cells (wild type phenotype) will not. Time of growth on each medium were generally based on time required for reasonably sized individual wild type colonies to be observed (for media probing growth defects) or for suppressive phenotypes to be discerned (for media where wild type cells do not grow). See also Table S3 and Figure S4.

The only allele that did not grow was *rpb1-TL1X*, which carried the largest portion of the Pol I domain inserted into *RPB1*. Consistent with this observation, a shorter Pol I TL chimera (termed *rpb1-TL1*) exhibited a severe growth defect relative to the WT *RPB1* allele (Figure 4B). The Pol III TL did not dramatically affect growth of the strain (Figure 4A and 4B).

Growth defects caused by the *rpb1-TL1* allele were partially rescued by E1103G substitution. We additionally observed suppression of *rpb1-TL1X* lethality by E1103G substitution (Figure 4B). These results suggest that defects in *rpb1-TL1* and E1103G counteract each other, similar to native *rpb1* gain-of-function (like E1103G) and loss-of-function alleles within the Pol II TL (Kaplan et al., 2012). To demonstrate that the E1103G mutation functions as a gain-of-function allele, irrespective of the TL sequence, it was important to test whether the TL1 chimeras behaved as gain-of-function or loss-of-function mutations in Pol II.

We used several established *in vivo* assays to determine whether the chimeric constructs induced loss- or gain-of-function phenotypes in Pol II. We found that *rpb1-TL1* indeed conferred loss-of-function phenotypes, and these were partially suppressed in *rpb1-TL1 E1103G*, consistent with suppression of growth defects noted above (Figure 4C). Previously, we have shown that Pol II TL loss-of-function alleles manifest WT phenotypes for Spt-reporter assays (Spt^+) and relative resistance to MPA (MPA^r), but confer suppression of the *gal10*Δ*56* mutation (e.g., *rpb1-N479S* in Figure 4C)(Kaplan et al., 2012; Kaplan et al., 2008). Opposite phenotypes (strong Spt^-, MPA^s but no strong suppression of *gal10*Δ*56* outside of *rpb1-G1097D*) are common to known Pol II gain-of-function mutants, including the hyperactive *rpb1-E1103G* strain [Figure 4C and (Kaplan

et al., 2012; Kaplan et al., 2008)]. We observed that the *rpb1-TL1* chimeric mutant manifested phenotypes similar to known Pol II loss-of function TL alleles (strong suppression of *gal10*Δ*56,* Spt^+, and MPA^r, Figure 4C). Thus, *rpb1-TL1* impairs Pol II transcription *in vivo;* however, the growth defects of *rpb1-TL1* were rescued by the E1103G substitution, suggesting that this mutation acts as a gain-of-function in the chimera irrespective of TL sequence.

To further test this conclusion, we examined genetic interactions with a known Pol II loss-of-function allele, *rpb1-N479S*, to probe *rpb1-TL1* and *rpb1-TL1/E1103G* phenotypes. We observed lethality for the combination of *rpb1-N479S* with *rpb1-TL1*. Furthermore, the genetic suppression that E1103G conferred on *rpb1-TL1* was lost in combination with *rpb1-N479S* (Figure 4B and 4C). All of these data are consistent with E1103G acting as a gain-of-function mutation in Pol I/Pol II chimeric alleles, just as it does in a fully Pol II context.

We repeated these assays using constructs that carried Pol III/Pol II chimeric alleles (Figure 4A). We observed little, if any, effect of the Pol III trigger loop on growth phenotype on rich medium, though there is a slight growth defect in our plasmid shuffle assay (Figure 4B). E1103G did not confer strong phenotypes to *rpb1-TL3* except increased suppression of *gal10*Δ*56*. We note that combination of N479S and E1103G in the *rpb1-TL3* context showed a mild enhancement of growth defect and of *gal10*Δ*56* suppression. This result is in contrast to the behavior of E1103G in *RPB1* or *rpb1-TL1*/*TL1X* backgrounds where they counteract/suppress each other, underscoring the conclusion that the TL makes multiple complex interactions within and outside of the domain. The results gathered from the chimeric *rpb1* alleles are summarized in

supplemental Table S3. Altogether, these data suggest that the influence of amino acid substitutions on trigger loop function is context dependent and not strictly reliant on the sequence of the TL itself.

One likely site of interaction that could directly influence TL mobility is the bridge helix (BH). The TL is directly adjacent to BH residues and the mobility of both domains has been proposed to be critical for the RNA polymerase nucleotide addition cycle. To test this hypothesis, we constructed chimeric alleles of *RPB1* that carried both the Pol I TL and BH. First, we found that Pol II could functionally host Pol I bridge helix constructs (Figure S4E-S4G). Phenotypic analysis suggested that *rpb1-BH1* constructs might be loss-of-function for Pol II. However, rather than suppressing growth defects of *rpb1-TL1* constructs, *rpb1-BH1* domains exacerbated growth defects of *rpb1-TL1* constructs, consistent with their single mutant phenotypes, but inconsistent with mutual suppression as might be expected if each Pol I domain increased compatibility for the other within the Pol II context. Thus, the bridge helix does not appear to be the sole mediator of the observed incompatibility of the Pol I TL in Pol II.

Intra-molecular genetic interactions within Pol I TL convert E1224G from a loss-of-function to a gain-of-function mutation. The results above suggested that Pol I TL function is influenced by its enzymatic context and not solely its internal sequence. Furthermore, the data are consistent with the model that a glycine at position 1103/1224 likely has similar effects on TL dynamics regardless of TL sequence. These findings led to the hypothesis that the E1224G mutation in Pol I affects the TL just as the E1103G mutation affects Pol II, but with functionally distinct outcomes. We propose these different outcomes reflect different rate-limiting steps for Pol I and Pol II. It was shown

previously that the E1103G mutation improves nucleotide addition by Pol II, but impairs the translocation step in the nucleotide addition cycle (Larson et al., 2012). Since nucleotide addition is apparently rate-limiting for Pol II, *rpb1-E1103G* results in a gain-of-function phenotype. If the rate-limiting step for Pol I transcription elongation were translocation rather than catalysis, one would expect the *rpa190-E1224G* mutation to impair transcription elongation. To test this hypothesis, we constructed a series of intra-molecular *rpa190* double mutants to assess *rpa190-E1224G* context-specific behavior in Pol I.

Our analysis is based on the observations that Pol II E1103G substitution shows extensive intra-molecular genetic relationships with substitutions in other Pol II residues, both inside and outside of the TL. These relationships support a general model that increased TL dynamics can compensate for loss-of-function mutations, while exacerbating other hyperactive mutations (Kaplan et al., 2012). We combined several of our originally selected substitutions (Table 1) with E1224G to potentially gain insight into their effects on Pol I transcription. When combined with mutations that are expected to impair catalysis (N1203S^{N1082S}, H1206Q^{H1085Q}, F1207S^{F1086S}), the E1224G mutation in Pol I rescued growth defects (Table 3), resulting in a gain-of-function rather than loss-of-function. Also in agreement with Pol II data, combination of the E1224G mutation with another mutation in the hinge (L1222S^{L1101S}) was lethal. These data are consistent with the hypothesis that the primary effects of the *rpa190-E1224G* mutation on TL motion are similar between Pols I and II; however, the steps in transcription that are rate-limiting may differ between the two enzymes leading to distinct transcriptional outcomes.

Table 3. Intramolecular genetic interactions with *rpa190-E1224G*

***rpa190* alleles**	**% of WT growth rate +/- 1 SD**	**observed genetic interactions**
N1203S	inviable	Double mutant is viable, E1224G rescues lethality of N1203S
N1203S/E1224G	64 ± 7	
H1206Q	64 ± 13	E1224G rescues growth rate of H1206Q
H1206Q/E1224G	85 ± 3	
F1207S	100 ± 6	E1224G does not significantly affect growth of F1207S at 30°C; it rescues cold-sensitivity of F1207S but leads to ts-phenotype of the double mutant (at 37°C)
F1207S/E1224G	88 ± 6	
L1222S	59 ± 2	Double mutant lethality
L1222S/E1224G	inviable	

WT, wild-type; ts, temperature-sensitive.

DISCUSSION

TL function is not identical between Pol I and Pol II. Most studies of cellular RNA polymerases have focused on Pol II and bacterial RNAP. In this study, we constructed a collection of mutations in the TL region of the largest subunit of Pol I that had been previously characterized in analogous residues in the TL region of Pol II. Using both biochemical and simple phenotypic analyses, we observed substantial differences in the effects of the mutations in Pol I vs. Pol II. To learn more about the root of these observed differences, we focused our analysis on comparisons with the well-characterized, hyperactive *rpb1-E1103G* mutation in Pol II. In contrast to expectations, the analogous *rpa190-E1224G* mutation in Pol I led to impaired transcription elongation and loss-of-function.

There is a general assumption that functional data measured in one RNA polymerase system are applicable to most or all other related RNA polymerases. This expectation initially took hold when seminal studies of sequence conservation between divergent RNA polymerases were published (Allison et al., 1985). Based on sequence and

subsequent structural data, it has become widely accepted that functional studies of polymerase catalysis can be broadly applied [e.g. (Jennebach et al., 2012; Maoileidigh et al., 2011)]. Our genetic and biochemical data indicate that even minor differences in local protein sequence or structure within RNA polymerase active centers can lead to substantially different functional consequences for otherwise identical perturbations, and we propose that these differences extend to the rate-limiting steps in Pol I and Pol II transcription.

Potential models to explain the different effects of mutations in the trigger loop. Our results are consistent with the model that the TLs in Pol I and Pol II (and Pol III) have dynamics similarly influenced by E1103G and analogous residues, but minor differences in the rates of individual steps in the nucleotide addition cycle by the enzymes render each enzyme differentially sensitive to the mutation. Critically, *rpb1-E1103G* has been shown to alter both catalysis and translocation, with its net increase in transcription rate coming from effects on catalysis (Larson et al., 2012). Despite an increased transcription rate, E1103G is defective for translocation. This result has been explained by E1103G stabilizing the active site in the closed conformation, as inferred biochemically (Kireeva et al., 2008). The closed conformation should promote catalysis but has also been proposed to inhibit translocation (Feig and Burton, 2010; Larson et al., 2012). According to this interpretation, the E1224G substitution in Pol I would alter Pol I TL dynamics, just as E1103G does in Pol II; but if translocation were more limiting for Pol I than catalysis, the end result would be a slower enzyme. According to this model, any positive effects of *rpa190-E1224G* on catalysis would be outweighed by negative effects on translocation.

Upon closure of the TL, an almost identical sequence of nucleotide-interacting residues should be positioned for interactions with substrates for Pol I or Pol II. Differences in behavior of identical substitutions between the enzymes thus are quite perplexing. For example, the conserved phenylalanine in the nucleotide-interacting region of the trigger loop (position 1205 in A190; 1084 in Rpb1) would occupy the catalytic center when the trigger loop is closed. We observed opposite effects of mutations at this position in Pol I versus Pol II. It has been shown previously that position 1084 is sensitive to conditional epistasis in Pol II, as double mutant analysis indicated that gain-of-function characteristics of a similar substitution (F1084I) are not maintained in certain double mutant configurations (Kaplan et al., 2012). Residues proximal to F1084 control its gain-of-function or loss-of-function behavior. Consistent with the epistasis studies performed within Pol II, it is likely that minor differences in conformation between the active sites of Pol I and Pol II naturally create distinct enzymatic features that facilitate evolution of specialized metabolic roles.

Unique enzymatic features suit distinct cellular roles. Why would divergent enzymatic features evolve in closely related enzymes? The answer to this question may lie in the unique cellular roles for the RNA polymerases. Unlike most genes transcribed by Pol II, the transcription initiation rate is very high at the rDNA promoter. As a result, the space between transcribing Pol I complexes is small (*e.g.* Figure 3). This high polymerase density at the rDNA has several important consequences, two of which are highlighted by the mutational analyses performed here.

First, even transient perturbation of transcription elongation could induce potentially catastrophic “traffic jams” on the rDNA. Perhaps Pol I has evolved efficient chemical

properties to avoid such catastrophic events. Consistent with this hypothesis, the *rpa190-F1205H* mutation was dominant (data not shown) and caused a much greater growth defect than the *rpa190-E1224G* allele (Table 1). We previously characterized a mutation in *RPA135* that also reduced transcription elongation rate by impairing nucleotide addition, and like *rpa190-F1205H, rpa135(D784G)* mutant growth was poor (Schneider, et al., 2007). Almost exactly like the *rpa135* mutant, *rpa190-F1205H* mutants exhibit a series of defects both in transcription and rRNA processing (Figure 2 and data not shown).

Second, Gadal and colleagues proposed that high polymerase density favors Pol I transcription elongation [in essence, trailing Pol I complexes might "push" the neighboring polymerase (Albert et al., 2011)]. Trailing polymerases have been observed to promote elongation by leading polymerases in a number of contexts (Epshtein et al., 2003; Saeki and Svejstrup, 2009). Based on these studies, polymerase clustering would hypothetically suppress defects in Pol I translocation *in vivo*, explaining the observed *in vivo* tolerance of E1224G.

Misincorporation by RNA polymerase I. Another well-characterized effect of the *rpb1-E1103G* mutation involves transcriptional fidelity. The E1103G substitution in Rpb1 increases the rate of incorporation of non-cognate substrates *in vivo* and *in vitro* (Kaplan et al., 2008; Kireeva et al., 2008). *In vitro*, we detected no misincorporation with WT or E1224G mutant Pol I under a variety of experimental conditions. Fidelity mechanisms of RNAPs include substrate selectivity and removal of the misincorporated NTP during proofreading (Sydow and Cramer, 2009). The Cramer lab showed that Pol I has strong intrinsic cleavage activity, unlike Pol II (Kuhn et al., 2007). We suspect that proofreading

by Pol I is too efficient to permit detection of misincorporation *in vitro*. This interpretation is consistent with previous studies using RNA polymerase III, which also possesses intrinsic cleavage capabilities and failed to misincorporate *in vitro* (Alic et al., 2007).

The A12 subunit is the Pol I homologue of TFIIS (the gene product of *DST1*). Co-transcriptional cleavage of the nascent transcript during proofreading requires the A12 subunit of Pol I (Kuhn et al., 2007). We observed a synthetic lethal interaction between *rpa190-E1224G* and *rpa12Δ*. This interaction is consistent with the observation that *rpb1-E1103G* is lethal when combined with *dst1Δ*. TFIIS is known to influence fidelity of transcription by Pol II (Koyama et al., 2007), thus elevated misincorporation by the *rpb1-E1103G* polymerase might render *DST1* essential. A similar model might be possible for Pol I, if the *rpa190-E1224G* mutation results in increased misincorporation *in viv*o. However, unlike the E1103G mutation in Pol II, the *rpa190-E1224G* enzyme is more prone to pausing. If these pauses result in a significant amount of backtracking, A12 would be required for clearance of the resulting arrested complexes, therefore genetically analogous observations (E1103G/E1224G-cleavage factor genetic interactions) could have distinct underlying molecular mechanisms. One or both of these models could explain the lethality observed for the *rpa190-E1224G rpa12Δ* double mutant.

Growing list of differences between Pol I and Pol II. Despite the high degree of similarity between nuclear RNA polymerases, there is a growing list of functional differences between Pols I and II. Recent evidence has shown that three peripheral subunits of Pol I (A12.2, A34 and A49) are structural equivalents to trans-acting factors for Pol II (TFIIS, TFIIE and TFIIF) (Geiger et al., 2010; Jennebach et al., 2012; Kuhn et

al., 2007). Early studies showed that the nuclear polymerases exhibited differential sensitivities to buffer conditions or inhibitors (Roeder and Rutter, 1969). However, most of the highlighted differences between the polymerases were attributed to peripheral subunits or trans-acting factors, whereas the core enzymes were considered to be less diverged (Cramer et al., 2008).

Our study suggests that the list of differences between Pol I and Pol II can now extend into the most conserved domains of the enzymes – the active center. Despite high sequence identity, corresponding mutations in the TL have different consequences for the activity of related enzymes. The data presented here are consistent with the model that different steps in transcription elongation may be rate-limiting for RNA polymerases I and II. It is likely that the functional differences that exist between the nuclear RNA polymerases are critical for their unique, specialized cellular roles. Continued characterization of these differences will lead to a better understanding of eukaryotic RNA metabolism and its regulation.

EXPERIMENTAL PROCEDURES

Yeast strains, plasmids, media and growth conditions. Yeast strains and plasmids used in the study are listed in Supplemental Tables S4-S5. Standard techniques were used for growth and manipulation of yeast (Longtine et al., 1998; Sherman et al., 1986). Media used for analysis of TL swap variants were described previously (Kaplan et al., 2012).

Phenotypic Analysis of the Pol I TL mutants. Viability of the *rpa190* TL mutants was assessed by recovery of the segregants in tetrad dissection of the diploid *RPA190*/*rpa190Δ::HIS3Mx6* strain bearing respective *rpa190* alleles on a centromeric

plasmid. Additionally, survival for all of the *rpa190* mutants, except E1224G and F1205H, was confirmed by 5-fluoroorotic acid resistance in the standard "plasmid shuffling" technique (Sikorski and Boeke, 1991). The growth rates of the viable mutants were calculated using the GrowthCurve software (N. Rovinskiy, University of Alabama at Birmingham). Phenotypes were carefully analyzed under various conditions: 30°C, 23°C, 18°C, 37°C. The *rpa190-E1224G* and *rpa190-F1205H* mutants were additionally tested for survival on 6-azauracil (250 μg/ml) containing media (SD –Ura). No sensitivity under those conditions was observed unless specified in the text or figures.

Pol I purification. Either *RPA190* or *rpa190* mutant alleles were expressed from a low copy *CEN* plasmid (1-2 copies/cell, pRS315 derivative) in a strain carrying a chromosomal deletion of *RPA190* and a triple hemagglutinin $(HA)_3$-hexahistidine $(His)_6$ C-terminal epitope tag on the A135 subunit. Expression level of the mutant alleles and assembly into stable A135/A190 subcomplexes were verified by immunoprecipitation and Western blot (not shown). The cells were grown in 25 L of YEPD until their growth rate started to slow and harvested immediately. The WT or mutant polymerase was purified using a three step purification protocol (Ni-chelate, heparin sepharose and mono-Q) as described in (Schneider, 2012).

***In vitro* activity assays for Pol I elongation and pausing.** The transcription elongation assay was performed as per (Schneider, 2012). Each reaction contained 10 μCi α-^{32}P GTP and unlabeled NTPs at the concentrations indicated in the text or figure legends.

Electron Microscopy. Electron microscopy of Miller chromatin spreads was performed and analyzed as described previously (French et al., 2003).

Phenotypic Analysis of Chimeric *RPO21/RPB1* Strains. Sequences encoding variants of the TL domains from RNA Pol I and III and or bridge helixes from Pol I were cloned in place of the analogous region of *RPO21/RPB1* (*RPO21* is official designation, for simplicity we have chosen to use *RPB1* as is common practice) using *in vivo* gap repair or standard molecular techniques subsequent to PCR amplification. Plasmids were isolated, the sequences of the constructs were verified, and then retransformed into yeast. Plasmid shuffling permitted expression of the chimeric *RPB1* alleles in a strain carrying a chromosomal deletion of *RPB1*. Phenotypic analyses of the resulting strains were performed exactly as described previously (Kaplan et al., 2012). To control for expression of the chimeric Pol II complexes, Rbp1 levels relative to Rpb3 were measured by Western blot (Figure S4A-S4C). Since increases in cellular levels of Rpb1 were detectable after plasmid shuffle for chimera mutants, genetic analyses were performed with overexpression of *RPB1* on a 2µ (high copy) plasmid. Overexpression of Rpb1 from 2µ *RPB1* was determined to be equal to or greater than all *rpb1* chimera mutants (Figure S4B-S4C) while *in vivo* phenotypes of high copy *RPB1* were much weaker or dissimilar to those of *rpb1* chimeras, arguing against excess *rpb1* generically being responsible for observed chimera phenotypes.

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SUPPLEMENTAL DATA

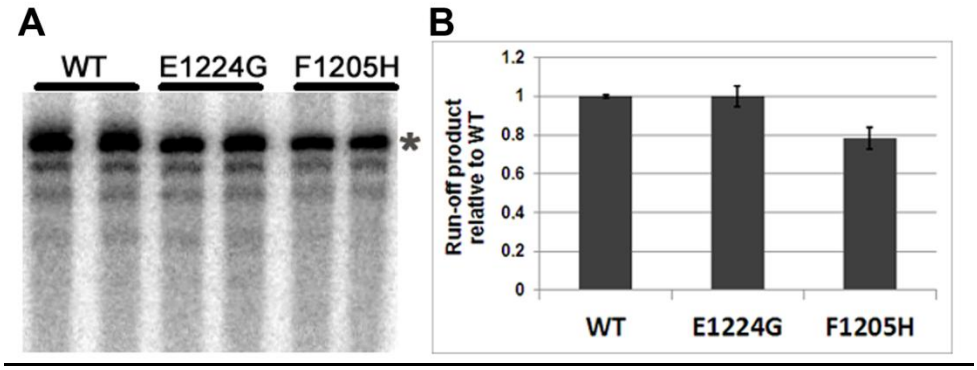


Figure S1. *In vitro* multiround transcription assay for Pol I, related to Figure 2
Activity of A190-E1224G and A190-F1205H Pol I complexes in multiround transcription assay. A) Promoter-dependent multiround transcription with purified components was performed exactly as described in (Bedwell et al., 2012). Yeast-derived pure Pol I (WT or mutant) was incubated with equi-molar concentrations of recombinant Rrn3 (Keener et al., 1998). The pre-initiation complex was assembled on the template (containing the rDNA promoter region and 336 nt of downstream DNA) using recombinant TBP and Core Factor, Upstream Activating Factor (UAF) purified from yeast, and pre-incubated Pol I-Rrn3. Transcription reactions were initiated with NTPs [200 μM of ATP, UTP and CTP, 15 μM of GTP and 10 μCi α-^{32}P-GTP (800 μCi/mmole)], incubated for 5 minutes and stopped by addition of excess phenol. RNA was ethanol precipitated, separated by 8% polyacrylamide gel electrophoresis and visualized by autoradiography. The runoff product is indicated by the *asterisk*. The reactions were performed in duplicate. Data shown are representative from one of three independent assays. **B)** Quantifications of the run-off product were done using Quantity One software, average values relative to the WT control from the three independent assays and the standard deviations are shown.

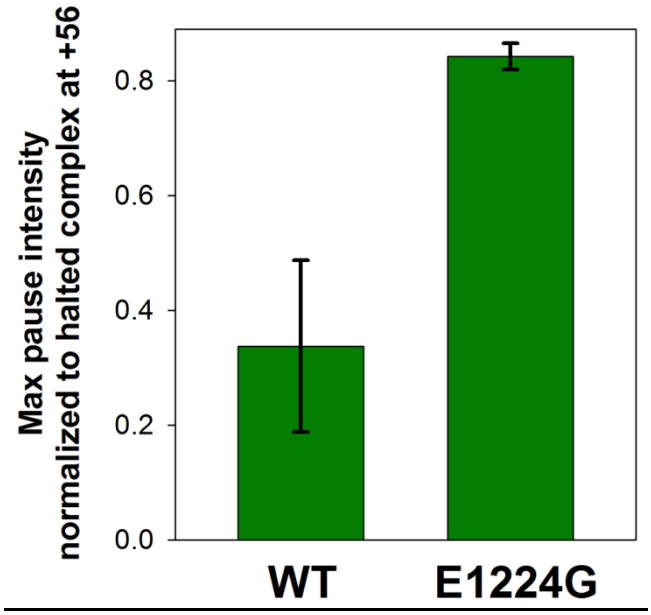


Figure S2. Quantification of *in vitro* pause intensity, related to Figure 2
Site specific pausing by A190-E1224G polymerase is enhanced *in vitro*. Three independent transcription elongation rate assays at low NTP concentrations were performed with WT and A190-E1224G Pol I (representative assay shown in Figure 2C). The major pause site intensity (indicated by an asterisk in Figure 2C) was quantified in each experiment and the values were normalized to intensity of halted elongation complexes at +56. The resulting values were plotted, with error bars = +/- 1 standard deviation.

Table S1. Total RNA synthesis rates of the *RPA190* and *rpa190-E1224G* strains, related to Table 1

Strain	Growth Rate (doublings/hr)	Total RNA, ng/µl	Predicted Synthesis Rate	Observed rRNA Synthesis Rate (from Figure S3 panel A)
WT	0.52	347.4	1	1
E1224G	0.51	288.3	0.82	0.94

Total RNA synthesis rates predicted for the *RPA190* and *rpa190-E1224G* strains. Since the rRNA synthesis rates measured by ^{3}H-methylmetionine incorporation pulse-and chase assay were done in SD-Met medium, we measured the total RNA synthesis rates and growth rates in SD-Met for a better comparison. Total RNA was extracted from exponentially growing cells and measured spectrophotometrically (NanoDrop ND1000). The predicted synthesis rate was quantified as (**Growth Rate) X (Total RNA)** and normalized to WT. These data are representative from one experiment, qualitatively similar results were obtained from repeated analyses from independent cultures grown on different days.

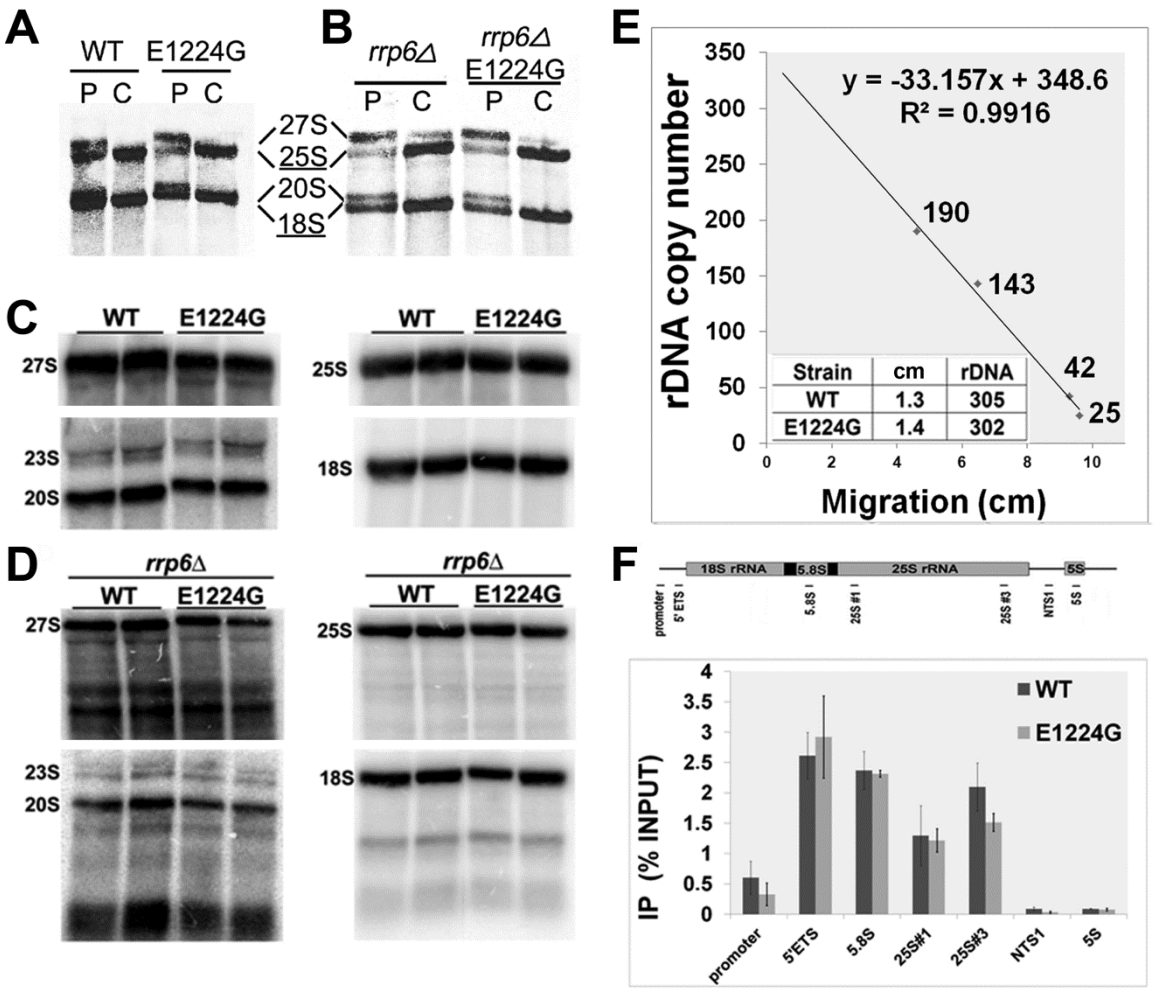


Figure S3. The *rpa190-E1224G* mutant is not hyperactive *in vivo*, related to Figure 3

A) Relative Pol I transcription rates were measured using the [^{3}H]methylmethionine incorporation pulse-and-chase assay as described in (Zhang et al., 2010). Since rRNA is co-transcriptionally methylated, this method is an effective way to quantify rRNA synthesis *in vivo*. The cells were grown in SD-Met medium and pulse-labeled with [^{3}H]methylmethionine for 5 minutes, and then chased with excess cold methionine to allow completion of rRNA processing. RNA was extracted from cells collected 4 minutes after pulse and 5 minutes after chase (Zhang et al., 2010). The RNA species were separated by gel-electrophoresis, transferred to a membrane and detected by autoradiography. The lanes indicated as P ("pulsed") contain rRNA pulse-labeled for 4 min; and lanes C ("chased") contains 5 min pulse-labeled rRNA followed by a 5 minute chase. Data shown are from one of two independent experiments. **B)** Same as panel A, except the metabolic labeling was done using the *rrp6*Δ *RPA190 (WT)* or *rrp6*Δ *rpa190-E1224G* cells. Rrp6 is a non-essential subunit of the nuclear exosome involved in degradation of unstable precursors and defective rRNA (Allmang et al., 2000). The *rrp6*Δ *rpa190-E1224G* double mutant does not accumulate rRNA degradation intermediates, precursors, or mature rRNA species when compared to the single mutants. This experiment is an additional control for co-transcriptional exosome-dependent decay of rRNA.

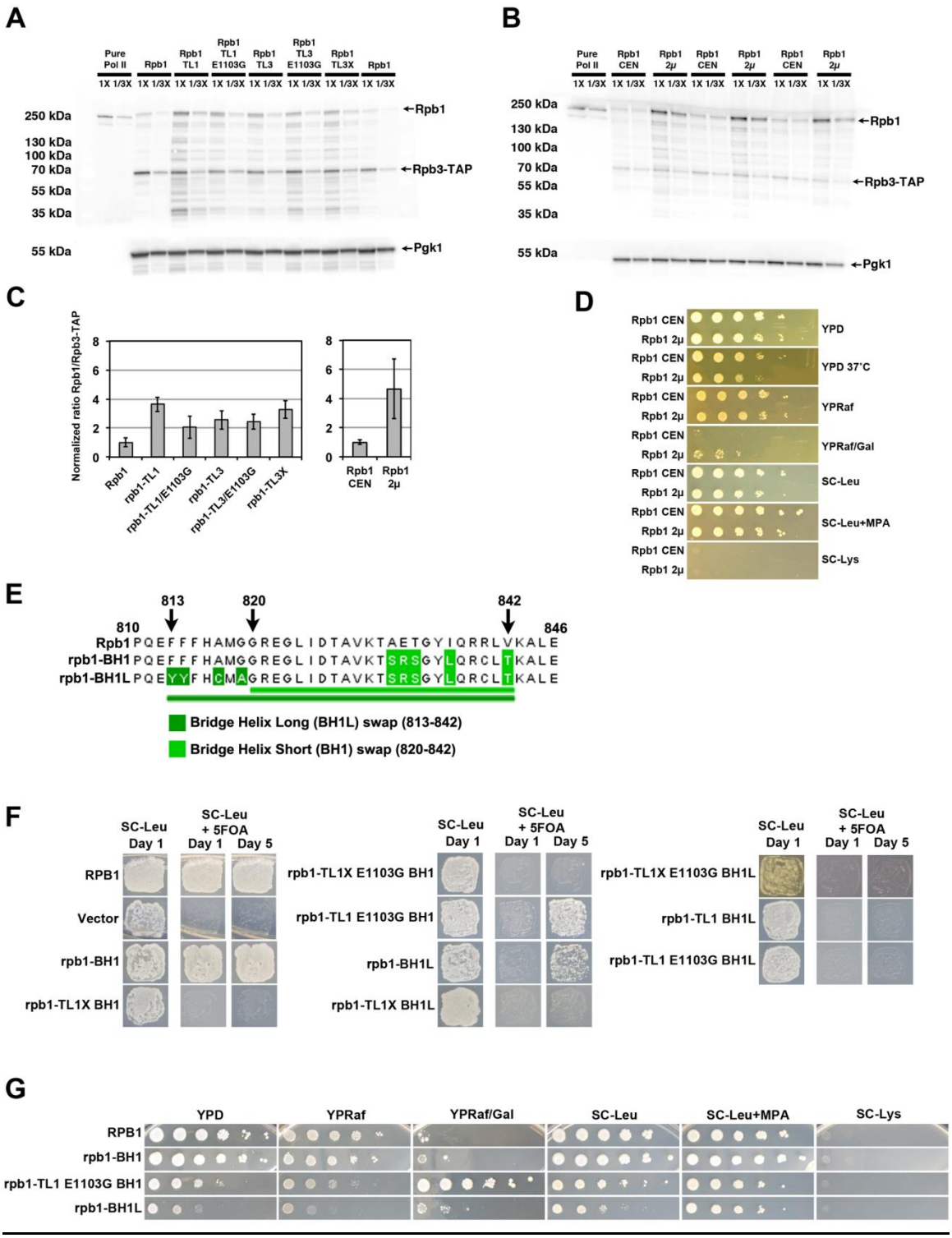
C) Northern Blot analysis of the rRNA isolated from the *RPA190* and *rpa190-E1224G* strains. The Northern blot analysis was performed as described in (Schneider et al., 2007). Total RNA was extracted from exponentially growing cells; equal amount of RNA was loaded onto the 0.8% agarose gel in duplicates and separated by electrophoresis. After electrophoresis, RNA was transferred to a membrane and analyzed by northern blot hybridization using ^{32}P-labeled oligonucleotide probes (described in the Table S2). The blot was visualized using phosphorimaging (The Storm, GE Healthsciences). The rRNA species detected by the probes are indicated on the figure. No significant difference in the signal between the mutant and the WT was observed. Data shown are from one of the three independent experiments. **D)** The *rrp6*Δ and the *rrp6*Δ *rpa190-E1224G* cells were processed as described for panel C. No significant difference in the signal between the *rrp6*Δ and the double mutant was observed, supporting the rRNA synthesis data (panels A and B). Data shown are from one of the two independent experiments. **E) The rDNA copy number of the *RPA190* and *rpa190-E1224G* strains** was determined based on the size of the chromosome XII separated from other yeast chromosomes using the Contour-clamped Homogenous Field Electrophoresis (CHEF) (as in (Zhang et al., 2009) and visualized with SYBR-Safe staining (Invitrogen, Carlsbad, CA). The migration distance of the chromosome XII of the reference strains (containing 190, 143, 42 and 25 rDNA copy numbers) was plotted versus the rDNA array size. The resulting linear plot (R^2=0.9916) yielded an equation [y=-33.2x+348.6] which was used to calculate rDNA copy number for the WT and *rpa190-E1224G* strains. We observed that the number of rDNA repeats in the mutant strain is not altered compared to WT. Since the number of the rDNA loci can potentially affect rRNA synthesis rate and rRNA abundance, this experiment was an additional control for the relative Pol I activity in *rpa190-E1224G*. **F) The Chromatin Immunoprecipitation (ChIP) analysis of Pol I occupancy over rDNA** was performed using polyclonal antibody against A190 subunit as described previously (Zhang et al., 2009). The bound DNA was measured using quantitative PCR and displayed as a ratio of precipitated to total DNA. The location of the primer sets used for the PCR on the rRNA gene is schematically depicted on the top of the panel. Each bar represents the average IP/input value for at least two 10-fold dilutions from at least two independent cultures. Error bars represent ± 1 SD. We observed no significant changes in Pol I occupancy of any region of the rDNA (promoter or throughout the coding region) relative to the WT control. Thus, given similar rRNA synthesis rates (panel A), and similar numbers of active genes (panel E and Figure 4), transcription initiation rates are approximately equal in the WT and mutant.

Table S2. Oligonucleotides used for the Northern Blot hybridization, related to Figures 3 and S3

Probe Target	Sequence	Reference
18S	5'-AGCCATTCGCAGTTTCACTG	this study
20S and 23S	5'-GCACAGAAATCTCTCACCGT	(Schneider *et al.,* 2007)
27S	5'-GCCTAGACGCTCTCTTCTTA	(Schneider *et al.,* 2007)
25S	5'-ACTAAGGCAATCCCGGTTGG	this study

Table S3. Summary of phenotypes observed in strains carrying chimeric alleles of *RPB1* (raw data shown in main text, Figure 4C)

Alleles	Phenotype				Interpretation
	Growth on YEPD	Suppression of *gal10Δ56*	Spt	MPA sensitivity	
RPB1	WT	not suppressed	Spt^+	Not MPA^s	WT
Common GOFs (e.g. E1103G)	N/A	weak or no suppression	Spt^-	MPA^s	GOF
Common LOFs (e.g. N479S)	N/A	strong suppression	Spt^+	MPA^r or not MPA^s	LOF
rpb1-TL1	Severe defect	strong suppression	Spt+	MPA^r	LOF
rpb1-TL1/E1103G	Mild defect	mild suppression	Spt+	Not MPA^s	E1103G suppresses *rpb1-TL1* growth phenotypes
rpb1-TL1X	Inviable				Inferred LOF
rpb1-TL1X/E1103G	Moderate defect	strong suppression	Spt^+	Not MPA^s	E1103G suppresses *rpb1-TL1X* inviability
rpb1-TL1/N479S	Inviable				Double mutant lethality
rpb1-TL1/E1103G/N479S	Severe defect	strong suppression	Spt^+	MPA^r	N479S suppresses growth suppression of *rpb1-TL1* by E1103G
rpb1-TL3	No defect	not suppressed	Spt^+	Not MPA^s	No obvious defect
rpb1-TL3/E1103G	Mild defect	weak suppression	Spt^+	Not MPA^s	E1103G slightly impairs *rpb1-TL3*
rpb1-TL3/N479S	No defect	weak suppression	Spt^+	Not MPA^s	N479S impairs *rpb1-TL3*
rpb1-TL3/E1103G/N479S	Mild defect	strong suppression	Spt^+	Not MPA^s	E1103G exacerbates effects of N479S on *rpb1-TL3*
rpb1-TL3X	Mild defect	not suppressed	Spt^+	Not MPA^s	Mild LOF



<u>Figure S4.</u> Chimeric alleles of *RPB1* accumulate excess Rpb1, but this is not the cause of chimera phenotypes; enzymes bearing the Pol I bridge helix and the Pol I trigger loop sequences do not mutually suppress impaired Pol II function, related to Figure 4. A) Western analysis for Rpb1 and Rpb3-TAP using anti-Rpb1 antibody (sc-25758, Santa Cruz Biotechnology) strains for WT and *rpb1-TL* chimera mutant strains. Extracts from equal cell

equivalents and 1/3 said amount were subjected to SDS-PAGE, immublotting and detection. Anti-Pgk1 (22C5D8, Life Technologies) blotting of same gel shown for loading control. **B)** Overexpression of Rpb1 via 2μ *RPB1* plasmid was analyzed relative to low copy CEN *RPB1* plasmid as in (A) for Rpb1, Rpb3-TAP, and Pgk1. **C)** Quantification of Western blotting using Bio-Rad Chemi-Doc system in conjunction with ImageQuant software (GE) shown in (A)(left graph, n≥4, average ratio Rpb1 signal/Rpb3-TAP signal ± standard deviation shown) or (B)(right graph same as left, n=3). **D)** Phenotypes of *rpb1-TL* chimera do not appear to derive from the Rpb1 overexpression observed in (A) for *rpb1-TL* mutant strains as the equal or greater overexpression observed in (B) does not result in phenotypes observed in Figure 4C. Very slight suppression of *gal10Δ56* is of a different quality from Pol II-Pol I/III chimeras and is much more similar to the appearance of papillae. These papillae may relate to *RPB1* being present in high copy, which may facilitate the genesis of dominant *rpb1* suppressors of *gal10Δ56*. **E)** Summary of chimeric *RPB1* bridge helix alleles used in this study. **F)** Plasmid shuffle results measuring viability of individual *rpb1* alleles. The assay is performed as described in the main text for Figure 5B. **G)** Dilutions of viable strains were plated on indicated growth media. Phenotypes were assessed as described in the main text for Figure 4C. *rpb1-BH1* did not show significant defects compared to WT. *rpb1-TL1/E1103G/BH1* suppressed *gal10Δ56* mutation and *rpb1-BH1L* was resistant to MPA: both phenotypes consistent with Pol II loss-of-function alleles.

Table S4. Strains used in this study, related to Figures 1-4

Strain	Description	Reference
DAS496 **(WT)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-RPA190***	this study
DAS702 **(E1224G)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-rpa190-E1224G***	this study
DAS701	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190-E1224G::LEU2Mx6***	this study
DAS715	same as DAS701, except *MAT***a**	this study
DAS479	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa135-D784G:: neurseothricin-r***	this study
DAS703	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa49Δ::KANMx6***	this study
DAS531	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa12Δ::URA4Mx6***	this study
DAS515	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***paf1Δ::HIS3Mx6***	(Zhang et al., 2009)
DAS607	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt5(1-797) - $(HA)_3$-$(His)_7$:HIS3Mx6***	(Viktorovskaya et al., 2011)
NOY2167	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***spt4Δ:HIS3Mx6***	(Schneider et al., 2006)
DAS704	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rrp6Δ::KANMx6***	this study
DAS206	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***trf4Δ:HIS3Mx6***	(Schneider et al., 2007)
NOY1075	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rrn3-S213P***	(Claypool et al., 2004)
DAS562	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***uaf30Δ::HIS3Mx6***	this study
DAS705	*MAT***a**/*α* diploid resulted from cross DAS701 x DAS479	this study
DAS706	*MAT***a**/*α* diploid resulted from cross DAS701 x DAS703	this study
DAS707	*MAT***a**/*α* diploid resulted from cross DAS715 x DAS607	this study
DAS708	*MAT***a**/*α* diploid resulted from cross DAS701 x DAS531	this study
DAS709	*MAT***a**/*α* diploid resulted from cross DAS701 x DAS515	this study
DAS711	*MAT***a**/*α* diploid resulted from cross DAS701 x NOY2167	this study
DAS710	*MAT***a**/*α* diploid resulted from cross DAS715 x DAS206	this study
DAS712	*MAT***a**/*α* diploid resulted from cross DAS702 x DAS704	this study
DAS713	*MAT***a**/*α* diploid resulted from cross DAS701 x NOY1075	this study
DAS714	*MAT***a**/*α* diploid resulted from cross DAS715 x DAS562	this study
DAS716 (***rrp6Δ***)	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190Δ::HIS3Mx6 rrp6Δ::KANMx6*** **carrying** ***pRS315-RPA190***	this study
DAS717 (***rrp6Δ*** **E1224G)**	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190Δ::HIS3Mx6 rrp6Δ::KANMx6*** **carrying** ***pRS315-rpa190-E1224G***	this study
NOY1071	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***fob1Δ::HIS3Mx6 rDNA copy number ~25***	(Cioci F, 2003)
NOY886	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***fob1Δ::HIS3Mx6 rpa135Δ::LEU2*** **with** ***pNOY117, rDNA copy number ~42***	(French et al., 2003)
NOY1051	same as NOY886, except ***rDNA copy number ~143***	(French et al., 2003)
NOY1064	same as NOY1071, except ***rDNA copy number ~190***	(Cioci F, 2003)
DAS721 **(F1205H)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-rpa190-F1205H***	this study
DAS764 **(L1222S)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-rpa190-L1222S***	this study
DAS765	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-***	this study

(N1203S/E1224G)	***(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-rpa190-N1203S/E1224G***	
DAS766 **(F1207S)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-rpa190-F1207S***	this study
DAS767 **(F1207S/E1224G)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-rpa190-F1207S/E1224G***	this study
DAS768 **(H1206Q)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-rpa190-H1206Q***	this study
DAS769 **(H1206Q/E1224G)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-rpa190-H1206Q/E1224G***	this study
DAS770	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS316-RPA190* and *pRS315-rpa190- L1222S/E1224G***	this study
DAS771	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS316-RPA190* and *pRS315-rpa190- G1218D***	this study
DAS772	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS316-RPA190* and *pRS315-rpa190- H1206Y***	this study
DAS773	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS316-RPA190* and *pRS315-rpa190- N1203S***	this study
CKY283	*MATa ura3-52 his3Δ200 leu2Δ1 or Δ0 trp1Δ63 met15Δ0 lys2-128∂ gal10Δ56 rpb1Δ::CLONATMX RPB3::TAP::KlacTRP1 [pRP112 RPB1 URA3 CEN]*	(Kaplan et al., 2008)
CKY1271	*MATa ura3-52 his3Δ200 leu2Δ1 or Δ0 trp1Δ63 met15Δ0 lys2-128∂ gal10Δ56 rpb1Δ::CLONATMX RPB3::TAP::KlacTRP1 [rpb1-TL1/E1103G/N479S LEU2 CEN]* further referred as to CKY283 derivative carrying pCK1367	this study
CKY1272	CKY derivative carrying pCK1369	this study
CKY1273	CKY283 derivative carrying pCK1374	this study
CKY1274	CKY283 derivative carrying pCK1378	this study
CKY1275	CKY283 derivative carrying pCK1379	this study
CKY1276	CKY283 derivative carrying pCK1380	this study
CKY1277	CKY283 derivative carrying pCK1383	this study
CKY1278	CKY283 derivative carrying pCK1384	this study
CKY1279	CKY283 derivative carrying pCK1385	this study
CKY1280	CKY283 derivative carrying pCK1386	this study
CKY1281	CKY283 derivative carrying pCK1387	this study
CKY1282	CKY283 derivative carrying pCK1391	this study
CKY1283	CKY283 derivative carrying pCK1393	this study
CKY1284	CKY283 derivative carrying pCK1394	this study
CKY1285	CKY283 derivative carrying pCK859	this study
CKY1286	CKY283 derivative carrying pCK960	this study
CKY1287	CKY283 derivative carrying pCK856	this study
CKY1288	CKY283 derivative carrying pCK964	this study
CKY1340	CKY283 derivative carrying pCK1143	this study

Table S5. Plasmids used in this study, related to Figures 1-4

pRS315	pBluescript, *CEN6, ARSH4, LEU2* (Sikorski and Hieter, 1989)
pRS316	pBluescript, *CEN6, ARSH4, URA3* (Sikorski and Hieter, 1989)
pRS315-*RPA190*	pRS315 derivative carrying wild type *RPA190*
pRS316-*RPA190*	pRS316 derivative carrying wild type *RPA190 (used for "plasmid shuffle" experiments)*
pRS306-*rpa190-E1224G*	pRS306 ("suicide vector") derivative carrying *rpa190-E1224G* used for the integration of *rpa190-E1224G* on the chromosome
pRS315-*rpa190-E1224G*	pRS315 derivatives carrying corresponding *rpa190* mutant alleles
pRS315-*rpa190-F1205H*	
pRS315-*rpa190-N1203S*	
pRS315-*rpa190-N1203S/E1224G*	
pRS315-*rpa190-H1206Y*	
pRS315-*rpa190-H1206Q*	
pRS315-*rpa190-H1206Q/E1224G*	
pRS315-*rpa190-F1207S*	
pRS315-*rpa190-F1207S/E1224G*	
pRS315-*rpa190-G1218D*	
pRS315-*rpa190-L1222S*	
pRS315-*rpa190-L1222S/E1224G*	
pCK plasmids:	pRS315 derivatives with corresponding *RPB1* alleles unless otherwise noted
pCK859	*RPB1* (Kaplan et al., 2012)
pCK960	*rpb1 E1103G* (Kaplan et al., 2012)
pCK856	*rpb1 N479S* (Kaplan et al., 2012)
pCK964	*rpb1 N479S/E1103G* (Kaplan et al., 2012)
pCK1143	pRS425 (2µ *LEU2*) derivative carrying *RPB1*
pCK1366	*rpb1-TL1/N479S*
pCK1367	*rpb1-TL1/E1103G/N479S*
pCK1368	*rpb1-TL1X/E1103G/N479S*
pCK1369	*rpb1-TL3/N479S*
pCK1371	*rpb1-TL1X/N479S*
pCK1372	*rpb1-TL1X BH1*
pCK1373	*rpb1-TL1X BH1L*
pCK1374	*rpb1-TL1 E1103G BH1*
pCK1375	*rpb1-TL1X E1103G BH1*
pCK1376	*rpb1-TL1 BH1L*
pCK1377	*rpb1-TL1 E1103G BH1L*
pCK1378	*rpb1-TL1*
pCK1379	*rpb1-TL1/E1103G*
pCK1380	*rpb1-TL3*
pCK1381	*rpb1-TL3/E1103G*
pCK1382	*rpb1-TL1X*
pCK1383	*rpb1-BH1L*
pCK1384	*rpb1-TL1 E1103G BH1*
pCK1385	*rpb1-TL1X/E1103G*
pCK1386	*rpb1-BH1*
pCK1387	*rpb1-TL1*
pCK1390	*rpb1-TL3*
pCK1393	*rpb1-TL3/E1103G*
pCK1394	*rpb1-TL3/E1103G/N479S*
pCK1395	*rpb1-TL1X E1103G BH1L*
pCK1397	*rpb1-TL3X*

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MUTATIONS IN THE TRIGGER LOOP OF RNA POLYMERASE I AFFECT TRANSCRIPTION ELONGATION AND RIBOSOME BIOGENESIS

by

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INTRODUCTION

The ribosome is a central component of the protein synthesis apparatus in the cell. Ribosome biogenesis demands the coordinated work of hundreds of proteins and several classes of non-coding RNAs. In *Saccharomyces cerevisiae* (hereby referred to as "yeast") the 40S ribosome subunit is composed of the 18S ribosomal RNAs (rRNA) and 32 ribosomal proteins. The 60S subunit has more components containing three RNA molecules - 25S, 5.8S and 5S rRNAs in association with 45 ribosomal proteins (Spahn et al., 2001; Verschoor et al., 1998). Proper assembly of such a complex machine is directed by more than 200 proteins and multiple small nucleolar RNAs (snoRNAs) [reviewed in (Fatica and Tollervey, 2002; Fromont-Racine et al., 2003; Granneman and Baserga, 2005)]. Every minute more than 2000 ribosomes are synthesized and exported from the nucleus into the cytoplasm turning ribosome metabolism into the major energy consuming process in the growing cell (Warner, 1999). For these reasons, ribosome synthesis is tightly linked to cell growth and proliferation.

The earliest step in ribosome production is the transcription of ribosomal DNA (rDNA) by RNA polymerase I (Pol I). The yeast rDNA locus is located on chromosome XII as a series of tandem repeats [~150-200 copies per haploid genome in wild type (WT) yeast], only half of which are actively transcribed. Pol I generates a 35S transcript, which is cleaved and processed through a series of intermediates into mature 18S, 5.8S and 25S rRNA species (Figure 1A). The fourth rRNA is produced by RNA polymerase III, which transcribes the 5S rRNA gene found within the rDNA locus.

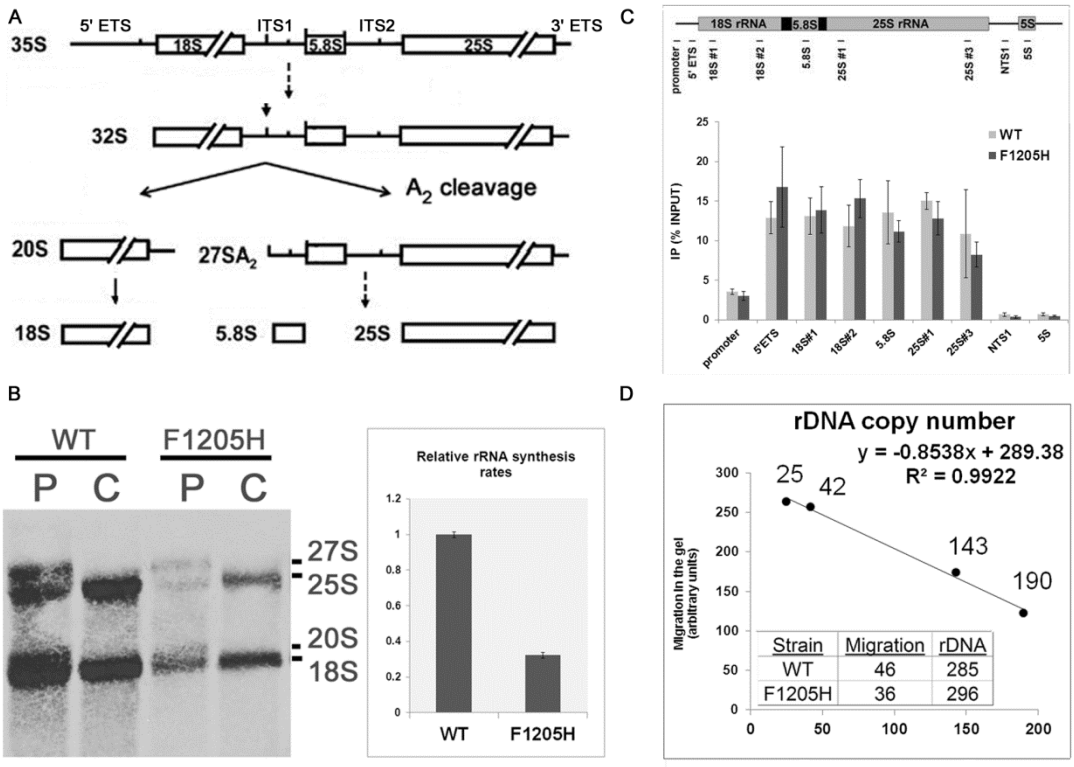
The maturation of 35S pre-rRNA and the assembly of ribosomal subunits is a complex, multi-step process that begins in the nucleolus and is completed in the cytoplasm (reviewed in Venema et al., 1995; Venema and Tollervey, 1999). The steps involved include endo- and exonucleolytic digestion, pre-rRNA modifications, folding of the transcript to adopt proper secondary and tertiary structures and the association of the ribosomal and non-ribosomal proteins with the rRNA [reviewed in (Fromont-Racine et al., 2003; Tschochner and Hurt, 2003)]. Several protein sub-complexes work as discrete assembly subunits that bind in defined steps to the 35S pre-rRNA (Perez-Fernandez et al., 2007). Thus, the 35S transcript forms a large RNA-protein (RNP) complex that is converted into precursors of the small and large subunits (Dragon et al., 2002; Osheim et al., 2004). The final steps of ribosome maturation happen after the subunit precursors are exported to the cytoplasm generating functional 40S and 60S particles. Despite significance of ribosome biogenesis to cell growth and proliferation, there is much to be learned about the mechanisms by which cells control and coordinate ribosome formation.

One of the possible ways by which ribosome assembly is coordinated became clearer when the co-transcriptional nature of rRNA processing was discovered in yeast (Kos and Tollervey, 2010; Osheim et al., 2004). While it has long been known that the processomes form on the nascent transcripts (Mougey et al., 1993), for decades it was proposed that cleavages and modifications of the rRNA precursor are post-transcriptional (Trapman et al., 1975; Trapman and Planta, 1975; Udem and Warner, 1972). However, recent studies of yeast Pol I nascent transcripts by electron microscopy (EM) clearly showed that at least the "separating" A_2 cleavage (Figure 1A) occurs co-transcriptionally in approximately half of the transcripts (Osheim et al., 2004). Furthermore, this study

suggested that other events in rRNA processing, such as RNA folding, modifications and early assembly of pre-ribosomal subunits may also occur on the emerging transcript. The discovery of the co-transcriptional nature of rRNA processing was later validated by the high resolution kinetic labeling of the pre-rRNA in the Tollervey lab (Kos and Tollervey, 2010). "Co-transcriptionality" allows functional coupling between different steps in RNA biogenesis and provides the potential for order and control over assembly of RNP by RNA polymerases [reviewed in (Perales and Bentley, 2009)]. The folding pathway of a growing RNA chain may vary greatly, depending on the speed of elongation, on co-transcriptional interactions of the nascent RNA with other factors and, in particular, on polymerase pausing [reviewed in (Pan and Sosnick, 2006)].

Importantly, the first direct support for the model that Pol I transcription elongation rate is linked to efficient rRNA processing came from a study of the elongation defective Pol I mutant (Schneider et al., 2007). We found that the *rpa135-D784G* allele (bearing a substitution of aspartate to glycine in position 784 of the second largest subunit of Pol I, A135) slows Pol I transcription elongation and results in decreased rRNA synthesis and faulty processing. Accumulation of aberrant rRNA intermediates in the mutant promoted massive degradation by the RNA surveillance machinery. Intriguingly, the effects of the D784G mutation on the large subunit pre-rRNAs were more dramatic than on the small subunit pre-rRNA creating imbalance in the ratio of the 40S to 60S particles and hindering proper ribosome assembly. Recently, another Pol I mutant lacking A49 subunit was shown to have defects in transcription elongation and ribosome assembly (Albert et al., 2011).

To find further support for the model that Pol I elongation is coupled with ribosome biogenesis, we studied a collection of Pol I mutants in order to compare their biochemical properties to the effects on rRNA production and ribosome biogenesis. Detailed analysis of one of the mutants, *rpa190-F1205H*, definitively supports the functional coupling between Pol I transcription and ribosome assembly. We found that several other Pol I mutants with severely decreased growth rates have defects in ribosome assembly, likely due to a slow elongation rate. We show that rRNA processing can tolerate a slight reduction in Pol I transcription elongation rate but not beyond a point. We hypothesize that there is a threshold in transcription elongation rate beyond which the speed of transcription becomes too slow, impeding proper folding/processing of the nascent transcript and recruiting degradation machinery, thus, hampering proper ribosome biogenesis.



<u>Figure 1</u>. Transcription elongation is impaired in the *rpa190-F1205H* mutant. **A**) The scheme represents the major rRNA processing pathways in yeast (modified from Schnieder et al., 2007). Positions of two internal transcribed spacers (ITS1 and ITS2) and 5′ and 3′ external transcribed spacers (ETS) are shown. The location of the A_2 cleavage site is dipicted by the arrowhead. The steps that involve more than one event are shown as dotted arrows. **B**) Relative rRNA synthesis rates were measured using the [^{3}H]methylmethionine incorporation pulse-and-chase assay. The lanes indicated as P ("pulsed") contain pulse-labeled rRNA; and lanes C ("chased") contain pulse-labeled rRNA chased with the non-radioactive methionine. The bands corresponding to the 18S and 25S rRNA were cut from the membrane and quantified with the scintillation counter. The resulting synthesis rates were normalized to WT. Data shown on the right panel are the average values from the three independent experiments ± SD. **C**) The ChIP analysis of Pol I occupancy over rDNA was performed using polyclonal antibody against A190 subunit. The bound DNA was measured using quantitative PCR and displayed as a ratio of precipitated to total DNA. The location of the primer sets used for the PCR on the rRNA gene is schematically depicted on the top of the panel. Each bar represents the average of at least two 10-fold dilutions of immunoprecipitated DNA expressed as a percentage of total input DNA. Error bars represent ±SD. Experiment was repeated three times. **D**) The rDNA copy number was determined based on the size of the chromosome XII separated from other yeast chromosomes using the CHEF. The migration distance of the chromosome XII of the reference strains (containing 190, 143, 42 and 25 rDNA copy numbers) was plotted versus the rDNA array size. The resulting linear plot (R^2=0.9922) yielded an equation which was used to calculate rDNA copy number for the WT and *rpa190-F1205H* strains.

MATERIALS AND METHODS

Yeast strains and growth media. Yeast strains used in the study are listed in Table 1. Standard yeast manipulation techniques and media were used for the study (Sherman et al., 1986; Zhang et al., 2009).

Polysome profile analysis was carried out as described in (Schneider et al., 2006) with changes. The sucrose gradients 5 - 55% containing 50 mM Tris acetate (pH 7.6), 50 mM ammonium chloride, 12 mM $MgCl_2$ and 100 mM DTT were prepared in the polyallomer centrifuge tubes (14 X 89 mm, Beckman Instruments Inc.). Yeast cells were grown in 50 ml of rich media at 30°C. When cultures reached OD_{600} = 0.3-0.5, cycloheximide was added to the final concentration 0.1 mg/ml and cells were immediately harvested. All subsequent steps were done at 4°C. Cells were washed in the extraction buffer (20mM Tris-HCl pH 8.0, 140 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml cycloheximide , 1 mg/ml heparin, 0.5 mM DTT and 1% Triton X-100) and frozen at -80°C. On the day of experiment, cells were disrupted in the extraction buffer using the bead beater (FastPrep-24, M.P. Biomedicals) and 0.5 mm disruption glass beads (Research Products International Corp.). The lysates were cleared by centrifugation (17000g for 5 min), diluted to 100 absorbance units (at OD_{260}) for each sample and 200 µl of the extracts were loaded on top of the sucrose density gradients. The gradients were centrifuged at 30000 rpm for 5 hours in the SW41 rotor (Beckman Instruments Inc.) and installed in the bottom up gradient fractionator (ISCO), connected to fast protein liquid chromatograph (FPLC; AKTA purifier) so that the pumps of the FPLC would push the gradients to the FPLC detector. The A_{254} was measured continuously from top to bottom and the obtained profiles were analyzed using the Unicorn 5.11 software.

***In vivo* analysis of the Pol I mutants** including the chromatin immunoprecipitation (ChIP) assay, H^3-methylmethionine incorporation pulse-and-chase assay, contour-clamped homogenous field electrophoresis (CHEF), Nothern blot analysis and calculation of the total RNA synthesis rate were performed exactly as in (Viktorovskaya et al., 2013).

Electron Microscopy analysis of the Miller chromatin spreads of the *rpa190-F1205H* was executed as described previously (French et al., 2003).

Table 1. Yeast strains used for the study.

Strain	Description	Reference
DAS496 **(WT)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-RPA190***	(Viktorovskaya et al., 2013)
DAS702 **(E1224G)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6*** **carrying** ***pRS315-rpa190-E1224G***	(Viktorovskaya et al., 2013)
DAS721 **(F1205H)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6 carrying pRS315-rpa190-F1205H***	(Viktorovskaya et al., 2013)
DAS764 **(L1222S)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100 RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6 carrying pRS315-rpa190-L1222S*	(Viktorovskaya et al., 2013)
DAS766 **(F1207S)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6 carrying pRS315-rpa190-F1207S***	(Viktorovskaya et al., 2013)
DAS768 **(H1206Q)**	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6 carrying pRS315-rpa190-H1206Q***	(Viktorovskaya et al., 2013)
DAS716 (***rrp6Δ***)	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190Δ::HIS3Mx6 rrp6Δ::KANMx6*** **carrying** ***pRS315-RPA190***	(Viktorovskaya et al., 2013)
DAS (***rrp6Δ*** **F1205H)**	*MAT? ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190Δ::HIS3Mx6 rrp6Δ::KANMx6*** **carrying** ***pRS315-rpa190-F1205H***	this study
OV202	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100* ***rpa190-F1224H***	this study
DAS217	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100*	(Anderson et al., 2011)
NOY1071	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100 fob1Δ::HIS3Mx6 rDNA copy number ~25*	(Cioci F, 2003)
NOY886	*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100 fob1Δ::HIS3Mx6 rpa135Δ::LEU2 with pNOY117, rDNA copy number ~42*	(French et al., 2003)
NOY1051	*same as NOY886, except rDNA copy number ~143*	(French et al., 2003)
NOY1064	*same as NOY1071, except rDNA copy number ~190*	(Cioci F, 2003)

RESULTS

Pol I transcription elongation is defective in the F1205H mutant. We have proposed a model that the rate of transcription elongation by Pol I is functionally connected to rRNA processing and ribosome assembly (Schneider et al., 2006; Schneider et al., 2007). The *rpa135-D784G* Pol I mutant exhibits a ribosome biogenesis defect and a reduction in the net transcription elongation rate consistent with the model (Schneider et al., 2007). To further correlate the biochemical properties of Pol I mutants with the effects on rRNA biogenesis, we analyzed another Pol I mutants defective in transcription elongation. Previously we isolated a Pol I mutant bearing the F1205H substitution in the A190 subunit, which was shown to elongate slower than WT *in vitro* and manifested severe growth defects (Viktorovskaya et al., 2013). Therefore, we focused on this mutant to further analyze consequences of the F1205H substitution on the ribosome production.

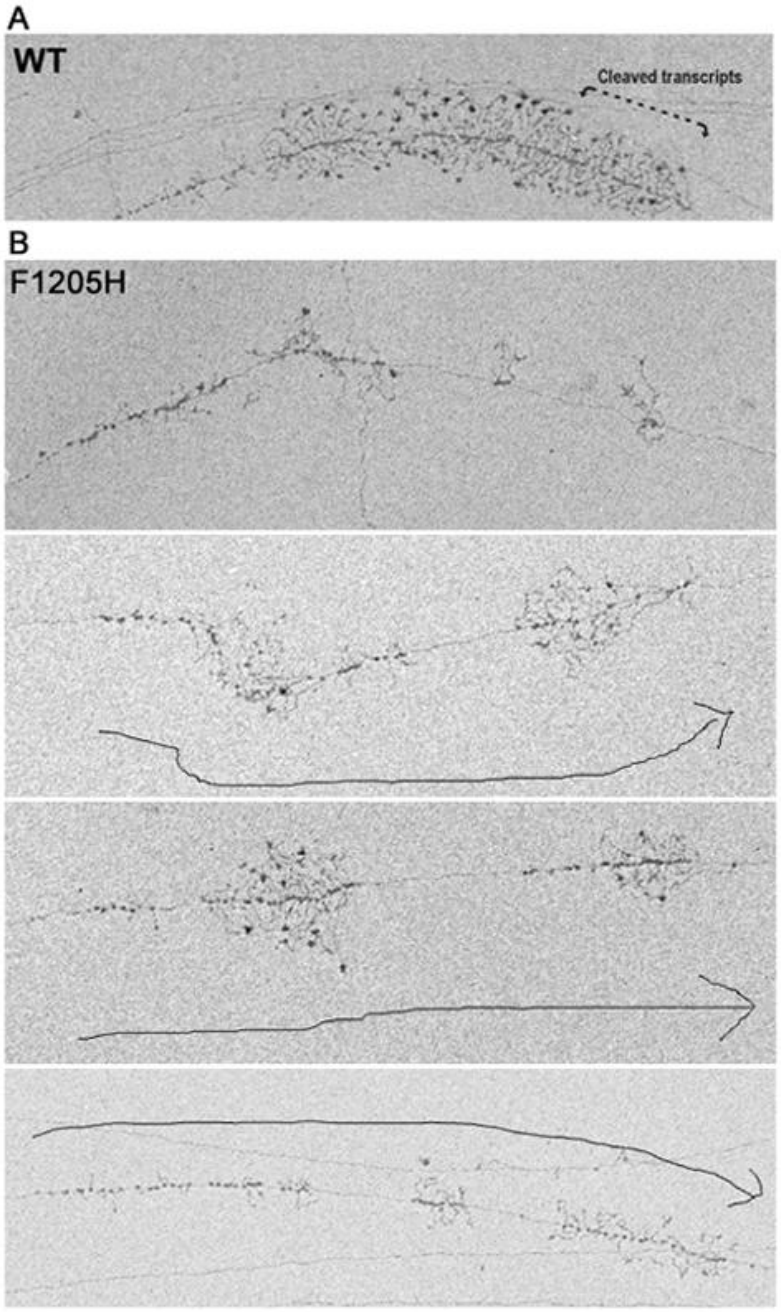
To examine the F1205H-mediated effects on Pol I activity *in vivo* we measured rRNA synthesis rates in the mutant and WT strains using an H^3-methylmethionine incorporation pulse-chase assay. Since rRNA is co-transcriptionally methylated, one can calculate the amount of radioactive label integrated into the nascent transcripts (Zhang et al., 2009). We found that F1205H polymerase produces approximately 3-fold less rRNA compared to WT (Figure 1B). This number is very close to the relative total RNA synthesis rate predicted from the growth rate of the mutant and the total cellular RNA abundance per cell mass (39% of the WT activity).

The occupancy of the Pol I complex over rDNA was measured by ChIP assay in the mutant and WT strains. We observed that Pol I association with the promoter and the

coding region is not changed in the mutant compared to WT (Figure 1C). In addition, the number of the rDNA repeats was not altered in the *rpa190-F1205H* strain as measured by the CHEF assay (~285 repeats in the WT and ~296 repeats in the mutant; Figure 1D). Altogether, the 3-fold decrease in the rRNA synthesis rate observed in the *rpa190-F1205H* mutant is not caused by transcription initiation defects or a decrease in the size of the rDNA array. These results indirectly support the interpretation that transcription elongation by F1205H polymerase is impaired *in vivo* consistent with its biochemical activity (Viktorovskaya et al., 2013).

To further analyze the effects of the F1205H substitution on Pol I transcription, we performed EM analysis of Miller chromatin spreading of the *rpa190-F1205H* mutant (Figure 2). We observed gaps in the Pol I occupancy over the active rDNA repeats as well as polymerase pile-ups prior to the gap regions, indicative of pauses or transcriptional arrests. The locations of these gaps were random in different rDNA units suggesting no site specific preference. This pattern of Pol I occupancy is a strong evidence of severely defective transcription elongation (Schneider et al., 2007). Thus, consistent with the *in vitro* data, F1205H substitution generates dramatic defects in transcription elongation *in vivo* (Viktorovskaya et al., 2013).

F1205H causes processing defects and degradation of rRNA precursors. Previous studies have revealed that slow transcription elongation rate by Pol I might be accompanied by defects in rRNA processing and massive degradation of the aberrant intermediates (Schneider et al., 2007; Zhang et al., 2009). We examined the Miller spreads of the *rpa190-F1205H* strain to study efficiency of the co-transcriptional processing and the signs of degradation. EM analysis revealed defects in rRNA



<u>Figure 2</u>. EM analysis of the Miller chromatin spreads for the *rpa190-F1205H* mutant. The representative EM images of the actively transcribed rDNA units from the WT (**A**) and the *rpa190-F1205H* (**B**) strains are shown. The 3' and 5' polymerase pileups along with co-transcriptional degradation of the nascent transcripts and decreased processome assembly are detected in the mutant. The directionality of Pol I transcription for the *rpa190-F1205H* active repeats is indicated by arrows**.** The EM image of the WT strain is modified from (Viktorovskaya et al., 2013; Chapter 2) since the data for the WT, *rpa190-F1205H* and *rpa190-F1205H* cells were collected at the same time.

processing since multiple elongation complexes lacking the nascent transcripts were detected in the *rpa190-F1205H* mutant (Figure 2).

To study the rRNA processing in the mutant more thoroughly, northern analysis was performed to determine the abundance of the processing precursors (35S, 20S, 23S, 27S pre-rRNAs) as well as the presence of the aberrant intermediates (Figure 3A). We found no accumulation of aberrant 23S rRNA precursor in the mutant suggesting that the "separating" A_2 cleavage is not affected by F1205H (Figure 3A, B). However, we observed a decrease in the ratios of the other precursors to the mature products in the mutant compared to WT strain. The 20S/18S ratio was approximately 3-fold lower in the mutant than in WT. A more dramatic effect was detected for the large subunit precursor: the 27S/25S ratio was more than 4-fold decreased when compared to WT. The decreased ratio of precursors to mature products suggested premature degradation. Indeed, we observed a smear in the lane when probing for the 27S pre-rRNA (but not for the 25S) revealing selective degradation of the 27S intermediate consistent with EM data.

Additional evidence for the increased rRNA degradation caused by the F1205H substitution comes from a study of *rpa190-F1205H rrp6*Δ double mutant. Rrp6 is a non-essential subunit of the nuclear exosome involved in degradation of unstable precursors and defective rRNAs (Allmang et al., 2000). The rRNA synthesis rates of the *rrp6*Δ *rpa190-F1205H* double mutant was slightly higher than expected if defects caused by each of the single mutants were mutually independent (Figure 3C). Indeed, deletion of *RRP6* partially restored F1205H-mediated rRNA synthesis defects from ~30% (Figure 1B) to ~60% of wild type *RPA190* activity on the *rrp6*Δ background (Figure 3C). We

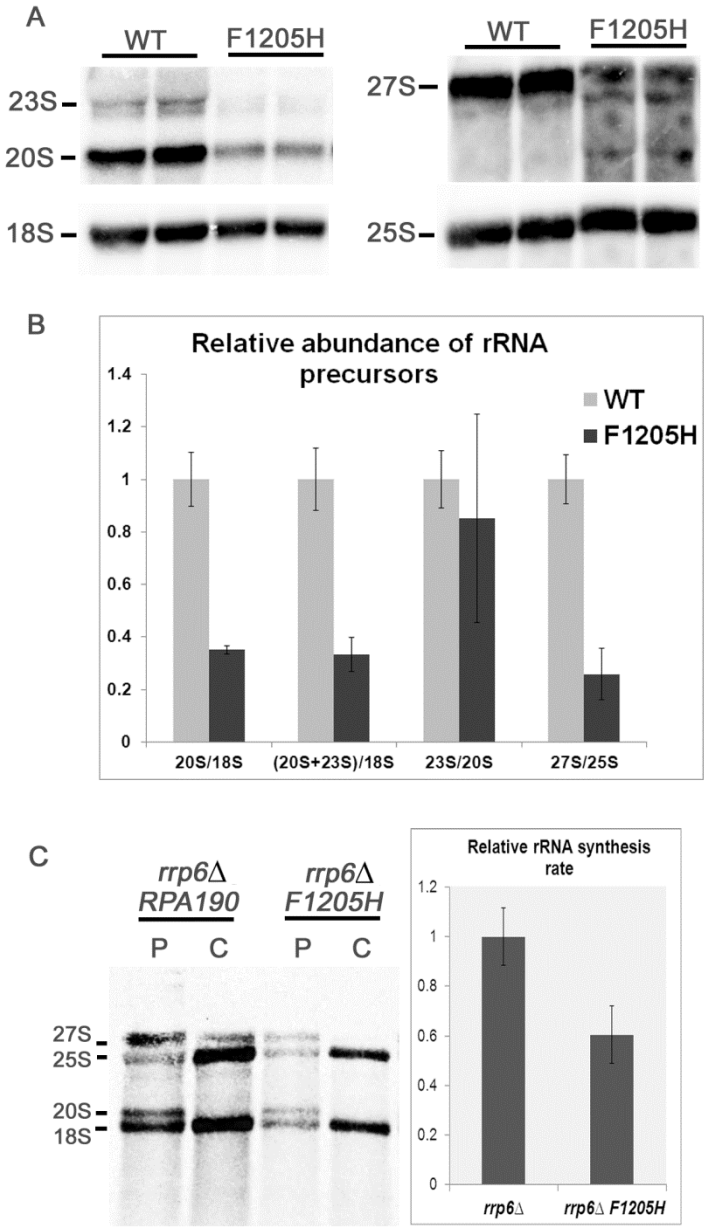


Figure 3. rRNA processing is impaired in the the *rpa190-F1205H* mutant. **A**) The Nothern blot analysis was performed as described in Viktorovskaya et al., 2013. The rRNA species detected by the probes are indicated on the figure. Data shown are from one of the three independent experiments. **B**) The radioactive signals corresponding to 20S, 23S, 27S, 18S and 25S RNAs from the Nothern blot experiments (panel A) were quantified using the Quantity One software. Each bar represents the indicated ratio averaged from the three independent experiments and normalized to the WT value. Error bars represent ± SD. **C**) Relative rRNA synthesis rates of the *rrp6*Δ *RPA190* or *rrp6*Δ *rpa190-E1224G* cells were measured using the [^{3}H]methylmethionine incorporation pulse-and-chase assay as described for Figure 1 B.

conclude that exosome-mediated degradation is involved in elimination of aberrant transcripts produced by the mutant Pol I, as it was shown for the *rpa135-D784G* allele. Altogether, these data are consistent with the model that the efficiency of transcription elongation by Pol I affects rRNA processing and surveillance.

The *rpa190-F1205H* mutant has defects in ribosome assembly. Since we detected significant impairment in rRNA maturation in the F1205H mutant, we tested whether or not there are major consequences of the substitution on the pools of ribosomal subunits. To study ribosome assembly in the *rpa190-F1205H* strain we performed sucrose gradient analysis of the polysome profiles. We revealed a significant reduction of the free 60S subunit relative to 40S in the mutant (Figure 4A). Moreover, we discovered the F1205H-mediated appearance of "halfmers" which represent monosomes and polysomes with an extra 40S subunit not bound by 60S. Similar ribosome assembly defects were described previously for the *rpa135-D784G* mutant (Schneider et al., 2007). F1205H substitution causes results in elongation rate and correlated with defective rRNA processing, which results in enhanced degradation of the nascent transcripts, reduced rRNA production and aberrant ribosome formation.

The growth rate of the Pol I mutants correlated with the ribosome biogenesis defects. Previously we characterized a Pol I mutant, the *rpa190-E1224G* allele demonstrating mild decrease in transcription elongation rate and enhanced pausing (Viktorovskaya et al., 2013). Unlike *rpa190-F1205H* or *rpa135-D784G*, the *rpa190-E1224G* mutant had no defects in rRNA synthesis and maturation despite decelerated transcription elongation. These data suggest that optimal transcription elongation rate by Pol I is

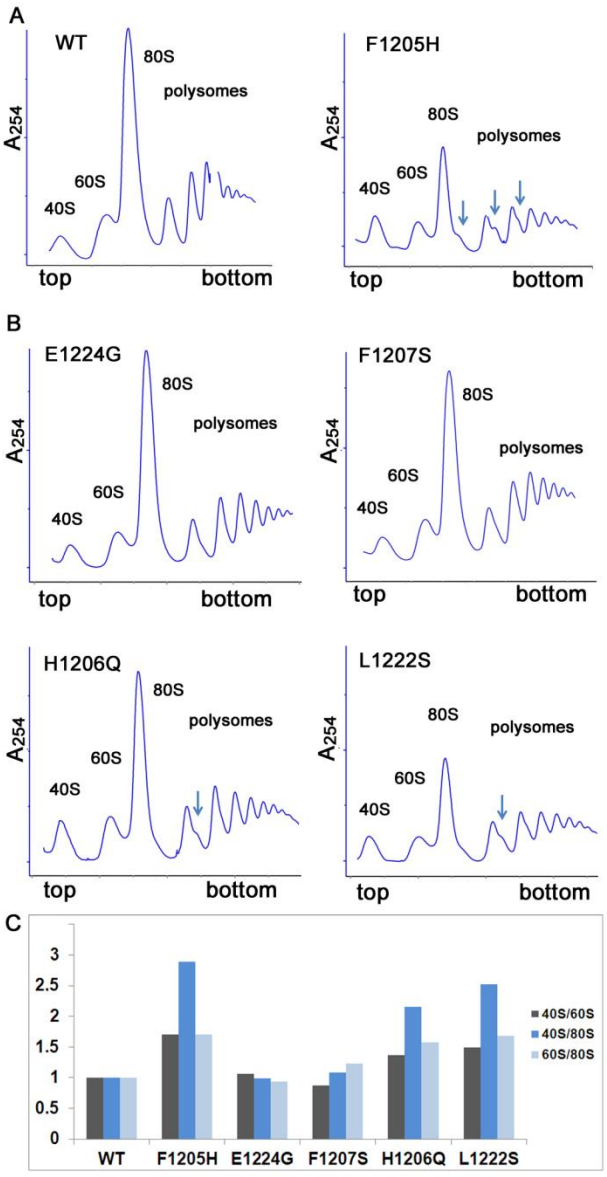


Figure 4. Sucrose gradient analysis of ribosome profiles in the Pol I mutants. **A** and **B**) The extracts from the growing WT, *rpa190-F1205H, rpa190-E1224G, rpa190-F1227S, rpa190-H1206Q* and *rpa190-L1222S* cells were analyzed by sucrose gradient centrifugation. Absorbance at A_{254} was measured from top to the bottom of the gradients. Positions of 40S, 60S, 80S and polysomes are indicated. The halfmers are shown by the blue arrowheads. The experiment was repeated at least twice for the WT, *rpa190-F1205H* and *rpa190-E1224G* strains, and two more independent times for all six strains simultaneously. Data shown are representative from one of the experiments. Note, that the absorbance for the WT polysomes has a small area without signal due to detection defect, which does not affect interpretation or quantification of the data. **C**) The abundance of the absorbance signal was quantified for the peaks corresponding to the 40S, 60S and 80S species using the UNICORN 5.11 software, the indicated ratios calculated and normalized to the WT values.

required for proper rRNA maturation. We hypothesize that moderate changes in Pol I activity can be tolerated by the cell (as for the *rpa190-E1224G* allele), whereas rRNA processing and ribosome assembly becomes impaired beyond a certain Pol I velocity (like in the cases of the *rpa190-F1205H* or *rpa135-D784G* mutants). To test this hypothesis, we investigated if there is a correlation between the growth rates [indirectly representing the rRNA synthesis rates and Pol I activity *in vivo*] and ribosome biogenesis defects in a series of Pol I mutants.

We used a collection of previously generated Pol I trigger loop mutant alleles in addition to F1205H: H1206Q, F1207S and L1222S substitutions in A190 which had various degree of growth defects (Viktorovskaya et al., 2013). H1206Q and L1222S manifested severe growth phenotypes whereas F1207S was only slightly affected. We performed sucrose gradient analysis of the polysome profiles for these three trigger loop mutants. In addition, we included a well-characterized *rpa190-E1224G* mutant with a mild phenotype as a control (Viktorovskaya et al., 2013).

As expected, the most dramatic effect on ribosome biogenesis was observed for the two mutants with the most severe phenotypes - L1222S and H1206Q. As in the case of F1205H, L1222S and H1206Q resulted in the altered 60S/40S ratio and the appearance of "halfmers" (Figure 4B, C). The ribosome profile for the *rpa190-E1224G* mutant looked like WT, consistent with its unaffected synthesis and processing of rRNA (Viktorovskaya et al., 2013). As in case of E1224G, the F1207S substitution also did not cause defects in ribosome assembly in agreement with its growth rate (Viktorovskaya et al., 2013).

Pol I occupancy was not altered in the H1206Q and L1222S Pol I mutants (data not shown). These data indicate that growth rate defects of the two mutants are likely due to the reduced transcription elongation rates causing the decreased rRNA synthesis and faulty processing similar to the *rpa190-F1205H* allele. Altogether, these results suggest that the growth rate phenotype correlated with the magnitude of the effects on ribosome biogenesis in the Pol I mutants probably resulting from the defects in elongation. Thus, we identified two distinct classes of phenotypes of the elongation defective Pol I mutants suggesting the existence of a threshold in the elongation rate below which ribosome biogenesis is hampered. Further detailed studies of the Pol I mutants *in vivo* and *in vitro* will allow to test this hypothesis and establish the boundaries for the optimal elongation rates. Altogether, our data further support the model that transcription by Pol I and rRNA biogenesis are functionally connected.

DISCUSSION

Confirmation of the model that Pol I transcription elongation is functionally coupled to ribosome assembly. Our previous characterization of the *rpa135-D784G* allele was the first evidence that established a direct link between the speed of Pol I transcription and rRNA processing/ribosome assembly (Schneider et al., 2007). In addition, it was recently shown that Pol I mutant lacking A49 subunit is impaired in transcription elongation and ribosome assembly (Albert et al., 2011). In this study we extended this list of Pol I mutants to include *rpa190-F1205H* and potentially *rpa190-H1206Q* and *rpa190-L1222S* alleles. We have shown that these three mutants have

ribosome assembly defects. Furthermore, F1205H results in impaired rRNA processing, enhanced degradation of the transcripts and decreased rRNA synthesis. All of the described defective phenotypes most likely are generated by the decrease in the net elongation rate of the mutant enzyme (Viktorovskaya et al., 2013). Consistent with the impaired ribosome assembly, *rpa190-H1206Q* and *rpa190-L1222S* are likely to exhibit defects in rRNA biogenesis similar to those observed for *rpa190-F1205H.*

The decrease in *in vitro* transcription elongation rate of F1205H polymerase was not as dramatic as observed for *rpa135-D784G* [40% versus 10% of WT activity, respectively; Figure 2A, (Schneider et al., 2007; Viktorovskaya et al., 2013)], nevertheless the effects of these mutations *in vivo* were similar. Remarkably, the *rpa190-F1205H* mutant was prone to pausing/arrests as indicated by the presence of the polymerase pile-ups and polymerase-free gaps throughout the actively transcribed rDNA repeats (Figure 2). Altogether, this study supplied further evidence to the model that certain transcription elongation rates of Pol I provide proper transcript maturation and ribosome subunit formation.

The two classes of Pol I elongation defective phenotypes. In order to expand our knowledge about the functional coupling of Pol I transcription and rRNA maturation, we characterized a collection of Pol I mutants. There are two distinct classes of the phenotypes for the mutants with decreased Pol I transcription elongation rates based on the severity of the defect. The first class of mutants includes the *rpa135-D784G, rpa190-F1205H, rpa190-H1206Q* and *rpa190-L1222S* alleles which resulted in severe phenotypes leading to unbalanced production of the ribosomal subunits as discussed above.

On the other hand, there is a different set of mutant phenotypes with mild transcription elongation defects which do not result in significant rRNA processing impairments (French et al., 2003; Viktorovskaya et al., 2013). EM analysis of the Miller chromatin spreading of the *top2*Δ strain yielded a pattern referred to as the "double gradient". This pattern of the active rDNA unit is explained by the slow moving elongation complexes relative to the rate of the co-transcriptional cleavage event. Similar "double gradient" pattern was observed for the elongation defective *rpa190-E1224G* mutant. Unlike the *rpa135-D784G* allele, the E1224G substitution did not alter rRNA processing efficiency regardless of decelerated transcription elongation [(Viktorovskaya et al., 2013) and this study]. Moreover, such "double gradient" phenotype may in fact be interpreted as enhancement of the A_2 cleavage event since almost no nascent transcripts that escape the co-transcriptional cleavage were detected in contrast to WT (Viktorovskaya et al., 2013). The simplest interpretation is that these mutant polymerases move on the rRNA genes with a permissive velocity that does not disrupt events critical for proper nascent transcript maturations, and may be even facilitating certain co-transcriptional events. Another mutant *rpa190-F1207S*, showed similar growth rate phenotypes and absence of ribosome biogenesis defects, however, whether or not this mutant also has mild defects in transcription elongation remains to be tested using EM and biochemical studies.

A threshold in transcription elongation rate *in vivo*. Surprisingly, the consequences on rRNA processing for the F1205H versus the E1224G substitutions are dramatically diverse whereas the former enzyme is only modestly slower *in vitro* than the latter [40% versus 60% of WT rate in the presence of 200 μM NTPs, respectively (Viktorovskaya et al., 2013)]. That observation suggests that *in vivo* there is a threshold in Pol I

transcription elongation rate, within which rRNA production stays unaffected, but beyond which rRNA folding/maturation dramatically fails. Interestingly, both E1224G and F1207S mutants that express the mild phenotypes, are cold-sensitive (Viktorovskaya et al., 2013) signifying that their elongation rates might cross the threshold at low temperatures. Indeed, it was shown that lower temperature affects transcription elongation rates more dramatically than ribosome formation rates in bacteria so that decrease in the temperature can rescue ribosome assembly defects in case of hyperactive rRNA production (Lewicki et al., 1993).

Determination of the *in vitro* elongation rates under various NTP concentrations for all of the trigger loop mutants will allow calculation of the kinetic parameters of the mutant enzymes, such as maximal velocity and K_m for substrate. Our preliminary data (not shown) suggests that maximal velocity is decreased in the F1205H, but not E1224G mutant. In that case, a relatively small difference in the transcription elongation rate between the F1205H and E1224G observed *in vitro* is likely to be significantly exaggerated in the cell. If confirmed, these data explain the more profound effect of F1205H compared to E1224G observed *in vivo*. Similar mechanisms might be the basis for differentiation between the two distinct classes of the elongation-defective Pol I mutants. Future detailed analysis of the catalytic properties of the Pol I mutants will aim to establish the boundary between the optimal and sub-optimal speed of elongation.

A threshold for the optimal elongation rate or a window? So far, we have characterized six point mutations in Pol I which resulted (or which are predicted to result) in the slower transcription elongation rate than WT (*rpa135-D784G*, *rpa190-F1205H*, *rpa190-E1224G*, *rpa190-H1206Q*, *rpa190-F1207S* and *rpa190-L1222S*). No mutants

with increased rate of transcription elongation have been identified yet. Theoretically, it seems very appealing to characterize the effects of a hyperactive Pol I allele on rRNA maturation. Since ribosome biogenesis is energy consuming, it is intriguing to study the consequences of rRNA overproduction on cell metabolism. Moreover, considering functional coupling of Pol I transcription elongation with rRNA processing, one might expect hyperactive Pol I to affect rRNA folding and recruitment of the processing machinery as well. Support to such hypothesis comes from the experiments with the bacterial rRNA genes transcribed by the T7 phage RNA polymerase (Lewicki et al., 1993). Since the T7 phage RNA polymerase moves five times faster than the bacterial enzyme, the ribosome subunits assembled on the T7 polymerase transcripts were inactive. However, the ribosome assembly was rescued when bacterial cultures were grown at the lower temperature which reduces the transcription elongation rate by T7 RNA polymerase. Thus, in bacteria the formation of active ribosomal subunits *in vivo* requires a fine adaptation of the transcription rate of rRNAs and the assembly process. Similar to bacterial ribosome assembly, we hypothesize an existence of a window of optimal elongation rates with both lower and upper limits beyond which the rRNA maturation is hindered in yeast. Further studies aimed to detect Pol I hyperactive mutants will test that hypothesis.

Potential mechanisms by which transcription elongation coordinates rRNA processing and ribosome assembly. Pol I transcription elongation and rRNA maturation can be coupled via programmed pausing encoded by *cis*-elements in the rDNA coding region. Transcriptional pausing of Pol I can potentially be regulatory, serving as check-points for proper transcript folding into its complex secondary-tertiary structure, rRNA

interactions with snoRNPs and assembly proteins or growth-dependent control of transcription elongation. Such pauses are well-known for bacterial RNA polymerase [reviewed in (Landick, 2009)]. Recently a universal pause sequence was described, that affects various multi-subunit RNA polymerases *in vitro*, including Pol I (Bochkareva et al., 2012). Furthermore, we routinely detect sequence specific NTP-dependent pauses *in vitro* for WT Pol I (Viktorovskaya et al., 2013). These data suggest that the Pol I complex has some sequence preference for pausing. We are currently working on determining the functional relevance of such pausing *in vivo*.

Another mechanism by which transcription elongation may be linked with rRNA processing is co-transcriptional recruitment or activation of trans-acting factors. Recently several factors that affect Pol I transcription elongation and processing of rRNA were characterized in yeast, such as the Spt4/5 and the Paf1C complexes (Anderson et al., 2011; Lepore and Lafontaine, 2011; Schneider et al., 2006; Zhang et al., 2009; Zhang et al., 2010). These and other elements of Pol I elongation complex may modulate pausing, speed of transcription and recruitment of the processome components, ribosomal proteins and assembly factors. Altogether, our data undoubtedly support and expand the model that Pol I transcription elongation orchestrates of the ribosome biogenesis in cells.

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CONCLUSION

"**Nothing in Biology Makes Sense Except in the Light of Evolution**".
Theodosius Dobzhansky, 1973.

The past few decades have shed much light on our understanding of eukaryotic rDNA transcription and ribosome biogenesis. Recently, interest in rRNA production has grown due to its roles in cancer biology (Drygin et al., 2010). The research presented here aimed to expand our knowledge of Pol I transcription and evolution of the transcription apparatus. We studied how catalytic properties of Pol I and trans-acting factors control rRNA synthesis, their roles in comparison with other RNA polymerases and their contribution to ribosome assembly.

The major questions that I sought answers to are: to what extent is rDNA transcription by Pol I different from the other polymerase systems? What are the similarities and divergences between Pol I and Pol II transcription machineries? Are the Pol I specific peripheral surfaces and unique transcription factors primarily responsible for the obvious functional divergences? Or do the differences between the paralogs extend to the most conserved parts of the enzymes within the catalytic core? To answer some of these questions, we compared the roles of the conserved active site residues of Pol I and Pol II. We generated point mutations within the trigger loop domain of Pol I. Analysis of the effects of different substitutions within the trigger loop led to the remarkable conclusions. We have shown that despite high sequence conservation, the trigger loop of Pol I has

important functional divergences from Pol II's trigger loop (Viktorovskaya et al., 2013). This functional dissimilarity is not solely dependent on the sequence of the trigger loop, but rather likely results from different rate-limiting steps in nucleotide addition catalyzed by the paralogs. These new data shift the paradigm that the active centers and the catalytic steps are quasi-identical in multi-subunit RNA polymerases.

Our astonishing results have raised multiple additional questions. We have shown that the trigger loop sequence was not solely responsible for the observed opposite effects of the conserved substitutions (Viktorovskaya et al., 2013). If not the trigger loop, than which regions of the polymerase generate the unique catalytic features of Pol I? The recently solved X-ray crystal structure of Pol I complex suggests that the bridge helix has a Pol I-specific "kink" which might affect nucleotide entry or polymerase translocation during nucleotide addition (Engel et al., 2013; Fernandez-Tornero et al., 2013). If so, the kinked bridge helix might be responsible for the differences in catalysis between Pols I and II as we predicted based on the functional studies of the trigger loop mutants (Viktorovskaya et al., 2013). In addition, multiple sequence variations dispersed throughout the catalytic core might affect the geometry of the active center and polymerase activities. In support of this hypothesis, multiple amino acid residues proposed to be critical for evolution of Pol I were identified using *in silico* sequence analyses (Carter and Drouin, 2009). These residues are all clustered near the active center and RNA:DNA hybrid interacting region but were not restricted to a sole particular domain. Thus, it is necessary to determine the roles of the polymerase core elements as well as the specific peripheral subunits in Pol I activity in future. Detailed studies revealing the mechanisms of the nucleotide addition cycle for Pol I and its comparison to

Pol II are needed to test our model concerning potential differences in the rate-limiting steps in catalysis of the related enzymes. Altogether, these studies will establish the degree of functional conservation of the paralogs and shed light on Pol I evolution.

Our discovery of the important functional divergence between Pol I and Pol II catalysis leads to an even bigger question: why eukaryotes have evolved three homologous RNA polymerases? What is the benefit of having three specialized enzymes compared to an ancestral single enzyme? What factors influenced RNA polymerase evolution? The answers to these questions are probably hidden in the very particular biological roles of the three RNA polymerases and thus, the unique requirements and selective pressure encountered by the paralogs. rDNA transcription by Pol I might serve as a model to learn more about RNA polymerase evolution and enzyme specialization in general.

rDNA transcription by Pol I has several characteristics absent or extremely rare for the other eukaryotic transcription apparatuses. First of all, Pol I transcription is exceptionally robust. It accounts for more than 60% of total nuclear transcription (Warner, 1999). According to calculations, Pol I initiation must occur every 5 seconds in growing yeast under standard conditions (Reeder and Lang, 1997). In addition, high rates of transcription initiation by Pol I demand fast and effective promoter escape and result in extremely high polymerase density of approximately 50 - 60 elongation complexes per 35S rRNA gene (French et al., 2003). In yeast the full size of the pre-rRNA transcript is approximately 6.7 kb, which is considerably longer than the average yeast Pol II and III transcripts. To complete the transcript synthesis, Pol I elongates throughout the 35S rRNA gene approximately 60 nucleotides per second. Thus, Pol I must be highly

processive and resistant to pauses or arrests. Critically, Pol I transcription elongation is functionally coupled with ribosome biogenesis (Schneider et al., 2007). Ongoing studies continue to identify factors that affect transcription elongation by Pol I and rRNA biosynthesis [reviewed in (Schneider, 2012)].

One of the most intriguing features of rDNA transcription is co-transcriptional control of the ribosome biogenesis. What are the mechanisms by which cell coordinates ribosome assembly and what is the contribution of the rate of transcription elongation to formation of the active ribosomal particles? We have previously shown that the rate of Pol I transcription elongation is functionally coupled with rRNA processing and ribosome assembly (Schneider et al., 2007). The studies presented here have further validated and expanded the previously proposed model (Chapter 3). We have shown that various Pol I mutants exhibit ribosome assembly defects which are likely to correlate with their transcription elongation rates. Furthermore, based on the analysis of multiple Pol I mutants we propose the existence of a threshold in the velocity of Pol I transcription for efficient rRNA processing. We suggest that rRNA processing is not affected within a range of elongation rates; however, beyond a certain rate of elongation, rRNA production and ribosome biogenesis are dramatically impaired. This novel hypothesis updates the existing model and approaches a better understanding of the tight connection between Pol I transcription and ribosome production. We need to analyze multiple Pol I mutants in order to observe the contribution of specific steps in transcription to ribosome metabolism. Isolation and characterization of Pol I speed-up alleles seems especially intriguing since no hyperactive Pol I mutants have been described to date. Ongoing

studies revealing mechanisms that link Pol I transcription and rRNA processing/ribosome assembly are critical to our understanding of ribosome biogenesis and cell proliferation.

Remarkably, the functional coupling between the rate of transcription elongation and ribosome assembly also exists in bacteria (Lewicki et al., 1993). Thus, some of the mechanisms by which this coupling occurs are likely shared between eukaryotes and prokaryotes and might have been present in the common ancestor. So, what are these mechanisms that connect the rate of elongation by Pol I and ribosome assembly? Two major processes are known to affect co-transcriptional RNP complex formation in bacterial RNA polymerase or in Pol II: programmed transcriptional pausing and controlled recruitment of the interacting proteins to the nascent transcripts ([reviewed in (Perales and Bentley, 2009)].

One factor that might play both of these roles, connecting Pol I elongation and rRNA processing is the Spt4/5 complex. Spt4/5 is known to directly affect Pol II elongation and orchestrate processing of mRNA precursors via co-transcriptional recruitment of the capping, splicing, polyadenylation and mRNA localization factors (Chen et al., 2009; Lindstrom et al., 2003; Shen et al., 2010; Wada et al., 1998). It has been shown that Spt4/5 complex promotes promoter-proximal pausing of Pol II in higher eukaryotes which serves as an important regulatory check-point in mRNA production (Missra and Gilmour, 2010). In addition to the roles in Pol II transcription, Spt5 homologs are universal elongation and processivity factors known to regulate activities of bacterial and archaeal RNA polymerases. We have demonstrated that this list of polymerases should also include Pol I.

The initial studies defined that Spt4/5 affects transcription elongation by Pol I and rRNA processing (Anderson et al., 2011; Schneider et al., 2006). It has been shown that mutations in *spt5* result in altered transcription elongation rate by Pol I and a change in polymerase distribution over the rDNA (Anderson et al., 2011). These data suggest that yeast Spt4/5 might affect Pol I pausing. In addition, Spt4/5 is proposed to recruit components of the rRNA processing and surveillance machinery and affect rRNA maturation (Anderson et al., 2011; Lepore and Lafontaine, 2011). However, the mechanism by which Spt4/5 functions in rRNA synthesis remained unknown.

Considering multiple cellular roles of Spt4/5 (Hartzog and Fu, 2013), *in vivo* studies were unable to differentiate between the direct versus indirect effects of Spt4/5 on rRNA synthesis and maturation. A discovery of the physical association of Spt5 with Pol I complex presented here is the first evidence that Spt4/5 affects Pol I transcription directly (Viktorovskaya et al., 2011). Acting as an assembly platform, Spt4/5 might provide a direct link between the Pol I elongation complex and the rRNA "processome". Association of Spt5 and Pol I was later supported by two independent studies from different research groups (Beckouet et al., 2011; Lepore and Lafontaine, 2011). These following studies confirm the significant contribution of my work to the field of Pol I transcription. We need to determine the Spt4/5-mediated effects on rDNA transcription by measuring Pol I transcription efficiency *in vitro* (using fully reconstituted or nuclear extract-based transcription assays) in the presence of the Spt4/5 complex. These experiments will establish the roles of Spt4/5 in control of Pol I transcription elongation rate and polymerase processivity. Once the mechanisms by which Spt4/5 affects Pol I

transcription are revealed in yeast, the conservation of these mechanisms will be examined in mammalian cells.

Spt4/5 is one of the very few components of the Pol I elongation complex identified to date. Establishing the composition of Pol I elongation complex, identification of the factors unique for Pol I elongation or shared with other polymerases, defining their roles in rRNA synthesis are the important future tasks. There is still much to be learned about multiple trans-acting factors that regulate Pol I transcription and ribosome biogenesis.

Altogether, the data I presented here underline the significance of intrinsic polymerase properties and extrinsic factors involved in Pol I transcription elongation. The new prospective in the field of Pol I transcription as a consequence of our studies have already raised multiple questions to be addressed in future like a domino effect. The answers to these questions will led to the deeper understanding of the mechanisms of Pol I transcription and shed some light to the evolutionary relationships between the RNA polymerase machineries.

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