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REVIEW ARTICLE

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Shaping the chromatin landscape at rRNA and tRNA genes, an emerging new role for RNA polymerase II transcription?

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Abstract

Eukaryotic genes must be condensed into chromatin while remaining accessible to the transcriptional machinery to support gene expression. Among the three eukaryotic RNA polymerases (RNAP), RNAPII is unique, partly because of the C-terminal domain (CTD) of its largest subunit, Rpb1. Rpb1 CTD can be extensively modified during the transcription cycle, allowing for the co-transcriptional recruitment of specific interacting proteins. These include chromatin remodeling factors that control the opening or closing of chromatin. How the CTD-less RNAPI and RNAPIII deal with chromatin at rRNA and tRNA genes is less understood. Here, we review recent advances in our understanding of how the chromatin at tRNA genes and rRNA genes can be remodeled in response to environmental cues in yeast, with a particular focus on the role of local RNAPII transcription in recruiting chromatin remodelers at these loci. In fission yeast, RNAPII transcription at tRNA genes is important to re-establish a chromatin environment permissive to tRNA transcription, which supports growth from stationary phase. In contrast, local RNAPII transcription at rRNA genes correlates with the closing of the chromatin in starvation in budding and fission yeast, suggesting a role in establishing silent chromatin. These opposite roles might support a general model where RNAPII transcription recruits chromatin remodelers to tRNA and rRNA genes to promote the closing and reopening of chromatin in response to the environment.

KEYWORDS
chromatin, histone, RNA polymerase II, transcription

1 | INTRODUCTION

The eukaryotic genome is compacted into chromatin, a super-complex composed of DNA, (mostly nascent) RNA, histones, and other proteins. The chromatin serves multiple purposes: genome compaction, genome stability, and transcriptional regulation. The compaction of eukaryotic genomes can be quite extensive: untangled, the human genome can stretch up to two meters, all of which fit in the nucleus of most cells of our body. During mitosis and meiosis, an even higher level of DNA condensation is achieved to allow for chromosome segregation. Despite this prominent role in eukaryotes, it has been suggested that chromatin did not initially evolve for this purpose since the chromatin-free bacterial genomes can also be subject to high levels of DNA condensation. Instead, increased genome stability was proposed as the primary driver for chromatin apparition in eukaryotes (Madhani, 2013). Regardless of its evolutionary origin, chromatin is undoubtedly essential in protecting the genome: compacted DNA is less sensitive to DNA damage

(Nair et al., 2017), is protected from the integration of foreign genetic elements (viruses and transposons), is less likely to undergo harmful recombination (Okita et al., 2019), and suppresses the expression of parasitic genes (Almeida et al., 2022). However, the need for dense chromatin structure to support genome compaction and protection is balanced by the requirement to open up chromatin to allow for DNA replication, for DNA repair, and for transcription of the thousands of genes of the eukaryotic genome. Such chromatin remodeling is mediated by chromatin remodeler complexes, histone modifiers, and other factors, including transcription itself.

In eukaryotic cells, transcription is carried on by three conserved RNA polymerase (RNAP) transcription complexes, designated as RNAPI, RNAPII, and RNAPIII. Each of these complexes transcribes different classes of RNA molecules from specific gene promoters: RNAPI and RNAPIII are highly specialized in transcribing the long ribosomal RNA (rRNA) species and small stable noncoding RNA (ncRNA) molecules—including the transfer RNA (tRNAs), respectively. In contrast, RNAPII is more versatile and transcribes a large portion of the yeast genome, from messenger RNA (mRNA) to various ncRNA, the latter being sometimes so unstable that they are barely detectable in wild-type cells (Tisseur et al., 2011). Another feature that distinguishes RNAPII from the other RNAP complexes is the unique C-terminal domain (CTD) of its largest subunit, Rpb1. Rpb1 CTD structure is intrinsically disordered and its length varies from species to species. However, its amino acid sequence made of repetitions of consensus YSPTSPS heptapeptide is highly conserved among eukaryotes. Rpb1 CTD can be extensively modified during the transcription cycle, participating in the co-transcriptional recruitment of specific interacting proteins, including chromatin remodeling factors, recently reviewed in Singh et al. (2022).

How chromatin can be remodeled during transcription has been extensively studied in the context of genes transcribed by RNAPII. In contrast, less is known about the chromatin surrounding the genes transcribed by the CTD-less RNAPI and RNAPIII even though the chromatin at these loci is also known to be remodeled to adapt rRNA and tRNA synthesis to cellular needs. This piece aims to review recent advances in our understanding of how the chromatin encompassing tRNA genes (tDNAs) and rRNA genes (rDNAs) can be remodeled in response to environmental cues in yeast. It emphasizes on the emerging new role of local RNAPII transcription initiated from cryptic promoters in driving these chromatin modifications.

Take-away

- Local RNAPII transcription brings chromatin modifications to tRNA and rRNA genes.
- RNAPII at tRNA genes is important for growth from stationary phase.
- RNAPII at rRNA genes correlates with the closing of the chromatin.
- An emerging view: RNAPII supports chromatin remodeling at tRNA and rRNA genes.

2 | tDNA ARCHITECTURE IN *S. pombe* AND *S. cerevisiae*

tDNAs are very small (70–120 nt) genes that localize throughout the *Saccharomyces cerevisiae* genome. In *Schizosaccharomyces pombe*, most of the nuclear tDNAs are similarly scattered across the chromosomes while about 30% of them organize in dense gene clusters at centromere boundaries (Kuhn et al., 1991; Takahashi et al., 1991). tRNA transcription by RNAPIII requires only two general transcription factors: TFIIIB and TFIIIC. TFIIIC is recruited to tDNAs through interaction with two specific intragenic promoter elements called the A and B boxes (Figure 1a). Due to its high affinity to the tDNA, TFIIIC acts as a pioneer transcription factor and can open the chromatin in vitro (Burnol et al., 1993). It is also an efficient chromatin insulator that can limit the spread of heterochromatin (Noma et al., 2006; Simms et al., 2008). To promote RNAPIII transcription, TFIIIC recruits TFIIIB, a complex composed of the TATA-binding protein, Brf1, and Bdp1. Although a TATA box upstream tDNAs is rare in *S. cerevisiae*, it is widespread in fission yeast, providing a second mode of recruitment for TFIIIB (Hamada et al., 2001). TFIIIB recruits RNAPIII to initiate tRNA transcription, which causes TFIIIC to partially dissociate from the internal promoter elements. Meanwhile, TFIIIB remains stably bound and can reinitiate a new round of transcription by recycling RNAPIII (Arimbasseri et al., 2013).

In growing yeast, tDNAs are all highly occupied by RNAPIII and are usually found in nucleosome-depleted regions flanked by positioned nucleosomes (Shukla & Bhargava, 2018). Even in conditions of nutritional stress where Maf1-dependent repression rapidly shutdowns RNAPIII recruitment by TFIIIB, tDNAs remain occupied by TFIIIC (Harismendy, 2003; Roberts et al., 2003), possibly to preserve the genes from encroaching nucleosomes and allow rapid reinitiation of transcription upon exit from starvation. The constitutively high chromatin occupancy of RNAPIII and related transcription factors was proposed to be sufficient to exclude nucleosomes from the loci, suggesting that tDNAs were not subject to chromatin-based regulations. However, that view has been challenged by recent data showing that TFIIIC is released from the tDNA after extended nutritional starvation (Galic et al., 2019). Therefore, tDNAs occupied by neither RNAPIII nor TFIIIC may become nucleosomal in conditions of prolonged starvation (Figure 1b).

Supporting the idea that tDNAs can be nucleosomal in yeast, depletion of the RSC (remodel the structure of chromatin) complex leads to a gain of nucleosome density on tDNAs (Mahapatra et al., 2011; Parnell et al., 2008; Roberts et al., 2006). RSC is an abundant and essential paralog of the canonical SWItch/Sucrose Non Fermentable remodeler that uses adenosine triphosphate to translocate DNA to shift or eject nucleosomes (Cairns et al., 1996). The RSC complex is recruited to the chromatin through multiple interactions with acetylated histone H3 (Chatterjee et al., 2011, 2015; Chen et al., 2020; Kasten et al., 2004) and with Rpb5, a common subunit to

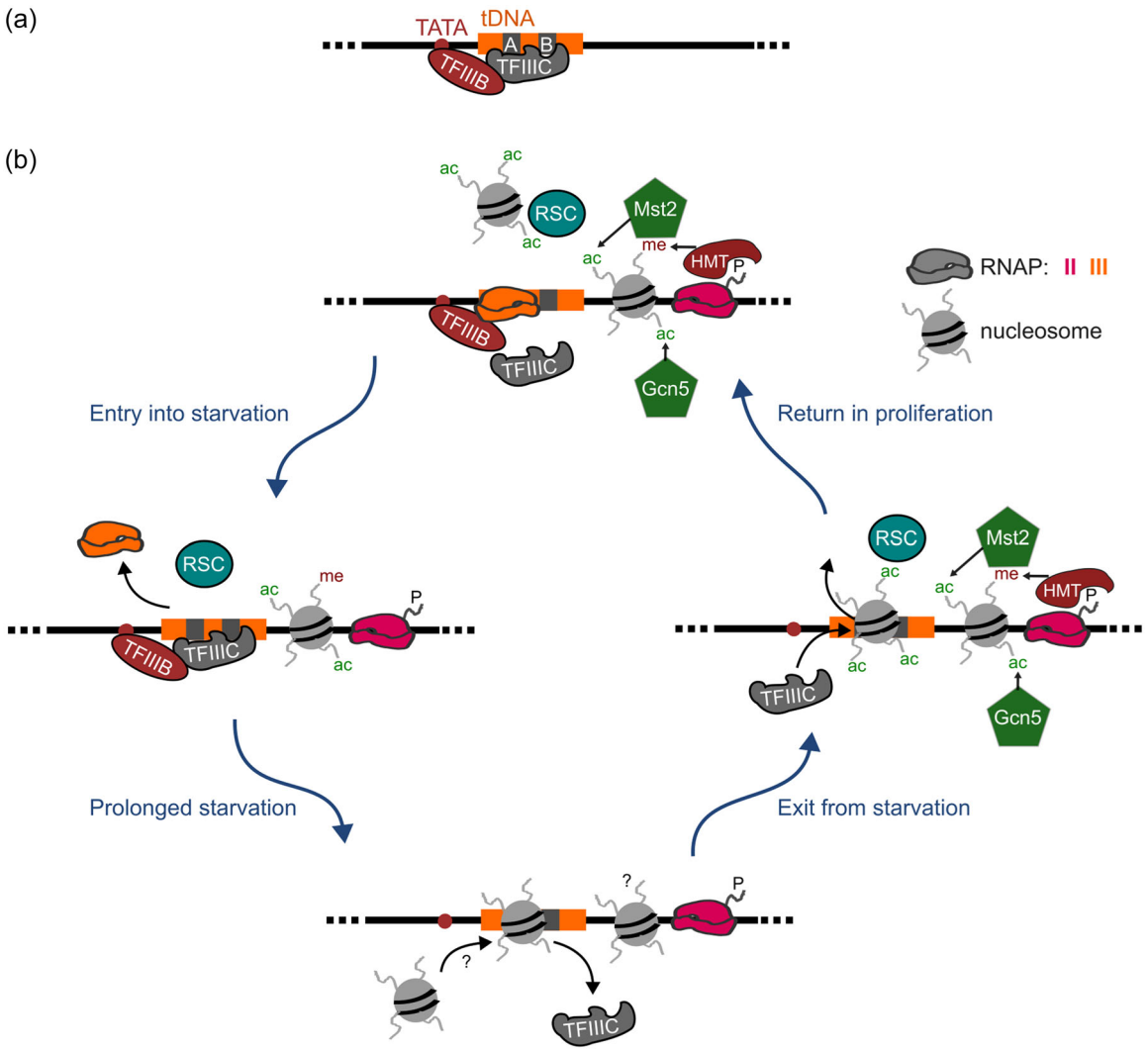

FIGURE 1 Chromatin architecture of tDNA. (a) tDNAs promoter elements (TATA-, A-, and B-boxes) and associated transcription factors. (b) A model for chromatin-based regulation of tRNA expression in fission yeast: in proliferation, TFIIIB and RNAPIII occupancy are high on the tDNA. Flanking nucleosome are methylated (me), possibly accounting for low level of RNAPII antisense transcription that recruits histone methyltransferase (HMT) through interaction with Rpb1 phosphorylated (P) C-terminal domain. H3K36 methylated histones and the Pcr1 transcription factor (not shown) participate in the recruitment of the histone acetyltransferase Mst2 and Gcn5, respectively. Histone acetylation (ac) might favor the activity of the RSC chromatin remodeler in maintaining the tDNA depleted from nucleosomes. In nutrient starvation, Maf1-dependent repression (not shown) releases RNAPIII from the chromatin. TFIIIC is more tightly associated with the internal promoter elements. TFIIIC and the RSC complex maintain the tDNA nucleosome depleted so that reactivation of tRNA transcription can occur rapidly. However, in prolonged starvation, TFIIIC dissociates, potentially allowing nucleosomes to invade the tDNA. Upon exit from nutritional starvation, RNAPII-dependent remodeling of the chromatin (methylation and acetylation) is especially important to re-establish a chromatin environment permissive for RNAPIII transcription. RSC, remodel the structure of chromatin.

the RNAPI, RNAPII, and RNAPIII complexes (Soutourina et al., 2006). Accordingly, acetylated histones, typically found at RNAPII-transcribed genes, compose the nucleosomes flanking tDNAs in both budding yeast (Galic et al., 2019; Rufiange et al., 2007) and fission yeast (Yague-Sanz et al., 2023). Therefore, histone acetylation might participate in RSC recruitment and/or activity at these loci. How histone acetyltransferases (HAT) are recruited to tDNAs and whether their recruitment is regulated is still largely unclear. Our efforts to answer these questions in fission yeast highlighted the unexpected contribution of local RNAPII transcription.

3 | RNAPII AND CHROMATIN REMODELING AT tDNAs

We recently found that MNase-resistant, H3-containing nucleosomes (or nucleosome-like particles) occupy tDNAs in an RNAPII mutant in fission yeast where the 29 serines in position 2 of the Rpb1 CTD consensus repeats are mutated into alanine (Yague-Sanz et al., 2023). The "CTD S2A" mutant mimics the lack of serine 2 phosphorylation, a modification associated with RNAPII elongation. Indicating that the phosphorylation of Rpb1 CTD S2 is largely

dispensable for transcription, the CTD S2A only causes defects in the expression of specific protein-coding genes, none of which could readily explain the dramatic chromatin change at tDNAs (Cassart et al., 2012; Coudreuse et al., 2010; Materne et al., 2015, 2016).

Supporting the possibility of a more direct role of RNAPII transcription in shaping tDNA chromatin in fission yeast, Rpb1 was found at tDNAs where low level of RNAPII-specific antisense transcription can be detected (Castel et al., 2014; Yague-Sanz et al., 2023). The recruitment of RNAPII at the tDNA depends at least in part on the Pcr1 transcription factor that also recruits the Gcn5 HAT (Yague-Sanz et al., 2023). A second HAT, Mst2, is also crucial for acetylating histones at tDNAs, even though ChIP-seq experiments suggest that it does not robustly associate with the locus (Flury et al., 2017). Strengthening the connection with RNAPII transcription, Mst2 recruitment is dependent on methylated H3K36 (Flury et al., 2017), a mark deposited by the Set2 histone methyltransferase that is recruited to chromatin by elongating RNAPII phosphorylated on CTD serine 2 in both budding yeast (Hampsey & Reinberg, 2003; Krogan et al., 2003) and fission yeast (Boulanger et al., 2024). RNAPII transcription and histone acetylation by at least Gcn5 and Mst2 are required to maintain nucleosome depletion on tDNAs, a process especially important to reinitiate growth from the stationary phase (Yague-Sanz et al., 2023) (Figure 1b).

In *S. cerevisiae*, the involvement of RNAPII transcription in shaping tDNA chromatin has not been established. However, there are indications that a similar mechanism might be at play. Notably, the RNAPII-dependent methylation of H3K36 and H3K4 are present downstream of the tDNAs (Bhalla et al., 2019), and 12 RNAPII-related factors (including the Rpb1 CTD Ser2 kinase Bur1 and Ctk1) associate with the RNAPIII transcription complex (Trotta, 2019). Furthermore, recent research using cross-linking and cDNA analysis (CRAC) has revealed the presence of RNAPII-specific transcription in both the sense and antisense directions around tDNA in *S. cerevisiae* (Aiello et al., 2022). Together, these findings indicate that RNAPII is also present at tRNA genes in budding yeast and recruits RNAPII-related factors that could potentially influence the chromatin organization at these loci. Besides tDNAs, rRNA genes also appear to be occupied and transcribed by the RNAPII. Hence, subsequent sections of this review will explore the possibility that the CTD-containing RNAPII modulates the chromatin environment of yet another class of gene: the RNAPI-transcribed rRNA genes.

4 | rDNA ORGANIZATION IN YEAST

RNAPI has only one essential function: to transcribe ribosomal RNA genes (rDNA) to produce the long ribosomal RNA precursor (35S rRNA in yeast) that is further processed into the ribosome components 18S, 5.8S, and 25S rRNA (Nogi et al., 1991). The rDNA must be abundantly transcribed to support high level of protein synthesis by the ribosome. Indeed, in rapidly growing yeast cells, rDNA transcription accounts for a large portion (estimated at about 60% in yeast) of all the newly synthesized RNA (Warner, 1999). Such an exceptionally high expression level is only possible because the rDNA is amplified in multiple copies, which are typically organized in tandem repeats. For instance, the *S. cerevisiae* genome contains approximately 150 rDNA repeats in a single locus localized on chromosome XII (Petes, 1979), while the *S. pombe* genome contains 200–300 rDNA repeats shared between two loci localized on both ends of chromosome III (Wood et al., 2002). The repeats are separated by intergenic spacer (IGS) regions and, in *S. cerevisiae*, by copies of the RNAP III-transcribed 5S rRNA gene (Figure 2a,b).

Extensive work in budding yeast has shown by electron microscopy and biochemical assays that even in proliferating cells where ribosomes are limiting for growth, only about half the rDNA repeats are actively transcribed by the RNAPI. The active and non-active rDNA repeats are intervened; they do not organize into linearly separated domains. They differ primarily by their chromatin structure, with the inactive repeats being packaged by nucleosomes whereas the active (open) repeats are being mostly occupied by RNAPI. Whether nucleosomes are present on active rDNA repeats along with RNAPI has been under debate, with conflicting findings either reporting a complete absence of nucleosomes (Conconi et al., 1988; Dammann et al., 1993) or the presence of dynamic, unphased, nucleosomes (Jones et al., 2007). These conflicting results and interpretations have been reviewed elsewhere (Hamperl et al., 2013; Németh & Längst, 2008), and the general consensus is that nucleosomes also assemble at active rDNA repeats but more loosely and with reduced density compared to closed rDNA repeats.

The balance between active and inactive rDNA repeats is highly dynamic, with repeats stochastically closing or opening up without any change in growth conditions. These events were estimated to occur frequently in yeast, every 5–10 min on average (Dammann et al., 1995). The closed to open rDNA chromatin ratio is also changed in response to the environment. For instance, the closed state increases from about 50% to 80% when yeast cells enter stationary phase upon nutritional starvation (Sandmeier, 2002). Multiple factors participate in the switch between active and inactive states. For instance, DNA replication favors rDNA chromatin closing up during the S phase of the cell cycle, whereas RNAPI transcription opposes nucleosome deposition (Wittner et al., 2011). Histone modifications, chromatin remodeling, and local RNAPII transcription also contribute to the balance between active and inactive rDNA repeats.

5 | HISTONE MODIFICATIONS AND CHROMATIN REMODELING AT THE rDNA IN *S. cerevisiae*

The modification of lysine residues at the N-terminal tails of histone H3 and H4 of nucleosome at the rDNA dynamically modulates the chromatin structure, thereby influencing rDNA accessibility to RNA polymerase I (RNAPI) and the transcriptional machinery. In general, acetylation of histones within the rDNA promotes an open chromatin

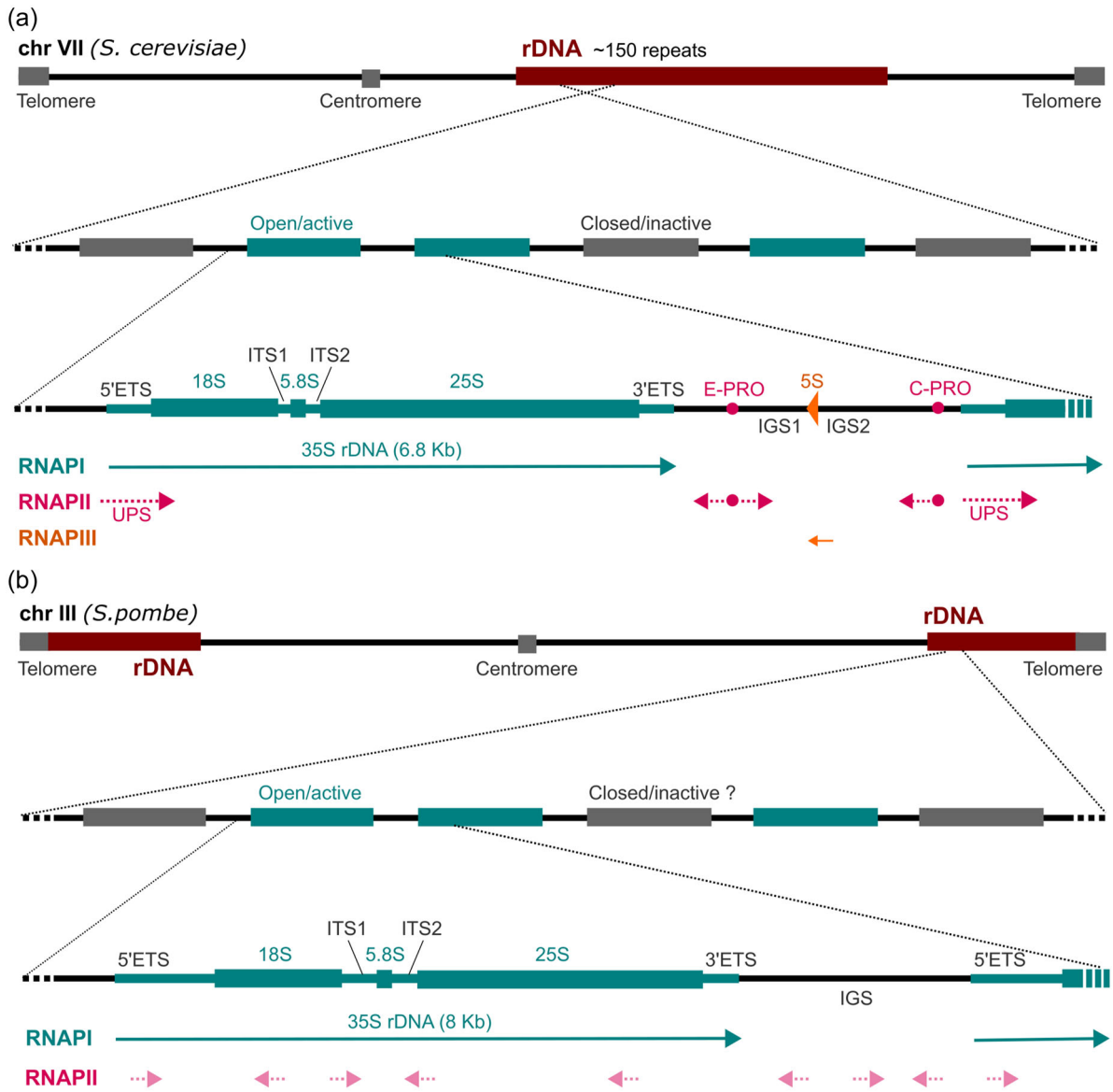

FIGURE 2 Genome organization of rDNA repeats in (a) *S. cerevisiae* and (b) *S. pombe*. Arrows denote directional transcription by the indicated RNAP, with the dotted line when the boundaries (transcription start and/or termination sites) of the transcriptional events are not clearly defined. C-PRO, cryptic promoter; E-PRO, EXP promoter; ETS, externally transcribed sequence; IGS, intergenic spacer; ITS, internally transcribed sequence; UPS, upstream initiating transcript.

conformation, facilitating transcription initiation and elongation. Conversely, deacetylation by histone deacetylases (HDAC) can counteract this effect, leading to transcriptional repression.

Among the HDAC involved in histone deacetylation at the rDNA, Rpd3 is required in *S. cerevisiae* to close rDNA chromatin in stationary phase. Despite this requirement, cells lacking Rpd3 still repress RNAPI transcription by decreasing the density of RNAPI on the rDNA repeats that remained open (Sandmeier, 2002), indicating that RNAPI recruitment at the rDNA is regulated by chromatin-independent mechanisms, recently reviewed in (Hirai & Ohta, 2023). RPD3 exists in two HDAC complexes, the 13-subunits Rpd3L complex and the smaller Rpd3S complex with only 5 subunits. Deleting specific subunits from either complexes leads to defect in nucleosome deposition on the rDNA when cells enter stationary phase, indicating that both complexes are involved in the closing of rDNA chromatin. However, the defect was milder with the deletion of the specific subunit of the less abundant Rpd3S complex (Johnson et al., 2013). In the context of RNAPII genes, Rpd3L is recruited to promoters in association with specific transcription factors (Kadosh & Struhl, 1997) or through interaction with trimethylated H3K4 (Lee et al., 2018), whereas Rpd3S is recruited to protein-coding gene bodies through interaction with methylated H3K36 (Carrozza et al., 2005). Strikingly, trimethylated H3K4 (Briggs et al., 2001) and H3K36 (Ryu & Ahn, 2014) have been reported at the rDNA, along with their respective histone methyltransferase (HMT) Set1 and Set2 (Sayou et al., 2017). Therefore, while the specific mode of recruitment of

RPD3 at the rDNA locus has not been demonstrated, it could depend on histone methylation by Set1 and/or Set2. How Set1 and Set2 are recruited to the rDNA has yet to be elucidated, but in the context of RNAPII genes, their recruitment has been tightly linked with active RNAPII transcription.

6 | RNAPII TRANSCRIPTION AT THE rDNA IN *S. cerevisiae*

The presence of histone modifications traditionally associated to RNAPII transcription at the rDNA raises the question of how these marks are deposited. Could RNAPII be a recruitment platform for the histone-modifying enzymes at this locus? Supporting this possibility, RNAPII can be active in the nucleolus as it can drive the expression of reporter genes with RNAPII promoters inserted between the rDNA repeats (Cioci et al., 2003; Fritze, 1997). RNAP II also physically associates with the rDNA in *S. cerevisiae* (Steinmetz et al., 2006). Perhaps even more surprising is the ability of RNAPII to transcribe the 35S rRNA in mutants deficient in RNAPI transcription (Siddiqi, 2001; Vu et al., 1999). At least three RNAPII promoter regions have been identified at the rDNA (Figure 2a): the E-pro (EXP bidirectional promoter) located in the IGS1 region (Ganley et al., 2005), the C-pro (cryptic promoter) located in the IGS2 region (Li et al., 2006), and the 35S rRNA promoter itself, from which RNAPII can produce UPS (UPStream-initiating) transcripts starting between 9 and 96 nucleotides upstream of RNAPI transcription start sites (Lesage et al., 2021; Vu et al., 1999).

In contrast with RNAPI that abundantly transcribes functional rRNA in nutritious conditions, RNAPII transcription from the rDNA in wild-type *S. cerevisiae* is kept at a very low level through multiple silencing mechanisms. The first layer of repression consists of histone H3 and H4 deacetylation by the NAD^+-dependent HDAC Sir2. Sir2 is recruited close to the E-pro in the IGS1 by the replication fork barrier and also localizes in another region that spans the IGS2 and the beginning of the 18S gene (Huang & Moazed, 2003). At these loci, Sir2 activity maintains the chromatin in a repressive configuration (Fritze, 1997), reducing the accessibility of the RNAPII promoters and efficiently repressing ncRNA expression from the IGS (Li et al., 2006; Smith & Boeke, 1997). Sir2 silencing appears mostly confined to the IGS regions; unlike Rpd3, it is not required to close the entire rDNA repeats (Johnson et al., 2013).

Even when Sir2 is present, RNAPII can still be recruited at the rDNA and initiate transcription, although at a much lower rate. Providing a second layer of silencing, RNAPII transcription is terminated precociously by the NNS complex (Nrd1-Nab3-Sen1) to prevent elongation into the rDNA. The exosome contributes to a third layer of repression by degrading any ncRNA eventually synthetized by RNAPII (Vasiljeva et al., 2008).

When RNAPII at the rDNA is derepressed (i.e., in the absence of Sir2 or Nrd1), H3K4 methylation by the Set1 HMT increases locally (Li et al., 2006; Vasiljeva et al., 2008). This correlation provides the first compelling evidence that RNAPII can serve as a recruiting platform for HMT at the rDNA, a concept already established in the context of protein-coding genes. There, Set1 is recruited co-transcriptionally through physical interaction with the CTD of the RNAPII subunit Rpb1 phosphorylated on the serines 5 (Buratowski & Kim, 2010; Ng et al., 2003). Similarly, H3K36 methylation at the rDNA could rely on Set2 recruitment through interaction with Rpb1 CTD phosphorylated on the serines 2 (Kizer et al., 2005). Suggesting that the Rpb1 phosphorylation is functionally important for the rDNA, the loss of the Rpb1 serine 2 kinase Ctk1 leads to contraction of the rDNA repeats, reduced RNAP I recruitment at rDNA, and reduced rRNA transcription—although some of these effects might be undirect (Bouchoux, 2004; Grenetier et al., 2006). By recruiting Set1 and Set2, RNAPII could also modulate its own silencing since RNAPII transcription at the rDNA is silenced by Set1 (Briggs et al., 2001; Bryk et al., 2002) and activated by Set2 (Ryu & Ahn, 2014).

Remarkably, RNAPII transcription at the rDNA can be derepressed in natural conditions, as was demonstrated recently for the capped and polyadenylated UPS transcript. UPS expression increases after a heat shock or when cells enter stationary phase, reflecting an anticorrelation between RNAP I and RNAP II transcription of the rDNA and a positive correlation between RNAPII transcription and the closing of rDNA genes (Lesage et al., 2021). Increased UPS transcription in these conditions could be a direct consequence of reduced recruitment of the RNAPI through cell signaling, as RNAPI usually outcompetes RNAPII for access to the 35S TSS and participates in RNAPII silencing (Cioci et al., 2003). What would be the role of RNAPII transcription at the rDNA in these conditions? We can speculate that the higher RNAPII density on the rDNA could participate in the CTD-dependent recruitment of the HMT Set1 and Set2, which would methylate the loosely associated histones in the open repeat. Subsequently, histone methylation could favor the recruitment of the Rpd3 HDAC, required to close the rDNA chromatin in stationary phase. Once established, the closed chromatin conformation could be maintained by a low level of RNAPII transcription (Figure 3).

7 | HISTONE MODIFICATIONS AND CHROMATIN REMODELING AT THE rDNA IN *S. pombe*

While *S. cerevisiae* Rpd3 localizes in the nucleolus and is critical in closing the rDNA chromatin, a similar role for the Rpd3 homolog Clr6 in *S. pombe* (whose nucleolar localization is debated at best) has not been demonstrated (Bjerling et al., 2002; Hirai et al., 2022). In contrast, the Clr3 HDAC appears to have a prominent role in rDNA chromatin remodeling in fission yeast. Clr3 primary molecular function is the deacetylation of H3K14. Yet, its deletion causes increased acetylation of H3K14, H3K9, H4K8, and H4K12 at the rDNA, indicating either a broader substrate specificity or, perhaps more likely, a cascade effect leading to general opening/deacetylation of rDNA repeats (Bjerling et al., 2002). Clr3 operates within the

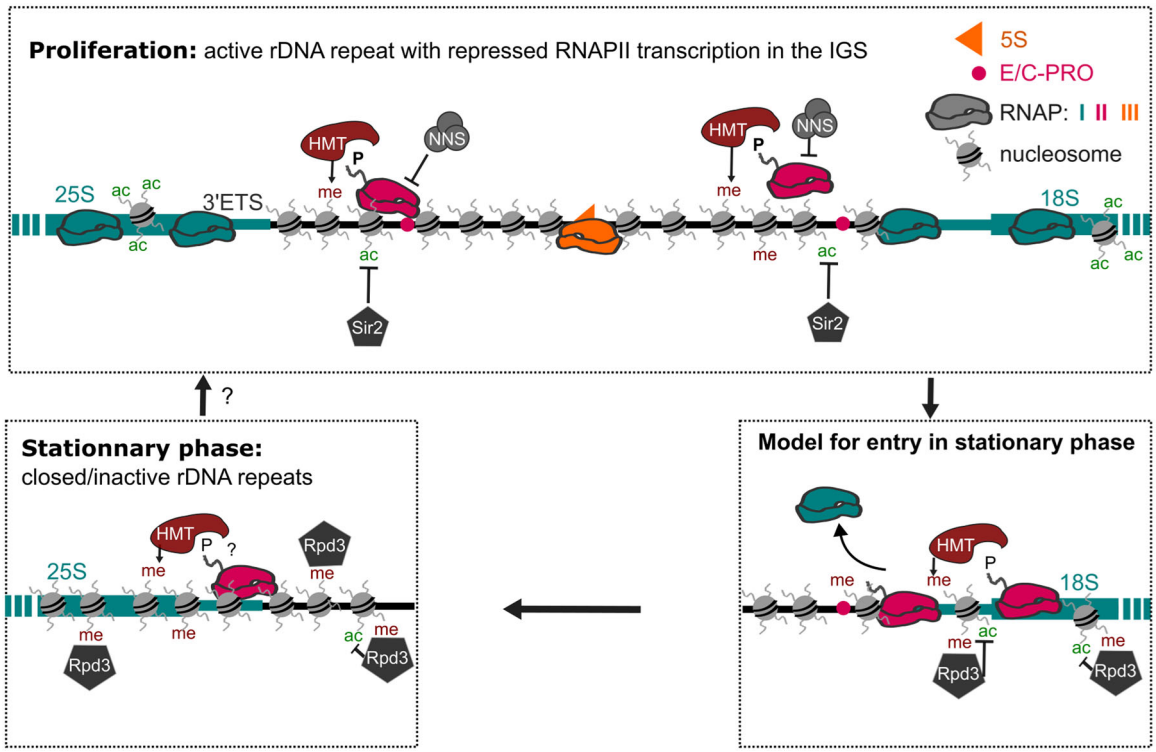

FIGURE 3 Model for the closing of rDNA chromatin in *S. cerevisiae*. During proliferation, open rDNA repeats are actively transcribed by RNAPI. Few, loosely bound, acetylated (ac) nucleosome are associated with the chromatin along the 35S gene. In contrast, nucleosomes are deacetylated by Sir2 in the intergenic spacer (IGS) region, which silence RNAPII transcription. Despite Sir2-dependent silencing, low level of pervasive RNAPII transcription from the E-PRO and C-PRO cryptic promoters exist, but is prematurely terminated by Nrd1-Nab3-Sen1 (NNS) pathway. When cells enter stationary phase, RNAPI is no longer recruited to the 35S promoter, which correlates with increased RNAPII transcription from the IGS into the 18S gene. Active RNAPII transcription might be involved in the establishment and/or maintenance of heterochromatin at the rDNA as the phosphorylated (P) C-terminal domain of RNAPII largest subunit recruits histone methyl transferase (HMT) proteins and methylated (me) histones participate in the recruitment of the Rpd3 histone deacetylase, which is required for the closing of rDNA chromatin in stationary phase. C-PRO, cryptic promoter; E-PRO, EXP promoter; ETS, externally transcribed sequence.

SHREC complex which localizes to all major heterochromatin loci in fission yeast, including the rDNA. It is essential to establish transcriptional gene silencing and for gross chromatin organization at the mat locus, one of the other heterochromatin locus silenced by Clr3 (Sugiyama et al., 2007).

In contrast to *S. cerevisiae* and more in line with other eukaryotic lineages, *S. pombe* retained the ability to assemble heterochromatin through the RNA interference (RNAi) pathway and the methylation of H3K9. RNAi-dependent heterochromatin formation in *S. pombe* is a complex multi-step process whose molecular basis has been recently reviewed in Grewal (2023). Briefly, it begins with the generation of small RNA molecules called small interfering RNAs (siRNAs) that are then loaded onto Argonaute proteins (Ago1 and Ago2 in *S. pombe*), forming the RNA-induced initiation of transcriptional silencing (RITS) complex. RITS guides the Clr4 H3K9 methyltransferase to specific genomic loci based on sequence complementarity. Methylated H3K9 recruits the chromodomain-containing heterochromatin protein 1 (HP1) homologs Swi6, Chp1, and Chp2 in *S. pombe*. Together, methylated H3K9 and HP1 constitute the hallmark of heterochromatin, and they recruit other factors, including the Clr3 HDAC (Yamada et al., 2005), to mediate a feed-forward loop leading to heterochromatin spreading.

In *S. pombe*, heterochromatin and H3K9 methylation are present at the rDNA even in proliferating cells, suggesting that, similarly to *S. cerevisiae*, some of the rDNA repeats are closed even when protein synthesis and ribosomes are limiting for growth (Cam et al., 2005). In glucose starvation however, H3K9 methylation at the rDNA increases within 30 and 90 min for H3K9me2 and H3K9me3, respectively, indicating a nutrient-dependent heterochromatinization of the chromatin at the rDNA repeats. Histone deacetylation by Clr3, exclusion of the histone H3K9 acetyltransferase Gcn5, and the FACT (facilitates chromatin transcription) histone chaperone are all required for this process (Hirai et al., 2022). Similarly to glucose starvation, entry into quiescence and nutrient starvation in general increase H3K9me2 which correlates with the dissociation of RNAPI from the rDNA (Joh et al., 2016; Roche et al., 2016).

The molecular requirements for H3K9 methylation within the rDNA locus in fission yeast are distinct from those in other heterochromatin domains. Several key components of the conventional RNAi pathway, such as the Clr4 histone methyltransferase and the argonaute protein Ago1, are essential for effecting H3K9 methylation at the rDNA site (Hirai et al., 2022; Roche et al., 2016). However, the RNAse III Dcr1, primarily responsible for processing

longer double-stranded RNA precursors into siRNAs, is not required for this process (Castel et al., 2014). In contrast, the loss of the CCR4-NOT complex dramatically reduces H3K9 methylation at the rDNA and other facultative heterochromatin islands (Sugiyama et al., 2016). CCR4-NOT can interact with both the Mmi1-Erh1 RNA binding complex and the RITS complex (Cotobal et al., 2015). It functions within the RNAi pathway by bringing facultative/regulated heterochromatin formation to nascent RNAPII transcripts containing cryptic intron or specific sequence motif called determinant of selective removal (DSR, UNAAAC). Therefore, while the precise mechanism of heterochromatin nucleation at the rDNA remains to be elucidated in *S. pombe*, it could depend on local rDNA transcription by RNAPII.

8 | RNAPII TRANSCRIPTION AT THE rDNA IN *S. pombe*

Although transcription by RNAPII within the rDNA repeats was never directly proven in *S. pombe*, it is supported by multiple evidence. ChIP against Rpb1 CTD serine 5 and serine 2 phosphorylation marks reveal that, like in *S. cerevisiae*, RNAPII also associates with the rDNA chromatin in *S. pombe* (Castel et al., 2014). Typically, Rpb1 CTD phosphorylation on the serine 5 and 2 correlates with initiating and elongating RNAPII, respectively, which suggests that RNAPII transcribes actively the rDNA. Moreover, Rdp1-independent sRNA originating from the antisense strand relative to the 35S further suggests that antisense transcription by Pol II is occurring at these loci (Yu et al., 2014). While these sRNAs are partially dependent on Dcr1 activity and have the typical length of siRNA (Castel et al., 2014), they do not enter the RNAi pathway in proliferative wild-type cells as they are targeted by the Trf4/Air2/Mtr4 Polyadenylation complex to degradation by the nuclear exosome (Bühler et al., 2008). However, in quiescent cells, Ago1-associated small RNAs originating from the rDNA increase dramatically, suggesting that these small RNAs might contribute to rDNA heterochromatinization in this condition (Joh et al., 2016). Transcription also occurs between the rDNA repeats in *S. pombe* as new ncRNAs transcribed from the IGS regions have been recently reannotated. These ncRNAs are stabilized in *rrp6* and *exo2* exonuclease mutants, suggesting that they are targeted for degradation (Atkinson et al., 2018).

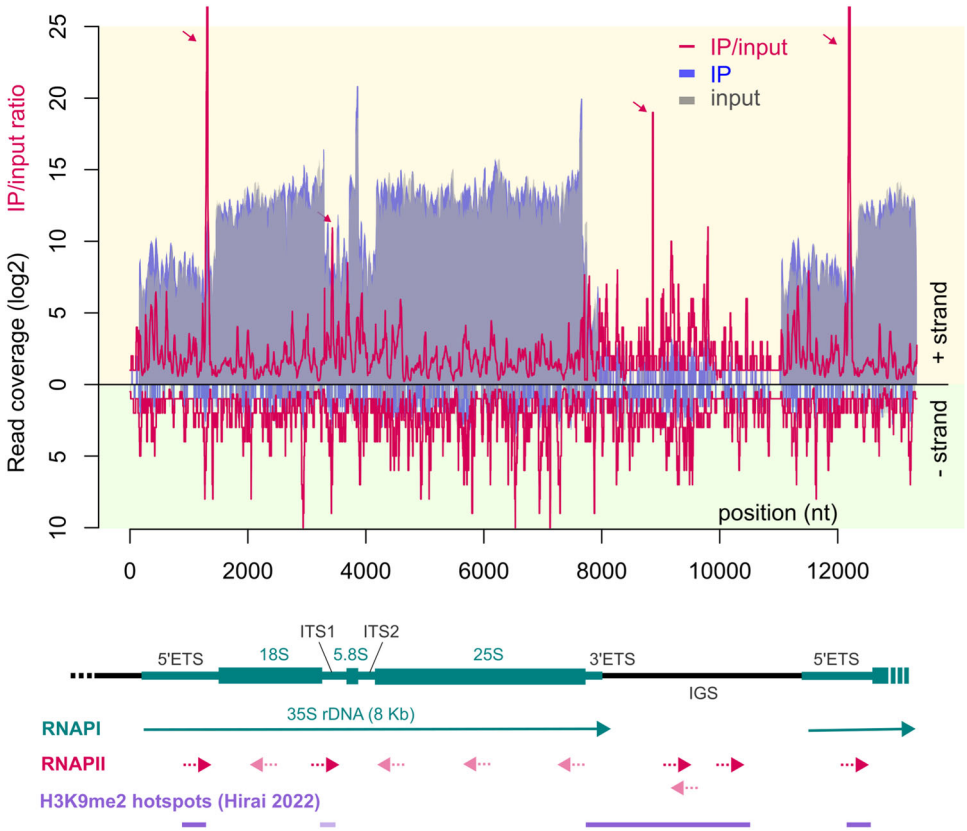

FIGURE 4 Nascent RNAPII transcription at the rDNA in *S. pombe*. Top: strand-specific enrichment ratio (IP/input, magenta line) on the rDNA for RNAPII-associated nascent RNA of a nascent transcript sequencing experiment (Wery et al., 2018). Read coverage in base 2 logarithm for RNA co-immunoprecipitated with RNAPII (IP) and input fractions are overlayed in blue and gray, respectively. For the IP/input ratio, a pseudocount of 1 was added to the denominator to stabilize the ratio and avoid division by zero. Arrows in magenta point to regions where the IP/input enrichment ratio peaks. Bottom: horizontal arrows denote directional transcription by RNAPI and RNAPII, with dotted line when the boundaries (transcription start and termination sites) of the transcriptional events are not clearly defined. Purple rectangle indicates the position of H3K9me2 hotspots as previously reported (darker purple) or apparent from the same ChIP-seq data (lighter purple in the ITS1) (Hirai et al., 2022). ETS, externally transcribed sequence; IGS, intergenic spacer; ITS, internally transcribed sequence.

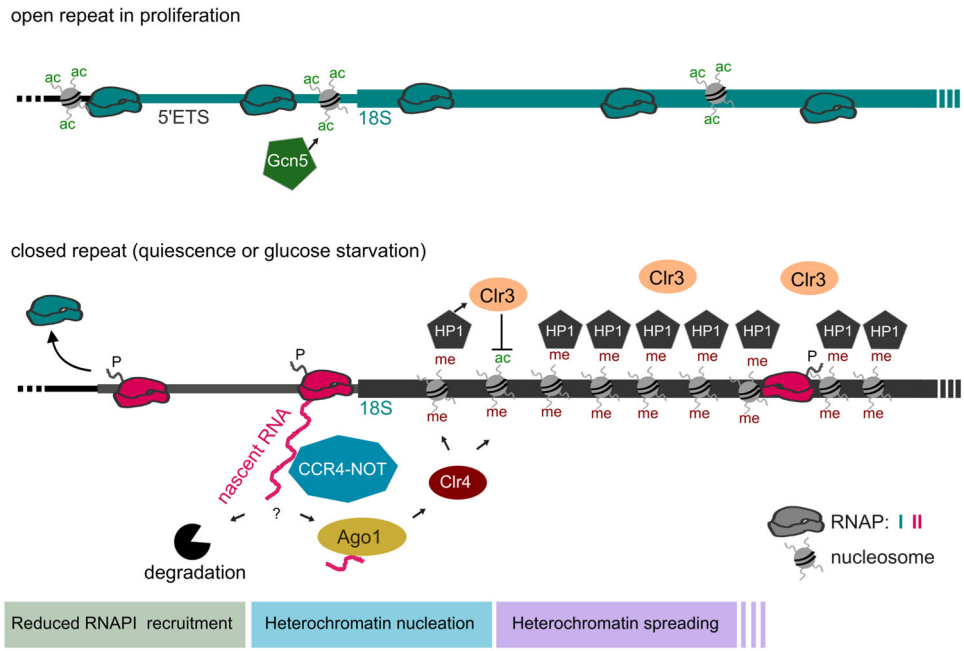

FIGURE 5 Model for rDNA heterochromatin formation in *S. pombe*. During proliferation, open rDNA repeats are actively transcribed by RNAPI. Few, loosely bound, nucleosome are associated with the chromatin along the 35S rDNA. These nucleosomes are acetylated by the Gcn5 histone acetyltransferase. In nutritional starvation, RNAPI dissociates from the rDNA. Pervasive RNAPII transcription within the rDNA pauses at sites of heterochromatin nucleation, such as in the 5′ETS where it might participate in the recruitment of the CCR4-NOT complex. CCR4-NOT and Dcr1-independent small RNAs recruit the Ago1-containing RNA-induced initiation of transcriptional silencing complex that targets H3K9 methylation by Clr4. The spreading of heterochromatin into the rDNA is mediated by the deacetylation of histones by the Clr3 histone deacetylase. ETS, externally transcribed sequence.

Together, the above results suggest the existence of rDNA transcription outside of the 35S gene in fission yeast. However, they do not directly address the question of polymerase specificity. To clarify this issue, I reanalyzed previously published nascent elongating transcript sequencing (NET-seq) data in which nascent RNAPII-associated transcripts are enriched through RNAPII co-immunoprecipation (Wery et al., 2018). Because the abundant tRNA and rRNA transcripts are known contaminants of NET-seq, reads mapping to these loci were informatically filtered out in the original NET-seq data processing pipeline (Churchman & Weissman, 2012). By including them in the reanalysis, we can confirm broad RNAPII-specific enrichment in antisense at the rDNA, as was previously suspected (Castel et al., 2014). In addition, despite the high level of background in the input, the signal corresponding to RNAPII nascent transcription is also highly enriched on specific regions in the sense direction relative to the 35S: in the 5′ETS, in the ITS1, and in the IGS region (Figure 4). These regions of peaking NET-seq signal might correspond to RNAPII pausing sites where RNAPII is typically captured with a higher probability (Mayer et al., 2017). Surprisingly, they perfectly overlay the highest H3K9 methylation peaks along the rDNA (Hirai et al., 2022), a correlation suggesting a role for nascent RNAPII transcription in heterochromatin nucleation at the rDNA in fission yeast (Figure 5).

9 | CONCLUSIONS, CHALLENGES, AND PERSPECTIVES

To summarize, local RNAPII transcription brings chromatin remodelers to tRNA and rRNA genes. That role at tDNAs in fission yeast is important to re-establish a chromatin environment permissive to RNAPIII transcription and to support growth from stationary phase (Yague-Sanz et al., 2023). In contrast, local RNAPII transcription at the rDNA correlates with the closing of the chromatin in budding and fission yeast, suggesting a role in establishing heterochromatin domains. These opposite roles might support a general model where RNAPII transcription brings chromatin remodelers to tDNA and rDNA to support both the closing (in nutrient starvation) and the reopening (upon exit from starvation) of the chromatin. However, many questions remain to be answered. For instance, could RNAPII transcription also be required for the reopening of rDNA chromatin? Does long-term starvation lead to tDNA condensation and if so, how is the process regulated?

Addressing these questions is challenging for at least three reasons. To begin with, rDNAs and, to a lesser extent, tDNAs, are repeated elements. Consequently, their study often involves observing populations that comprise individual repeats in different states. This is particularly true for rDNA repeats, which coexist in open or closed state. As an example of the consequences of this added

complexity, it remains unknown whether antisense RNAPII transcription at rDNA in fission yeast (Figure 4) takes place on open or closed chromatin.

Another challenge lies in the determination of polymerase specificity for the transcriptional events observed, especially since two or three different RNAP complexes coexist on the chromatin at tDNAs and rDNAs, respectively. Several approaches can be used to know whether a transcript is dependent on RNAPII transcription. First, the specific inhibition of the RNAPII complex using either a thermosensitive mutation (*rpb1-1*) or selective chemical inhibitors (such as amanitin) correlates with a decrease of the steady state level of (especially unstable) RNAPII-transcribed RNA species (Lesage et al., 2021). The drawback of these approaches is that they induce undirect effects, as the inhibition of RNAPII is rapidly lethal. Alternatively, the co-immonuprecipitation of nascent RNA with RNAPII with the CRAC or NET-seq techniques enriches RNAPII-specific transcripts without introducing chemical or genetic perturbations to the growing yeasts (Aiello et al., 2022; Yague-Sanz et al., 2023). However, these approaches are laborious, hard to generalize, and suffer from contamination from the highly abundant rRNA and tRNA molecules.

Finally, even more challenging is the difficulty is to distinguish between direct and indirect effects. Indeed, most actors discussed here as important in shaping rDNA and tDNA chromatin also have roles outside of these regions, hence any perturbation in those actors has the potential to affect rDNAs or tDNAs indirectly. To clarify the contribution, a possible approach consists of the specific depletion of these ubiquitous factors from the studied loci. For instance, blocking RNAPII antisense transcription at a specific tRNA gene by introducing a strong terminator sequence downstream of the tRNA resulted in a specific decrease of RNAPII recruitment at the corresponding tDNA, while other loci were unaffected (Yague-Sanz et al., 2023). Recently, Hirai et al. (2022) used an elegant genetic approach to specifically degrade the Gcn5 HAT in the nucleolus while keeping nuclear Gcn5 intact. Future work may exploit similar approaches to fully elucidate the role of the ever-ubiquitous RNAPII in shaping the tDNA and rDNA chromatin.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no data sets were generated during the current study.

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