Beyond rRNA: nucleolar transcription generates a complex network of RNAs with multiple roles in maintaining cellular homeostasis

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Nucleoli are the major cellular compartments for the synthesis of rRNA and assembly of ribosomes, the macromolecular complexes responsible for protein synthesis. Given the abundance of ribosomes, there is a huge demand for rRNA, which indeed constitutes ~80% of the mass of RNA in the cell. Thus, nucleoli are characterized by extensive transcription of multiple rDNA loci by the dedicated polymerase, RNA polymerase (Pol) I. However, in addition to producing rRNAs, there is considerable additional transcription in nucleoli by RNA Pol II as well as Pol I, producing multiple noncoding (nc) and, in one instance, coding RNAs. In this review, we discuss important features of these transcripts, which often appear species-specific and reflect transcription antisense to pre-rRNA by Pol II and within the intergenic spacer regions on both strands by both Pol I and Pol II. We discuss how expression of these RNAs is regulated, their propensity to form cotranscriptional R loops, and how they modulate rRNA transcription, nucleolar structure, and cellular homeostasis more generally.

The properties and biological functions of noncoding RNAs (ncRNAs) transcribed in the nucleus are well established (Zhou et al. 2002; Hung and Chang 2010; Morris and Mattick 2014; Nojima and Proudfoot 2022). In fact, genome-wide studies suggest that 68% of human genes encode transcripts classified as ncRNAs (Iyer et al. 2015). Considerable evidence indicates that ncRNAs are also transcribed in the nucleolus by both Pol I and Pol II (e.g., see Bierhoff et al. 2010; Lafontaine 2015; Abraham et al. 2020). In yeast, transcripts produced from rDNA intergenic spacers (IGSs) are transcribed by Pol II in both directions. In mice and humans, a significant group of transcripts is produced by Pol I in the rRNA sense direction (Mayer et al. 2006; Schmitz et al. 2010; Abraham et al. 2020), while others are transcribed by Pol II in the antisense direction (Grummt 2010; Zhao et al. 2016a; Abraham et al. 2020; Feng and Manley 2021). These transcripts influence nucleolar homeostasis by regulating ribosomal gene transcription and rDNA copy number, as well as by helping to maintain nucleolar structures and functions (Santoro et al. 2010; Schmitz et al. 2010; Pirogov et al. 2019; Yan et al. 2019; Vydzhak et al. 2020). In mammalian cells, a variety of IGS ncRNAs are also regulated by stress, such as heat shock, hypoxia, or transcriptional inhibition (Bierhoff et al. 2010; Santoro et al. 2010; Grummt and Längst 2013; Zhao et al. 2016a,b, 2018; Pirogov et al. 2019). Certain of these RNAs recruit specific regulatory proteins to the nucleolus to sequester or immobilize them in response to stress (e.g., see Audas et al. 2012), while others recruit chromatin-modifying complexes to silence rRNA expression (e.g., see Mayer et al. 2006).

A number of nucleolar RNAs are prone to formation of nucleic acid structures known as R loops (Nadel et al. 2015; Zhao et al. 2018; Velichko et al. 2019; Abraham et al. 2020; Feng and Manley 2021). These structures consist of RNA:DNA hybrids that arise when the nascent RNA and template DNA strand hybridize, leaving the nontemplate strand as a single-strand "loop." R loops play distinct roles in a number of cellular processes (Li and Manley 2006; Ginno et al. 2012; Skourti-Stathaki and Proudfoot 2014; Crossley et al. 2019; García-Muse and Aguilera 2019), including controlling rRNA transcription in yeast and human cells (Nadel et al. 2015; Abraham et al. 2020; Niehrs and Luke 2020; Feng and Manley 2021). R loops can act as modulators of genome dynamics and function in transcriptional regulation. However, when they form unnaturally they have been linked to a range of human diseases (Richard and Manley 2017; Crossley et al. 2019; Niehrs and Luke 2020; Marabitti et al. 2022). Failure to prevent formation of or to

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remove such "unscheduled" R loops can result in problematic transcriptional elongation as well as in DNA double-strand breaks (DSBs), leading to hyperrecombination and genomic instability (Santos-Pereira and Aguilera 2015; Sollier and Cimprich 2015; García-Muse and Aguilera 2019).

R loops are prevalent and occupy ~5% of the human genome (Sanz et al. 2016; Niehrs and Luke 2020) and ~8% of the yeast genome (Chan et al. 2014; Sanz et al. 2016; Wahba et al. 2016). They are abundant at actively transcribed genomic loci, and especially at rRNA genes in the nucleolus (Chan et al. 2014; Niehrs and Luke 2020). In yeast cells, about half of all R loops map to the rDNA locus (Wahba et al. 2016). However, how these R loops contribute to rRNA gene transcription and how they can be resolved or lead to genomic instability have not been as well documented. It is likely that R loops form within the rDNA gene body during transcription by Pol I to synthesize the pre-RNA (Chan et al. 2014; Santos-Pereira and Aguilera 2015). However, it has also been suggested that R loops arise from the ncRNAs transcribed from the rDNA IGS region (Yan et al. 2019; Abraham et al. 2020; Vydzhak et al. 2020; Feng and Manley 2021).

In this review, we summarize and discuss features and transcription of nucleolar non-rRNAs. We focus on how such RNAs and relevant protein factors contribute to nucleolar R loop formation and turnover, as well as how these RNAs influence transcription of rRNA. We discuss this in terms of the remarkably complex transcriptional network that exists in the nucleolus, involving both Pol I and Pol II regulatory transcripts, and how this network contributes to nucleolar homeostasis and beyond; e.g., potentially linking nucleoli to the mitochondrial stress response (Coelho et al. 2002; Bonawitz et al. 2008; Poole et al. 2012). A number of ncRNAs function in the nucleolus but are transcribed in the nucleus (e.g., Pirogov et al. 2019; Yan et al. 2019), but here we focus only on those transcribed in the nucleolus.

Nucleoli express multiple ncRNAs

rRNA-encoding genes are the most heavily transcribed in the cell. rDNA loci are organized in tandem repeats located on several chromosomes, numbering between ~100 and 300 per cell, all of which come together to form nucleoli. Within the Pol I transcribed pre-rRNA (designated 35S in yeast and 47S in mammals), mature rRNAs are separated by 5′ and 3′ external transcribed spacers (5′ETSs and 3′ETSs) and internal transcribed spacers 1 and 2 (ITS1 and ITS2), with individual rRNA genes embedded between IGSs (Fig. 1). Strikingly, multiple non-rRNAs are transcribed from both the sense and antisense strands by both Pol I and Pol II not only within the IGSs but also overlapping the pre-rRNA itself (Bierhoff et al. 2010, 2014b; Grummt 2010; Santoro et al. 2010; Schmitz et al. 2010; Abraham et al. 2020). These RNAs respond to multiple cues, including a variety of stresses, by regulating rRNA synthesis and by altering nucleolar morphology, reflecting changes in protein dynamics. Transcription of these RNAs can be induced by DNA damage, cellular stress, and embryonic development, as well as in cancers and by stem cell differentiation (Bierhoff et al. 2010, 2014a; Jacob et al. 2013; Savić et al. 2014; Larsen and Stucki 2016; Zhao et al. 2016a,b; Kresoja-Rakic and Santoro 2019). Below we discuss the plethora of non-rRNAs transcribed from the rDNA locus, highlighting both differences from yeast to mammals and the related but distinct functions of these transcripts. Several excellent reviews dealing with related issues have been published in the last few years (Lindström et al. 2018; Pirogov et al. 2019; Yan et al. 2019; Vydzhak et al. 2020; Mamontova et al. 2021).

Transcripts originating from rDNA spacer promoters

Perhaps the earliest non-rRNA nucleolar transcription was detected and characterized in mice. Transcription by Pol I initiating from so-called spacer promoters (SPs; ~2 kb upstream of the rRNA promoter) in the rRNA sense direction was shown to produce an ~2-kb-long transcript (see Fig. 2; Kuhn and Grummt 1987). This IGS-rRNA is subsequently processed into upstream IGS-rRNAs close to the SP, as well as promoter-associated pRNAs that cover the actual rDNA promoter region, including the rDNA upstream control element (UCE) (Fig. 2; Mayer et al. 2006; Santoro et al. 2010; Savić et al. 2014; Wehner et al. 2014; Agrawal and Ganley 2018). The mechanism of IGS-

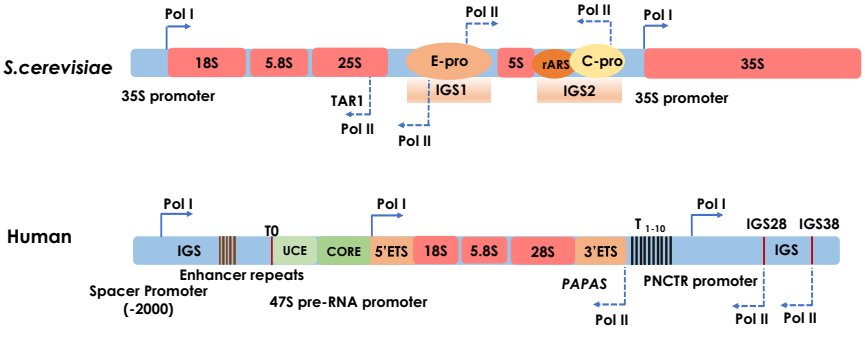

Figure 1. Diagram of one repeat of the yeast and human rDNA arrays. The *top* panel depicts the yeast 35S rDNA. The locus contains 35S and 5S rRNA-coding regions and two intergenic spacer regions (IGS1 and IGS2). Positions of Pol I and Pol II promoters (C-pro and E-pro) are indicated. The *bottom* panel shows the human locus consisting of the 47S 13.3-kb coding region and 30-kb IGS region. Coding and IGS regions are shown with pre-RNA promoters (UCE and CORE), variable size enhancers, the promoter-proximal terminator T0, and terminators T_{1-10}. Three Pol I promoters (spacer promoter, 47S pre-rRNA promoter, and PNCTR promoter) and possible Pol II promoters are indicated.

rRNA transcript processing is not fully understood but is discussed below.

IGS-rRNA levels are subject to regulation; for example, changing during stem cell differentiation. Intriguingly, 5′ and internal IGS-rRNA transcripts upstream of the rDNA promoter (−2 kb) were found to decrease in abundance in mouse embryonic stem cells (mESCs) differentiated to neural progenitor cells (NPCs). However, pRNAs, which function to establish an rDNA heterochromatin state (see below), were shown to be processed only upon mESC differentiation, and unprocessed transcript levels were higher in mESCs than in NPCs (Savić et al. 2014; Leone et al. 2017). The unprocessed IGS-rRNAs abolished the association of TTF1 (transcription terminator factor 1) with TIP5 (TTF1-interacting protein 5), thereby maintaining the euchromatin rDNA state (Fig. 2). In mESCs, processing of IGS-rRNA into pRNA is impaired but is reactivated upon differentiation, although the levels of pRNAs between mESCs and NPCs are unchanged (Savić et al. 2014). IGS-rRNA transcripts that partially cover the UCE and rRNA core promoter are highly conserved throughout metazoans, indicating conservation of function (Mayer et al. 2008; Wehner et al. 2014; Agrawal and Ganley 2018).

pRNAs

pRNAs have been reported to vary from 100 to 300 nt in length and to play conserved roles in modulating rRNA transcription. Even though mouse and human pRNA sequences are less conserved (Mayer et al. 2006; 2008; Guetg et al. 2012; Wehner et al. 2014; Agrawal and Ganley 2018), pRNA sequence alignment from primates shows 40%–73% similarity (Wehner et al. 2014; Agrawal and Ganley 2018). Two conserved motifs were reported within the pRNA sequence—at its 5′ and 3′ ends (Wehner et al. 2014)—and these contribute to pRNA stem–loop secondary structure formation (Mayer et al. 2008; Wehner et al. 2014). These stem–loops, which are found in both humans and mice, are crucial for recruitment of TIP5 to nucleoli and for establishment of rDNA heterochromatin (Fig. 2; Mayer et al. 2008; Schmitz et al. 2010; Savić et al. 2014). In addition, 5′ pRNAs, covering the UCE and T0 (promoter-proximal binding site for TTF-1), play a key role in rDNA methylation and transcriptional silencing. Mutating T0 abrogates pRNA function in rDNA transcription (Schmitz et al. 2010). Truncated pRNAs, retaining T0 but lacking its stem–loop hairpin, showed efficiency similar to that of full-length pRNA in rDNA methylation but were incapable of removing the euchromatin histone marker H3K4me3 or of extending the heterochromatin marker H4K20me3 (Mayer et al. 2006).

pRNAs function to silence rRNA transcription by additional mechanisms. For example, pRNAs recruit the chromatin remodeling complex NoRC, which targets HDAC1, to the rDNA promoter region and further represses Pol I transcription in response to stress (Fig. 2; Zhou et al. 2002; Mayer et al. 2008). Depletion of pRNAs by antisense RNA reduced rDNA promoter CpG methylation, which, coupled with decreased H4K20 and increased H3K4 methylation, activates Pol I transcription (Mayer et al. 2006). It is also notable that pRNAs are transcribed from SPs upstream of unmethylated or hypomethylated rDNA repeats, and thus further propagate the

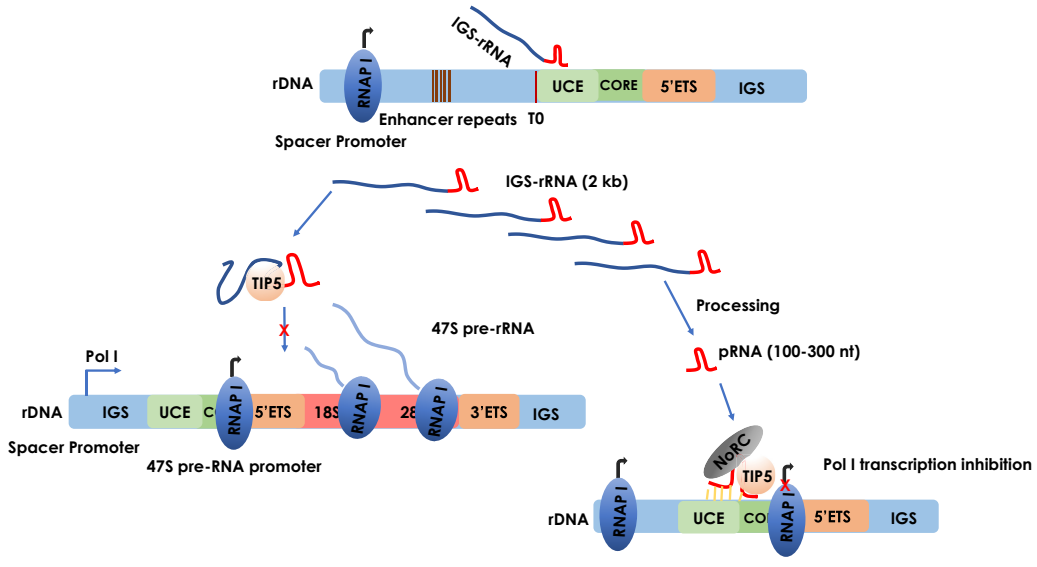

Figure 2. Transcripts from mammalian rDNA spacer promoter are transcribed by Pol I. IGS-rRNA is transcribed by Pol I from spacer promoters (SPs), terminating at the rDNA upstream termination site T0, producing a 2-kb-long transcript. IGS-rRNAs are processed into 100- to 300-nt pRNAs. Unprocessed IGS-rRNAs bind TIP5, abolishing its interaction with TTF1, thereby maintaining the euchromatin rDNA state (see the text). However, pRNAs associate with TIP5 and recruit the chromatin remodeling complex NoRC, repressing 47S pre-rRNA transcription. pRNAs can also form R loops over the promoter/UCE region, which also contributes to repression.

heterochromatin state and silencing of rDNA transcription (Tseng et al. 2008; Santoro et al. 2010).

Mature pRNAs are produced and regulated by RNA processing. The RNA helicase DHX9 plays a key role in processing IGS-rRNA into pRNA, likely involving the activity of the 5′-to-3′ exoribonuclease Xrn2, which was identified as a DHX9-associated protein (Leone et al. 2017). Maturation of pRNAs is important for ESC differentiation, and loss of DHX9 blocks stem cell differentiation (Leone et al. 2017). Notably, pRNA degradation also involves the nuclear exosome, as depletion of exosome subunit ExoSC3 results in pRNA accumulation (Santoro et al. 2010).

pRNAs have been reported to form distinct RNA:DNA hybrid structures. In vitro studies provided evidence that pRNAs can form an RNA:DNA triplex via Hoogsteen base-pairing between the duplex DNA and RNA (Bierhoff et al. 2010). In this study, synthetic pRNAs were incubated with rRNA promoter DNA, and formation of an RNA:DNA hybrid was monitored by gel shift assays. Evidence that the structure detected was a triplex and not an R loop was provided by its observed sensitivity to RNase A but not RNase H1 (Bierhoff et al. 2010). However, a more recent study showed that levels of pRNAs are significantly up-regulated in the absence of the single-strand DNA binding replication protein A complex (RPA) and that this reflects increased formation of R loops, as determined by sensitivity to RNase H1 and RNase H ChIP assays (Feng and Manley 2021). These results suggest that pRNAs may form different RNA:DNA hybrid structures, perhaps simultaneously or in response to different cellular conditions. Alternatively, these findings may reflect differences in assays; for example, in vitro compared with in vivo conditions.

PNCTR

Pyrimidine-rich noncoding transcript (PNCTR) is another nucleolar lncRNA. The primary transcript is >10 kb long, which, like pRNAs, is transcribed from the rDNA IGS in the rRNA sense direction, initiating ~7.5 kb downstream from the pre-rRNA 3′ end (Fig. 3; Yap et al. 2018). The predominant 10-kb PNCTR and a less abundant 3-kb transcript are produced by Pol I, as their synthesis was reduced by Pol I inhibition (by the Pol I-specific inhibitor CX-5461). Interestingly though, accumulation of the 10-kb transcript was increased by Pol II inhibition (by treatment with the Pol II inhibitor DRB). A possible explanation is that while Pol I generates full-length PNCTR, Pol II might form a transcription "barrier" of some sort, preventing full-length transcription and giving rise to the 3-kb transcript (Fig. 3). Consistent with this idea, inhibition of either Pol I or Pol II decreased levels of the 3-kb transcript, and there is also known to be considerable antisense Pol II transcription in the rDNA locus (see below). However, the mechanism by which Pol II inhibition stabilizes or enhances accumulation of the 10-kb transcript is unclear.

Although localized in the perinucleolar compartment (PNC), PNCTR functions as a regulator of pre-mRNA splicing. The RNA contains a number of pyrimidine tract-binding protein 1 (PTBP1)-specific (UC)n motifs and functions by sequestering PTBP1, a splicing regulator, in the PNC (Fig. 3). A significant percentage of PTBP1 can be trapped by PNCTR in the PNC, consequently limiting PTBP1 levels and thereby dysregulating PTBP1-controlled splicing events (Bubenik and Swanson 2018; Yap et al. 2018). PTBP1 typically functions as a splicing repressor, so PNCTR can be viewed as an activator of PTBP1-

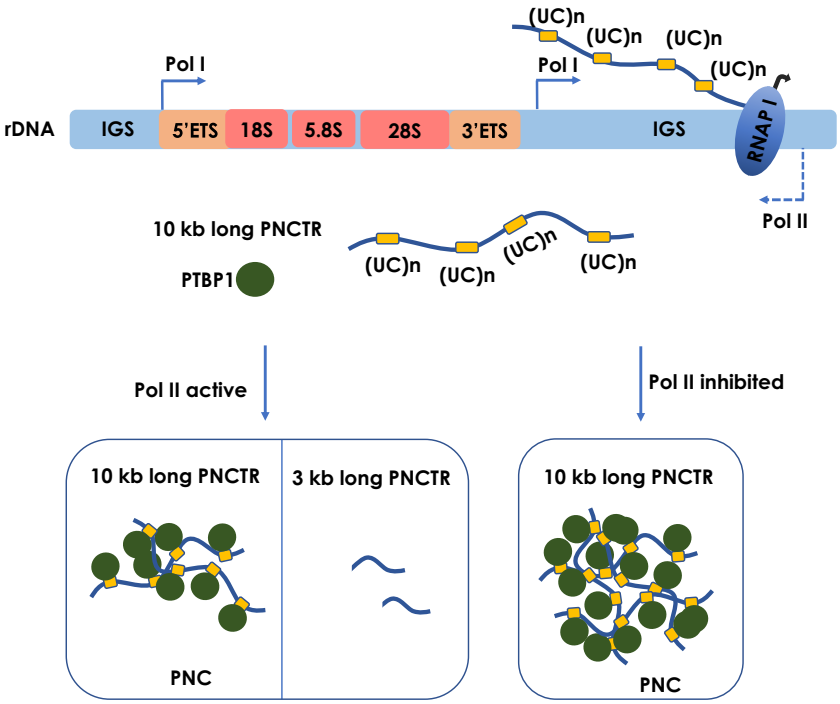

Figure 3. PNCTR is transcribed by Pol I. Transcription initiates ~7.5 kb downstream from the pre-rRNA 3′ end and gives rise to a 10-kb-long full-length transcript. A truncated 3-kb transcript is also produced, possibly reflecting a "barrier" formed by antisense Pol II transcription (see the text). When Pol II is inhibited, levels of the 3-kb transcript decrease and 10-kb transcript levels increase. PNCTR is enriched in (UC)n motifs and functions by sequestering the splicing regulator PTBP1 in the perinucleolar compartment (PNC), thereby modulating RNA splicing.

controlled splicing events. The protein interactome of the (UC)n-enriched region of PNCTR was reported recently, and proteins involved in RNA splicing and its regulation, as well as other processes such as DNA repair, were identified (Yap et al. 2022). Expression of PNCTR is essential for cell survival, as depletion of PNCTR by siRNA in HeLa cells results in programmed cell death with induction of cleaved caspase 3 (Yap et al. 2018). High levels of PNCTR in PNC bodies have been detected in a variety transformed cell lines and are linked with splicing changes of PTBP1-regulated transcripts that contribute to increased cell growth and proliferation (Yap et al. 2018; Statello et al. 2021).

PAPAS

In contrast to pRNA and PNCTR RNAs, the lncRNA PAPAS (promoter and pre-rRNA antisense) is transcribed by Pol II in the antisense orientation of pre-rRNA (Fig. 4; Bierhoff et al. 2010). PAPAS transcripts are less abundant than pRNAs, likely in part because they are normally degraded by the nuclear exosome (Bierhoff et al. 2010). They are composed of a heterogeneous population of RNAs 12–16 kb in length that cover a portion of the IGS just 3′ to the 47S pre-rRNA, the 3′ ETS, pre-rRNA-coding region, 5′ ETS, and rRNA promoter and UCE/enhancer region (Bierhoff et al. 2010; Zhao et al. 2016b). Transcription of PAPAS, which is inhibited by the Pol II inhibitor α-amanitin but not by CX-5461, is not completely characterized, as the promoter and transcription start and termination sites are still elusive (Bierhoff et al. 2010, 2014a) and it is possible that there are multiple transcripts across this region. Consistent with this, Bierhoff et al. (2010) failed to map the 5′ end of PAPAS by RACE, suggesting there might be multiple transcription start sites (Fig. 4). Indeed, as the entire full-length PAPAS transcript has not been isolated, it is possible that there are multiple initiation and termination sites generating a collection of RNAs. It is also unknown whether PAPAS RNAs are polyadenylated like most Pol II transcripts.

Additional studies have examined PAPAS transcriptional regulation. PAPAS is up-regulated in growth-arrested cells but down-regulated in rapidly proliferating cancer cells (Zhao et al. 2018). High levels of PAPAS transcripts were also observed following certain cellular stimuli, such as heat shock, hypotonic stress, or serum starvation (Zhao et al. 2016a,b; 2018). Notably, increased Pol II occupancy at the 3′ end of the 28S rRNA-coding region was discovered following hypo-osmotic stress or heat shock, suggesting that PAPAS transcription may initiate near the 3′ end of the 47S pre-rRNA-coding region (Zhao et al. 2016a). A recent study showed that the RPA complex inhibits PAPAS expression. Depletion of RPA subunits significantly increased PAPAS levels in HeLa cells in an R loop-dependent manner (Feng and Manley 2021). The data indicate that under these conditions, PAPAS forms R loops overlapping the rRNA promoter/UCE region, and suggest that these contribute to PAPAS stability. Note that as with pRNAs, the region of PAPAS shown to form R loops has also been suggested to form triplex structures in vitro (Zhao et al. 2018). The same possible explanations as discussed above in the section concerning pRNAs may underlie these findings.

PAPAS function appears to dampen rRNA transcription when necessary. Given that PAPAS is transcribed antisense to pre-rRNA, it is not unexpected that PAPAS is transcribed from rRNA loci not expressing 47S RNA (Bierhoff et al. 2010). More generally, increased PAPAS levels correlate with reduced pre-RNA transcription in growth-arrested cells or in cells subject to certain stresses (Zhao et al. 2016a,b, 2018; Feng and Manley 2021).

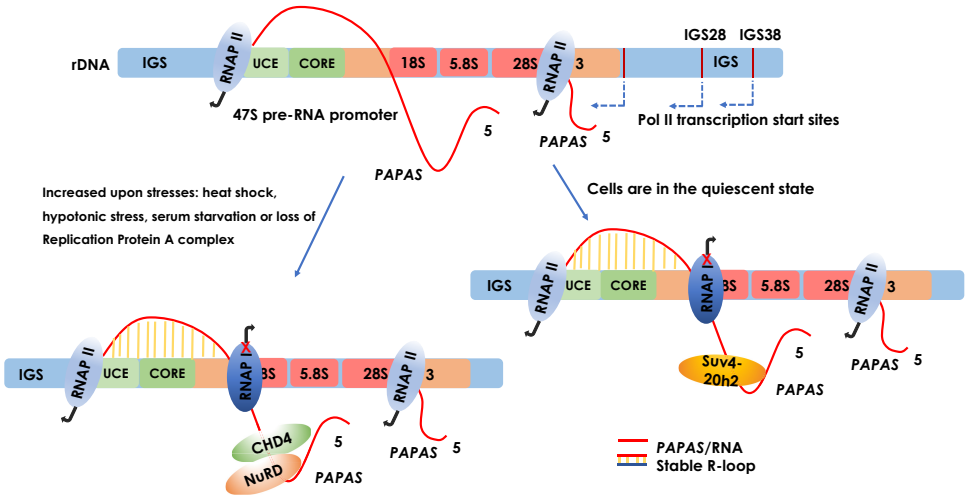

Figure 4. PAPAS RNAs are transcribed by Pol II. Transcription initiates from uncharacterized promoters likely located in rDNA IGS regions and continues across the 47S-coding and promoter regions. PAPAS transcripts can form R loops at the rDNA promoter/UCE regions, which contributes to repression of 47S transcription. In quiescent cells, PAPAS recruits Suv4-20h2 and establishes the repressive mark at the rDNA. Under stresses (e.g., heat shock, hypotonic stress, serum starvation, or the loss of RPA), PAPAS recruits the CHD4/NuRD complex that represses rDNA transcription.

This likely reflects PAPAS function in recruiting the repressive chromatin remodeling CHD4/NuRD complex through its unstructured A-rich region (see Fig. 4; Zhao et al. 2018) and in forming R loops at rDNA promoter and UCE elements (Feng and Manley 2021). In quiescent or growth-inhibited cells, pre-rRNA transcription is suppressed, but PAPAS is up-regulated and recruits the H4K20 methyltransferase Suv4-20h2 to the rDNA promoter, triggering H4K20 trimethylation and contributing to heterochromatin formation (Fig. 4; Bierhoff et al. 2014a,b).

TAR1 is a yeast nucleolar protein-coding gene

Nucleoli have long been known to be the site of pre-rRNA synthesis and, as we discuss here, more recently of transcription of many other noncoding RNAs. However, in nucleoli of *S. cerevisiae* and closely related species, rDNA can also serve as template for an mRNA. *Transcript antisense to ribosomal RNA 1* (*TAR1*) was discovered in a genome-wide study using transposon tagging with a *lacZ* reporter to identify previously uncharacterized ORFs. *TAR1* is transcribed antisense to the 25S rRNA-encoding region in the rDNA loci (see Fig. 1; Coelho et al. 2002). The transcript is polyadenylated and encodes a small 124-amino-acid polypeptide (Coelho et al. 2002). Interestingly, overexpressed Tar1 localizes to mitochondria and can suppress a mutation in the mitochondrial RNA polymerase that causes a respiration-deficient petite phenotype (Coelho et al. 2002). *TAR1* RNA levels are decreased by the Pol II repressor Sir2, as its expression is increased in a *SIR2*-null strain (Coelho et al. 2002; Li et al. 2006). Consistent with these findings, *TAR1* expression is tightly regulated and very low under normal growth conditions but up-regulated by glucose depletion, correlating with enhanced mitochondrial respiratory function, and down-regulated by mitochondrial dysfunction (Bonawitz et al. 2008). A yeast two-hybrid screen revealed that Tar1 interacts with Coq5, an enzyme involved in biogenesis of CoQ, providing another link with mitochondrial function (Bonawitz et al. 2008). It was further proposed that Tar1 functions in the yeast retrograde response linking mitochondrial defects to changes in gene expression, in which variations in *TAR1* copy number by generation of extrachromosomal circles, and hence in Tar1 levels, plays an important role (Poole et al. 2012). However, studies providing further insight into Tar1 function are lacking.

It is intriguing that *TAR1*-like genes appear to exist only in *S. cerevisiae* and closely related species. This may reflect unusual properties of budding yeast mitochondria (discussed by Poole et al. 2012). However, in any case, the existence of *TAR1* both further highlights the complexity of nucleolar transcription and illustrates a possible link between mitochondria, the primary energy source in the cell, and nucleoli, where ribosome biogenesis is a major energy sink. The importance of such a link, and whether mitochondrial/nucleolar interplay exists in other species, remain to be established.

Small antisense rRNAs

Genome-wide studies have detected small antisense rRNAs in different organisms. For instance, in *C. elegans*, antisense ribosomal siRNAs (risiRNAs) were identified and found to play a role in pre-rRNA transcription inhibition by activating the nuclear RNAi-mediated gene silencing pathway (Zhou et al. 2017a,b). risiRNAs are 22-nt ncRNAs, starting with a 5′ guanosine and complementary to 18S and 26S rRNAs. risiRNAs are a class of so-called 22G-RNAs that are produced by RNA-dependent RNA polymerase (RdRP), associate with an Argonaute (Ago) protein (Halic and Moazed 2009), and accumulate upon stress such as exposure to low temperature or UV radiation (Zhu et al. 2018). 22G-siRNAs are transported from the cytoplasm to the nucleus by an Ago-containing NRDE complex (Burkhart et al. 2011), typically inhibiting Pol II transcription and inducing trimethylation of H3K9 and H3K27 (Guang et al. 2008, 2010; Mao et al. 2015). risiRNAs act similarly except they are targeted to the nucleolus and inhibit rRNA transcription (Zhou et al. 2017b), possibly without altering histone methylation (Liao et al. 2021). The Ago protein NRDE-3 binds risiRNAs, forming risiRNA/NRDE complexes that translocate from the cytoplasm to the nucleolus, where they associate with and degrade pre-rRNAs (Zhou et al. 2017a,b; Zhu et al. 2018; Liao et al. 2021). This pathway can be activated by certain stresses or by mutations (*susi* mutations) (Zhu et al. 2018) that allow accumulation of aberrant rRNAs (Ustianenko et al. 2016; Zhou et al. 2017a; Zhu et al. 2018).

rRNA–siRNAs also accumulate in other organisms. These include the fission yeast *S. pombe*, the fungus *N. crassa*, and the vascular plant *A. thaliana*. In all cases, the underlying mechanisms appear similar to those just described for *C. elegans* (for review, see Zhou et al. 2017a; Yan et al. 2019). Notably, all these organisms contain RdRPs, which appear essential for synthesis of rRNA–siRNAs, explaining their apparent absence in vertebrates. It is possible that PAPAS-like RNAs have assumed their function in silencing rRNA transcription in response to stress or perhaps to defects in quality control (although this has not been investigated). It is also not inconceivable that PAPAS transcripts might be processed to generate risi-like RNAs, but there is no evidence to support this.

Transcripts from yeast E-pro and C-pro (rDNA noncoding promoters)

In yeast, the 35S and 5S ribosomal RNA genes are separated by IGS1 and IGS2. Notably, these IGS regions harbor two Pol II promoters: E-pro and C-pro. E-pro is a bidirectional Pol II promoter located in IGS1, and C-pro is located in IGS2 (see Fig. 1; Santangelo et al. 1988; Kobayashi and Ganley 2005; Li et al. 2006). E-pro transcription is involved in rDNA amplification and maintenance of rDNA copy number and is repressed by Sir2 (Fritze et al. 1997; Imai et al. 2000; Kobayashi and Ganley 2005; Saka

et al. 2013). *SIR2* disruption/repression (Iida and Kobayashi 2019) leads to increased E-pro transcription in both the ribosomal cohesion-associated region (CAR)/autonomously replicating sequence (rARS) direction and in the replication fork barrier (RFB) direction (see Fig. 1; Kobayashi and Ganley 2005). These two transcripts were named IGS1-F (transcribed in the CAR/rARS direction) and IGS1-R (transcribed in the RFB direction) (Houseley et al. 2007). Both are unstable, and IGS1-R, although not IGS1-F, is degraded by the TRAMP4/exosome complex, as the levels of IGS1-R are increased in *trf4*Δ mutant strains (Houseley et al. 2007). IGS1-R transcripts interact with the RBPs Nrd1 and Nab3 (Arigo et al. 2006; Thiebaut et al. 2006), which recruit the TRAMP/exosome complex and facilitate turnover (Houseley et al. 2007). Additionally, Nrd1/Nab3 together with the RNA:DNA helicase Sen1 (NNS complex) function to terminate transcription of Pro-E transcripts (Vasiljeva et al. 2008). These three mechanisms—Sir2 deacetylation, TRAMP4/exosome turnover, and NNS termination—keep E-pro transcript levels low, which in turn contributes to maintaining stable rDNA copy numbers. However, when these pathways are repressed/disrupted, E-pro transcription/transcript levels increase, resulting in fluctuations in copy number due to increased homologous recombination (for review, see Nelson et al. 2019; Vydzhak et al. 2020). This mechanism of rDNA copy number control is not shared among other well-studied eukaryotes (Nelson et al. 2019), which likely explains why IGS lncRNAs in other species have not been suggested to function like the E-pro RNAs.

C-pro also generates ncRNAs transcribed in the rRNA antisense direction. Like E-pro, C-pro activity is subject to Sir2-dependent silencing, as C-pro transcript levels are significantly increased in the absence of Sir2 (Santangelo et al. 1988; Li et al. 2006; Houseley et al. 2007; Foss et al. 2019). Functionally, in *sir2* mutant strains, C-pro transcription—specifically, elongating Pol II—displaces the Mcm2-7 replicative helicase complex from its loading site, the rARS, and causes it to bind a nearby site in a nucleosome-free region (Foss et al. 2019). Interestingly, by an unknown mechanism, this leads to premature activation of DNA replication. Notably, and whatever the exact mechanism, these findings provide an explanation for the link between transcriptional silencing and the late DNA replication that characterizes heterochromatic regions such as silenced rRNA loci.

Additional IGS lncRNAs function in nucleolar protein sequestration

In addition to the RNAs discussed above, a number of other mammalian IGS ncRNAs have been described, and their function in nucleolar homeostasis explored. Several human IGS lncRNAs are produced by Pol I from the same strand as rRNA transcripts (see Fig. 1; for review, see Pirogov et al. 2019; Vydzhak et al. 2020). These RNAs function in sequestering proteins containing a nucleolar detention sequence (NoDS) such as VHL, HSP70, DNMT1, and MDM2/PML (Mekhail et al. 2005, 2007; Audas et al. 2012) to the nucleolus under a variety of conditions; e.g., heat shock or induction of acidosis (Audas et al. 2012, 2016; Jacob et al. 2012, 2013). IGS28 RNA is synthesized from the rDNA IGS28 region at low pH, is up-regulated during acidosis, and functions to sequester or immobilize NoDS-containing proteins in nucleoli (Audas et al. 2012). Notably, two distinct IGS ncRNAs—IGS16 and IGS22 RNAs—are up-regulated upon heat shock, but not acidosis, and function in a complementary way to sequester HSP70 in the nucleolus during heat shock (Audas et al. 2012; Jacob et al. 2013). Disruption of one IGS locus does not affect the function of the other IGS RNA, as they use different promoters responding to different stresses (Audas et al. 2012).

Sequestration of susceptible proteins by the IGS RNAs appears to involve sequence-specific RNA binding. The NoDS motif constitutes an RNA binding domain that interacts directly with the IGS RNAs, and such interactions are required for sequestration (Audas et al. 2012). This RNA-mediated sequestration or immobilization, based on photobleaching experiments, leads to reversible accumulation of target proteins in amyloid bodies (A bodies) (Audas et al. 2016; Wang et al. 2019). The relevant regions of these lncRNAs appear to be "low-complexity" extended dinucleotide repeats, which are suggested to nucleate formation of A bodies such that the NoDS is now referred to as the amyloid-converting motif (ACM) (Wang et al. 2018). Since the IGS RNAs appear to function largely in *cis* (Audas et al. 2012), it is conceivable that they form R loops that contribute to sequestration (discussed in Vydzhak et al. 2020). In any case, these RNAs play an important role in regulating protein activity by nucleolar sequestration.

IGS antisense lncRNAs modulate rRNA synthesis via R loop 'shields'

Recently, another layer of rRNA transcriptional control by non-rRNA lncRNAs was proposed. Analysis of strand-specific transcription of IGSs revealed transcription in both sense and antisense directions to produce sense intergenic ncRNAs (sincRNAs) and antisense intergenic ncRNAs (asincRNAs). sincRNAs appear to be transcribed by Pol I, as they are sensitive to low doses of the general transcriptional inhibitor actinomycin-D, while asincRNAs are transcribed by Pol II, as their levels are reduced by flavopiridol or α-amanitin (Abraham et al. 2020). Furthermore, elongating forms of Pol II (phosphorylated on serine 2 of the C-terminal domain of the Pol II largest subunit [S2-P]) were detected within nucleoli by immunofluorescence and along rDNA by ChIP, while Pol II with S2-P and/or S5-P, which is associated more with initiating Pol II (Hsin and Manley 2012), was detected across the rDNA locus. The highest levels of S5-P were observed at IGS regions IGS28 and IGS38, suggesting that these regions may contain asincRNA TSSs (Fig. 1). These results both confirm the well-established presence of transcribing Pol II in mammalian nucleoli (e.g., see Bierhoff et al. 2010) and suggest the existence of additional Pol II transcribed IGS lncRNAs.

Abraham et al. (2020) proposed a novel function for asincRNA transcription, which is to generate an R loop

"shield" on rDNA that functions to block sincRNA transcription, which otherwise in some way interferes with pre-rRNA synthesis and processing. Supporting this model, levels of sincRNAs are increased when asincRNA/R loops are repressed (e.g., by α-amanitin, RNase H, or heat shock), and this in turn correlates with reduced rRNA expression and nucleolar disorganization. That these effects are due at least in part to sincRNAs is supported by the fact that ASOs targeting these RNAs can alleviate inhibition. Finally, Senataxin (SETX), the human homolog of yeast Sen1, appears to function in maintaining the asincRNA "shield," as its loss reduced asincRNA/R loops, increased sincRNAs, and reduced pre-rRNA transcription.

The above findings raise a number of interesting questions concerning how the asincRNA/sincRNA interplay fits with the documented functions of other nucleolar lncRNAs, and more generally how this complex network of non-rRNA transcripts might cooperate to maintain nucleolar homeostasis. One notable example is the relationship between asincRNAS and PAPAS transcripts, which was not addressed by Abraham et al. (2020). Both of these distinct groups of antisense transcripts are transcribed by Pol II and therefore inhibited by α-amanitin. Inhibition of PAPAS should increase pre-rRNA transcription by reducing PAPAS-mediated heterochromatinization (Bierhoff et al. 2014a,b; Zhao et al. 2018), while reduced asincRNA transcription correlates with decreased rRNA expression, apparently by "unleashing" repressive sincRNAs. The physiological significance of these apparently contrasting responses to reduced Pol II activity is unknown. On the other hand, heat shock also decreases asincRNA expression but increases PAPAS levels (Zhao et al. 2016b), both of which correlate with reduced rRNA expression, suggesting possible complementary or redundant functions. Another intriguing issue is how sincRNAs relate to the multiple other known Pol I transcribed IGS lncRNAs (see above). Are they in some cases the same? Are all these RNAs distinct, and each with its own function(s)? Finally, the roles of R loops and SETX in modulating rRNA expression are complicated by the findings of Abraham et al. (2020). Their results suggest that SETX is required to form the asincRNA/R loop "shield" that blocks the repressive effects of sincRNAs. This is in contrasts to other results suggesting that formation of nucleolar R loops is in general enhanced by loss of SETX (Feng and Manley 2021; Jurga et al. 2021), and that formation of R loops (e.g., involving pRNAs and PAPAS) is associated with silencing rather than enhancement of pre-rRNA transcription (Zhao et al. 2018; Feng and Manley 2021).

Taken together, these studies demonstrate the existence of a complex network of ncRNAs and R loops at the rDNA locus that participate in control of rRNA synthesis in response to different cellular growth conditions and stresses in ways that remain to be fully understood.

Conclusions

The complicated network of nucleolar transcribed non-rRNAs described here is remarkable in many respects. These RNAs, with one species-specific exception, are all noncoding, are transcribed in both sense and antisense direction relative to pre-rRNA, fine-tune rRNA transcription, and contribute to normal cell growth and differentiation, as well as when dysregulated to cancer progression and metastasis. In response to various cellular stresses, including rDNA damage and R loop accumulation leading to genomic instability, heat shock, osmotic stress, and other stresses including mitochondrial stress, nucleolar ncRNA levels are often elevated and take different roles to combat these conditions. These include many of the known functions of lncRNAs, including recruitment of chromatin-modifying enzymes, sequestration of regulatory proteins such as transcription and splicing factors, and formation of RNA structures such as R loops. These RNAs arise from both Pol I and Pol II transcription, and a notable feature is that sense transcription is almost always by Pol I, while antisense transcription is by Pol II. This could reflect in some way heavy loading of pre-rRNA transcribing Pol I molecules on the sense strand, although why Pol II is apparently restricted to the antisense strand is unclear. Given our expanding appreciation of this RNA network and its roles in nucleolar regulation and maintaining cellular homeostasis, it is likely that more exciting and unexpected discoveries regarding transcription in nucleoli will be forthcoming.

Competing interest statement

The authors declare no competing interests.

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