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DISSERTATION

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Roles of the Novel 5'-Polynucleotide Kinase Nol9 in Ribosome Synthesis

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Verfasserin / Verfasser:	Katrin Heindl
Matrikel-Nummer:	0105648
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Betreuerin / Betreuer:	Javier Martinez

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Abstract

In a dividing cell, about 80% of the cellular RNA consist of ribosomal RNAs (rRNAs), the core components of the ribosomes. Efficient protein production relies on the sufficient availability of ribosomes; therefore indefectible rRNA processing is fundamental to every cell.
The 18S, 5.8S and 28S rRNAs are transcribed in the nucleolus by RNA polymerase I as a single polycistronic precursor RNA and liberated by a complex series of endo- and exonucleolytic cleavage events. Eventually, they are assembled together with the 5S rRNA and a plethora of ribosomal proteins to form the ribosomes.

In my PhD work, I identified the first nucleolar polynucleotide kinase Nol9/Grc3 and investigated its involvement in two different aspects of rRNA biogenesis: 1. RNA Polymerase I transcription termination; and 2. processing of the large subunit rRNAs. In yeast, Grc3 is essential for efficient transcription termination in the Rat1-dependent 'torpedo mechanism' thereby enabling rapid recycling of RNA polymerase I. In human cells, Nol9 is required for the processing of 5.8S and 28S rRNAs, the components of the large 60S ribosomal subunits, most likely at a Xrn2-dependent processing step.
These findings intrigue a potential interplay between the polynucleotide kinase Nol9/Grc3 and the 5'-3' exonuclease Xrn2/Rat1. Given the involvement of the nucleolus in a growing number of RNA metabolic pathways and the fact that Nol9/Grc3 can efficiently phosphorylate single and double stranded RNA and DNA substrates, Nol9/Grc3 potentially has additional roles in RNA metabolism.

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Zusammenfassung

In einer sich teilenden Zelle nehmen ribosomale RNAs 80% der gesamten zellulären RNA ein. Sie sind die Hauptkomponente der Ribosomen, grossen Komplexen aus RNA und Proteinen, die als “Protein-Fabriken” der Zelle eine zentrale Aufgabe ausüben. Die korrekte und effiziente Biosynthese der ribosomalen RNA ist daher grundlegend für jede Zelle.
Im Nukleolus werden drei der vier ribosomalen RNAs (rRNAs), 18S, 5.8S und 28S, gemeinsam von der RNA Polymerase I zu einem einzelnen polyzistronen Precursor transkribiert. In einer geordneten Aufeinanderfolge von endo- und exonukleolytischen Aktivitäten maturieren diese rRNAs und werden letztendlich gemeinsam mit der 5S rRNA und einer Vielzahl von ribosomalen Proteinen zum Ribosom zusammengefügt.

Während meiner Doktorarbeit habe ich die erste nukleoläre Polynukleotid-Kinase Nol9/Grc3 identifiziert und ihre Funktion studiert. Nol9/Grc3 ist in zweierlei Hinsicht an der Maturierung von ribosomalen RNAs beteiligt: 1. *S. cerevisiae* Grc3 ist essenziell für eine effiziente Beendigung der Transkription von rRNA Genen als Teil des Rat1-abhängigen ‘Torpedo-Mechanismus” und somit wesentlich für die rasche Wiederverwendung der RNA Polymerase I. 2. Humanes Nol9 hat eine Funktion bei der Prozessierung der 5.8S und 28S ribosomalen RNA aus ihrem gemeinsamen Precursor, möglicherweise in einem Xrn2-abhängigen Prozessierungsschritt. Diese Ergebnisse beleuchten ein moegliches Zusammenspiel der Polynukleotid-Kinase Nol9/Grc3 und der 5’-3’ Exonuklease Xrn2/Rat1.
Berücksichtigt man die wachsenden Berichte über zusätzliche Aufgaben des Nukleolus im RNA Metabolismus abseits der Maturierung ribosomaler RNA und die Aktivitaet von Nol9/Grc3 an sowohl einzel- als auch doppelsträngigen RNA- und DNA-Substraten, zeichnen sich mögliche weitere Funktionen für die Polynukleotid-Kinase Nol9/Grc3 ab.

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INTRODUCTION

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Ribosomes are large ribonucleoproteins (RNPs) that translate the genetic information from RNA into protein. Protein production is fully dependent on the sufficient availability of ribosomal subunits as dictated by the instantaneous demand of the cell. The synthesis of these subunits requires the concerted action of all three RNA polymerases; RNA Pol I transcribes the 35/45S precursor of 18S, 5.8S and 25/28S rRNAs. RNA polymerase III generates 5S rRNA, while RNA polymerase II transcribes the mRNAs for the ribosomal proteins. All together, up to 80% of the RNA synthesis capacity of a cell can be channeled into the biogenesis of ribosomes. A plethora of non-ribosomal proteins are therefore devoted to the proper and equimolar synthesis of the individual components of a ribosome (Andersen et al, 2005).

JR Warner has compiled data from yeast cells that reveal the impressive demands a growing yeast cell has to meet (Warner, 1999); the ratio of RNA to DNA culminates to 50:1 with rRNAs contributing 80% of the total RNA in a fast growing yeast cell. As much as 60% of total transcription is devoted to rRNAs, 50% of RNA polymerase II transcription and 90% of mRNA splicing are assigned to ribosomal proteins, producing 2000 ribosomes per minute.
Overall, ribosome biogenesis is one of the main limitations to cell growth.

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1. THE rDNA LOCUS

To synthesize vast numbers of ribosomes, the cell needs to produce extensive amounts of rRNAs and ribosomal proteins. Whereas protein production can be upregulated on the level of transcription and translation, the RNA output relies solely on transcription. Therefore, rDNA genes are typically arranged as head-to-tail tandem repeat arrays in discrete chromosomal clusters, or Nucleolus organizer regions (NORs), ranging from 100 to 1000 rDNA repeats in total (Prokopowich et al, 2003). The human rDNA genes are located on the p-arm of the acrocentric chromosomes 13, 14, 15, 21 and 22 in a telomere-to-centromere orientation (Figure 1A) (Henderson et al, 1972). This positioning separates rDNA loci physically from genes transcribed by RNA polymerases II and III. Further reinforcement of this isolation is provided by heterochromatic repetitive satellite DNA adjacent to rDNA loci (Choo et al, 1990; Henderson et al, 1972; Shiels et al, 1997; Tagarro et al, 1994). Typically, 20 to 40 rDNA copies are clustered on each chromosome; however, as these loci are predominant sites of recombination, number and location vary among and even within species (Caburet et al, 2005).

Canonical rDNA units consist of the coding region and a non-coding intergenic spacer (IGS) (Figure 1B) (Sylvester et al, 1986). Throughout all eukaryotic species, the genes encoding the 18S, 5.8S and 25/28S rRNAs are organized into a single transcription unit comprising a 5' external transcribed spacer (ETS), 18S rDNA, internal transcribed spacer 1 (ITS1), 5.8S rDNA, ITS2, 25/28S rDNA and 3'ETS (Sylvester et al, 2004; Sylvester et al, 1986). The coding regions are separated by IGS, which contain the promoter and terminator regions as well as the spacer promoter and terminator. The majority of the approximately 30kb long IGS, however, is devoid of regulatory elements, but consists of simple sequence repeats and transposable elements (Sylvester et al, 2004). Several of these repetitive elements comprise short sequences homologous to stretches in the rDNA promoter and enhance transcription from both the spacer and the rDNA promoters (Putnam & Pikaard, 1992; Reeder, 1984).

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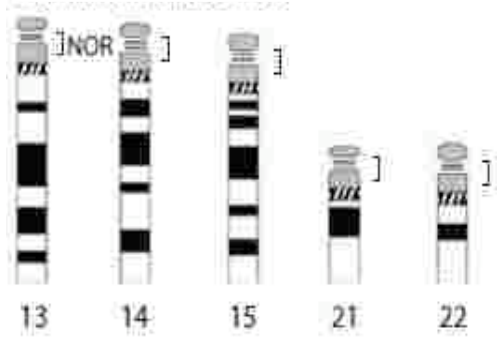
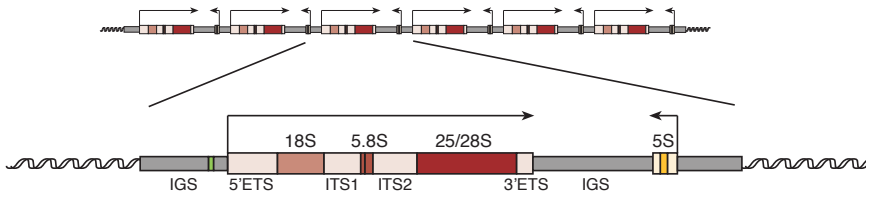
A - human chromsomes



B - canonical rDNA unit



C - non-canonical rDNA repeats

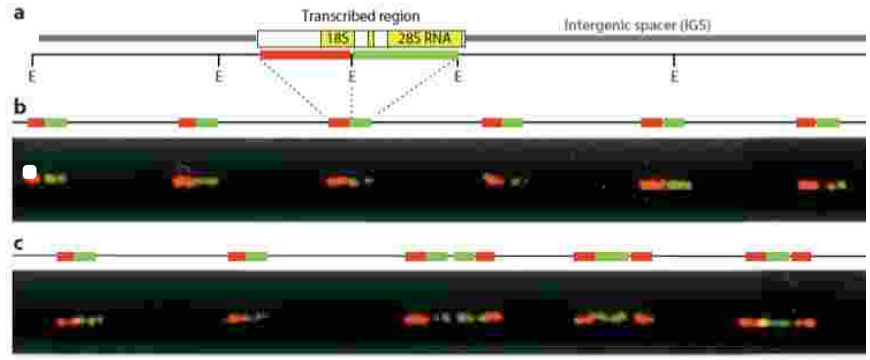


Figure 1. The rDNA locus. **A** Human rDNA genes are clustered on 5 acrocentric chromosomes, forming secondary constrictions. Due to their role in nucleolar assembly they are also called Nucleolus Organizer Regions (NORs) (Picture from (McStay & Grummt, 2008). **B** rDNA genes are organized in arrays of tandem repeats. They consist of the coding region, containing a 5' external transcribed spacer (ETS), 18S, internal transcribed spacer (ITS) 1, 5.8S, ITS2, 25/28S and 3'ETS, and the separating intergenic spacers (IGS). Yeast encodes the 5S gene in the opposite direction downstream of the coding region within the IGS . **C** rDNA repeats are a prominent spot for recombination and rearrangement resulting in a mosaic of canonical and non-canonical, palindromic rDNA repeats (Figure from (McStay & Grummt, 2008). a, illustration of one rDNA unit and the location of the in situ probes; b, canonical rDNA repeats; c, canonical and non-canonical rDNA repeats.

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Including the IGS, the human rDNA unit spans 44kb, the transcription unit itself is 13kb in length and gives rise to a 45S transcript (Sylvester et al, 2004). The 18S and 28S rRNAs have highly conserved segments that are invariant in length and secondary structure, but may fluctuate in nucleotide sequence and fulfill catalytic functions (Noller, 1984; Raue et al, 1988). Additionally, a number of variable segments are described, whose size and sequence may vary even within one organism. To date, no function could be assigned to these variable stretches.
Interestingly, NORs contain a mosaic of canonical and non-canonical rDNA repeats (Caburet et al, 2005). Approximately one third of all repeats form palindromic, non-canonical structures (Figure 1C).

The human 5S rRNA is encoded on chromosome 1 in an array of 100 copies, transcribed by RNA polymerase III and transported into the nucleolus, where it is assembled together with 5.8S and 28S rRNAs into the large ribosomal subunit (Little et al, 1989). The yeast 5S rRNA coding region, in contrast, is located within the IGS of the rDNA locus in the opposite orientation (Figure 1B); however, its transcription and processing is completely uncoupled from the 18S-5.8S-28S unit (Drouin & de Sa, 1995).

1.1. rDNA epigenetics

rDNA copy number correlates positively with the genome size and ranges from 100 in yeast to 1000 in plants (Prokopowich et al, 2003). Nevertheless, the genome seems to encode more rDNA units than actually needed; a significant fraction of rDNA genes remains transcriptionally silent (Grummt & Pikaard, 2003; Santoro, 2005). Two different classes of rDNA clusters seem to co-exist, being either entirely inactive or consisting of a mosaic of inert and actively transcribed rDNA units (Raska et al, 2004). Doubling the rDNA amount in cultured lymphoblasts by addition of a 14p+ chromosome does not lead to an increase of rRNA transcripts, suggesting that rDNA inactivation is a tightly regulated mechanism (Tantravahi et al, 1981).

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Even in proliferating cells with a high demand of ribosomes, a significant fraction of rDNA is silenced.

Inactive and active NORs are characterized by two different epigenetic states based on chromatin structure (McStay & Grummt, 2008). The specific chromatin structure is maintained throughout cell cycle and is inherited from one generation to the next.

1.1.1. Nucleosome pattern

Active NORs coincide with undercondensed chromatin forming 'secondary constrictions'. Compared to the adjacent heterochromatic satellite DNA, NORs are 10 fold less condensed (Heliot et al, 1997) appearing as a gap on chromosome spreads. Inactive NORs, in contrast, are packaged similarly to the surrounding heterochromatin and appear as condensed foci devoid of Pol I, its specific factors and secondary constrictions (McStay & Grummt, 2008).

The state of nucleosome packaging of active NORs is not completely clear. Early reports demonstrated a complete absence of nucleosomes at transcribed rDNA units, while silenced regions display regularly spaced nucleosomes (Conconi et al, 1989). Recent studies revised the model of a nucleosome-free rDNA unit in yeast and suggested a dynamic chromatin structure with unphased nucleosomes at actively transcribed rRNA genes (Jones et al, 2007; Tongaonkar et al, 2005). Intergenic spacers are constitutively nucleosomal in both active and silenced gene copies (Dammann et al, 1993).

The nucleosome consists of a histone octamer comprising two copies each of histones H2A, H2B, H3 and H4 (Kornberg & Lorch, 1999). A number of modifications within the N- and C-terminal tails confer transcription silencing or activating features to histones, depending on the pattern of the respective acetylation, methylation, phosphorylation or SUMOylation (Bartova et al, 2008). The rDNA unit comprises several terminator elements within IGS, one of them, T_0, upstream of the actual transcription start site of the coding region (Figure 3) (Reeder, 1999). TTF-I, a multifunctional protein implicated in transcription termination, binds to T_0 and thereby triggers structural alterations of the surrounding chromatin correlating with transcription activation (Henderson & Sollner-Webb, 1986; Langst et al, 1998;

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Langst et al, 1997; McStay & Reeder, 1990). TTF-I recruits Cockayne syndrome protein B (CSB) to the promoter-proximal termination element (Bradsher et al, 2002), a DNA-dependent ATPase implicated in chromatin remodeling (Beerens et al, 2005; Citterio et al, 2000). CSB in turn associates with G9a, a histone methyltransferase, generating mono- and dimethylated lysines at position 9 in H3 (H3K9me and H3K9me2, respectively) (Tachibana et al, 2002). H3K9 methylation has established roles in heterochromatin formation (Bannister et al, 2001; Peters et al, 2003; Rice et al, 2003). However, in the case of rDNA, H3K9me2 characterizes actively transcribed regions (Langst et al, 1998; Langst et al, 1997). CSB together with G9a therefore might promote transcription elongation by depositing a specific histone modification pattern. Interestingly, TTF-I also interacts with Nucleolar remodeling complex (NoRC), which is known to induce nucleosome sliding (Strohner et al, 2001). NoRC acts as a repressor of rDNA transcription by recruiting DNA methylases, Histone deacetylases and methyl transferases, which are factors linked with epigenetic silencing (Frescas et al, 2007; Santoro et al, 2002; Strohner et al, 2004; Zhou et al, 2002). In the nucleosome, approximately 147bp of DNA are wrapped around the core protein octamer (Richmond & Davey, 2003). At potentially active rDNA genes, nucleosome distribution covers base pairs -150 to the transcription start site, while silent genes display occupancy until 22bp into the transcribed region (Li et al, 2006b; Simpson, 1991). The different position may either facilitate or disfavor transcription factor binding, respectively. Indeed, in the silencing nucleosome position, the core promoter lies within the nucleosome, thus preventing transcription initiation (Li et al, 2006b). NoRC was identified as the major determinant shifting the nucleosome into the silencing position by recruiting histone-remodeling factors in an RNA-dependent manner (Li et al, 2006b; McStay & Grummt, 2008).

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In summary, TTF-I associates with the promoter-proximal terminator and recruits both the transcription activating complex CSB-G9a and the repressor NoRC (McStay & Grummt, 2008). The balance between CSB-G9a and NoRC association to rDNA repeats determines the ratio between active euchromatic and repressed heterochromatic genes.

1.1.2. DNA methylation

rDNA genes are exceptionally rich in methylated CpG dinucleotides, a structure conferring an epigenetic mark associated with gene silencing (Klose & Bird, 2006; Meehan & Stancheva, 2001). Methylated DNA is predominantly present in promoters and enhancers of inactive rRNA genes (Stancheva et al, 1997). A variety of different studies have confirmed the correlation between methylated rDNA promoters and gene silencing in mouse (Bird et al, 1981a; Bird et al, 1981b; Santoro & Grummt, 2001). In addition, compounds counteracting CpG methylation are able to re-activate silenced rDNA copies (Santoro & Grummt, 2001). Interestingly, methylation of a single CpG islets at position -133 is sufficient to impair binding of the Pol I-specific transcription factor UBF and therefore prevents transcription (Santoro & Grummt, 2001).

In human cells, rDNA promoters exhibit either a mosaic methylation pattern or are largely devoid of methylation, also referred to as 'hypomethylated' (Ghoshal et al, 2004). Highly proliferative cell lines of human liver carcinoma show significant hypomethylation of rDNA promoters, suggesting similarity to the mouse mechanism (Ghoshal et al, 2004; Majumder et al, 2006). Two different classes of hypomethylated promoters co-exist; the first fraction is completely devoid of methylated CpG islets and associates with the transcription factor UBF and Pol I and thus most likely marks active rRNA genes (Brown & Szyf, 2007). In contrast, the second class is unmethylated only at the core promoter and lacks Pol I association. This latter portion might represent a 'primed' promoter with UBF already associated to allow for a rapid switch from inactive to active transcription.

1.2. rDNA in genome stability

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Individuals who have lost up to 20% of their rDNA sequence due to Robertsonian translocation, which eliminates rDNA from two acrocentric chromosomes, can develop normally (Stahl et al, 1983). In yeast, only half of the rDNA loci are actively transcribed (Dammann et al, 1993; French et al, 2003), a mutant strain bearing only one seventh of the wild type rDNA copy number is still viable (Takeuchi et al, 2003).

This raises the question of an additional role of rDNA loci aside ribosome production, potentially in the maintenance of genome integrity (Kobayashi, 2008). rDNA loci are subjected to repeated recombination, which renders the copy number highly fluctuant (Chindamporn et al, 1993; Cowen et al, 2000; Rustchenko et al, 1993). In yeast, this fluctuation is related to the Fob1 protein, which inhibits replication fork progression at the replication fork barrier present within rDNA units (Kobayashi, 2003). As a consequence, Fob1p causes DNA double strand breaks and thus induces recombination (Burkhalter & Sogo, 2004; Kobayashi et al, 2004). In a FOB1 mutant yeast strain, rDNA copy number variation is abolished (Kobayashi, 2003). Another factor involved in rDNA stability is the histone deacetylase Sir2p (Fritze et al, 1997; Gottlieb & Esposito, 1989; Imai et al, 2000). Yeast strains depleted of Sir2p display high variation in rDNA copy number (Kobayashi et al, 2004). Sir2p is a repressor of a non-coding bidirectional promoter (Kobayashi & Ganley, 2005). In the absence of Sir2p, transcription from this promoter destabilizes cohesin associated to rDNA resulting in unequal sister chromatid recombination and consequently, highly variable rDNA copy number. Both Fob1p and Sir2p are proteins related to cellular aging. One *S. cerevisae* cell buds approximately 10 daughter cells before it shows the first signs of senescence, characterized by cell enlargement, extension of the cell division cycle and sterility (Jazwinski, 2001). 10 buddings later, the mother cell dies. One trigger of senescence was reported to be extrachromosomal rDNA circles (ERC) (Sinclair & Guarente, 1997). These protrusions from rDNA loci accumulate specifically in the mother cell and are promoted by rDNA instability. FOB1 mutant strains show less ERC due to the inactivation of rDNA recombination and as a consequence, a lifespan increase by 60% (Defossez et al, 1999; Takeuchi et al, 2003). Contrarily, SIR2 mutants promote recombination and subsequently accumulation of ERC and show a decreased lifespan of 50% (Kaeberlein et al, 1999). The senescence phenotype can be rescued to a certain extent by insertion of an additional SIR2 copy. As a conclusion, Kobayashi hypothesized, that rDNA stability dictates the general stability of the whole genome (Kobayashi, 2008). Although the preliminary results are intriguing, solid evidence is needed to emphasize this model, especially since asymmetric cell

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division is not universal and therefore the model is not applicable to all species, in particular multicellular organisms. However, cell lines of various human carcinoma displayed hypomethylation of rDNA promoters, possibly leading to re-activation of silenced genes (Majumder et al, 2006). This hypomethylation correlates with decreased genomic stability and therefore suggests a role for silenced rDNA copies in genome integrity potentially by rendering the region less accessible to recombination.

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2. rRNA TRANSCRIPTION

Nuclear transcription is accomplished by three different machineries in eukaryotes that are structurally and functionally related, RNA polymerases I, II and III (Cramer et al, 2008). rDNA genes are solely transcribed by RNA polymerase I.

In Miller spreads, the occupancy of fully extended rDNA repeats by transcribing RNA polymerase I can be visualized (Figure 2) (Chooi & Leiby, 1981). Along each rDNA unit, the nascent transcripts protrude from the rDNA template in an arborescent or "Christmas tree"-like manner (Miller & Beatty, 1969). In highly proliferative cells, rDNA genes are engaged with up to one active RNA polymerase I every 130bp.

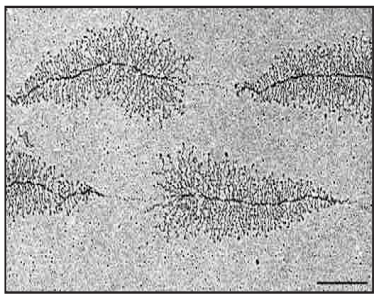

Figure 2. Electron micrographs of active rDNA units. rRNA transcription can be visualized by 'Miller spreads' in *Xenopus* oocytes. As RNA polymerase I transcribes along the DNA, depicted as the horizontal axis, the nascent rRNA precursors protrude from the template. Multiple RNA polymerases are engaged to one rDNA gene at once, resulting in the characteristic 'Christmas tree'-appearance (Picture from (Raska et al, 2006b).

2.1. RNA polymerase I

The core of mammalian RNA polymerase I (Pol I) is a large complex of at least 12 subunits with 2 or 3 associated factors (Hanada et al, 1996; Hannan et al, 1998; Russell & Zomerdijk, 2006). Of these, 5 polypeptides are common to all RNA polymerases, 2 are shared with RNA polymerase III and 7 factors are Pol I-specific (Reeder, 1999). In contrast, for yeast RNA Pol I fourteen subunits have been identified (Gadal et al, 1997; Song et al, 1994); 5 core subunits similar to the prokaryotic α, β, β' and ω polypeptides; 5 proteins common to all three RNA polymerases; and 4 specific subunits (Nomura et al, 2004). There exists a significant degree of sequence conservation between the homologous mammalian and yeast subunits, as well as between the subunits of RNA Pol I, II and III (Cavanaugh et al,

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2004). A number of proteins have been shown to closely interact with RNA polymerase I (Cavanaugh et al, 2004), among them the transcription factors UBF and SL1 (see 2.2.). Some of the RNA Pol I associated factors are also known as pre-requisites in Pol II transcription, for example TFIIH (Conaway & Conaway, 1993; Iben et al, 2002).

2.2. Transcription initiation

The mammalian rDNA promoter is of bipartite structure consisting of two functionally homologous elements (Figure 3, left panel); a core promoter adjacent to the transcription start site and an upstream control element (UCE) approximately 100nt further upstream (Haltiner et al, 1986; Learned et al, 1986). The core promoter is sufficient for *in vitro* transcription (Read et al, 1992).

RNA polymerase I transcription requires prior formation of a pre-initiation complex (PIC) at the rDNA promoter (Bell et al, 1988; Grummt, 2003; Moss & Stefanovsky, 2002; Paule & White, 2000). Binding of the dimeric Upstream binding factor (UBF) and the selectivity factor SL1 to the core promoter and UCE recruits initiation-competent RNA polymerase I, which is characterized by the presence of TIF-IA (Bodem et al, 2000; Miller et al, 2001). SL1 is a 300kDa protein complex that confers promoter-specificity to the Pol I transcription machinery (Comai et al, 1992). It comprises TATA-binding protein (TBP) and Pol I-specific TBP-associated factors (TAF_Is) (Comai et al, 1994; Denissov et al, 2007; Gorski et al, 2007; Heix et al, 1997; Zomerdijk et al, 1994). While TBP is a subunit common to all three nuclear transcription machineries, TAF_Is do not display homology to the TAFs of RNA polymerase II or III (Comai et al, 1994; Radebaugh et al, 1994). The TAF_Is mediate the specific interaction between the promoter sequence and RNA polymerase I by binding to UBF and TIF-IA, the characteristic component of initiation-competent Pol I (Bodem et al, 2000; Miller et al, 2001; Yuan et al, 2002). UBF is a highly abundant transcription factor ($5x10^5$ molecules per cell) comprising several DNA-binding high mobility group (HMG) domains (Jantzen et al, 1990). Two isoforms, UBF1 and UBF2, form hetero- or homodimers that bind to the UCE and possibly also to the core

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promoter (O'Mahony & Rothblum, 1991). UBF association induces bending of the DNA creating a UBF-DNA platform referred to as 'enhancerosome' (Bazett-Jones et al, 1994; O'Mahony et al, 1992a; Putnam et al, 1994). This disc is 19nm in diameter and thus enables interaction between the UCE-bound UBF and the SL1 associated to the core promoter (Bazett-Jones et al, 1994; Ridsdale et al, 1998).

In yeast, the transcription factor composition driving RNA polymerase I is different (Figure 3, left bottom panel) (Nomura et al, 2004). Instead of the dimer UBF, the yeast multiprotein complex Upstream activation factor (UAF) binds to the upstream element. While UBF displays general DNA binding due to its HMG domains, UAF associates sequence-specifically and thus solely with the upstream element. At the core promoter, the trimeric core factor (CF) functionally resembles mammalian SL1 mediating recruitment of the transcription competent form of RNA polymerase I via interaction with the TIF-IA homolog Rrn3p (Bodem et al, 2000).

F**igure 3. RNA polymerase I transcription initiation and termination**. Yeast and mammalian mechanisms for transcription initiation and termination of Pol I are significantly similar. In mammals, the upstream intergenic spacer (IGS) comprises the spacer promoter (SP) and its Terminator (T_0) as well as the bipartite rDNA promoter. The Upstream control element (UCE) and core promoter are recognized by the transcription factors Upstream binding factor (UBF) and Selectivity factor (SL1). UBF binding induces DNA bending, which promotes interaction between the transcription factors and, ultimately, recruitment of the initiation-competent, TIF-IA-containing RNA polymerase I subpopulation. Similarly, the yeast promoter region is of bipartite structure, consisting of the Upstream Element (UE), binding site for the Upstream Activator Factor (UAF), and the core promoter, which recruits Core factor and eventually Rrn3p-associated RNA polymerase I.

The yeast terminator region comprises a T-rich element, 2 terminators (T_1) and (T_2) separated by the Reb1 binding site and a replication fork barrier (RFB). Reb1 pauses the transcribing RNA polymerase I, while the upstream T-rich element destabilizes RNA-template interaction. The RNAse III enzyme Rnt1p cleaves within a hairpin structure 3' to the 25S rRNA and thereby creates the entry point for the 5' to 3' exonuclease Rat1p which chases after the polymerase. All the above mechanisms cooperate in terminating RNA Pol I at T_1. In case of read-through, transcription is terminated at T_2 or by Fob1p associated with RFB. Mammals have a more complex terminator pattern, yet termination might employ similar mechanisms. 10 terminators are located within IGS, which recruit the roadblocks TTF-I. PRTF promotes polymerase release.

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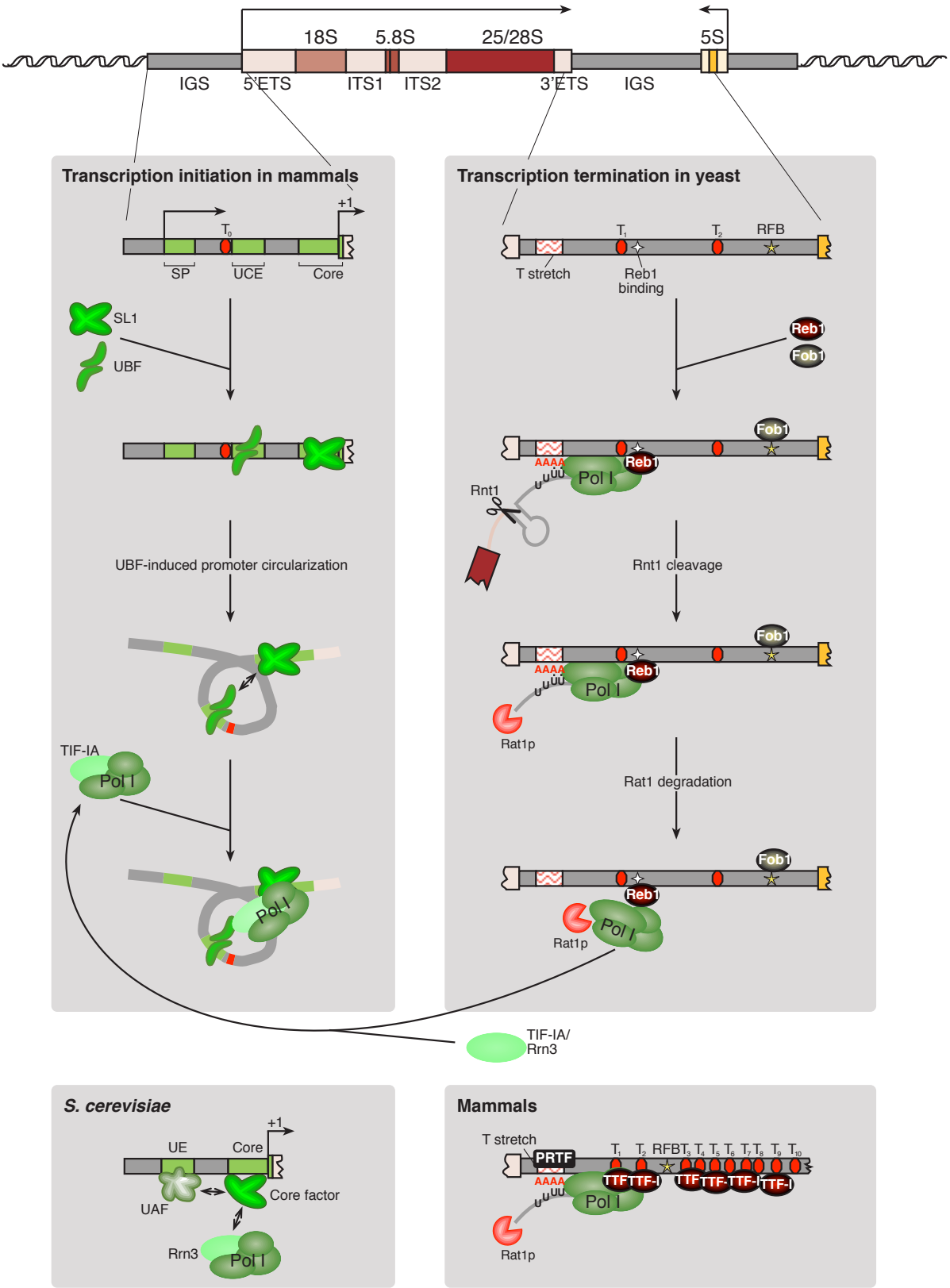
Figure 3. RNA polymerase I transcription initiation and termination



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2.3. Transcription termination

Transcription termination is crucial for the release of RNA polymerases from their transcripts. Defective termination can lead to interference with transcription of downstream genes and also depletes the pool of available RNA polymerases, ultimately preventing reinitiation and new transcription (Greger et al, 2000). The three nuclear polymerases employ different strategies for transcription termination, yet share common features (Richard & Manley, 2009). Transcription termination is studied best for RNA polymerase II, which is intimately linked with mRNA 3' processing (Buratowski, 2005). Although RNA Pol I termination involves fewer factors than Pol II termination, the mechanism have surprising similarities (Kawauchi et al, 2008; Richard & Manley, 2009).

Ninety percent of yeast RNA Polymerase I terminate at the first terminator, T_1, which is located right upstream of the Reb1 binding site (Figure 3, right panel) (Ju et al, 1990; Lang et al, 1994; Lang & Reeder, 1993). Reb1p is suggested to act as a roadblock, pausing the polymerase. A-U base pairing between the template and transcript resulting from a T-rich stretch further upstream, also termed 'transcript release element' destabilizes the transcribing polymerase (Lang & Reeder, 1995). Together, pausing and destabilization lead to polymerase disengagement and thus termination. The remaining 10% of RNA polymerase I terminate at the fail-safe terminator, T_2, situated 250nt downstream of the 25S 3'end (Prescott et al, 2004; Reeder et al, 1999) or at the Replication fork barrier (RFB) where Fob1p acts similarly to Reb1p at T_1 (Takeuchi et al, 2003).

In mammals, the major terminator sequence is termed 'Sal-box' and was first identified in mouse (Grummt et al, 1985; Kuhn et al, 1988). It consists of an 18bp stretch repeated 10 times within the IGS (Figure 3, bottom right panel). Humans contain a shorter, 11bp element (Pfleiderer et al, 1990). The terminator element is recognized by Transcription termination factor I (TTF-I), which is functionally homologous to yeast Reb1p, bends DNA and thus induces RNA polymerase I pausing (Bartsch et al, 1987; Bartsch et al, 1988; Evers & Grummt, 1995). Upstream of the Sal-box, a T-rich sequence acts as a release element, similar to the yeast one

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(Richard & Manley, 2009). The release factor PTRF promotes release of terminated transcripts from RNA polymerase I (Jansa & Grummt, 1999).

In addition to Rep1p-induced Pol I-pausing, efficient rRNA transcription termination was shown to require Rat1p in yeast (El Hage et al, 2008; Kawauchi et al, 2008). For RNA polymerase II a so-called 'torpedo-mechanism' of termination was proposed (Connelly & Manley, 1988). As part of the cleavage and polyadenylation process, the nascent mRNA is cleaved generating an entry point for the 5' to 3' exonuclease Rat1p (Tollervey, 2004). Rat1p degrades the trailing transcript that is still engaged with the elongating RNA Pol II complex until it reaches the transcription machinery and disengages it – similar to a torpedo. In rRNA transcription, the RNase III protein Rnt1p cleaves the precursor shortly after the 3' end of 25S rRNA (Figure 3, right panel) (Kufel et al, 1999). This allows Rat1p to attack the exposed 5' end of the downstream nascent transcript and trace RNA Polymerase I by degrading the RNA (El Hage et al, 2008; Kawauchi et al, 2008). Ultimately, the transcription machinery is kicked off the DNA.

Because of the absence of Reb1p over the rDNA terminator region and since inactivation of Reb1p, Rat1p or Rnt1p alone is not sufficient to display a strong termination defect, rather than being mutually exclusive, it is very likely that the Reb1p-mediated pausing and the Rnt1p/Rat1p-dependent torpedo mechanism cooperate (El Hage et al, 2008; Kawauchi et al, 2008). Hence, Rnt1p promotes entry of Rat1p, which degrades the trailing transcript until it catches up with RNA polymerase I that is paused by Reb1p.

Interestingly, RNA polymerase II and I display very similar transcription termination mechanisms. Not only do they share important factors and mechanisms (for example the Rat1p-mediated torpedo model), but they both rely on co-transcriptional processing of the transcript, that is cleavage by the mRNA 3' end formation machinery or Rnt1p, respectively (Richard & Manley, 2009).

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Mammals contain an additional Sal-box, T_0, upstream of the rDNA coding region (Grummt et al, 1986; Pfleiderer et al, 1990), which seems to not only terminate spacer transcripts, but also enhance transcription from the rDNA promoter (see

chapter 1.1.1.) (Bateman & Paule, 1988; Grummt et al, 1986; Henderson et al, 1989). Yeast rDNA also includes a T_0 element; however, it is oriented in the wrong direction.

2.4. Transcription regulation

The number of ribosomes available in a cell determines the protein production rate. Rather than through rRNA degradation, the ribosome number is primarily regulated on the level of synthesis, especially via the transcription rate of RNA polymerase I which can be regulated in response to different stages of development and/or cell cycle (Antonetti et al, 1993; Larson et al, 1993; Larson et al, 1991; O'Mahony et al, 1992b). While nucleated erythrocytes and spermatocytes are inactive in rRNA synthesis, dividing cells rely on a large pool of ribosomes (Reeder, 1999).

rDNA expression is regulated on two levels; long-term regulation is achieved by applying epigenetic mechanisms and modifying DNA as well as histones, which render complete rDNA stretches inactive (see chapter 1.1.) (Grummt, 2007; Grummt & Pikaard, 2003; Lawrence & Pikaard, 2004; McStay, 2006). Flexible adaptation of rDNA transcription is channeled through a variety of different signaling pathways monitoring nutrient and growth factor availability or cell cycle stages. These signaling cascades converge at the RNA polymerase I transcription machinery (Grummt, 2003; Mayer & Grummt, 2005; Russell & Zomerdijk, 2006) by affecting assembly of the pre-initiation complex, promoter escape, elongation and transcription termination as well as by posttranslational modifications of transcription factors (Russell & Zomerdijk, 2005). Rather than altering the number of active rRNA genes, the developing mouse oocyte, for example, doubles RNA polymerase I activity to adapt to increased demands for ribosomes (Tian et al, 2001).

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2.5. Additional roles of the RNA polymerase I transcription machinery

Only 7-10% of RNA polymerase I in the nucleolus are actually engaged in rDNA transcription (Dundr et al, 2002a; Dundr et al, 2002b). This poses the question of whether RNA polymerase I fulfills additional functions in the nucleolus. ChIP analyses showed that Pol I as well as SL1 associates to sequences within the IGS approximately 2kb upstream of the pre-rRNA transcription start site (Mais et al, 2005).

Two to seven spacer promoters, with 90% homology almost perfect duplications of rDNA promoters, are localized within the human intergenic spacer (Reeder, 1984). RNA polymerase I produces transcripts terminating just upstream of the rDNA promoter, which was shown to enhance transcription levels in *Xenopus* possibly by delivering Pol I to the rDNA promoter (Figure 3) (De Winter & Moss, 1986). However, this model is controversial (Reeder, 1984). Transcripts originating from the promoter within the IGS are implicated in heterochromatin formation and thus rDNA silencing (Mayer et al, 2006; Moss et al, 1980). Typically, these transcripts do not accumulate *in vivo*, presumably due to rapid processing into shorter intermediates of 150 to 300nt termed promoter-associated RNAs (pRNAs) and subsequent degradation (Kuhn & Grummt, 1987; Morgan et al, 1983; Paalman et al, 1995). pRNAs are stabilized by NoRC and govern the silencing function of NoRC mentioned in 1.1.1. (McStay & Grummt, 2008).

Apart from its function in Pol I transcription initiation, UBF is thought to be an 'architectural' transcription factor implicated in the maintenance of secondary constrictions. Indeed, UBF binds extensively across rDNA repeats (O'Sullivan et al, 2002), but also to large arrays of ectopic heterologous UBF-binding sequences outside of the nucleolar rDNA loci (Mais et al, 2005). Just as rDNA genes, these arrays form secondary constrictions, recruit SL1, TIF-IA and even RNA polymerase I and are therefore called Pseudo-NORs (Andersen et al, 2005; Dundr et al, 2002a; Dundr et al, 2002b; Mais et al, 2005; Prieto & McStay, 2007; Schneider & Nomura,

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2004). UBF contains several high mobility group (HMG) boxes, involved in DNA binding and also bending (Jantzen et al, 1990). Hence, a UBF dimer can organize nucleosome-free DNA into a 360° loop, a structure resembling the nucleosome in terms of mass and DNA content (Bazett-Jones et al, 1994; Putnam et al, 1994). While UBF binds to active rDNA units and persists throughout mitosis, it is completely absent from silent genes (Roussel et al, 1993; Wright et al, 2006). UBF can disrupt binding of Histone H1 to the core nucleosomes, an association that stabilizes compact, higher-order chromatin structures (Catez et al, 2006; Kermekchiev et al, 1997). Thereby, UBF promotes nucleosome destabilization and chromatin decompaction and renders the rDNA accessible to the RNA polymerase I transcription machinery. In summary, UBF is an important factor for the maintenance of the active state of rDNA repeats yet is not involved in the establishment of euchromatic features because it cannot bind to methylated promoters (see chapter 1.1.2.) (Santoro & Grummt, 2001) and thus convert repressing marks into activating ones.

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3. rRNA PROCESSING

The 18S, 5.8S and 25/28S rRNAs are transcribed as a single, polycistronic precursor (Fatica & Tollervey, 2002). In an ordered series of endo- and exonucleolytic cleavages the mature rRNAs are liberated and in part concurrently assembled into the 40S and 60S ribosomal subunits. In addition, rRNAs are subjected to extensive modifications.

Precursor processing is probably preceded or accompanied by the association of ribosomal proteins (r-proteins) and accessory, non-ribosomal proteins (Figure 4) (Granneman & Baserga, 2004; Henras et al, 2008). In Miller spreads, terminal knobs marking the 5', most extended ends of the transcripts are detected corresponding to rRNA processing complexes. These complexes are the precursors to the mature ribosomal subunits and contain, among accessory proteins and enzymatic activities required in rRNA processing, many of the r-proteins (Granneman et al, 2010). Composition of the terminal knob is highly dynamic. Initially about 15nm in size, they grow into a larger knob of 40nm as transcription proceeds and are cleaved off co-transcriptionally to release the precursor to the 40S ribosomal subunit (Dragon et al, 2002; Gallagher et al, 2004; Granneman & Baserga, 2005; Hoang et al, 2005; Osheim et al, 2004). A precursor to the large 60S ribosomal subunit has also been identified in yeast (Bassler et al, 2001; Nissan et al, 2004).

A number of similarities between pro- and eukaryotic rRNA processing have been described (Hage & Tollervey, 2004). rRNAs of the small (SSU) and the large ribosomal subunit (LSU) are co-transcribed as a single precursor, and the SSU rRNA is encoded upstream of the LSU rRNAs. rRNAs are processed similarly and fold presumably into analogous, tightly packed core structures. Also, several ribosomal proteins are conserved throughout species. Differences between pro- and eukaryotic rRNA maturations are mainly linked to complexity. Whereas bacteria only use a limited number of chaperones and trans-acting factors, yeast requires approximately 200 proteins and 70 small RNAs but no chaperones (Hage & Tollervey, 2004).

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Figure 4. Co-transcriptional assembly of proteins on rRNA

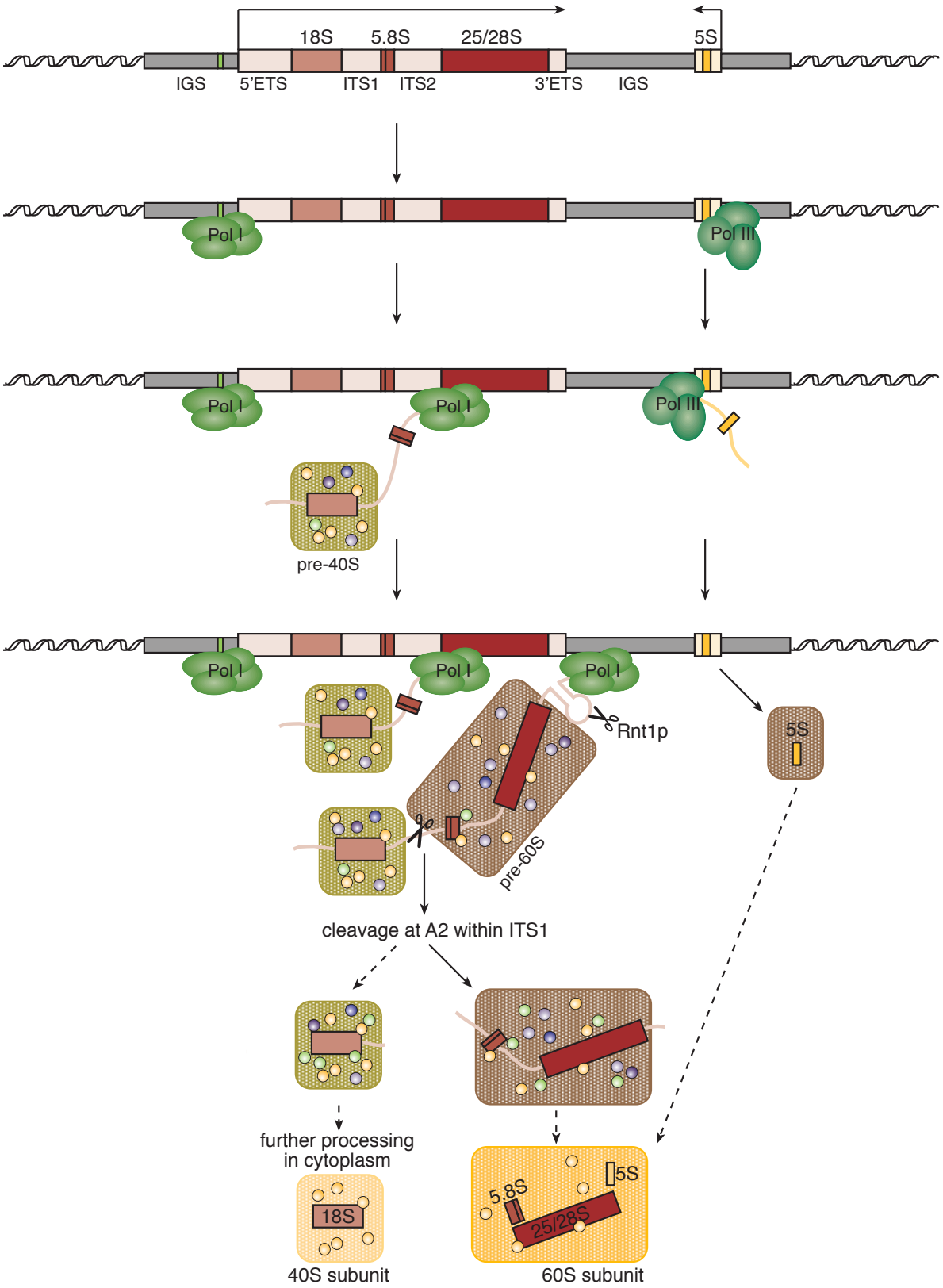

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Figure 4. Co-transcriptional assembly of proteins on rRNA. RNA polymerase I (Pol I) transcribes the polycistronic precursor to 18S, 5.8S and 25/28S rRNA. Already co-transcriptionally, ribosomal and non-ribosomal proteins (depicted as circles) associate with the nascent transcript to form precursors to 40S and 60S ribosomal subunits (rectangles). 5S rRNA is transcribed independently by RNA polymerase III and assembled together with 5.8S and 25/28S to form the 60S subunit. For more details on the processing of rRNA, see Figure 5.

3.1. rRNA processing in the yeast *Saccharomyces cerevisiae*

Most of our knowledge on ribosomal RNA processing has been obtained using the model organism *Saccharomyces cerevisiae* (Fatica & Tollervey, 2002; Granneman & Baserga, 2004; Nazar, 2004; Venema & Tollervey, 1999). More than 80 ribosomal proteins, 150 accessory proteins and a multitude of snoRNAs co-operate in the biogenesis of the ribosome.

S. cerevisiae RNA polymerase I terminates 210nt downstream of the 3' end of 25S rRNA (Kempers-Veenstra et al, 1986; Kufel et al, 1999). The RNAse III like endonuclease Rnt1p co-transcriptionally cleaves 14nt downstream of 25S, liberating the 35S precursor (Elela et al, 1996; Kufel et al, 1999). It was generally believed, that the onset of rRNA processing follows cleavage by Rnt1p, however, a recent study has revealed, that rRNA processing possibly commences prior to transcription termination and 3'ETS cleavage in yeast (Kos & Tollervey).

In a first wave of processing events at A_0, A_1 and A_2, the 5'ETS is removed and the precursor split into the 20S and $27SA_2$ pre-rRNAs, which contain the rRNAs for the small and large ribosomal subunits, respectively (Figure 5) (for details see Venema & Tollervey, 1999). The 20S precursor is subsequently exported to the cytoplasm, where processing at D liberates the mature 18S (Moy & Silver, 1999; Vanrobays et al, 2001). While maturation of 18S rRNA is achieved by only four successive endonucleolytic events, processing of the large subunit rRNAs 5.8S and 25S is more complex and proceeds via two different pathways resulting in either a short or a long, 5' extended form of 5.8S. Around 90% of $27SA_2$ precursors are cleaved by the endonuclease MRP at A_3 (Schmitt & Clayton, 1993), followed by 5' to 3' exonucleolytic degradation to B_{1S} through Rat1p or Rrp17p (Henry et al, 1994;

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Oeffinger et al, 2009). The precise mechanism that defines the stopping point for the exonucleases and therefore the 5' end of $27SB_{1S}$ is still unknown. A possible model involves proteins bound to the precursor acting as 'road-blocks'. In the second pathway, the ITS1 is removed by one endonucleolytic cleavage at B_{1L}, 6nt upstream of B_{1S}, resulting in the slightly longer, 5'-extended $27SB_{1L}$ intermediate (Henry et al, 1994; Schmitt & Clayton, 1993). The reason for the existence of two 5.8S rRNA species is still elusive. Both the $27SB_{1S}$ and $27SB_{1L}$ are subsequently processed in the same manner again. The short remainder of the 3'ETS upstream of B_2 is removed by the 3' to 5' exonuclease Rex1p, generating the 3' end of the mature 25S rRNA (Kempers-Veenstra et al, 1986; van Hoof et al, 2000). Cleavage within ITS2 at C_2 splits the $27SB_{2S/L}$ intermediate into the 7S and 25.5S precursors, from which a battery of exonucleases liberate the mature 5.8S and 25S rRNAs, respectively (Allmang et al, 1999; Faber et al, 2002; Geerlings et al, 2000; Mitchell et al, 1996). Possibly, 3' end formation of 5.8S is only completed in the cytoplasm (Thomson & Tollervey, 2010), similar to 18S maturation (Moy & Silver, 1999; Vanrobays et al, 2001).

At the same time, the released spacer sequences are degraded by the exosome and Rat1p. In the absence of Rat1p, its mainly cytoplasmic counterpart Xrn1p can substitute in all the above-mentioned steps (Geerlings et al, 2000; Johnson, 1997).

Figure 5. rRNA processing in yeast. rRNA processing follows a strict order of endo- and exonucleolytic cleavages best studied in *S. cerevisiae*. The 35S primary transcript is cleaved co-transcriptionally by Rnt1p, followed by processing steps at A_0, A_1 and A_2 that generate the 20S and $27SA_2$ intermediates. While 20S is exported to the cytoplasm were cleavage at D liberates the mature 18S rRNA of the 40S ribosomal subunit, processing of $27SA_2$ is more complex and involves two alternative pathways. In most cases, cleavage by MRP at A_3 and subsequent degradation by a 5' to 3' exonuclease generate the 5' end of the final 5.8S rRNA; in the second pathway, a 5' extended 5.8S form results from cleavage at B_{1S}. Both $27SB_{1S}$ and $27SB_{1L}$ are subjected to the exosome to remove the remaining stretch of the 3'ETS. Subsequently, an endonuclease splits $27SB_{2S/L}$ into $7S_{S/L}$ and 25.5S and the exosome and Rat1p rapidly generate the mature $5.8S_{S/L}$ and 25S, respectively, which associate with the independently transcribed and processed 5S and ribosomal proteins into the 60S ribosomal subunit.

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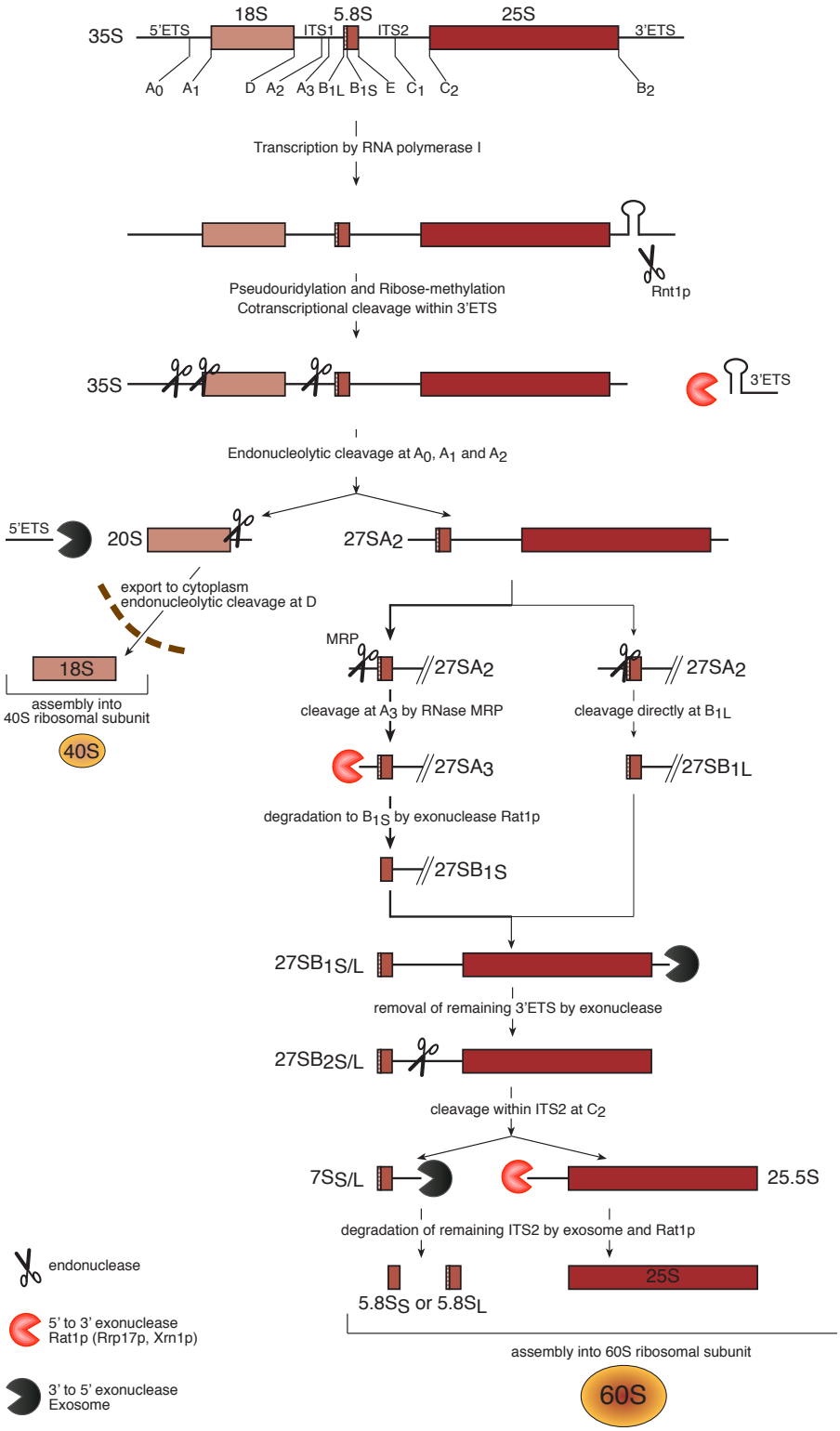
Figure 5. rRNA processing in yeast



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Interestingly, half of the 35S precursor is removed along the processing path. The spacer sequences contain information to assure the correct order of processing and are required for their own removal (Musters et al, 1990). Therefore, the secondary structure is important. The 3'ETS forms a hairpin structure that is recognized by Rnt1p (Elela et al, 1996). The cleavage site in ITS2, C_2, is theoretically distributed throughout the whole spacer, because only correct folding of ITS2 into a specific secondary structure allows cleavage and thus progression of the rRNA maturation pathway (Cote et al, 2002). Each processing step induces conformational changes of the precursor permitting the next processing step. In addition to the cis-acting elements in the spacer regions, rRNA processing also depends on a variety of different accessory proteins, containing nucleases, helicases, chaperones, modifying enzymes, GTP/ATPases or transport factors (Venema & Tollervey, 1999). A puzzling observation was that enzymes such as the exosome, Rat1p or RNase MRP, play a role in the processing pathway, but are not essential (Kawauchi et al, 2008). This is due to several parallel, redundant processing activities that ensure efficient rRNA maturation and thus act as fail-safe mechanisms.

rRNA processing in other eukaryotic species is substantially similar, yet individual steps may vary.

3.2. rRNA processing in mammals

As opposed to the yeast counterpart, mammalian rRNA processing is not well studied; in fact, most of the knowledge is based on speculation derived from presumptive conservation of yeast components (Gerbi & Borovjagin, 2004a).

The principal steps in rRNA maturation are identical to those in yeast (Figure 6). The 18S, 5.8S and 28S rRNAs are encoded in the same order as the yeast counterparts and transcribed into a single 45S polycistronic transcript with flanking and separating ETS and ITS sequences. This precursor is processed in a step-wise order through various intermediates similar to the yeast 35S precursor (Scherrer et al, 1963; Warner et al, 1966). The exact order of events appears to be flexible to a

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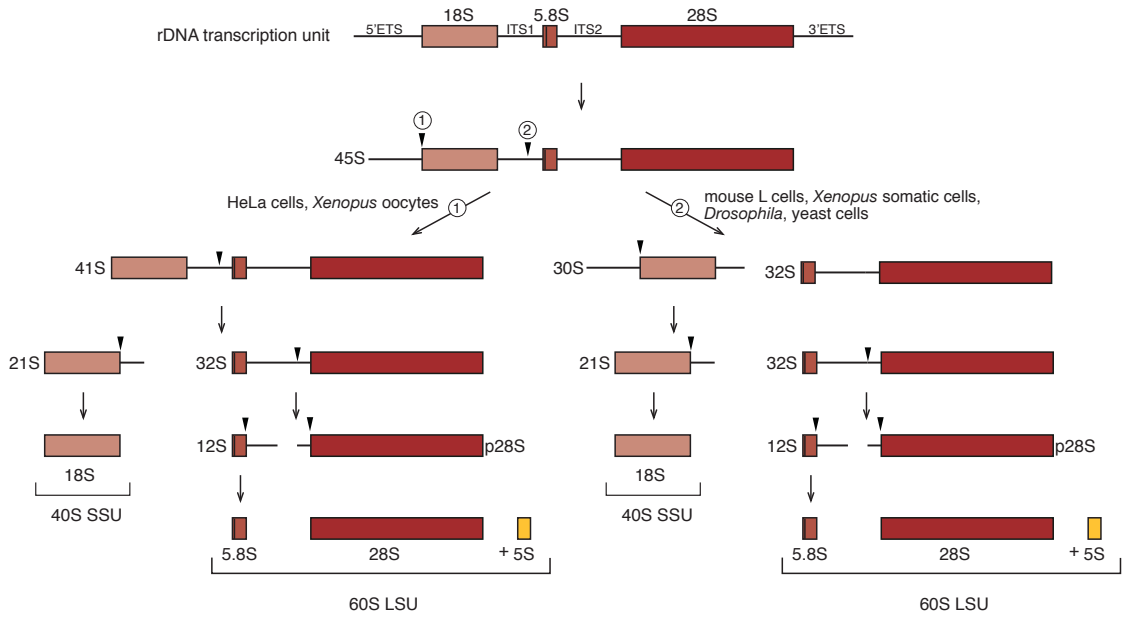

Figure 6. rRNA processing in mammals. Mammalian rRNA processing is thought to be similar to the yeast mechanism. However, a certain flexibility seems to apply to the otherwise strict processing order. The 45S precursor is either cleaved 5' of 18S and then within the ITS1, or vice versa. In both cases, the downstream processing events follow the same order again, as described for Figure 5. The two alternative pathways are found predominantly in the indicated cell types. ETS, external transcribed spacer; ITS, internal transcribed spacer; SSU, small ribosomal subunit; LSU, large ribosomal subunit. Arrow heads mark the next processing sites. (Figure adapted from Hadjiolova et al, 1993).

certain extent (Hadjiolova et al, 1993). Pathway '1' follows in principle the yeast rRNA processing scheme, explained above (Figure 5). By removing the 5'ETS the 45S is converted into the 41S precursor, which is subsequently cleaved within ITS1 to separate the precursor into 21S and 32S, intermediates to the SSU and LSU rRNAs, respectively. In contrast, pathway '2' commences with the endonucleolytic cleavage in ITS1 before the 5'ETS is removed and thus creates a 30S intermediate in addition to 32S. Upon removal of the 5'ETS, 21S is generated. Processing events downstream of 21S and 32S are shared by both pathways. Trimming the 3' end of 21S liberates the mature 18S rRNA. At the same time, cleavage within ITS2

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separates 32S into 12S and precursor to 28S (p28S), which form into 5.8S and 28S rRNAs after degradation of the remaining spacer sequence. Pathway '1' is primarily used by HeLa cells (Wellauer & Dawid, 1973), while pathway '2' is preferred by *Xenopus* somatic cells (Loening et al, 1969; Wellauer & Dawid, 1974), mouse L cells (Wellauer et al, 1974) and *Drosophila* cells (Levis & Penman, 1978). However, the order does not always underlie strict rules. *Xenopus* oocytes were shown to use either one pathway, sometimes the pathways even vary or co-exist in the same cell (Bowman et al, 1981; Dudov et al, 1978; Savino & Gerbi, 1990; Wellauer & Dawid, 1974; Winicov, 1976)

3.3. rRNA modification

A second major branch in the maturation of rRNAs comprises covalent modifications. Already co-transcriptionally, a myriad of RNA-protein (RNP) complexes directs extensive pseudouridylation and ribose-methylation (Fatica & Tollervey, 2002; Venema & Tollervey, 1999).

Modifications are guided through base-pairing of small nucleolar RNAs (snoRNAs), that assemble into RNP complexes, with rRNA precursors (Kiss et al, 2006). Yeast snoRNAs are oftentimes excised from introns of r-proteins or translation factors (Tollervey & Kiss, 1997) involving the exonuclease Rat1p (Petfalski et al, 1998).

snoRNAs are divided into two different classes based on their secondary structure and the modification they mediate (Balakin et al, 1996). Methylation-inducing Box C/D snoRNAs fold into a characteristic helix-asymmetric bulge-helix conformation, a motif that also occurs in rRNAs (Vidovic et al, 2000; Watkins et al, 2000). The 9-21nt long guide sequence is immediately upstream of the Box D and binds to the complementary sequence within the rRNA precursor. The 5th nucleotide within this sequence on the opposite strand is methylated by the snoRNP subunit Nop1p/Fibrillarin (Galardi et al, 2002; Niewmierzycka & Clarke, 1999; Wang et al, 2000). Pseudouridylation is guided by the H/ACA snoRNAs (Ganot et al, 1997a). Their characteristic conformations comprise two hairpin structures flanked by two hinge regions, 'H' and 'ACA' (Balakin et al, 1996; Ganot et al, 1997b). Either one or

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both hairpins contain the guide sequence targeting one uridine for isomerization by the snoRNP component Cbf5p/dyskerin (Lafontaine et al, 1998; Zebarjadian et al, 1999).

Two snoRNAs display additional functions (Venema & Tollervey, 1999); the C/D box snoRNA U14 mediates methylation within 18S (Dunbar & Baserga, 1998) and the H/ACA snoRNA snR10 guides pseudouridylation within 25S (Ni et al, 1997). Both snoRNAs are also required for the early cleavages 5' and 3' of 18S rRNA at A1 and A2, respectively (Li et al, 1990; Tollervey, 1987); U3 and snR30 snoRNAs do not function in modification, but solely in processing (Hughes & Ares, 1991; Morrissey & Tollervey, 1993).

Interestingly, mutations preventing modifications have no or only little effect on growth (Parker et al, 1988; Qu et al, 1999; Tollervey & Kiss, 1997). The definite role of modifications thus is still enigmatic. Suggested roles include determining conformations, facilitating protein binding, rRNA stability and ribosome function (Decatur & Fournier, 2002; Decatur et al, 2007).

Base-modifications are independent of snoRNAs but are mediated by protein-only enzymes. For example, dimethylation of two Adenosine residues in 18S rRNA is achieved by the protein Dim1p (Lafontaine et al, 1994).

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4. THE NUCLEOLUS

The nucleolus is a self-organizing, dynamic structure within the cell nucleus dedicated to ribosome synthesis (Andersen et al, 2005; Birnstiel et al, 1963; Lam et al, 2005; Raska et al, 2006b; Scherrer et al, 1963). The first observation of this 'ovaliform body' dates back to the 18th century (Fontana, 1781). Due to its prominent structure and visibility by light microscopy, the nucleolus has been studied extensively. In mammalian cells, up to 25% of the nuclear volume can be occupied by one to four nucleoli, the size and number reflecting the instantaneous demand for ribosomal RNA (Hernandez-Verdun, 2006).

4.1. Nucleolus formation

Nucleoli form around clusters of rDNA genes during late telophase, therefore rDNA loci are also termed Nucleolus Organizer Regions (NOR) (McClintock, 1934). Theoretically, in a diploid human cell, 10 nucleoli can emerge, yet typically, only 1 to 4 nucleoli are observed. Firstly, nucleoli can only form on actively transcribed NORs, however, as mentioned above, not all rDNA clusters are transcriptionally active. Secondly, nucleoli fuse throughout interphase.

Nucleolus formation is ultimately dependent on active rDNA transcription. At the onset of mitosis, RNA polymerase I-associated transcription factors are phosphorylated, leading to inhibition of transcription and eventually dissociation of nucleoli (Sirri et al, 2002). At the end of metaphase, previously active rDNA repeats form prominent chromatin structures distinct from those that were inactive before and the rest of the chromosome. These 'secondary constrictions' correlate with association of the RNA polymerase I machinery in late metaphase (Heliot et al, 1997).

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While nucleoli of multicellular organisms disperse and disassemble in an ordered fashion as the cells enter mitosis, yeast nucleoli remain intact during mitosis and are segregated only at late stages of division (Hernandez-Verdun, 2006).

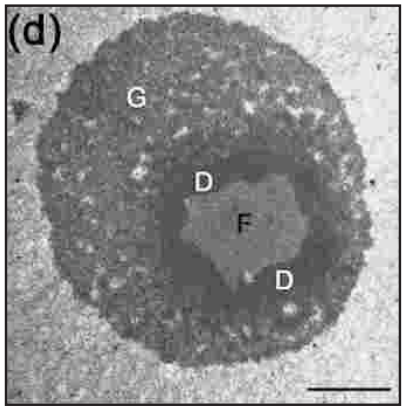

Figure 7. Electron micrograph of the nucleolus. The nucleolus can be separated into three different substructures by their appearance in EM. The lightly stained core is composed of thin fibrils and thus called Fibrillar center (F). The dark ring structure surrounding F is called Dense fibrillar component (D). Both F and D are embedded in the Granular component (G). (Picture from Raska et al, 2006b).

4.2. Nucleolar substructures

The nucleolus can be divided into three substructures according to their appearance in electron microscopy (Figure 7 and 8): The fibrillar centers (FC) are surrounded by the dense fibrillar components (DFC) and embedded in the granular component (GC) (Lam et al, 2005). Many nucleolar proteins accumulate mainly in one or two of these sub-compartments, manifesting distinct functions for FC, DFC and GC (Schwarzacher & Mosgoeller, 2000). Depending on the cell type and stage, arrangement of the three major components of the nucleolus diversifies. The morphology of nucleoli is directly connected to their functional status and can rapidly change in response to altered demands (Hernandez-Verdun, 2006).

The innermost component, termed fibrillar center or FC, consists of a meshwork of 4-5nm thick fibrils where rDNA, RNA polymerase I, and other components of the transcription machinery are localized (Recher et al, 1969). Transcription of the pre-rRNA takes place at the periphery of the FC. Just as the nucleolus, the size and number of FCs vary with the demand for ribosome production (Lafarga et al, 1994; Navascues et al, 2004). Whereas active cells can have several large nucleoli with up to 100 small FCs (Koberna et al, 2002; Ochs & Smetana, 1989; Raska et al, 1983), dormant lymphocytes harbor only one nucleolus with a single large FC (Raska et al, 1983; Smetana et al, 1968; Smetana et al, 1967). Similarly, the size of the DFC reflects the rRNA processing state. The DFCs are composed of densely packed fibrils

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Figure 8. Nucleolar substructures

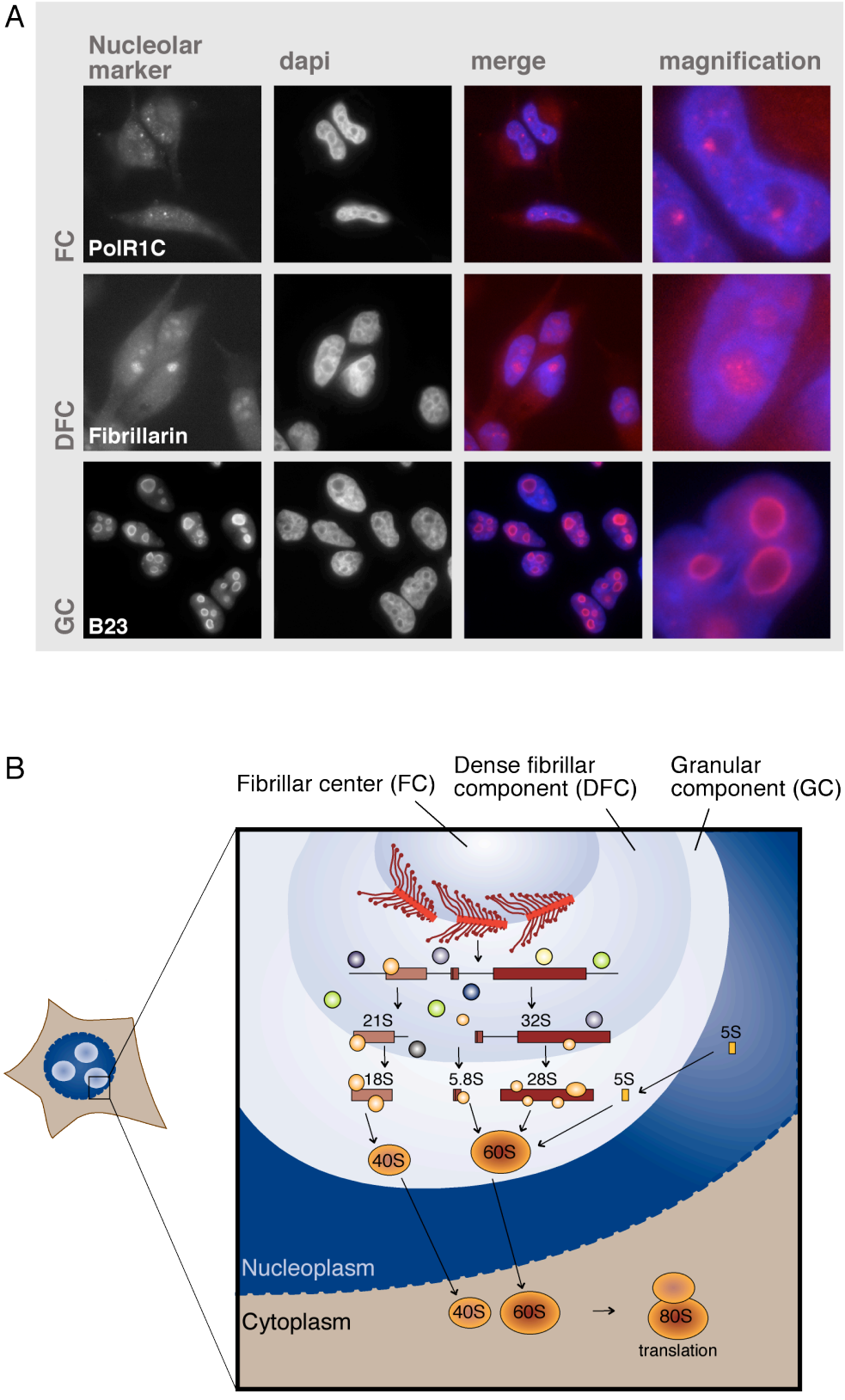

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Figure 8. Nucleolar substructures. **A** In dapi staining, the nucleolus appears as largely unstained, oval structure within the nucleus. The three nucleolar subregions have different protein compositions, which allows for their distinction in immunofluorescence (IF). PolR1C, Fibrillarin and B23 are nucleolar markers for Fibrillar center (FC), dense fibrillar component (DFC) and Granular component (GC), respectively. IF was carried out on HeLa cells. **B** The three nucleolar subregions have different roles in rRNA processing. rDNA is transcribed at the periphery of FC by Pol I, generating the rRNA precursor which is subjected to an ordered series of processing steps in the DFC. Ultimately, mature 18S, 5.8S and 28S are liberated and assembled together with ribosomal proteins into the 40S and 60S ribosomal subunits in the GC.

with a diameter of 3-5nm and are the main site of rRNA precursor processing. While progressing through the DFCs, the polycistronic rRNA transcript is subjected to an ordered series of processing events, removing spacer sequences and liberating the mature 18S, 5.8S and 28S rRNAs. The GC, a region containing small 15nm granules, functions mainly in ribosome subunit assembly and storage. Plant cell nucleoli have a fourth structure referred to as vacuole or cavity (Shaw & Brown, 2004). It is important to bear in mind that the tripartite model of the nucleolus equals an oversimplification; the composition, size and number of nucleoli is highly dynamic and therefore does not allow strict categorization.

4.3. Nucleolar Proteins

Despite the lack of a membrane, nucleoli are very robust structures that remain assembled even after nuclei disruption and under conditions that disintegrate most other sub-nuclear structures (Lam, 2006). Over 700 proteins stably associate with the human nucleolus (Andersen et al, 2002; Scherl et al, 2002). Whereas defined nuclear localization signals are known to direct import and export to or from the nucleus, targeting of nucleolar proteins is largely a black box (Carmo-Fonseca et al, 2000). Due to the lack of a physical barrier surrounding the nucleolus, theoretically, any soluble molecule can simply diffuse into and out of the nucleoli. Handwerger et al. showed that nucleoli are permeable to macromolecules of up to 2000 kDa in size (Handwerger et al, 2005). The mean residence time of most nucleolar proteins averages at a few tens of seconds (Misteli, 2001). Due to this sieve-like feature of

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nucleoli, targeting of nucleolar proteins is not necessarily directed by a cis-acting signal element as generally valid for cellular compartments, but might instead be determined by other factors that retain specific molecules within the nucleolar region. Currently it is accepted, that rDNA loci and their transcripts act as such 'nucleolar building blocks' (Jacobson et al, 1995). In addition, several nucleolar localization signals (NoLS) have been proposed (Kubota et al, 1999; Michael & Dreyfuss, 1996; Russo et al, 1997; Sheng et al, 2004; Song & Wu, 2005). They seem to be generally rich in arginine and lysine residues; yet do not share obvious consensus sequence or structure.

Nucleostemin, a protein preferentially expressed in neuronal stem cells (Tsai & McKay, 2002), is the first example involving a protein retention mechanism (Tsai & McKay, 2005). In a GTP-driven cycle, nucleostemin is retained in the nucleolus when associated with GTP. A GTP-switch in the nucleolus drives re-localization of nucleostemin-GDP to the nucleoplasm providing regulation of nucleostemin activity. Similarly, B23 is also sensitive to GTP levels, yet was not shown to bind GTP by itself (Finch et al, 1993). In addition, the phosphorylation status of B23 affects the integrity of the nucleolus (Louvet et al, 2006). Mutations in the phosphorylation sites of B23 induce reorganization of the sub-nucleolar components and dispersion. The above mentioned are the first implications of regulatory mechanisms for nucleolar localization.

For ribosome biosynthesis, the cell needs to produce exactly equimolar amounts of ribosomal RNAs and proteins. This seems to be achieved on the level of post-translational regulation; proteomic studies have revealed nucleolar accumulation of r-proteins upon proteasome inhibition (Andersen et al, 2005). In addition, ubiquitination and the proteasome play a role in ribosome biogenesis (Stavreva et al, 2006). The current working model for the mutual fine-tuning of the pool of different ribosomal components suggests that ribosomal proteins are overproduced, and those in excess, i.e. not incorporated into a maturing ribosomal subunit, are targeted by ubiquitination for proteasome-mediated degradation (Andersen et al, 2005).

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4.4. The multi-functionality of the nucleolus

In recent years, functions beyond ribosome biogenesis have been assigned to the nucleolus (Boisvert et al, 2007; Olson et al, 2002; Pederson, 1998; Raska et al, 2006a). Proteomic studies of HeLa and Arabidopsis nucleoli have revealed proteins related to cell cycle (3.5% of the identified proteome), DNA damage repair (1%) and pre-mRNA processing (5%) (Andersen et al, 2005; Pendle et al, 2005; Scherl et al, 2002). A variety of publications link RNA editing (Sansam et al, 2003), DNA damage repair (van den Boom et al, 2004), telomere metabolism (Kieffer-Kwon et al, 2004; Zhang et al, 2004), tRNA processing (Paushkin et al, 2004; Wolin & Matera, 1999), assembly and processing of the signal recognition particle (Jacobson & Pederson, 1998) and regulation of protein stability (Mekhail et al, 2004; Rodway et al, 2004) to the nucleolus. Nucleolar disruption is a common feature of stress responses involving p53, for example following ionizing radiation (Rubbi & Milner, 2003). Also, the small interfering RNA (siRNA) pathway has been connected with the nucleolus (Li et al, 2006a; Pontes et al, 2006). Many of the proteins of the siRNA machinery localize together with siRNAs to plant nucleoli, among them RDR2, DCL3, AGO4 and NRPD1b, a subunit of RNA polymerase IVb. This indicates a potential role of the nucleolus in siRNA synthesis as well as storage and/or sequestration of the RNA-induced silencing complex. In addition, co-localization of miRNA-206 with the 28S rRNA in the granular components has been reported (Politz et al, 2006).

4.4.1. Viruses

A wide variety of viruses interact with the nucleolus in one or the other way (Greco, 2009; Hiscox, 2007). RNA viruses have a cytoplasmic replication strategy. Upon transformation, the genome of a positive strand RNA virus is translated by the host ribosomes, producing the viral RNA-dependent RNA polymerase. This polymerase transcribes the genomic RNA strand, and subsequently the primary transcript to produce new genomic RNA. Although these mechanisms are in general cytoplasmic, viral replication is decreased in enucleated cells (Evans & Simpson, 1980; Wilhelmsen et al, 1981).

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Furthermore, viral capsid proteins have been shown to localize to the nucleolus of cells infected with RNA viruses (Dove et al, 2006; Haupt et al, 2005; Hiscox et al, 2001; Michel et al, 1990; Rikkonen et al, 1992; Rowland et al, 1999; Taliansky & Robinson, 2003; Tijms et al, 2002; Timani et al, 2005; Wang et al, 2002b; Wurm et al, 2001; Young et al, 2005). For many years it was generally believed that this phenomenon was coincidental due to diffusion and random retention of capsid proteins by nucleolar factors, especially since capsid proteins have a high affinity for RNA. Other viral proteins, however, actively localize to the nucleolus by mimicking host nucleolar localization signals, indicating that the targeting of viral proteins to the nucleolus has specific functional relevance (Rowland & Yoo, 2003). Indeed, disrupting nucleolar trafficking of positive strand RNA viruses affects virulence (Fazakerley et al, 2002; Lee et al, 2006; Mori et al, 2005).

Several viruses severely alter the architecture and composition of the host nucleolus (Dove et al, 2006; Miyazaki et al, 1996). Especially the highly abundant nucleolar proteins B23 and nucleolin are targets for relocalization. Poliovirus infection redistributes nucleolin from the nucleolus to the cytoplasm and inactivates UBF, thereby interrupting transcription by RNA polymerase I (Banerjee et al, 2005).

Hepatitis Delta Virus (HDV) encodes a single protein on a circular genomic RNA of negative orientation. Replication of HDV RNA takes place in the nucleolus, usurping RNA polymerase I and its transcription factor SL-1 (Huang et al, 2008; Li et al, 2006c). Hepatitis C virus (HCV) core protein enhances transcription of the rDNA loci by RNA polymerase I (Kao et al, 2004). Since HCV uses an internal ribosome entry site (IRES)-mediated translation system for their mRNA (Hellen, 2009; Pelletier & Sonenberg, 1988), elevation of ribosome levels potentiates virus expansion. In addition, nucleolin is relocated to the cytoplasm, where it binds to the IRES and possibly acts as a chaperon for the pre-initiation complex formation to favor viral mRNA over host mRNAs (Lu et al, 2004; Yu et al, 2005).

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The DNA human papilloma virus (HPV) recruits RNA polymerase I and its upstream binding factor to viral replication centers; UBF was shown to participate in and enhance viral replication and seems to be associated to the ends of the viral genome,

implicating a role in DNA synthesis (Lawrence et al, 2006; Okuwaki et al, 2001; Rodrigues et al, 1996).

As a consequence of these findings, the nucleolus is becoming more and more interesting for the design of antiviral strategies.

4.4.2. Cancer

Tumor cells are generally rapidly proliferating cells demanding high numbers of ribosomes. Since size and amount of nucleoli reflect their activity, rapidly growing cells display characteristically larger and more nucleoli, which are therefore used as classification parameters in cancer diagnostics (Maggi & Weber, 2005). Additional prognostic information is provided by the presence of the perinucleolar compartment (PNC), a structure physically connected with nucleoli and enriched for RNA transcripts as well as RNA-binding proteins and RNA polymerase III (Pollock & Huang). Over the past years, PNCs have been closely linked to malignant transformation and their presence positively correlates with breast cancer progression (Kamath et al, 2005; Kopp & Huang, 2005).

4.4.3. mRNA quality control

Although the nucleolus has been extensively studied, many questions are still unsolved.

In the 1960s, Harris investigated heterokaryons, cells containing multiple, genetically distinct nuclei of HeLa cells and chicken erythrocytes (Harris, 1967). Erythrocytes phenotypically have a condensed nucleus, lack nucleoli and are transcriptionally inert. When combined to HeLa cells to form a heterokaryon, transcription is re-activated and a nucleolus formed. Synthesis of chicken protein, however, is not initiated until complete formation of a chicken nucleolus, although the HeLa nucleolus theoretically contributes ribosomes for translation. The requirement of an own nucleolus within the chicken nucleus indicates a distinct nucleolar role in gene expression apart from generating ribosomes. The author suggests that the nucleolus is essential for mRNA export (Harris, 1967). This model was criticized harshly, yet supporting data has accumulated over the years. mRNA

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transport-defective yeast strains display a disrupted and fragmented nucleolus (Kadowaki et al, 1994; Schneiter et al, 1995); furthermore, yeast mutants of RNA polymerase I accumulate polyA positive RNA in their nucleoli (Kadowaki et al, 1995). The Arabidopsis nucleolus contains a variety of components of the mRNA exon junction complex (EJC) (Pendle et al, 2005), among them the well-studied RNA-binding protein eIF4IIIA (Shibuya et al, 2006). The EJC marks spliced mRNAs and is part of the recognition mechanism for premature translation termination. If a ribosome encounters a stop codon on a mRNA upstream of an associated EJC, the mRNA is targeted for nonsense-mediated decay (NMD) (Nicholson et al, 2009). Once in the cytoplasm, mRNAs seem to be immune to NMD (Stephenson & Maquat, 1996). Subsequently, it is likely that detection of premature stop codons is a nuclear, potentially nucleolar process, where mRNAs are scanned for their integrity in a pioneer round of translation (Raska et al, 2006a). Indeed, various translation factors localize to human and plant nucleoli (Andersen et al, 2005; Scherl et al, 2002), and very early studies implicated cases of protein translation in pea nucleoli (Birnstiel et al, 1961; Birnstiel & Hyde, 1963); however reproducing these findings in HeLa cells led to controversial results (Iborra et al, 2001; Iborra et al, 2004; Nathanson et al, 2003). As a conclusion, several reports provide evidence, that the nucleolus might be involved in mRNA quality control, yet further studies are definitely required.

The future will hopefully provide a clearer picture of the fragmentary knowledge about the many roles of the nucleolus and potentially disclose even more. However, since nucleoli are atypical compartments lacking the physical barrier provided by a membrane but rather are open systems, proteins can diffuse more or less freely. This challenges the results acquired by proteomics (Andersen et al, 2005; Andersen et al, 2002; Pendle et al, 2005; Scherl et al, 2002), the basis of many speculations about the multi-functionality of the nucleolus.

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Role of the RNA/DNA kinase Grc3 in transcription termination by RNA polymerase I

Priscilla Braglia[1], Katrin Heindl[2]*, Alexander Schleiffer[3], Javier Martinez[2]+ & Nick J. Proudfoot[1]++*

[1]Sir William Dunn School of Pathology, University of Oxford, Oxford, UK, [2]Institute of Molecular Biotechnology of the Austrian Academy of Sciences, and [3]Department of Bioinformatics, Research Institute of Molecular Pathology/Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria

Transcription termination by RNA polymerase I in *Saccharomyces cerevisiae* is mediated by a 'torpedo' mechanism: co-transcriptional RNA cleavage by Rnt1 at the ribosomal DNA 3′-region generates a 5′-end that is recognized by the 5′–3′ exonuclease Rat1; this degrades the downstream transcript and eventually causes termination. In this study, we identify Grc3 as a new factor involved in this process. We demonstrate that *GRC3*, an essential gene of previously unknown function, encodes a polynucleotide kinase that is required for efficient termination by RNA polymerase I. We propose that it controls the phosphorylation status of the downstream Rnt1 cleavage product and thereby regulates its accessibility to the torpedo Rat1.

Keywords: Grc3; kinase; Pol I; transcription termination

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INTRODUCTION

The enzyme RNA polymerase (Pol) I is dedicated to the synthesis of ribosomal RNA (rRNA). Despite using distinct transcription systems, Pol I and Pol II show close parallels in the process of transcription termination (El Hage *et al*, 2008; Kawauchi *et al*, 2008), as they both terminate by a 'torpedo' mechanism (Kim *et al*, 2004; West *et al*, 2004). On ribosomal DNA (rDNA), co-transcriptional cleavage of the transcript by Rnt1 releases the 35S pre-rRNA, but also generates a free 5′-end in the downstream Pol I-associated 3′-transcript. This acts as a substrate for the 5′–3′ exonuclease Rat1 that degrades the 3′-transcript. This process is associated with destabilization of the transcription complex and consequent termination (El Hage *et al*, 2008; Kawauchi *et al*, 2008).

Eukaryotic Clp1 is a component of the messenger RNA (mRNA) cleavage and polyadenylation machinery (Minvielle-Sebastia *et al*, 1997; de Vries *et al*, 2000). Human Clp1 (hClp1) has been identified as a RNA kinase and phosphorylates the 5′-end of both synthetic short interfering RNAs and transfer RNA (tRNA) 3′-exons during tRNA splicing (Weitzer & Martinez, 2007). A potential enzymatic role for hClp1 in Pol II transcription termination has also been proposed, in which it acts to maintain a 5′-phosphate on the downstream cleavage product generated by mRNA 3′-end processing. This might provide Rat1 (Xrn2 in mammals) with a favourable substrate for exonucleolytic degradation and consequent Pol II termination (Weitzer & Martinez, 2007).

Although the human protein is an RNA kinase, no such activity has been identified in *Saccharomyces cerevisiae* Clp1 (Noble *et al*, 2007; Ramirez *et al*, 2008). A polynucleotide kinase-active Clp1 has been characterized in Archaea, suggesting the ancestral Clp1 possessed enzymatic activity (Jain & Shuman, 2009). It is possible that this activity was lost during yeast evolution, as the tRNA ligase Trl1 has intrinsic kinase activity.

We looked for other potential RNA kinases in yeast that are functionally related to Clp1. From this work, we identified Grc3 by bioinformatic analysis (Supplementary Fig S1 online).

GRC3 is an essential gene in *S. cerevisiae* and its transcription is cell cycle regulated (El-Moghazy *et al*, 2000), but its function is unknown. A genome-wide study has shown that Grc3 is associated with rRNA processing, and in particular with the removal of internal transcribed sequence 2 (ITS2; Peng *et al*, 2003). Grc3 was also observed, together with ribosomal proteins, in a protein fraction that was isolated by affinity purification of Rai1 (Sydorskyy *et al*, 2003), which co-purifies with and enhances

[1]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
[2]Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr-Gasse 3, and
[3]Department of Bioinformatics, Research Institute of Molecular Pathology/Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr Bohr-Gasse 7-A, Vienna 1030, Austria
*These authors contributed equally to this work
+Corresponding author. Tel: +43 1 79044 4840; Fax: +43 1 79044 110; E-mail: javier.martinez@imba.oeaw.ac.at
++Corresponding author. Tel: +44 1865 275566; Fax: +44 1865 275556; E-mail: nicholas.proudfoot@path.ox.ac.uk

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Rat1 exoribonuclease activity (Xue *et al*, 2000; Xiang *et al*, 2009). Interestingly, pyrophosphatase activity has also been identified in *Schizosaccharomyces pombe* Rai1 (Xiang *et al*, 2009).

These molecular connections led us to investigate a possible role for Grc3 in Pol I transcription termination. In particular, we considered the possibility that the phosphorylation status of the 5′-end generated by Rnt1 cleavage on rRNA is fine-tuned by RNA kinase and phosphatase activities. Rat1 is closely related to the exonuclease Xrn1 (Kenna *et al*, 1993), which preferentially hydrolyses substrates with a 5′-monophosphate end (Stevens, 1980). This end is normally generated by the RNase III-like activity of Rnt1 (Gan *et al*, 2008) but other modifying enzymes could also be involved.

In this study, we show that Grc3 is a polynucleotide kinase, is present on rDNA and that its inactivation reduces the efficiency of termination by Pol I. We propose that Grc3 kinase activity is required to maintain the phosphorylated status of the downstream Rnt1 cleavage product, which in turn allows the torpedo activity of Rat1 to efficiently terminate Pol I transcription.

RESULTS

Grc3 is a polynucleotide kinase

In *S. cerevisiae*, no polynucleotide kinase activity has been identified except for tRNA ligase Trl1. Although hClp1 is an RNA kinase, yeast Clp1 lacks kinase activity (J. Martinez, unpublished results; Noble *et al*, 2007; Ramirez *et al*, 2008). Performing National Center for Biotechnology Information–protein basic local alignment search tool (NCBI–BLASTP) searches within the NCBI non-redundant database, we identified two protein families related to Clp1 orthologues (Supplementary Fig S1 online): a group of hypothetical proteins from Eubacteria and Archaebacteria and a eukaryotic protein family including *S. cerevisiae* Grc3. These proteins share a carboxy-terminal domain of unknown function and a central nucleotide-binding domain containing a highly conserved structural element of the NTPase fold, the P-loop/Walker A box (Fig 1A).

To determine whether yeast Grc3 has kinase activity, we expressed and purified the glutathione-*S*-transferase-tagged protein and performed an *in vitro* kinase assay using radiolabelled single- or double-stranded RNA or DNA as substrates (Fig 1B). As a positive control, we tested T4 polynucleotide kinase in parallel. Phosphorylation was detected as a mobility shift on a polyacrylamide denaturing gel. As shown in Fig 1B, Grc3 displayed polynucleotide kinase activity on both single- and double-stranded RNA and on single-stranded DNA alone, but not double-stranded DNA alone. Therefore, these results support our bioinformatic analysis and confirm that Grc3 is a polynucleotide kinase.

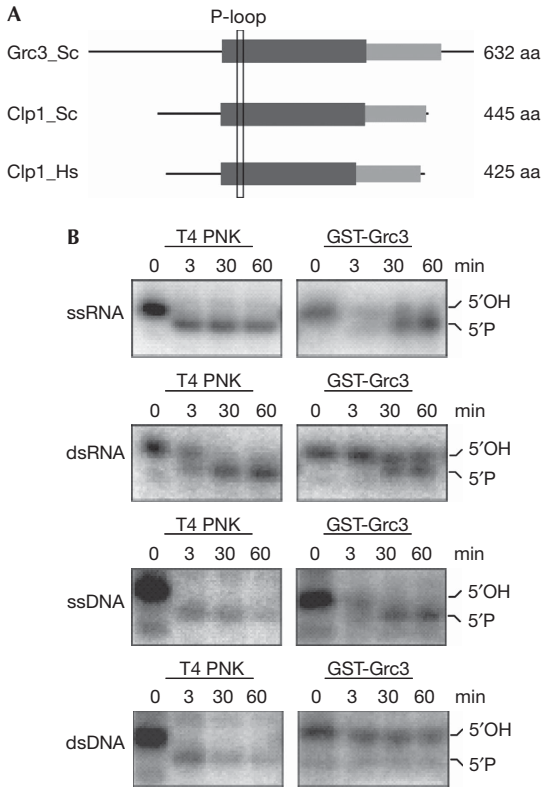

Fig 1 | Grc3 is a polynucleotide kinase. (**A**) Domain architecture of Clp1 and Grc3. The Clp1 domain architecture is derived from Noble *et al* (2007) and was transferred to Grc3 using an alignment. The central kinase domain is depicted in dark grey, the carboxy-terminal domain in light grey. Location of the P-loop/Walker A box is indicated with a rectangle. (**B**) Kinase assays of glutathione-*S*-transferase (GST)-tagged Grc3. Purified Grc3 was incubated with the indicated substrates (single- or double-stranded RNA or DNA) for the indicated time. Grc3 is active on RNA or single-stranded DNA substrates. T4 polynucleotide kinase (PNK) was assayed in parallel as a control. Phosphorylation was monitored by electrophoresis. aa, amino acid; ds, double-stranded; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; ss, single-stranded.

Grc3 ChIPs on rRNA encoding and terminator regions

Genome-wide studies suggest that Grc3 is involved in rRNA processing (Peng *et al*, 2003), which implies that it is present in the nucleolus. To determine whether this protein is recruited onto rDNA, we prepared a 3 × haemagglutinin (3HA–*GRC3*)-tagged strain and performed chromatin immunoprecipitation (ChIP) analysis across the Pol I transcription unit. As a control, we also probed the coding region of *ISY1*, a Pol II-transcribed gene. ChIP signals were detected above background across the rDNA gene (Fig 2A,B; regions 18,2 and 25,3), as well as over the terminator region (2 and 6+7). No significant enrichment was detected upstream to the transcription start site or further downstream in the 35S 3′-regions (US5S and 8). ChIP signals were relatively low but reproducible, consistent with a transient association of Grc3 with chromatin as an enzyme involved in rRNA processing. However, the presence of Grc3 over the terminator region prompted us to test further whether Grc3 is involved in 35S 3′-processing and/or Pol I transcription termination.

Grc3 depletion stabilizes 3′-ETS Pol I transcripts

We first determined whether the levels of 3′-external transcribed sequence (ETS) transcripts were influenced by Grc3 using a strain in which *GRC3* is under the control of a regulatable promoter (Tet Off). The addition of doxycycline severely affected cell growth and *GRC3* mRNA levels dropped to about 20% of the

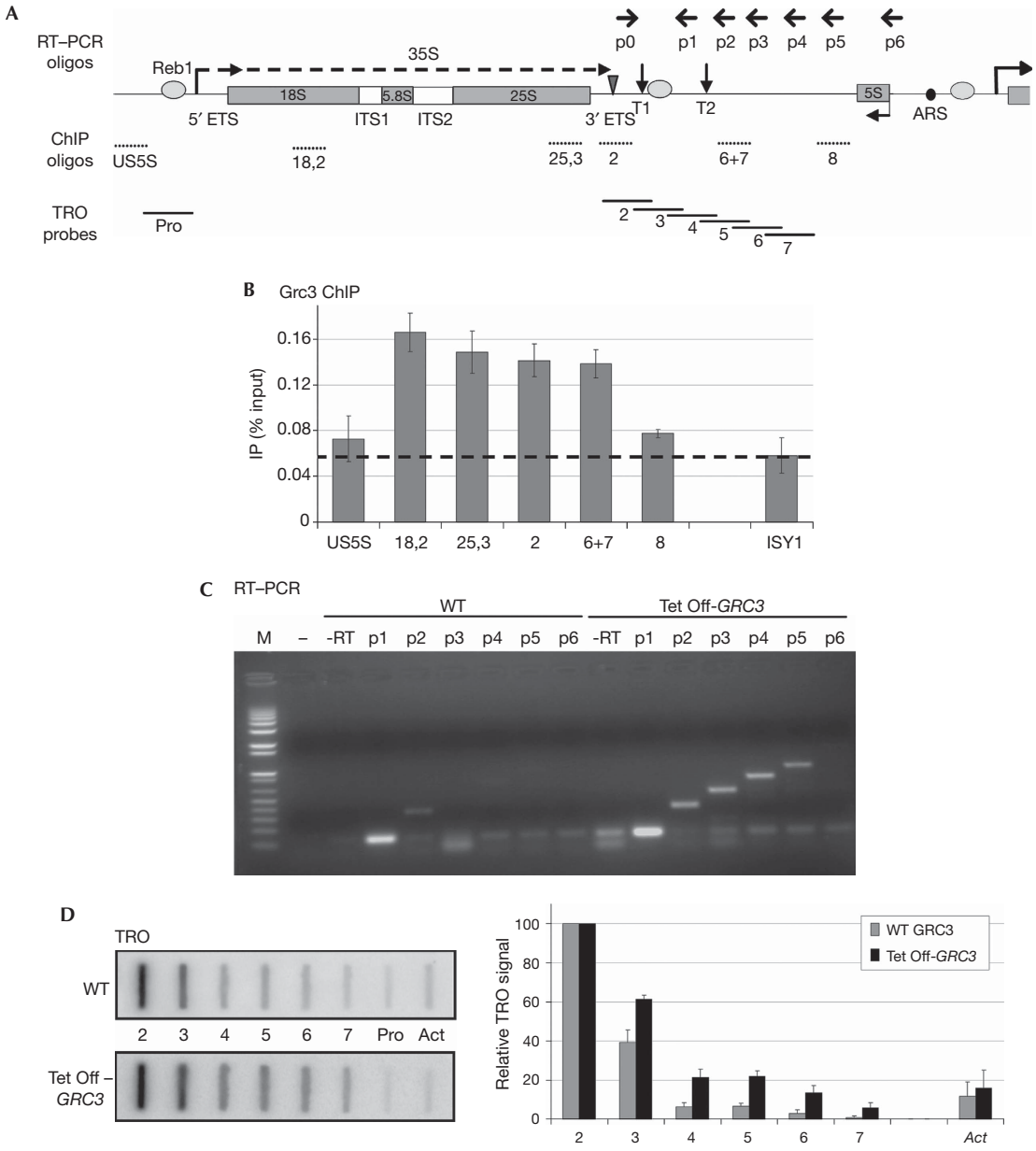

Fig 2 | Grc3 depletion results in defective transcription termination by RNA polymerase I. (**A**) Schematic of a *Saccharomyces cerevisiae* rDNA repeat. In addition to the sequence encoding 18S, 5.8S and 25S rRNA (grey rectangles), the Pol I transcription unit includes ETSs and ITSs; the 35S primary transcript is shown as a dashed arrow. Grey ovals denote binding sites for Reb1, the triangle denotes the Rnt1 cleavage site and vertical arrows indicate T1 and T2 terminator elements. 5S rDNA, transcribed by Pol III in the opposite orientation, and ARS are shown. Primers used for RT–PCR are shown above, chromatin immunoprecipitation (ChIP) oligonuleotides and TRO probes below. (**B**) ChIP analysis of 3HA-tagged Grc3 along rDNA. Specific enrichment (above dashed line) is visible over the rRNA encoding and terminator sequence. Pol II-transcribed *ISY1* is shown as a control. An average of two independent experiments is shown, error bars indicate s.d. values. (**C**) RT–PCR analysis of transcripts downstream to the Rnt1 cleavage site in WT or Grc3-depleted (Tet Off-*GRC3*) cells. Reverse transcription was primed with oligonuleotides p1–p6, PCR with the common forward primer p0 and the indicated reverse primers. Grc3 depletion results in stabilization of the Pol I transcripts over the 35S 3′-region. Oligonucleotide p1 and no reverse transcriptase (−RT) or no cDNA were used as a template in control PCR reactions. M, molecular weight marker (Invitrogen 1 kb Plus). (**D**) TRO analysis of Pol I transcripts over the 35S 3′-region in WT or Tet Off-*GRC3* cells. Grc3 depletion results in a Pol I termination defect. Quantification of the signals is shown in the right-hand panel. Background signal (Pro) was subtracted to each probe value. Data were then normalized towards probe 2, set to 100%. The Pol II-transcribed actin gene (*Act*) is shown as a control. The average of three independent experiments is shown, error bars indicate s.d. values. ARS, autonomously replicating sequence; ETS, external transcribed sequence; HA, haemagglutinin; ITS, internal transcribed sequence; RT–PCR, reverse transcriptase PCR; rDNA, ribosomal DNA; rRNA, ribosomal RNA; TRO, transcriptional run on; WT, wild type.

wild-type (WT) level after 5 h (data not shown). We extracted total RNA from WT or Tet Off-*GRC3* cells, performed reverse transcription with oligonucleotides p1–p6 (Fig 2A) and PCR with the same oligonucleotides and a common forward primer p0, annealing downstream to the Rnt1 cleavage site. With this method we detected transcripts produced by Pol I downstream to the main terminator element T1. On rDNA, a small proportion of polymerases read through this signal, mostly terminating at the downstream T2 element (Lang *et al*, 1994; El Hage *et al*, 2008). In the WT strain, reverse transcriptase PCR (RT–PCR) signal was detectable with oligonucleotide p1 (upstream to T2), very weak with p2 and not present at all further downstream (Fig 2C; left). By contrast, on depletion of Grc3, 3′-extended transcripts were visible (oligonucleotides p2–p5), up to the *5S* gene (Fig 2C; right). We conclude that Grc3 is involved in Pol I transcription termination or in stabilization of 3′-transcripts that extend beyond the normal termination site.

Grc3 depletion causes defective Pol I termination

Although RT–PCR measures steady-state RNA levels, transcriptional run on (TRO) analysis monitors nascent transcription and, thus, provides a map of the active polymerase density indicating where transcription termination occurs. Using TRO analysis, we further compared WT and Tet Off-*GRC3* strains, using probes covering the rDNA terminator region (outlined in Fig 2A). As a control, we also used a probe upstream to the Pol I transcriptional start site (Pro), providing background signal, and over the actin gene (*Act*), transcribed by Pol II. All data were quantified relative to probe 2, overlapping the T1 terminator, as previously described (Jones *et al*, 2007). In WT, we obtained the expected termination profile with a strong signal over probe 2, reduced signal over probe 3 and near to background signal downstream (Fig 2D). On depletion of Grc3, we observed a proportionally higher signal over the downstream probes (3–7), indicating a termination defect. We conclude that not only RNA stability, but also Pol I termination itself is affected by the loss of Grc3.

Pol I termination requires Grc3 kinase activity

Next, we aimed to determine whether the effect of Grc3 on Pol I termination is mediated by its polynucleotide kinase activity. We mutated specific conserved residues in the Walker A box of Grc3, based on the sequence homology with hClp1. In particular, we produced K252A and S253A mutants because the corresponding mutations abolish the kinase activity in hClp1 (Weitzer & Martinez, 2007).

We assessed the kinase activity of the mutants using purified glutathione-*S*-transferase-tagged Grc3 (WT, K252A or S253A) with a mixture of single- and double-stranded RNA substrate in the kinase assay, as before. The WT protein rapidly phosphorylated the RNA (Fig 3A), whereas the mutant K252A showed partial activity only after overnight incubation, and S253A was almost completely inactive.

To test these mutants *in vivo*, we replaced the endogenous *GRC3* promoter with the regulatable *GAL1* promoter, repressed its transcription in glucose and expressed Grc3, WT or mutant forms, from a centromeric plasmid. As shown in Fig 3B, control cells transformed with the empty plasmid showed scarce growth, both on plate and in liquid culture as expected, because *GRC3* is essential. Also transformation with WT Grc3 produced viable cells with nearly normal growth phenotype. Both K252A and S253A mutation strongly affected cell growth, the latter more severely, confirming that we had mutated critical residues in the Grc3 active site.

Next, we tested Pol I transcription termination of the K252A mutant by TRO (Fig 3C). The S253A mutant was too severely growth-retarded to allow further analysis. However, quantitative analysis of K252A produced a Pol I termination profile similar to that obtained after Grc3 depletion. Thus, a clear termination defect (higher polymerase density over probes 3–7) was obtained, strongly suggesting that the kinase activity of Grc3 is required for efficient termination. We conclude that Grc3 polynucleotide kinase activity has an important role in determining efficient termination by Pol I.

DISCUSSION

We have shown that Grc3, an essential yeast protein of previously unknown function, is a polynucleotide kinase with similarities to Clp1. Furthermore, this enzyme seems to have a physiological role in Pol I transcription termination, as kinase-inactive Grc3 mutants produce increased read-through transcription in the rDNA 3′-ETS.

How is a polynucleotide kinase involved in the termination process? Pol I transcription termination involves co-transcriptional RNA cleavage by Rnt1, followed by degradation of the downstream cleavage product by the 5′–3′ exonuclease Rat1. This ‘torpedoes’ Pol I and eventually causes termination by releasing it from the DNA template (El Hage *et al*, 2008; Kawauchi *et al*, 2008). The phosphorylation status of the downstream Rnt1 cleavage product might have a critical role in its recognition by Rat1. Substrates with 5′-monophosphate ends are strongly preferred to non-phosphorylated 5′-ends by the 5′–3′ exonuclease Xrn1 (Stevens, 1980). It is likely that Rat1, closely related to Xrn1, has similar specificity. We propose that Grc3 acts to maintain the phosphorylated status of the downstream RNA after Rnt1 cleavage, possibly by counteracting phosphatase activity (Fig 4). This equilibrium might regulate the kinetic and overall efficiency of transcription termination by Pol I. In support of this model, Grc3 has been observed to immunoprecipitate with Rai1, the Rat1-activating partner (Sydorskyy *et al*, 2003). Previous results have connected Grc3 with rRNA processing (Peng *et al*, 2003). Interestingly, Rat1 is also the RNA exonuclease involved in 5′-trimming of rRNA-processing intermediates (Kufel *et al*, 1999). Therefore, our studies predict a functional coupling between these two activities.

METHODS

Clp1/Grc3 protein family collection. hClp1 (NP_006822.1) was used as a query for NCBI–BLASTP searches within the NCBI non-redundant database (Altschul *et al*, 1997). To expand the protein family, significant hits (*E*-values $< 1 \times 10^4$) were used as queries in further searches. Selected proteins were aligned using MUSCLE (Edgar, 2004).

Expression and purification of Grc3. The open reading frame of Grc3 (WT, K252A or S253A) was cloned into pDEST20 using the Gateway technique (Invitrogen) and expressed in sf9 insect cells. After collection, cells were lysed in 100 mM NaCl, 50 mM Tris–HCl (pH 8), 5 mM $MgCl_2$, 0.1 mM 4-(2-aminoethyl) benzene-sulphonyl fluoride hydrochloride, 1 mM dithiothreitol and sonicated. Purification was performed using glutathione Sepharose 4B (GE Healthcare). Protein was eluted with 20 mM glutathione,

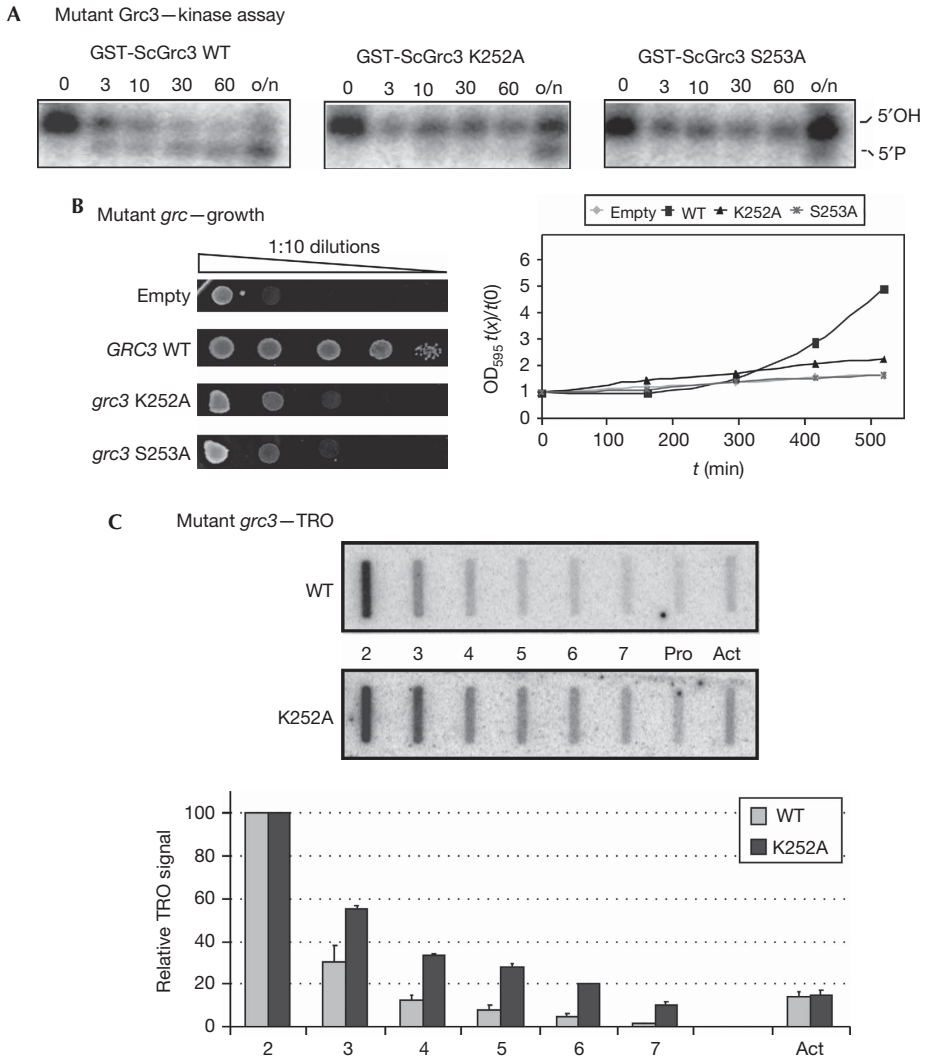

Fig 3 | Kinase-inactive Grc3 mutants are defective in cell growth and RNA polymerase I termination. (**A**) Kinase assays of WT and mutant Grc3. Purified GST-tagged Grc3 (WT, K252A or S253A) was incubated with a 2:1 mixture of single- and double-stranded RNA for the indicated time (in min; o/n, overnight). Substrate phosphorylation was monitored by electrophoresis. Grc3 kinase activity is strongly delayed in the K252A and absent in the S253A mutant. (**B**) Growth phenotype of cells expressing WT or mutant Grc3 (K252A or S253A) from a centromeric plasmid. Endogenous *GRC3* transcription was repressed in glucose. Cell proliferation is severely affected by Grc3 mutation. Cells transformed with an empty plasmid are shown as a control. Left panel: serial dilutions drop plate. Right panel: growth curves in liquid culture. (**C**) TRO analysis of cells expressing WT or K252A mutant Grc3. The kinase-inactive Grc3 mutant is defective in Pol I termination. Probes and quantification as in Fig 2D. GST, glutathione-*S*-transferase; Pol I, RNA polymerase I; TRO, transcriptional run on; WT, wild type.

concentrated on Vivaspin 500 columns (Sartorius) and dialysed against 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 10% glycerol and 0.1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride.

***In vitro* kinase assay.** Kinase assays were performed as described previously (Weitzer & Martinez, 2007) in the presence of 5 mM ATP at 30 °C. Substrates were 21-nucleotides long and either single-stranded or annealed to a complementary oligonucleotide to create blunt double strands. The RNA substrates were 3′-end-labelled with ^{32}P pCp using T4 RNA Ligase (Amersham) and then dephosphorylated with alkaline phosphatase (Roche) to obtain 5′, 3′-hydroxylated RNA substrates. The DNA substrates were labelled with ^{32}P cordycepin and recombinant terminal deoxynucleotidyl transferase (Promega). Phosphorylation was monitored on 15% polyacrylamide/urea gel.

Yeast strains and plasmids. Strains used were pGAL-3HA-GRC3 (MAT-a; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1; P_{GAL1}-3HA-GRC3 KANMX6) and untagged W303-1a (MAT-a; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1) (Fig 2B) and WT R1158 (MAT-a; his3-1; leu2-0; met15-0; URA::CMV-tTA) and Tet Off-GRC3 (MAT-a; his3-1; leu2-0; met15-0; URA::CMV-tTA; kan^R-$tetO_7$-TATA-GRC3) (Fig 2C,D). *GRC3* was repressed by

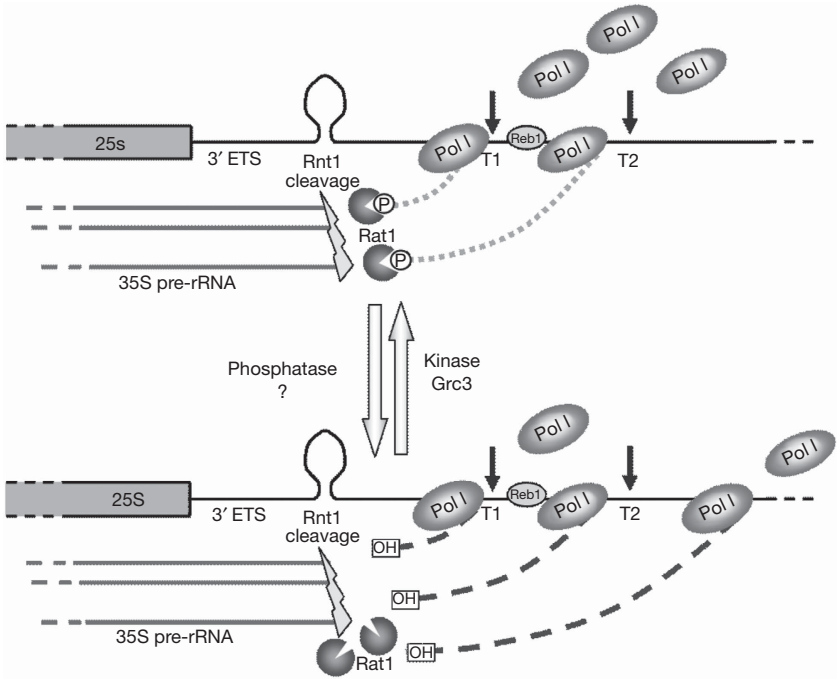

Fig 4 | Model of Grc3 kinase activity involvement in the process of transcription termination by Pol I. Rnt1 co-transcriptionally cleaves the transcript RNA at a stem loop structure in the 3′-ETS. The phosphorylation status of the Rnt1 cleavage 3′-product is controlled by the equilibrium between a putative phosphatase activity and the RNA kinase Grc3. The phosphorylated 5′-end (top) is recognized by the 5′–3′ exonuclease Rat1 that 'torpedoes' Pol I and thus promotes transcription termination. When Grc3 is absent or inactive, the equilibrium is shifted and the 3′-transcripts present a 5′-hydroxyl end (bottom). This is suboptimal substrate for the 'torpedo' Rat1. As a consequence, the 3′-transcripts are stabilized and Pol I termination is impaired. ETS, external transcribed sequence; Pol I, RNA polymerase I; rRNA, ribosomal RNA.

treating the cells with 10 μg/ml doxycycline for 5 h. pGAL-3HA-GRC3 strain was transformed with a modified pCM252 (lacking the Tet-O box repeats) containing *GRC3* open reading frame (WT or mutant) cloned at the *Stu*I site (Fig 3). Standard media and growth conditions were used.

ChIP. The ChIP analysis was performed as described previously (Kawauchi *et al*, 2008), using Anti-HA Clone F7 antibody (Santa Cruz Biotechnology). Background signal from the untagged strain was subtracted to 3HA-GRC3 signal at each position. Oligonucleotide sequences have been described previously (Kawauchi *et al*, 2008).

RT–PCR. RT–PCR was performed with Superscript III RT (Invitrogen) on 400 ng RNA priming the reaction with a mixture of oligonucleotides p1–p6. A total of 28 cycles of PCR were performed with the communal oligonulceotide p0 and each of the p1–p6 oligonucleotides. The PCRs with oligonucleotide p1 and no RT were used as a negative control. Primer sequences have been described previously (Kawauchi *et al*, 2008).

TRO. The TRO analysis and probes have been described previously (Kawauchi *et al*, 2008). Five micrograms of each single-stranded DNA probe was immobilized on Hybond-N membrane (Amersham). Cells collected in log-phase were permeabilized with sarkosyl and then incubated in transcription buffer containing α-^{32}P UTP for 5 min to label nascent transcripts. Extracted RNA was then partly hydrolysed and hybridized to the membrane.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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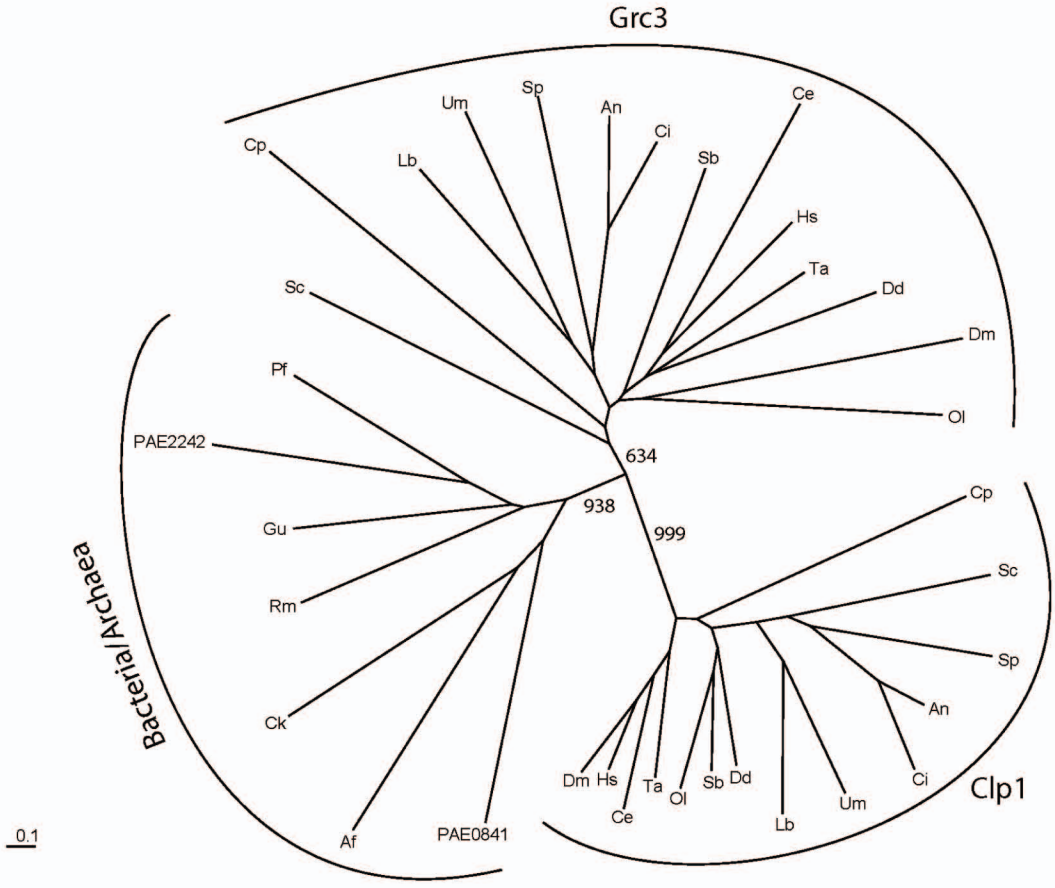

Phylogenetic tree of the Clp1/Grc3 protein family.
The tree is based on an alignment of selected Clp1 and Grc3 protein family members. Distances were calculated with PROTDIST and the tree was generated with NEIGHBOR of the PHYLIP package (Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle), using default parameters. TreeView was used for the graphical representation (Page, 1996). The length of the branch is the number of nucleotide changes that are expected in that particular branch in the tree. Support for the three major branches is provided by bootstrap probabilities (1000 replicates). Organism abbreviations and protein accessions are the following: Bacteria/Archaea group: *Archaeoglobus fulgidus* (Af, NP_070873.1), *Candidatus Korarchaeum cryptofilum* (Ck, YP_001737707.1), *Geobacter uraniireducens* (Gu, YP_001232219.1), *Pyrobaculum aerophilum* (Pb, NP_558890.1, NP_559872.1), *Pyrococcus furiosus* (Pf, NP_577841.1), *Rhodothermus marinus* (Rm, YP_003291740.1); Clp1/Grc3 group: *Aspergillus nidulans* (An, CBF90066.1/ XP_662203.1), *Caenorhabditis elegans* (Ce, NP_001040858.1/NP_502588.1), *Coccidioides immitis* (Ci, XP_001239523.1/ XP_001248112.1), *Cryptosporidium parvum* (Cp, XP_626224.1/ XP_627494.1), *Dictyostelium discoideum* (Dd, XP_638095.1/ |XP_642025.1), *Drosophila melanogaster* (Dm, NP_610876.1/ NP_611084.2), *Homo sapiens* (Hs, NP_006822.1/ Q5SY16.1), *Laccaria bicolor* (Lb, XP_001874797.1/ XP_001878778.1), *Ostreococcus lucimarinus* (Ol, XP_001416530.1/XP_001415935.1), *Saccharomyces cerevisiae* (Sc, NP_014893.1/NP_013065.1), *Schizosaccharomyces pombe* (Sp, NP_593741.1/NP_588473.1), *Sorghum bicolor* (Sb, XP_002451771.1/ XP_002457221.1), *Trichoplax adhaerens* (Ta, XP_002110634.1/ XP_002111887.1), *Ustilago maydis* (Um, XP_759217.1/ XP_761015.1).

Supplementary Figure 1

Nol9 is a novel polynucleotide 5'-kinase involved in ribosomal RNA processing

Katrin Heindl and Javier Martinez[1]

IMBA, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr-Gasse 3, 1030 Vienna, Austria

[1] Corresponding author: javier.martinez@imba.oeaw.ac.at

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ABSTRACT

In a cell, an enormous amount of energy is channeled into the biogenesis of ribosomal RNAs (rRNAs). In a multistep process involving a large variety of ribosomal and nonribosomal proteins, mature rRNAs are generated from a long polycistronic precursor. Here, we show that the nonribosomal protein Nol9 is a polynucleotide 5'-kinase that associates with the pre-60S ribosomal particles. Depletion of Nol9 leads to a severe impairment of ribosome biogenesis. In particular, the polynucleotide kinase activity of Nol9 is required for efficient generation of the 5.8S and 28S rRNAs from the 32S precursor. Upon Nol9 knock down, we also observe a specific maturation defect at the 5' end of the predominant 5.8S short form rRNA ($5.8S_S$), possibly due to the Nol9 requirement for 5'>3' exonucleolytic trimming. In contrast, the endonuclease-dependent generation of the 5'-extended, minor 5.8S long form rRNA ($5.8S_L$) is largely unaffected.
In summary, this is the first report of a nucleolar polynucleotide kinase with a role in rRNA processing.

Key words: nucleolus / polynucleotide kinase / ribosomal RNA / ribosomal RNA processing

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INTRODUCTION

Ribosomal RNA (rRNA) maturation represents a dramatic example of RNA processing, embracing a large number of enzymatic activities, small nucleolar RNAs (snoRNAs) and nonribosomal proteins (Henras et al, 2008). rRNA processing is best understood in the yeast *Saccharomyces cerevisiae* (Venema & Tollervey, 1999). Analogous processing events and the high conservation of the proteins involved suggest substantial similarities among several organisms, yet individual steps were shown to be variable (Gerbi & Borovjagin, 2004b).

Three out of four mature human rRNAs, 18S, 5.8S and 28S, are transcribed from one polycistronic transcription unit (Gerbi & Borovjagin, 2004b). In an ordered series of endo- and exonucleolytic events, external and internal transcribed spacers (ETS and ITS, respectively) are removed from the primary transcript and the mature rRNAs generated (Figure 1) (Hadjiolova et al, 1993). Immediately after transcription, external spacer sequences are degraded, generating first 45S and then 41S intermediates. A subsequent endonucleolytic cleavage within ITS1 splits the 41S precursor into the 21S and 32S rRNAs. The 21S is further processed into the mature 18S rRNA, the RNA component of the 40S small ribosomal subunit (SSU). Processing of the 32S is more complex, involving an elusive endonuclease activity that cleaves within ITS2. Eventually the mature 5.8S and 28S rRNAs are liberated and assemble, together with the independently transcribed and processed 5S rRNA, into the 60S large ribosomal subunit (LSU). Two forms of 5.8S have been described in yeast and mammals (Bowman et al, 1983; Rubin, 1974), a major short form ($5.8S_S$) and a long, 5'-extended form ($5.8S_L$).

Recent advances in large scale mass spectrometry and high troughput screens have revealed a multitude of proteins to be involved in rRNA processing (Andersen et al, 2002; Boisvert et al, 2010; Scherl et al, 2002), yet detailed studies on their individual roles are missing and important enzymatic activities are still elusive.

Our laboratory previously identified Clp1, an RNA 5'-kinase phosphorylating tRNA exons and siRNAs *in vitro* (Weitzer & Martinez, 2007b). Clp1 was initially described as a component of the mRNA 3' end formation and polyadenylation machinery (de

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Vries et al, 2000) and was later also implicated in the splicing of precursor tRNAs as a binding partner of the Sen endonuclease (de Vries et al, 2000; Paushkin et al, 2004). Bioinformatic analysis revealed a family of proteins closely related to Clp1, the "Grc3/Nol9 family" (Braglia et al, in preparation), that contains Walker A and Walker B motifs, both implicated in ATP/GTP binding (Walker et al, 1982). Interestingly, human Nol9 was previously detected in proteomic analyses of the nucleolus (Andersen et al, 2002; Scherl et al, 2002). Temperature sensitive mutants of the yeast homolog of Nol9, Grc3, showed an rRNA processing defect in a global screen for noncoding RNA processing (Peng et al, 2003), yet the role of Grc3 is not clarified.

Here, we identify Nol9 as a novel polynucleotide 5'-kinase and a component of the nuclear pre-60S particles in HeLa cells. We show that the kinase activity of Nol9 is required for efficient processing of the 32S precursor into 5.8S and 28S rRNAs and present evidence for two different processing pathways generating the two forms of 5.8S, similar to the situation in yeast. This is the first implication of a polynucleotide kinase activity in the rRNA maturation pathway.

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RESULTS

Nol9 is a polynucleotide kinase

To determine if Nol9 displays polynucleotide kinase activity, we expressed and purified GST-tagged human Nol9 from insect cells. Recombinant Nol9 phosphorylated single stranded (ss) and double stranded (ds) RNA and DNA substrates with high efficiency (Figure 2A). In order to analyze if Nol9 possesses additional enzymatic activities towards the 3' or 5' ends, we incubated GST-tagged Nol9 with dsRNA substrates displaying either a phosphate or a hydroxyl group at the 5' or 3' ends (Figure 2B). As a control we used the well-studied T4 polynucleotide kinase (T4 PNK) which harbors 3' phosphatase activity in addition to 5' polynucleotide kinase activity (Wang & Shuman, 2002). Nol9 was able to transfer a phosphate to 5' ends, but could not phosphorylate 3' ends or remove phosphates from either 5' or 3' ends of dsRNA substrates. We were also able to immunopurify Nol9 RNA 5'-kinase activity from nuclear extracts using specific antisera (Figure 2C). We conclude, that Nol9 is a 5' end specific RNA/DNA-kinase.

Nol9 co-sediments with pre-60S rRNP particles

Nol9 was previously found in high-throughput proteomic analyses of the human nucleolus (Andersen et al, 2002; Boisvert et al, 2010; Scherl et al, 2002), the main site of rRNA maturation (Boisvert et al, 2007). Already during transcription, the nascent rRNA precursors are packaged into precursor ribosomal particles (pre-rRNP), containing a multitude of ribosomal and nucleolar proteins (Granneman & Baserga, 2004; Warner & Soeiro, 1967). These complexes allow for efficient and accurate rRNA maturation and also ensure quality control. Two major forms of pre-rRNP particles are reported for HeLa nuclear fractions; the pre-40S rRNP, containing the maturing 18S rRNA, and the pre-60S rRNP particles, which include the 5.8S and 28S rRNAs and their precursors (Vaughan et al, 1967; Warner & Soeiro, 1967).

To investigate if Nol9 associates with pre-rRNPs, we separated nuclear extracts from HeLa cells on a 10 to 30% sucrose density gradient and collected 18 fractions

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by upward displacement. Each fraction was monitored by UV spectrometry at 254nm to determine protein content, yet, fractions 14 to 18 could not be measured due to the high sucrose concentration. Peaks comprising fractions 7 to 9 and 11 to 13 were observed, corresponding to the pre-40S and pre-60S rRNP particles, respectively (Figure 3A). Every fraction was tested for protein and RNA composition. Immunoblotting revealed, that Nol9 accumulated mainly in fractions 12 and 13 (Figure 3B). Nucleophosmin/B23, which co-localizes with ribosomal proteins of the 60S ribosomal subunit in HeLa nucleoli (Maggi et al, 2008), was also over-represented in these fractions together with the mature 28S rRNA (Figure 3C) and 12S and 32S RNA, the precursors of the 5.8S and 28S rRNAs (Figure 3D). RNA polymerase II was used as a control, since it is not part of either rRNP particle, and sedimented at lower weight fractions.

It is known that the composition of the pre-rRNP particles is dependent on the presence of RNA. Treating the nuclear extracts with RNase A prior to centrifugation resulted in a different UV absorption curve over the gradient, with all proteins accumulating in the earlier fractions and no obvious peaks for the pre-rRNP particles (data not shown). RNase A treatment did not alter sedimentation of RNA polymerase II (Figure 3E). Interestingly, Nol9 and B23 were largely shifted from fractions around 12 to lighter fractions (Figure 3E). This result confirms that Nol9 is part of the pre-60S rRNP particle and that this association is dependent on RNA.

Nol9 is required for rRNA processing

The above results together with the previous implication of the yeast homolog of Nol9, Grc3, in rRNA maturation by a large scale screen for proteins involved in noncoding RNA processing (Peng et al, 2003) strongly suggested a role for Nol9 in the processing of human rRNA. Therefore, we metabolically labeled HeLa cells with ^{3}H-methionine and chased rRNA maturation with non-radioactive methionine. Since precursor rRNAs are heavily methylated early on, their processing can be tracked over time. While 45S and 41S precursors are rapidly processed in wild type cells, the processing of 32S precursors is slow (Figure 4A, panels I and II). Knock down of the RNA-kinase Clp1 did not interfere with the progress of rRNA maturation (panel

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III). Strikingly, depleting the cells of Nol9 (91% knock down efficiency, determined by qPCR, data not shown) led to a drastic decrease of mature 28S levels after 240 minutes (panel IV and V), while the maturation of 18S was not affected. Additionally, processing of 45S and 41S precursors was slightly delayed. This suggests a role for Nol9 in the processing of rRNA, especially of the large subunit 28S rRNA.

Nol9 functions in the processing of large subunit rRNAs

To further investigate processing defects, we conducted a series of Northern blots, probing for different processing intermediates and products of the rRNA maturation pathway (Figure 4B). Three distinct strategies were used to knock down Nol9, leading to different knock down efficiencies of 70%, 83% and 97% (Figure 4C, lanes 3, 4 and 5, respectively). The effect on rRNA maturation correlated with the Nol9 knock down efficacy. Probes against the 5.8S (NB9) and 28S (NB40) showed elevated levels of the 32S processing intermediate and lower levels of the mature 28S and 5.8S rRNAs (Figure 4C, panels NB9 and NB40, and Figure 4D, panel NB9). As observed in the pulse chase experiment (Figure 4A), the early intermediates 45S and 41S precursor rRNA accumulated in correlation with the knock down efficiency of Nol9 (Figure 4C, panel NB11). Maturation of the 18S rRNA was not influenced by Nol9 knock down (Figure 4C, panel NB8). When we used probes against the U6 snRNA (data not shown) or the mitochondrial Glycine-tRNA (Figure 4D, NB48) as loading controls for the Northern blots, we observed unequal signals, although total RNA amounts loaded were always the same. We suggest, that this is due to an alteration of the overall composition of extracted RNA as a consequence of Nol9 depletion, resulting from the vast absence of the 28S and 5.8S rRNAs. Since rRNAs make up the majority of cellular RNAs, decreasing rRNA levels might result in a relative enrichment of other RNA classes in total RNA extracts. Acknowledging this theory, we normalized the 5.8S levels observed in Figure 4D (NB9) to those of the mitochondrial Glycine-tRNA (NB48). Nol9 knock down decreased the levels of $5.8S_S$ to 8% and of $5.8S_L$ to 13% of mock transfected cells.

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The 5.8S and 28S rRNAs are core components of the large 60S ribosomal subunit, whereas the 18S is assembled into the small 40S ribosomal subunit (Fatica &

Tollervey, 2002; Fromont-Racine et al, 2003; Venema & Tollervey, 1999). To investigate, if the levels of 40S SSU, 60S LSU and 80S ribosomes were imbalanced upon depletion of Nol9, we carried out HeLa cytoplasmic extract sedimentation studies. We observed a clear defect in 80S ribosome biogenesis in HeLa cells depleted of Nol9 as a direct effect of the vast absence of 60S ribosomal subunit (Figure 4E). This clearly indicates a requirement of Nol9 for the maturation of the large ribosomal subunit and hence the ribosome.

The polynucleotide kinase activity of Nol9 is required for rRNA processing

To differentiate, if the kinase activity of Nol9 itself is essential for rRNA maturation or Nol9 only serves as a scaffold protein within a larger complex, we generated kinase-inactive Nol9 mutants. Nol9 contains Walker A and a Walker B motifs which are both implicated in ATP/GTP binding (Walker et al, EMBO J 1982). Mutations within these domains often impair enzymatic activity (Wang & Shuman, 2001). The Walker A motif consists of the peptide stretch GxxxxGKS/T, which we altered by exchanging the lysine or serine (positioned at amino acid 312 and 313 in human Nol9, respectively) with an alanine (referred to as K312A or S313A, respectively). These mutations efficiently abolished kinase activity in recombinant Nol9 confirming that the Walker A motif is essential for the activity (Figure 5A). To investigate the role of the kinase activity of Nol9 in rRNA processing, we first depleted Nol9 from HeLa cells and then expressed either wild type or Walker A mutant human Nol9. 32S precursor levels were monitored by Northern blot using two different probes and subsequently quantified by signal intensity measurement. Upon Nol9 knock down the levels of 32S were increased by a factor of 4.1 and 2.2, respectively (Figure 5B and C). Whereas concurrent over-expression of wild type Nol9 diminished the elevated 32S levels to only 1.7 and 1.5 fold, respectively,

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both mutated forms of hNol9, K312A and S313A, could mitigate the effect of the Nol9 knock down on 32S levels only slightly (Figure 5B and C). The S313A mutation seemed to be more severe than the K312A mutation which showed a mild rescue effect on the levels of 32S precursor rRNA. 18S was used as a loading control, knowing that Nol9 does not alter its maturation. Wild type and mutant forms of

Nol9 were expressed with equal efficiency as determined by qPCR (Figure 5D), excluding that the above results were due to different expression levels. Therefore, our data strongly suggest, that the kinase activity of Nol9 is required for efficient rRNA processing.

Nol9 knock down affects the ratio between short and long 5.8S rRNA

In yeast, two alternate processing pathways are described to generate the 5'end of 5.8S rRNAs producing either the "short" ("$5.8S_S$") or the extended form of 5.8S rRNA bearing 6 additional nucleotides on the 5' end ("long" or "$5.8S_L$") (Faber et al, 2006; Henry et al, 1994; Schmitt & Clayton, 1993). In mammals, 5' heterogeneity of 5.8S rRNA was also described (Bowman et al, 1983; Khan & Maden, 1977; Smith et al, 1984). Aside the decrease of total 5.8S rRNA upon knock down of Nol9 (Figure 4D, panel NB9) we also observed an alteration of the ratio between the short and the long 5.8S rRNAs (Figure 6A). Whereas in wild type HeLa cells $5.8S_S$ comprised 70% and $5.8S_L$ only 30% of all mature 5.8S rRNAs, Nol9 knock down reproducibly shifted this ratio towards the long $5.8S_L$ form, resulting in ~55% $5.8S_S$ versus ~45% $5.8S_L$. Primer extension analysis using two individual primers near the 5' end of 5.8S (Figure 6B) confirmed this observation; the majority of transcripts terminated at the predicted 5' end of 5.8S in wild type RNA preparations, while a smaller fraction of 5'-extended transcript was detected (Figure 6C and D). Although we could not predict the exact sequence of the 5' extension from the primer extension analysis, it still allowed us to conclude that $5.8S_L$ was 5 nucleotides longer than $5.8S_S$ in HeLa cells and that Nol9 knock down decreased the short form but did not alter the levels of the long form of 5.8S (Figure 6D). Altogether, we conclude that, similar to the situation in yeast, in HeLa cells two alternate pathways for the generation of the 5' end of 5.8S co-exist, resulting in either the short or long 5.8S rRNA. Affecting the generation of the major short form is not completely rescued by an increase of the levels of the long form, suggesting that the pathway for $5.8S_L$ cannot complement the loss of the $5.8S_S$ maturation machinery. The polynucleotide kinase Nol9 is primarily involved in the generation of the major $5.8S_S$ form.

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DISCUSSION

We report the involvement of Nol9, a novel polynucleotide 5'-kinase, in the maturation of the 60S ribosomal subunit. Nol9 is a potent RNA/DNA 5'-kinase (Figure 2) associated with the pre-60S rRNPs in nuclear extracts (Figure 3). Absence of Nol9 decreases 5.8S and 28S rRNA levels to a great extent and increases the levels of the 32S, 41S and 45S precursors (Figure 4). Interestingly, the mere presence of Nol9 in the cell is not enough to ensure efficient rRNA processing; only the wild type, kinase-active form of Nol9 is capable to recover rRNA levels upon prior depletion of Nol9 (Figure 5). Nol9 specifically impairs maturation of the major $5.8S_S$ form, but does not seem to be involved in generating the minor $5.8S_L$ form (Figure 6). Since we could not identify the direct substrate for Nol9 in rRNA processing, we can only speculate on the role of the kinase activity. The obvious block of 32S processing implies that cleavage within ITS2, separating 5.8S and 28S, cannot occur. This can be due to (1) a direct effect of Nol9 on the endonuclease activity itself, or (2) an upstream quality control check that detects improperly completed 32S processing in ITS1 (Good et al, 1997) (Figure 7A). Either option would lead to a transient accumulation of 32S as well as the upstream precursors 41S and 45S followed by premature degradation, as we observed upon Nol9 knock down (Figure 4A).

As we show that the kinase activity of Nol9 itself is required for 32S processing (Figure 5B and C), Nol9 does not only serve as a scaffolding protein, but actually contributes to the rRNA maturation pathway with its kinase activity. Interestingly, the RNA-kinase Clp1, a homolog of Nol9, was found associated to the tRNA endonuclease complex (Paushkin et al, 2004). In yeast, the 3' end of 18S is generated by the endonuclease Nob1 (Pertschy et al, 2009), which binds to the putative Walker A-type kinase Fap7 (Granneman et al, 2005). Although to date no definitive role of a polynucleotide kinase within an endonucleolytic event is identified, the recurring observations of complexes containing both kinase and endonuclease activities are intriguing.

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rRNA processing follows a series of events, and only the correct order assures proper maturation of the ribosomes. For yeast it was shown, that the accurate removal of spacer sequences introduces conformational changes that allow the pathway to proceed to the next step (Cote et al, 2002). Most processing mutants only showed mild accumulations of precursors due to rapid and complete degradation by the exosome (Allmang et al, 2000), very similar to our observations for Nol9 depletion in HeLa cells. In yeast, the 5' end of the 32S equivalent is mainly produced by the combined action of an endonucleolytic cleavage event upstream and subsequent exonucleolytic digestion by Rat1. A second, less frequent pathway involves a single endonucleolytic cleavage generating a 32S form containing a 5' extension of a few nucleotides (Chu et al, 1994; Henry et al, 1994; Schmitt & Clayton, 1993). In metazoans, the mechanism is assumed to be similar (Tycowski et al, 1994). For HeLa cells the endonucleolytic cleavage site of the major pathway was mapped between nucleotides 942 and 949 within ITS1, ~150 nucleotides upstream of the 32S precursor (Idol et al, 2007). Figure 4C, panel NB11, shows a blot hybridized to a probe against the very 3' end of the spacer ITS1, visualizing all 5'-extended precursors of 32S RNA. A prominent smear can be observed (indicated by an asterisk), which resembles the 5'-elongated 32S RNA after the endonucleolytic cleavage of the main pathway in ITS1 but prior to complete exonucleolytic degradation. Comparing lanes 1 (wild type cells) and 5 (the most efficient depletion of Nol9) by linear density analysis, the pattern shows a very low signal intensity for wild type cells with peaks in the lower parts of the smear and the 41S and 45S RNAs (Figure 7B). RNAi depletion of Nol9 not only increased the signal intensities of 41S and 45S RNAs, but also shifted the intensity peak of the smear upwards indicating that the majority of the precursor is not efficiently 5' trimmed. This very 5' end might be the entry point for the RNA-kinase Nol9, providing the 5' phosphate and thus a favorable substrate for the subsequent 5'>3' exonucleolytic activity (Stevens, 1980). Knocking down Nol9 would impair this phosphorylation event, thereby blocking efficient 5' end trimming to produce the actual 32S precursor and consequently resulting in an rRNA maturation defect specifically of the large subunit rRNAs (Figure 7A (2)).

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Whereas in humans the endonuclease of the major pathway cleaving within ITS1 is elusive, this process is well characterized in yeast, where the RNase MRP cleaves the rRNA precursor at the corresponding A3 site, providing an entry point for Rat1 (Chu et al, 1994; Henry et al, 1994; Schmitt & Clayton, 1993). Efforts to show this function for metazoan MRP homologs have been unsuccessful so far. It is worth noting, that MRP belongs to the RNase P family and generates 5' phosphorylated cleavage products (Reilly & Schmitt, 1995). In case the endonuclease in metazoans is similar to yeast, Nol9 kinase activity would not be required. Since we do not observe a distinct cleavage intermediate upon Nol9 knock down but a smear of exonucleolytic degradation intermediates, our data could argue against a single entry point for the kinase Nol9, but rather suggest constant phosphorylation events after removal of each individual nucleotide (Figure 7A (2)). The following possibilities could support our hypothesis; either the metazoan exonuclease generates 5' hydroxyl ends or a phosphatase activity continuously de-phosphorylates the 5' ends and thus slows down 5'>3' degradation. The existence of a counteracting phosphatase activity in human cells has been a matter of speculation (Weitzer & Martinez, 2007a)Braglia *et al*., in preparation), yet to date, no such activity could be attributed to a known protein *in vivo* or reconstituted in an *in vitro* system. However, it is interesting to note, that Grc3, the yeast homolog of Nol9, was found in purifications of Rai1, an important partner and enhancer of the 5'>3' exonuclease Rat1 (Sydorskyy et al, 2003) and depletion of Rai1 or Grc3 impairs Rat1-dependent degradation of the 3' trailing transcript in RNA polymerase I termination (El Hage et al, 2008) Braglia et al, in preparation). In addition, we show that Nol9 depletion mainly impairs maturation of the major $5.8S_S$ form, but not of $5.8S_L$ (Figure 6). In yeast, $5.8S_L$ is generated by a distinct endonucleolytic event, but does not involve the action of an exonuclease (Faber et al, 2006). Our data present evidence that two independent processing pathways also co-exist in human cells generating either the short or long form of 5.8S. Nol9 seems to be involved in the exonuclease-dependent processing of $5.8S_S$ rRNA.

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Other possible substrates for Nol9 are the small nucleolar RNAs (snoRNAs). This class of RNAs guides rRNA modifications (Ganot et al, 1997a; Kiss-Laszlo et al, 1996;

Ni et al, 1997) and can be required for rRNA cleavage itself (Chu et al, 1994; Steitz et al, 1990). More than 90% of human snoRNAs are encoded in the introns of coding and non-protein-coding genes (Dieci et al, 2009). As for rRNAs, maturation of these snoRNAs involves the concerted action of endo- and exonucleases (Filipowicz & Pogacic, 2002). In yeast, Rat1 and Xrn1 are reported to provide the 5'>3' exonuclease activity generating the 5' end of intronic snoRNAs (Petfalski et al, 1998). Nol9 could be involved in generating the 5' ends of snoRNAs in a similar fashion as described above for the 5' end formation of 32S RNA (Figure 7A (3)). Since Nol9 depletion specifically blocks generation of only the 5.8S and 28S rRNA, but does not impair 18S maturation or RNA methylation as observed with metabolic labeling (Figure 4A), it is unlikely that Nol9 is involved in the general processing of snoRNAs.

In the light of the emerging evidence of new "un-traditional" roles of the nucleolus (Gerbi et al, 2003; Olson et al, 2002), further studies could reveal additional functions for Nol9. A growing number of RNPs and their components are reported to localize to the nucleolus in some stages of their life cycle, including SRP (Jacobson & Pederson, 1998) and RNase P (Jacobson et al, 1997), but also RNA editing (Desterro et al, 2003), genome integrity (Kobayashi, 2008) and RNA viruses (Hiscox, 2007) were previously linked to the nucleolus. Given that Nol9 can efficiently phosphorylate RNA and DNA substrates, a future line of research will be to find endogenous substrates of Nol9. It will therefore be interesting to reveal direct interacting partners of Nol9, which could lead to new roles for the polynucleotide 5'-kinase Nol9 in RNA metabolism.

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MATERIALS AND METHODS

Expression and Purification of wild type and mutant Nol9

The open reading frame of human Nol9 (NM_024654) was cloned into pDEST20 using the Gateway technique (Invitrogen) and expressed in sf9 insect cells. After harvesting, the cells were lysed in 100mM NaCl, 50mM Tris-Cl pH 8, 5mM $MgCl_2$, 0.1mM AEBSF, 1mM DTT and sonicated. Purification was performed using Glutathione Sepharose 4B (GE Healthcare). Protein was eluted with 20mM Glutathione pH 8, concentrated on Vivaspin 500 columns (Sartorius) and dialyzed against 30mM Hepes pH 7.4, 5mM $MgCl_2$, 100mM KCl, 10% Glycerol, 0.1mM AEBSF prior to use in kinase assays. The mutations were introduced using the QuikChange Site-directed mutagenesis kit (Agilent Technologies) and primers hNol9_K312A_rev (GGTATCTATTAAATGTTGACGCTCCAACATCCTGGG) and hNol9_K312A_fw (CCCAGGATGTTGGAGCGTCAACATTTAATAGATACC) or hNol9_S313A_rev (GGTATCTATTAAATGTTGCCTTTCCAACATCCTGGG) and hNol9_S313A_fw (CCCAGGATGTTGGAAAGGCAACATTTAATAGATACC).

In vitro kinase assay

Kinase assays were carried out as described in (Weitzer & Martinez, 2007b) in the presence of 5mM ATP.

Immunoprecipitation of endogenous Nol9

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Antisera were coupled to Protein A Sepharose 4FF (GE Healthcare) and incubated with HeLa cell extracts for 2 hours at 4°C. Kinase assays were carried out directly on the beads. Antisera were produced by Gramsch Laboratories, Germany. Clp1 antiserum 988 as described in (Weitzer & Martinez, 2007b). Nol9 antiserum 1687/1688 and 1689 were targeted against peptides EEAHKEKPYRRPKFC and CITPRNRESHNKILR, respectively. Pre-immune serum was taken from the rabbit prior to production of antiserum 1688.

Sucrose density studies

Ribosomal profiles were obtained as described in (Strezoska et al, 2000) on a 10-45% sucrose gradient.

Pre-ribosomes were analyzed as described in (Pestov et al, 2008) on a 10-30% sucrose gradient. Gradient was fractionated using downward replacement and UV measurement was carried out using the NanoDrop spectrophotometer. Fractions were analyzed for RNA and protein content by Northern blot and Western blot, respectively. Primary antibodies used: Nol9, affinity-purified antiserum 1687 (Gramsch Laboratories, Germany); Nucleophosmin/B23, B 0456 (Sigma), Polymerase II antibody H14 (Covance). RNAse A treatment was carried out incubating the nuclear extracts for 10 min at 30°C with 100µg/ml RNAse A (Promega).

Cell culture, knock down and transfection

HeLa cells were cultured as described in (Weitzer & Martinez, 2007b). siRNA-directed knock down was performed using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer and siRNAs against Clp1 (Dharmacon smart pool L-019895-00) or Nol9 (Dharmacon smart pool L-019418-02 for 97% knock down efficiency; where indicated we used single siRNA targeting the 3' UTR of Nol9, J-019418-18 for 70% knock down efficiency). Optionally, we used the pSUPER vector system (oligoengine) to produce a short hairpin RNA targeting the 3' UTR of Nol9 (using oligonucleotides GATCCCCCCGATGCCCAAACCACTTTTTCAAGAGAAAAG TGGTTTGGGCATCGGTTTTTA and AGCTTAAAAAACCGATGCCCAAACCACTTTTCTCTT GAAAAAGTGGTTTGGGCATCGGGG, as determined by RNAxs, (Tafer et al, 2008), resulting in 83% knock down efficiency. Knock down was generally performed two consecutive times to assure effectivity. Cells were transfected with the siRNA for three days, split and re-transfected for another three days. Knock down efficiency was determined by qPCR using standard conditions and QIAGEN QuantiTect Primer

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Assays (GAPDH for normalization, QT01192646; Nol9, QT00011305). In the rescue experiments, the pSUPER vector system was used for knock down. In the second round of transfection with the pSUPER vector we co-transfected wild type or mutant Nol9 cloned into the pcDNA-DEST53 Gateway vector (Invitrogen).

Metabolic labeling

Cells were labeled with L-[methyl-^{3}H]-methionine as described in (Strezoska et al, 2000).

Northern blot analysis

RNA was extracted from HeLa cells using TRIzol reagent (Invitrogen), transferred to a positively charged nylon membrane (Hybond N+, GE Healthcare) in 20xSSC by capillary forces, UV-cross linked and incubated with Ambion Ultrahyb oligo buffer. Hybridization to 5' (^{32}P)-phosphorylated oligonucleotides was carried out over night at 62°C, followed by consecutive washing steps in 5% SDS/5xSSC, 1% SDS/1xSSC and 0.1% SDS/1xSSC. Radioactive signals were analyzed by phosphorimaging. For analysis of the 5.8S rRNA, total RNA was separated on an 8% urea polyacrylamide gel (SequaGel system, National Diagnostics) and transferred to a nylon membrane in 0.5x TBE with 300mA for 2 hours. Probes used:

NB8 CCGGCCGTGCGTACTTAGACATGCATGGC,

NB9 CCGGGGCCGCAAGTGCGTTCGAAGTGTCG,

NB11 GGTCGATTTGGCGAGGGCGCTCCCGACG,

NB39 GGCGATTGATCGGCAAGCGACGCTCAGAC,

NB40 CTCCTTGGTCCGTGTTTCAAGACGGGTCGGG,

NB48 TACTCTTTTTTGAATGTTGTCAAAACTAGTTAATTGGAAGTTAACGGTAC TATTTATACTAAAAGAGT.

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For quantification of signal intensities in Northern blot analyses the ImageQuant analysis software by GE Healthcare was used.

Primer extension analyses

Primer extension analysis was adapted from (Beltrame & Tollervey, 1992). 1µg RNA from wild type or Nol9 knock down cells were shortly incubated with 0.5 pmoles primer (NB82 CTAGCTGCGTTCTTCATCGACG or NB86 CGCACGAGCCGAGTGATC) at 85°C for denaturation. Consequently, primer extension was performed with the Invitrogen Superscript III system for 50 minutes at 50°C in the presence of 1mM dNTPs. For the sequencing reaction, the indicated ddNTPs (A, ddATP; T, ddTTP; G, ddGTP; C, ddCTP) were added to the primer extension reaction at a final concentration of 0.25mM. Reactions were loaded on an 8% urea polyacrylamide gel and visualized by phosphorimaging.

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ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1 The 18S, 5.8S and 28S rRNAs are organized into a single polycistronic rDNA transcription unit, which also contains external transcribed spacers (ETS) on the 5' and 3' ends and two internal transcribed spacers (ITS). A series of endo- and exonucleolytic steps are required for proper maturation of the rRNAs. The mature 18S rRNA is eventually assembled into the 40S small ribosomal subunit (SSU); 5.8S and 28S rRNAs together with the independently transcribed and processed 5S rRNA are core components of the 60S large ribosomal subunit (LSU). Two forms of 5.8S are reported to co-exist; the major $5.8S_{S(hort)}$ and the 5' extended $5.8S_{L(ong)}$ form. Depicted are detectable intermediates of the major rRNA processing pathway in HeLa cells (derived from (Hadjiolova et al, 1993). Boxes represent rRNAs, triangles mark relevant endonucleolytic cleavage sites.

Figure 2 Human Nol9 is a polynucleotide 5'-kinase. (**A**) Nol9 phosphorylates single (ss) or double stranded (ds) RNA and DNA substrates. Purified GST-tagged hNol9 was incubated with the indicated substrates for the indicated time points. Phosphorylation was monitored by a mobility shift in electrophoresis. (**B**) Nol9 lacks 5'/3'-phosphatase or 3'-kinase activity on dsRNA substrates. Purified GST-Nol9 was incubated with the indicated RNA substrates displaying either 5' and 3' hydroxyl-, 5' phosphate and 3' hydroxyl-, or 5' hydroxyl- and 3' phosphate groups. Kinase activity was monitored over time. T4 polynucleotide kinase (PNK) was used as a control. Asterisk indicates radioactive label within substrate RNA. (**C**) Endogenous Nol9 phosphorylates RNA substrates. 5'-phosphorylation of dsRNA substrates after immunoprecipitation (IP) from HeLa nuclear extracts using antisera against Nol9 or Clp1.

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Figure 3 Nol9 co-fractionates mainly with the pre-60S particles. (**A**) HeLa nuclear extracts were separated on a 10-30% sucrose density gradient and divided into 18 fractions by downward displacement. UV absorption at 254nm of fractions 1 – 13 was measured to follow protein concentration and displayed in a graph. All fractions

were analyzed for RNA and protein content. (**B**) Fractions from (A) were loaded onto an SDS-PAGE to monitor Nol9 fractionation by immunoblotting using affinity-purified Nol9 antiserum. B23 and RNA Polymerase II were used as controls. (**C**) RNA was extracted from fractions in (A) and separated by electrophoresis, transferred to a membrane and stained with methylene blue to visualize 18S and 28S rRNAs. RNA from fraction 12 was greatly lost during this procedure. (**D**) After methylene blue staining, the membrane from (C) was hybridized with probe NB39 (see Figure 4B) to detect the 32S and 12S processing intermediates. (**E**) Nol9 sedimentation is RNA-dependent. Experiment was carried out as in (B) but the nuclear extracts were treated with RNase A prior to ultracentrifugation.

Figure 4 Depletion of Nol9 impairs ribosomal RNA processing. (**A**) Metabolic labeling of untreated (panel I) or mock transfected HeLa cells (panel II), or HeLa cells transfected with siRNAs targeting Clp1 (panel III) or Nol9 (panel IV). Cells were starved in methionine-free medium followed by addition of ^{3}H-methyl-methionine and chased in non-radioactive medium for the indicated time periods (in minutes). Panel V is shown to compare all four backgrounds after chasing for 240 min. (**B**) Illustration of the rRNA polycistronic transcript including the 5' and 3' external transcribed spacers (5'ETS and 3'ETS), the internal transcribed spacers 1 and 2 (ITS1 and ITS2) and the mature 18S, 5.8S and 28S rRNAs. Magnification of the 5.8S rRNA shows the short 5.8S$_S$ (white box) and the additional 5' extension of the long 5.8S$_L$ form (dashed line). Probes for Northern blot are indicated by grey boxes (NB8, NB9, NB11, NB39, NB40). (**C**) Nol9 depletion affects maturation of 28S but not 18S rRNA. 0.5μg total RNA containing wild type levels of Nol9 mRNA (lanes 1 and 2), or obtained from different Nol9 knock down experiments (efficiencies determined by qPCR, data not shown. 70% knock down efficiency, lane 3; 83%, lane 4; 97%, lane 5) were separated by electrophoresis, transferred to a membrane and hybridized with the indicated probes. (**D**) Nol9 is required for maturation of the 5.8S rRNA. 1μg RNA from mock transfected HeLa cells (lane 1) or cells transfected with siRNAs against Nol9 (lane 2) or Clp1 (lane 3) were hybridized with a probe against 5.8S (NB9); Mitochondrial tRNA^{Gly} (NB48) was used as control. Signal intensities were

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quantified, normalized to lane 1 (wild type) and displayed beneath the blot. (**E**) Depletion of Nol9 results in a severe decrease of 60S and 80S ribosomes. Ribosome profiles were obtained from HeLa cells transfected with a mock control or siRNAs against Nol9. Cytoplasmic extracts were separated on a 10-45% sucrose density gradient. UV absorption along the gradient was measured.

Figure 5 The kinase activity of Nol9 is required for efficient rRNA processing. (**A**) Nol9 Walker A mutations K312A and S313A abolish kinase activity. Purified GST-Nol9 (wildtype or mutant) was incubated with double stranded RNA substrates for indicated time points in minutes; o/n, over night; His-tagged Clp1 was used as control. (**B**) HeLa cells were first transfected with an empty vector (mock kd) or a short hairpin construct targeting the 3'UTR of Nol9 (Nol9 kd). In the second round of transfection, either an empty vector or a vector encoding the wild type Nol9, mutant version K312A or S313A of Nol9 were co-transfected. RNA was extracted, separated by electrophoresis, transferred to a membrane and hybridized using indicated probes. (**C**) Signal intensities of 32S precursor and 18S rRNA from the above Northern blots in (B) were quantified and displayed in a graph. (**D**) Nol9 is efficiently expressed from transfected vectors. qPCR analysis of Nol9 mRNA levels in the RNA extracts used in (B). Values were normalized to the mock knock down / empty vector control.

Figure 6

Nol9 knock down affects the ratio between $5.8S_S$ and $5.8S_L$. (**A**) Signal intensities of $5.8S_S$ and $5.8S_L$ in Figure 4D were measured, calculated ratios are depicted in a bar graph. (**B**) Genomic sequence of the ITS1-5.8S junction in the rDNA locus derived from NCBI HSU13369. Grey boxes depict location of primers used in primer extension. (**C**) 1µg RNA from wild type cells ("wt") or Nol9 knock down cells ("N"; identical to the RNA used in Figure 4C, lanes 1 and 5, respectively) were used in primer extension with the indicated primer (NB82 or NB86). In parallel, sequencing reactions were performed using ddNTPs (A, ddATP; T, ddTTP; G, ddGTP; C, ddCTP) M, decade Marker with size indications on the left side. The sequencing results are

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depicted on the right side of both gels, asterisks indicate unreadable results. According $5.8S_{S/L}$ sequences as derived from the NCBI database (HSU13369) are illustrated inside the rectangles. (**D**) Signal intensities of $5.8S_S$ and $5.8S_L$ from (C) were quantified and plotted against the y-axis. Calculated ratios between the short and long forms are stated within the columns.

Figure 7 A model for the function of Nol9 in rRNA processing. (**A**) Possible roles of the polynucleotide kinase Nol9 in rRNA processing. (1) Nol9 might act directly on the endonuclease cleaving within ITS2. (2) Nol9 together with an elusive phosphatase activity might regulate 5' end formation of 32S RNA by an exonuclease. (3) Nol9 might be required for snoRNA processing. Scissors indicate endonucleolytic cleavage sites, the "pacman" represents the exonuclease. RNAs are depicted in 5'>3' orientation. (**B**) Nol9 might have a role in the 5'>3' degradation creating the 5' end of 32S. Lanes 1 and 5 of the Northern blot from Figure 4C, panel NB11, were analyzed using the ImageQuant analysis software by GE Healthcare. The signal intensities of the area depicted on the left side were gradually measured and illustrated in a graph. Dashed lines mark the pixel position of the intensity peaks in the smear.

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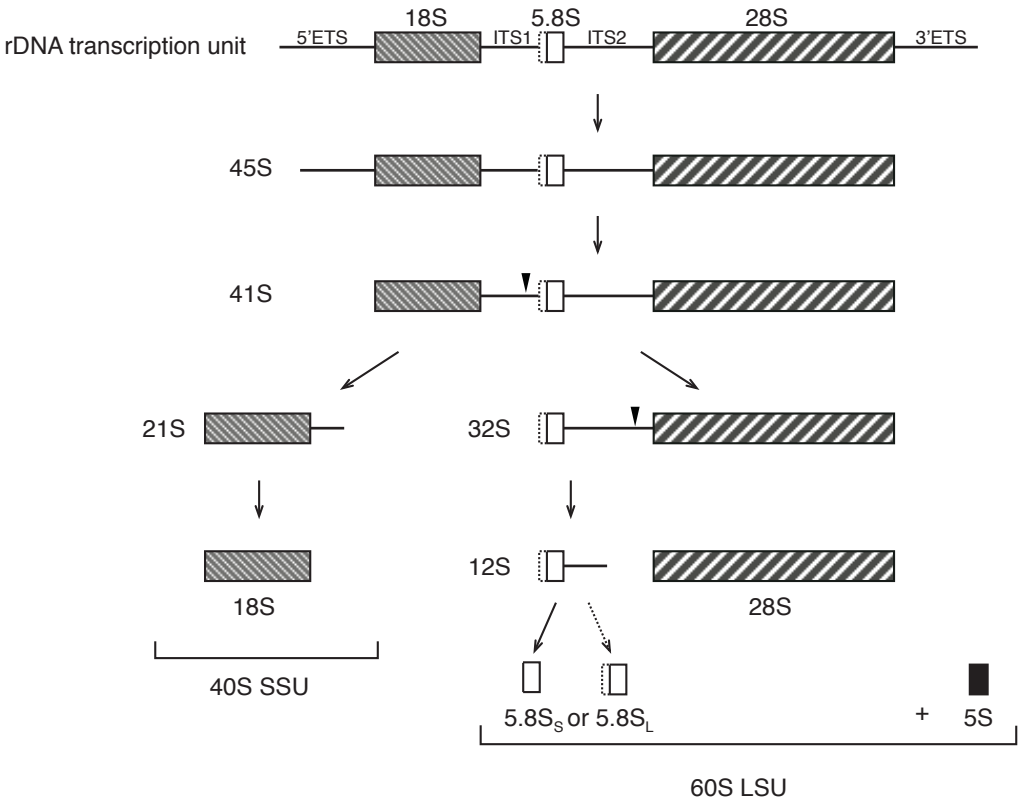
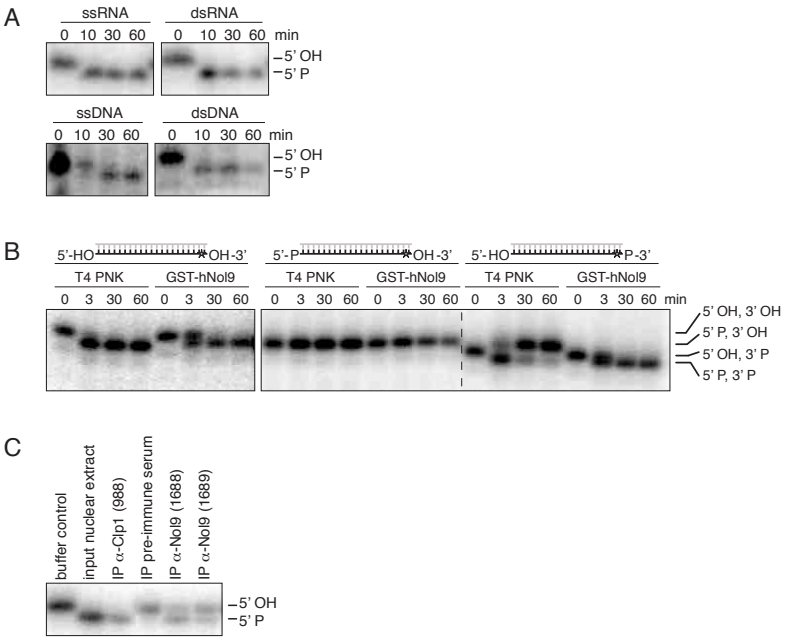

rDNA transcription unit
5'ETS
18S
ITS1
5.8S
ITS2
28S
3'ETS
45S
41S
21S
32S
18S
12S
28S
40S SSU
5.8S$_S$ or 5.8S$_L$
+
5S
60S LSU

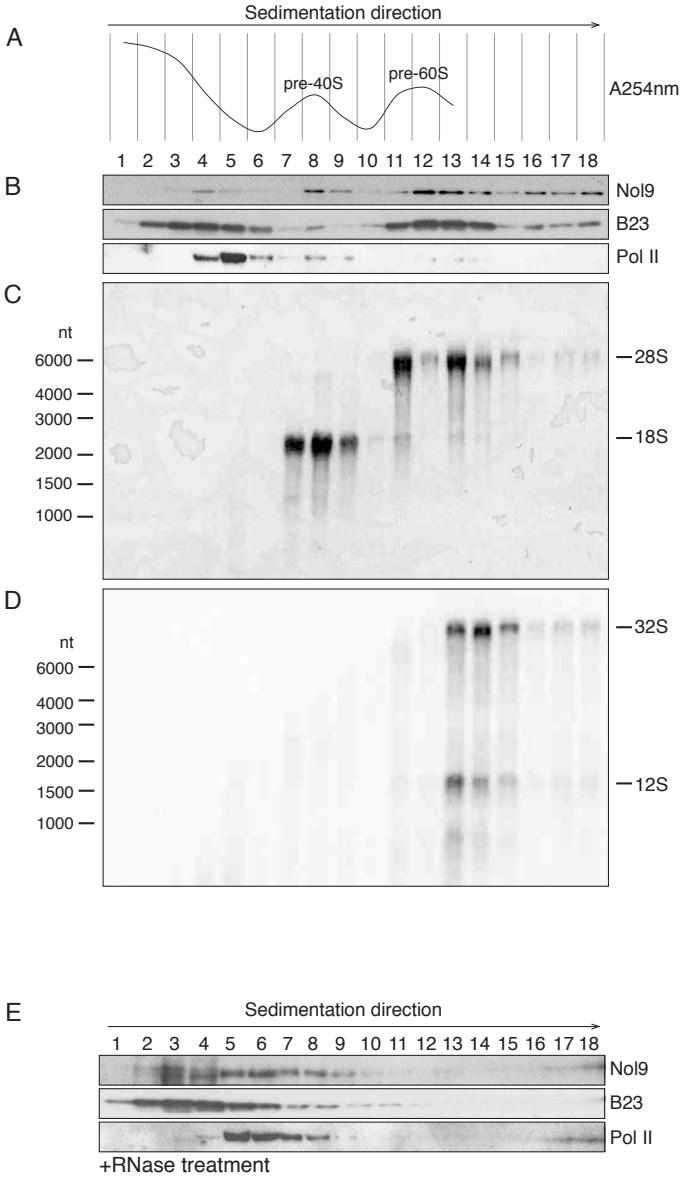
83

Figure 1


A
ssRNA
dsRNA
0 10 30 60 min
5' OH
5' P
ssDNA
dsDNA
B
5'-HO
OH-3'
5'-P
P-3'
T4 PNK
GST-hNol9
0 3 30 60 min
5' OH, 3' OH
5' P, 3' OH
5' OH, 3' P
5' P, 3' P
C
buffer control
input nuclear extract
IP α-Clp1 (988)
IP pre-immune serum
IP α-Nol9 (1688)
IP α-Nol9 (1689)
5' OH
5' P

84

Figure 2


A
Sedimentation direction
pre-40S
pre-60S
A254nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Nol9
B23
Pol II
C
nt
6000
4000
3000
2000
1500
1000
28S
18S
D
nt
6000
4000
3000
2000
1500
1000
32S
12S
E
Sedimentation direction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Nol9
B23
Pol II
+RNase treatment

85

Figure 3

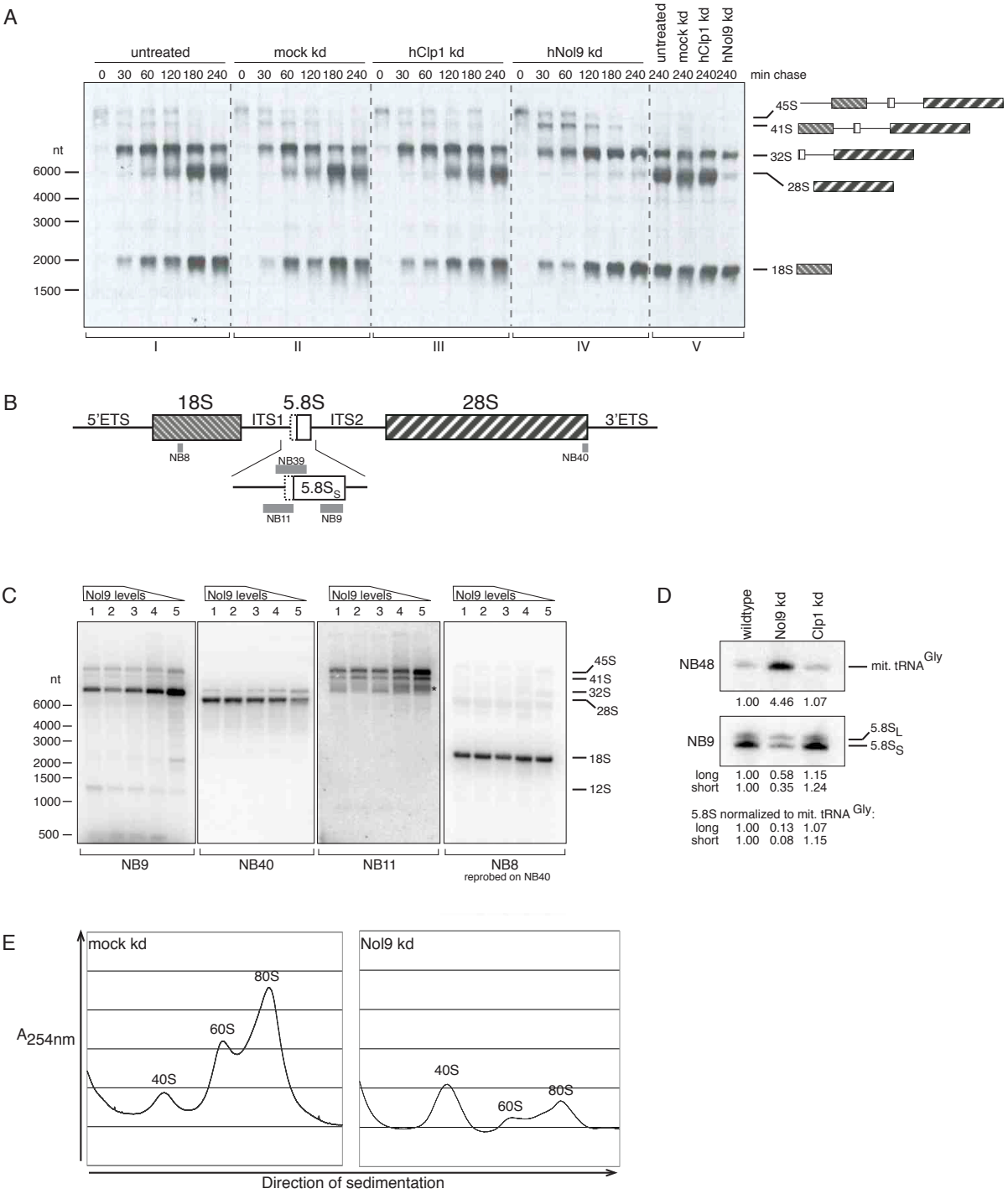
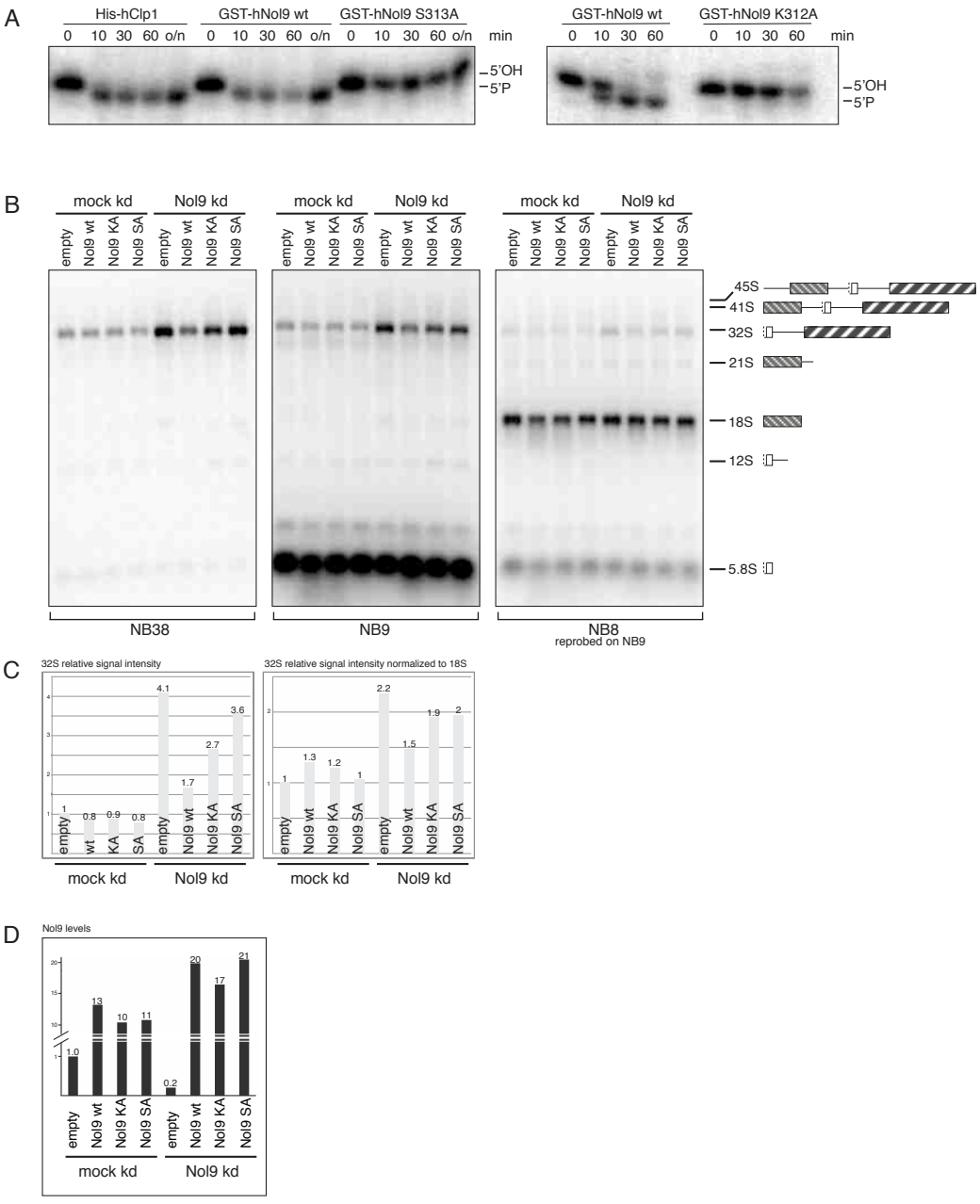

A
untreated
mock kd
hClp1 kd
hNol9 kd
0 30 60 120 180 240
min chase
nt
6000
4000
3000
2000
1500
45S
41S
32S
28S
18S
I
II
III
IV
V
B
18S
5.8S
28S
5'ETS
ITS1
ITS2
3'ETS
NB8
NB39
NB40
5.8S_S
NB11
NB9
C
Nol9 levels
1 2 3 4 5
nt
6000
4000
3000
2000
1500
1000
500
45S
41S
32S
28S
18S
12S
NB9
NB40
NB11
NB8
reprobed on NB40
D
wildtype
Nol9 kd
Clp1 kd
NB48
mit. tRNA^Gly
1.00 4.46 1.07
NB9
5.8S_L
5.8S_S
long 1.00 0.58 1.15
short 1.00 0.35 1.24
5.8S normalized to mit. tRNA^Gly:
long 1.00 0.13 1.07
short 1.00 0.08 1.15
E
mock kd
Nol9 kd
A254nm
80S
60S
40S
40S
60S
80S
Direction of sedimentation

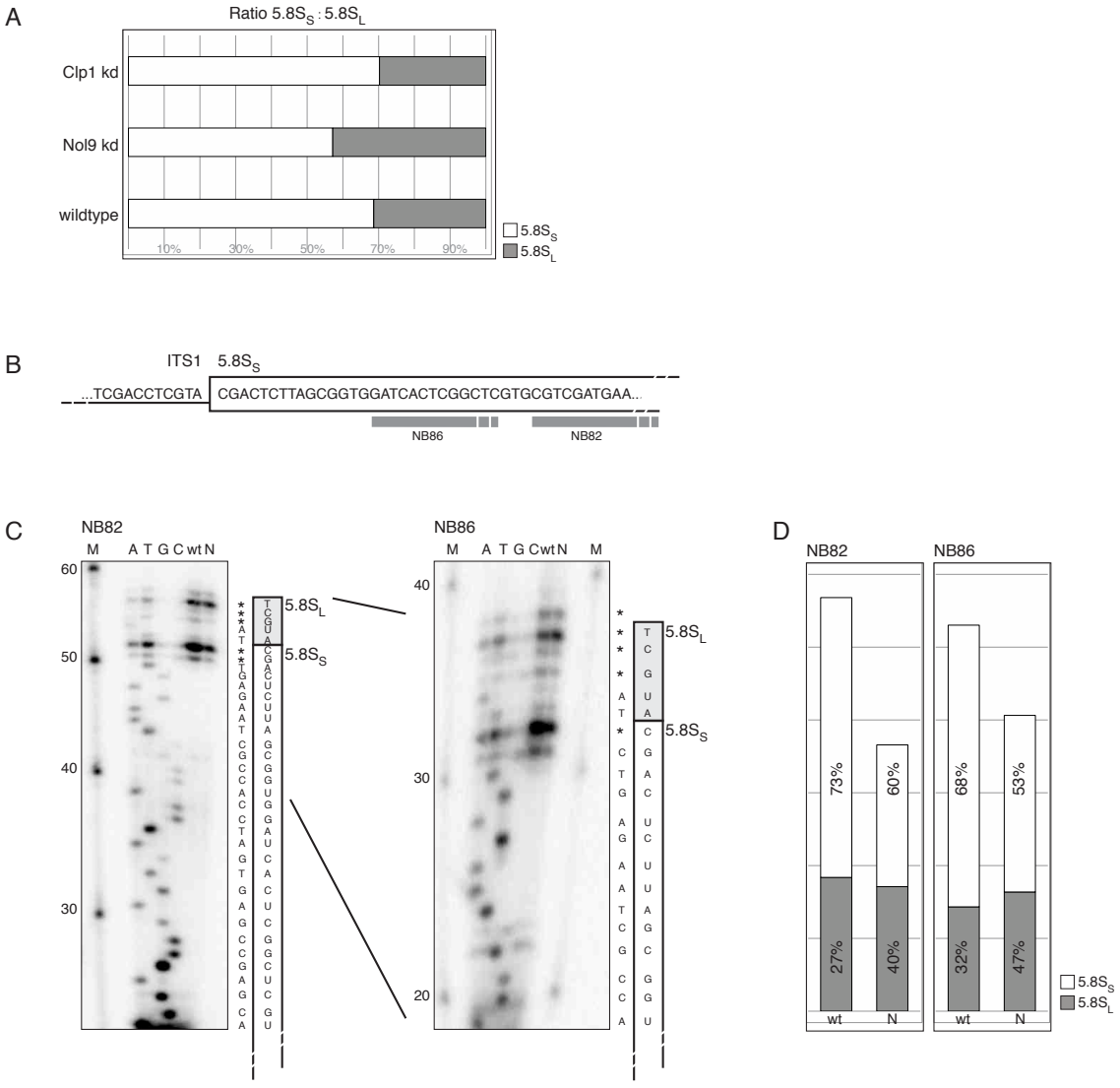
Figure 4

86


A
His-hClp1
GST-hNol9 wt
GST-hNol9 S313A
0 10 30 60 o/n 0 10 30 60 o/n 0 10 30 60 o/n min
5'OH
5'P
GST-hNol9 wt
GST-hNol9 K312A
0 10 30 60 0 10 30 60 min
5'OH
5'P
B
mock kd
Nol9 kd
empty
Nol9 wt
Nol9 KA
Nol9 SA
45S
41S
32S
21S
18S
12S
5.8S
NB38
NB9
NB8
reprobed on NB9
C
32S relative signal intensity
4.1
3.6
2.7
1.7
1
0.8
0.9
0.8
empty
wt
KA
SA
Nol9 wt
Nol9 KA
Nol9 SA
mock kd
Nol9 kd
32S relative signal intensity normalized to 18S
2.2
1.9
2
1.5
1.3
1.2
1
1
D
Nol9 levels
20
21
17
13
11
10
1.0
0.2

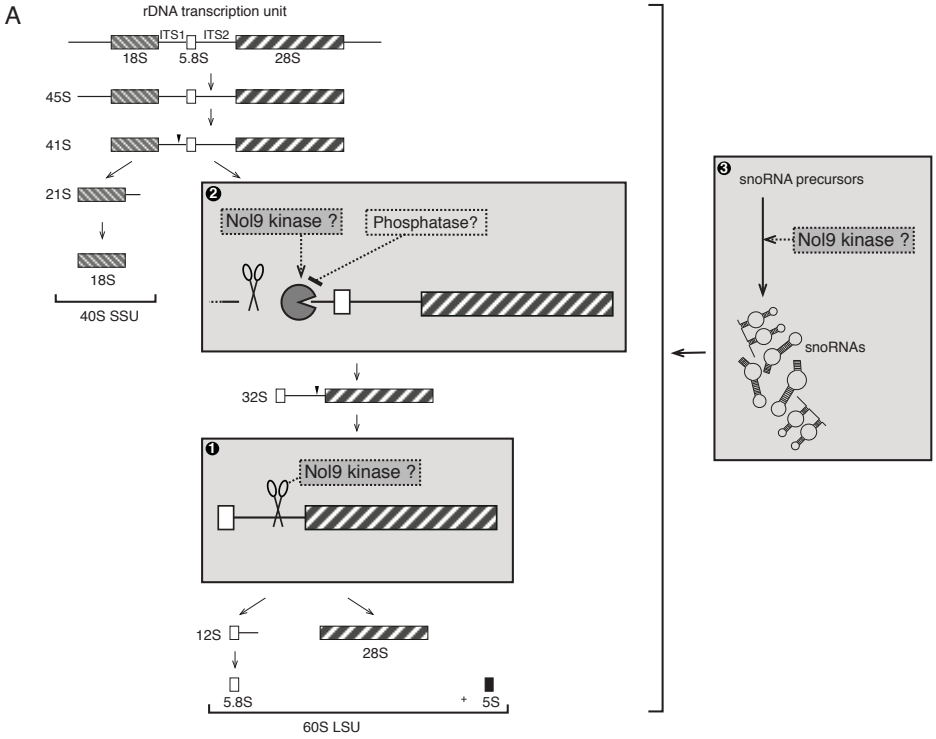
87

Figure 5


A
Ratio 5.8S$_S$: 5.8S$_L$
Clp1 kd
Nol9 kd
wildtype
10%
30%
50%
70%
90%
5.8S$_S$
5.8S$_L$
B
ITS1
5.8S$_S$
...TCGACCTCGTA
CGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAA...
NB86
NB82
C
NB82
M
A T G C wt N
60
50
40
30
5.8S$_L$
5.8S$_S$
NB86
M
A T G C wt N
M
40
30
20
5.8S$_L$
5.8S$_S$
D
NB82
NB86
73%
27%
60%
40%
68%
32%
53%
47%
wt
N
wt
N
5.8S$_S$
5.8S$_L$

88

Figure 6


A
rDNA transcription unit
ITS1
ITS2
18S
5.8S
28S
45S
41S
21S
18S
40S SSU
Nol9 kinase ?
Phosphatase?
32S
Nol9 kinase ?
12S
28S
5.8S
5S
60S LSU
snoRNA precursors
Nol9 kinase ?
snoRNAs

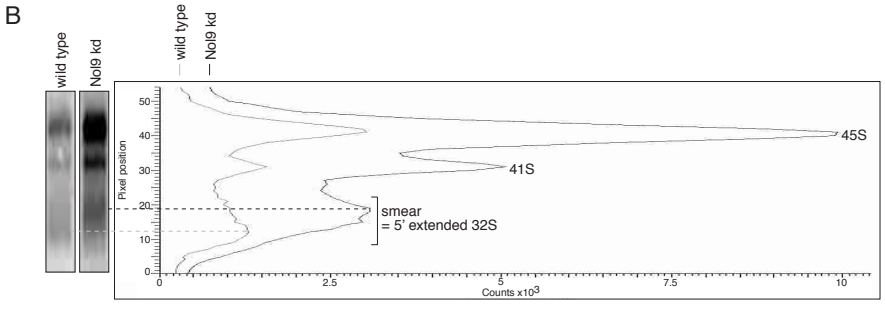

B
wild type
Nol9 kd
wild type
Nol9 kd
Pixel position
45S
41S
smear
= 5' extended 32S
Counts x10^3

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Figure 7

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DISCUSSION

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Nol9 versus Grc3 in rRNA processing

Using tetracycline-regulatable mutant strains, *S. cerevisiae* Grc3 was previously identified in a large-scale genomic screen for proteins involved in noncoding RNA processing (Figure 9) (Peng et al, 2003). In particular, the authors describe a 'distinctive impact on ITS2 processing' in the $tetO_7$-GRC3 strain, which becomes manifest in reduced levels of $27SA_2$, 25S and 7S, as well as an increase in 35S levels as observed by Northern blot (Figure 9A) (Peng et al, 2003). In addition, 3'-extended forms of 5.8S were detected, the exact nature of which was not specified, but was not identical to 7S precursors.

A closer look on the data by Peng et al. reveals that the prime impairment upon Grc3 depletion affects the 35S-separating cleavage within ITS1 (Peng et al, 2003); whereas 35S accumulates, levels of most downstream intermediates and products are decreased, including 20S and 18S, as well as $27SA_2$, 5.8S and 25S (Figure 9A); only 27SB accumulates. This intermediate comprises 5.8S, ITS2 and 25S and leads the authors to their main conclusion, that Grc3 is essential for processing in ITS2. The postulated reduction of 7S levels is not immediately visible. The authors compare three different RNA preparations from wildtype strains, which themselves show great variation in precursor pattern. However, the average intensity of the three resulting bands resembling 7S is not profoundly different from the $tetO_7$-GRC3 strain. The microarray data also show overall decreased levels over the whole rRNA precursor in Grc3 depleted cells (Figure 9B). The only exceptions are ITS2 and the very 3' end, which are overrepresented on the microarray. As shown in the 'Results' section, Grc3p is essential for efficient RNA Polymerase I termination, explaining the accumulation of 3' terminal sequences in the microarray (Braglia et al, 2010).

The accumulation of ITS2 sequences, however, cannot be explained. Overall, the GRC3 mutant phenotype is highly similar to a RAI1 mutant strain. The '*Ra*t1p-*i*nteracting protein' Rai1 associates with and stabilizes the 5' to 3' exonuclease Rat1 (Sydorskyy et al, 2003; Xue et al, 2000). In addition to RNA pol I termination (El Hage et al, 2008; Kawauchi et al, 2008), Rai1/Rat1 were also

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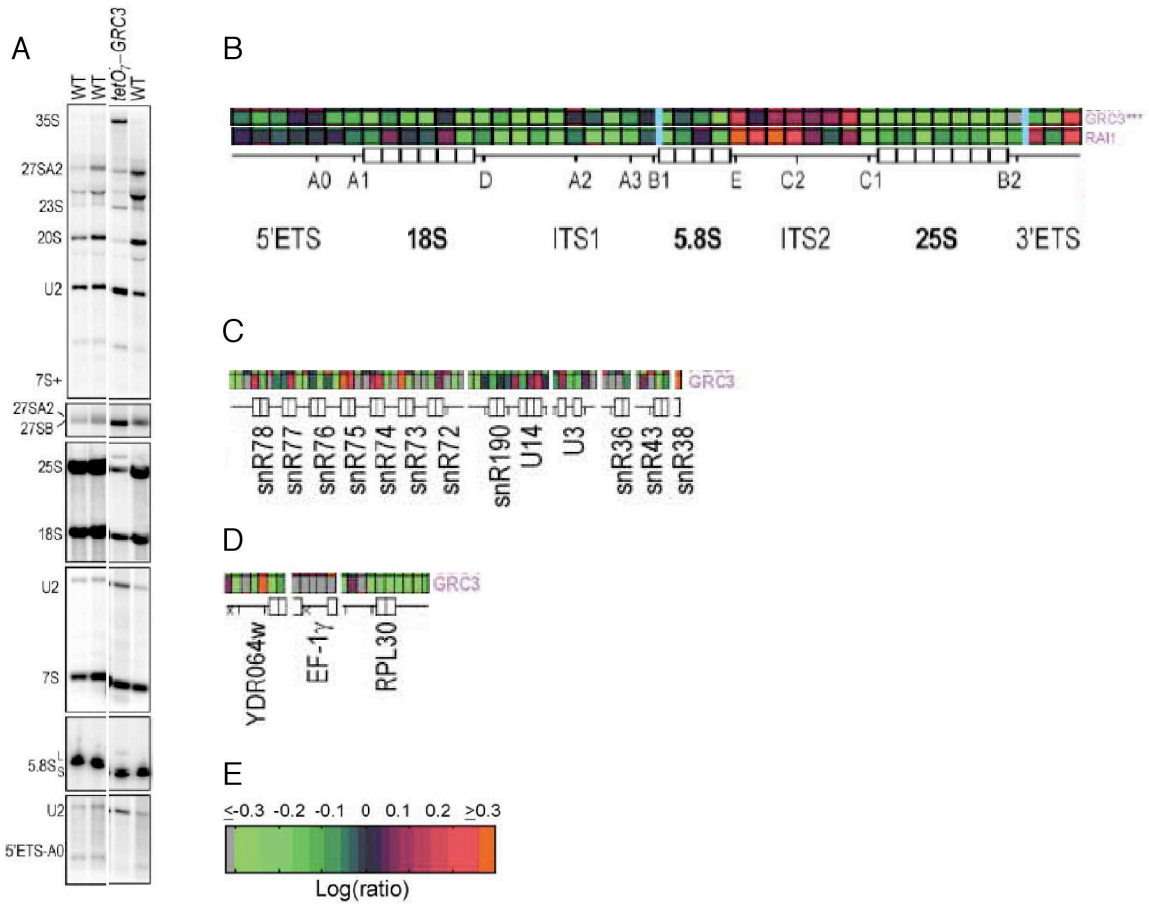

Figure 9. Grc3 depletion affects noncoding RNA processing. A Northern Blot analysis of rRNA processing in wildtype yeast strains or tetracycline-regulatable GRC3 mutants. **B-D** Microarray analysis of the relative levels of noncoding RNAs; **B**, rRNAs; **C**, small nucleolar RNAs; **D**, mRNAs. **E** Classification of the color code in B-D. Red indicates increased, green decreased relative abundance in the mutant. (Figures from Peng et al, 2003).

implicated in the generation of the 5' end of both 5.8S and 25S rRNAs (Geerlings et al, 2000; Henry et al, 1994). In addition, Rat1 seems to promote 3' to 5' exonuclease activity of the exosome (Fang et al, 2005). This explains the specific accumulation of overall ITS2 sequences in the Rai1 mutant background, however, raises the question, why no other spacer sequences were overrepresented, in particular the stretch between A_3 and B_{1S}, a known substrate of Rat1 (Henry et al, 1994).

The effects of Grc3 depletion on the levels of the analyzed subset of snoRNAs and mRNAs are not discussed (Figure 9C and D) (Peng et al, 2003). The pattern of abundance varies between the different RNAs tested and does not allow a general

conclusion for an involvement of Grc3 in the maturation of snoRNAs or the processing of mRNAs. The relative decrease represented by green boxes, especially in the case of the mRNAs (Figure 9D) might be a side effect of rRNA depletion.

The observations by Peng et al. are in notable contrast to the defects displayed by HeLa cells depleted of Nol9 (Peng et al, 2003). As shown in Figure 4, page 84, Nol9 knock down leads to an increase of 41 and 45S (comparable to yeast 33 and 35S, respectively; see Figure 6). The most striking phenotype was observed in the accumulation of 32S precursor (27SA in yeast) and decrease of 5.8S and 28S rRNAs (*S. cerevisiae* 5.8S and 25S), but 18S maturation was clearly not affected. Nol9 appears to be required for 5' end formation of 32S/5.8S, while Grc3 does not seem to impact this particular processing step.
It is possible, that the specific function of Grc3 and Nol9 is not fully conserved but has evolved differently; one common feature for the yeast and human kinases seems to be their relationship to Xrn2/Rat1. While GRC3 mutants show the same microarray pattern as RAI1 mutant strains (Peng et al, 2003) and defects in RNA polymerase I transcription termination (Figure 2, page 53), human Nol9 knock down specifically affects 5' end generation of 5.8S rRNA (Figure 7, page 87), a process most likely involving Xrn2, the human homolog of Rat1p (Amberg et al, 1992; Kenna et al, 1993; Zhang et al, 1999).

Grc3/Nol9 as regulator of Rat1/Xrn2 and consequently the torpedo mechanism of transcription termination?

As mentioned, both functions of Nol9/Grc3 within rRNA processing are closely linked to the 5' to 3' exonuclease Xrn2/Rat1p. Xrn1, a close homolog to Xrn2/Rat1, was shown to prefer 5' phosphorylated substrates (Stevens, 1980). Indeed, in our own hands, Xrn1 could not degrade RNAs displaying a 5' hydroxyl group (Figure 10A and B); addition of recombinant, wildtype but not kinase-inactive Nol9 rendered the substrate RNA susceptible to rapid digestion by Xrn1 *in vitro.*

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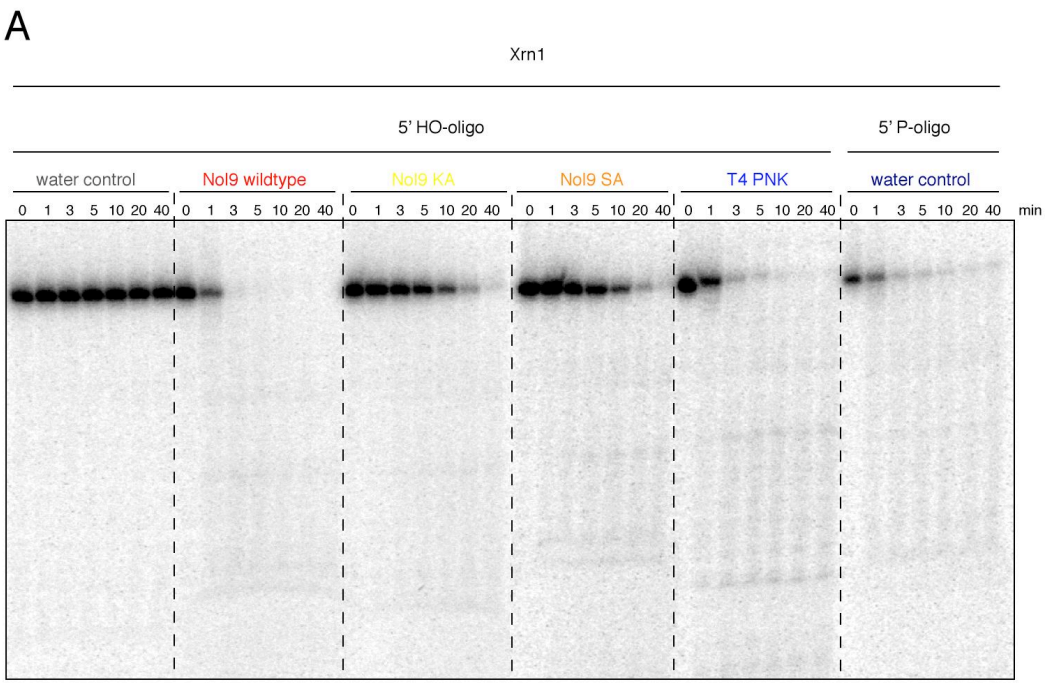

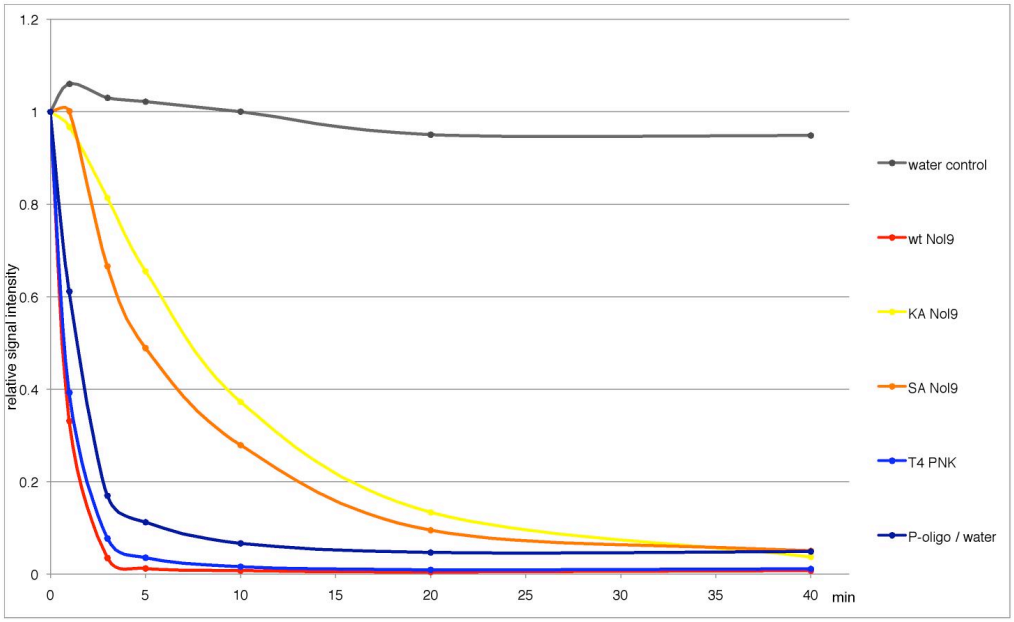

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Figure 10. Xrn1 acts on 5' phosphorylated substrates. **A** Xrn1 assay. Xrn1 (NEB) was incubated at 30°C with a 5' hydroxyl substrate in the presence of wildtype or mutant Nol9 (as indicated) for various time periods (buffer composition as in kinase assays, see 'Results' section) and degradation monitored on a 10% polyacrylamide gel. As a positive control, T4 PNK or 5' phosphorylated substrate was used; as negative control, water was added instead of a kinase. **B** Quantization of A by ImageQuant. Levels were normalized to the respective 0min time points.

Assuming, that Xrn1 and Xrn2/Rat1 share this preference, Nol9 could enhance exonuclease activity by providing 5' phosphorylated substrates. In most cases, the 5' phosphate on the substrates is generated by the preceding endonucleolytic activity. Therefore, our hypothesis implies the existence of an RNA 5' phosphatase, an activity that has not been observed to date. In this scenario, Nol9/Grc3 opposes the antagonizing action of the phosphatase and thereby adds a level of regulation to the exonuclease activity. This regulation might be important for processing mechanisms, where Xrn2/Rat1 do not fully degrade a substrate, but generate a very specific 5' end, as in the case of 5.8S or 25S.

It may also be of relevance as a fine-tuning mechanism for certain RNA species. RNA targets might be stabilized and protected from degradation by a phosphatase and need to be 'activated' by Nol9/Grc3-mediated phosphorylation to become Xrn2/Rat1 substrates. This could be the case for the promoter-associated RNA transcripts (pRNAS) that are required for establishing and maintaining repressive heterochromatin marks at the promoters of silent rDNA repeats (see chapter 1.1.1.) pRNAs are transcribed from promoters within the IGS of the rDNA unit and processed into 150-300nt long molecules that are complementary to rDNA promoter sequences (Mayer et al, 2006). These RNA fragments are carefully regulated and rapidly degraded unless stabilized by binding to NoRC, which in turn requires pRNA-association to bind chromatin and induce heterochromatin formation (Mayer et al, 2006). The exact mechanism of pRNA processing and turn over is still enigmatic. Therefore, it is highly speculative to propose, that pRNA stability might be influenced by the balance of a phosphatase and kinase that either protect or mark pRNAs for degradation, respectively. However, it would be interesting to determine, whether Nol9 depletion affects pRNA stability and as a consequence rDNA silencing, since over-expression of pRNAs displaced NoRC from nucleoli (Mayer et al, 2006).

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In any case, the observation of a polynucleotide kinase in close proximity to Xrn2/Rat1 is redundant. Clp1, a close homolog of Nol9, was identified as component of the mammalian RNA polymerase II-specific 3' end processing factor $CFII_m$ (de

Vries et al, 2000). The mammalian 3' end formation machinery comprises six multi-protein complexes that generate the 3' ends of mRNAs by cleavage at the poly(A) site and subsequent addition of poly(A) tails; CPSF, CstF, CFI_m, $CFII_m$, PAP and PAPB (Proudfoot, 2004). Transcription termination is intimately linked with 3' end formation (Buratowski, 2005; Proudfoot, 2004). Cleavage at the Poly(A) site not only precedes polyadenylation of the 3' product but also creates the entry point for 5' to 3' exonucleases at the 5' product. Xrn2/Rat1 degrade the trailing transcript and terminate RNA polymerase II via the torpedo mechanism (Kim et al, 2004; West et al, 2004).

It will be interesting to investigate whether Nol9 and Clp1 are essential for transcription termination of human RNA polymerases I and II, respectively, and to determine the exact role of a polynucleotide kinase in the Rat1/Xrn2-driven torpedo mechanism.

The multi-functionality of Nol9?

The past decade has linked the nucleolus to a multitude of cellular processes other than ribosome synthesis (see chapter 4.4). Nucleolar proteins that, to date, are solely implicated in rRNA processing or ribosome assembly might therefore have additional functions in other nucleolus-based processes. The RNA/DNA kinase Nol9 displays substrate-flexibility *in vitro*, phosphorylating the 5' end of single- and double-stranded RNA and DNA molecules. It would be interesting to determine other aspects in RNA and DNA metabolism apart from rRNA synthesis, where Nol9 is required. One very powerful tool to investigate RNA-protein interactions is UV cross-linking and immunoprecipitation (CLIP) (Granneman et al, 2009; Ule et al, 2005; Wang et al, 2009). This technique has been adapted for large RNA-protein complexes in yeast (Granneman et al, 2009) and could be used to screen for RNA species that interact with Grc3p and therefore are substrates for the kinase activity. However, the interaction of Grc3p with its substrates might be only very transient and therefore lead to false negatives.

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Nol9 in HDV replication

Replication of Hepatitis delta virus (HDV) takes place in the nucleolus (see chapter 4.4.1.). HDV contains a small, circular RNA genome, which replicates via a 'rolling cycle mechanism' (Branch & Robertson, 1984); the virus RNA serves as template for transcription of long antigenomic multimers (Figure 11A). Each unit of the multimer is defined by two cleavage sites of cis-acting, genomically encoded ribozymes, which release single units from the multimeric transcript (Ferre-D'Amare et al, 1998; Wadkins et al, 1999). The monomers are intramolecularly ligated to form circularized antigenomic RNA molecules that serve in turn as templates for genomic virus RNA circles in the exact same mechanism as used for their production. Ribozymes are RNA structures mediating autocatalytic cleavage in cis, thereby creating 5' hydroxyl and 2'-3' cyclic phosphate termini (Ferre-D'Amare et al, 1998; Wadkins et al, 1999). While the cleavages are catalyzed by the virally encoded genomic and antigenomic ribozymes, ligation depends on the host (Reid & Lazinski, 2000). It is possible that the virus hijacks the host tRNA ligase, an enzyme initially identified in tRNA splicing.

A small subset of eukaryotic transfer RNAs contains introns in their anticodon loops that need to be excised to produce functional tRNAs (Lowe & Eddy, 1997; Trotta et al, 1997; Valenzuela et al, 1978). The multimeric Sen endonuclease cleaves out the intervening sequence, generating 5' hydroxyl and 2'-3' cyclic phosphate groups, identical to ribozymes (Figure 11B) (Trotta et al, 1997). In *S. cerevisiae*, the trifunctional tRNA ligase Trl1p first heals the ends by transferring a phosphate from GTP to the 5' termini and opening the 2'-3' cyclic phosphates before sealing the ends in the actual ligation mechanism (Phizicky et al, 1986; Xu et al, 1990). In mammals, the tRNA ligase is still enigmatic. In fact, two distinct ligase activities have been identified biochemically. The first mechanism follows the same reactions as Trl1p, involving a kinase, cyclic phosphodiesterase and ligase step (Englert & Beier, 2005; Lowe & Eddy, 1997; Zillmann et al, 1991; Zillmann et al, 1992). Clp1 might serve as the kinase in mammalian tRNA splicing, implicating that, in the course of evolution,

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Figure 11. Similarities between HDV replication and tRNA splicing.

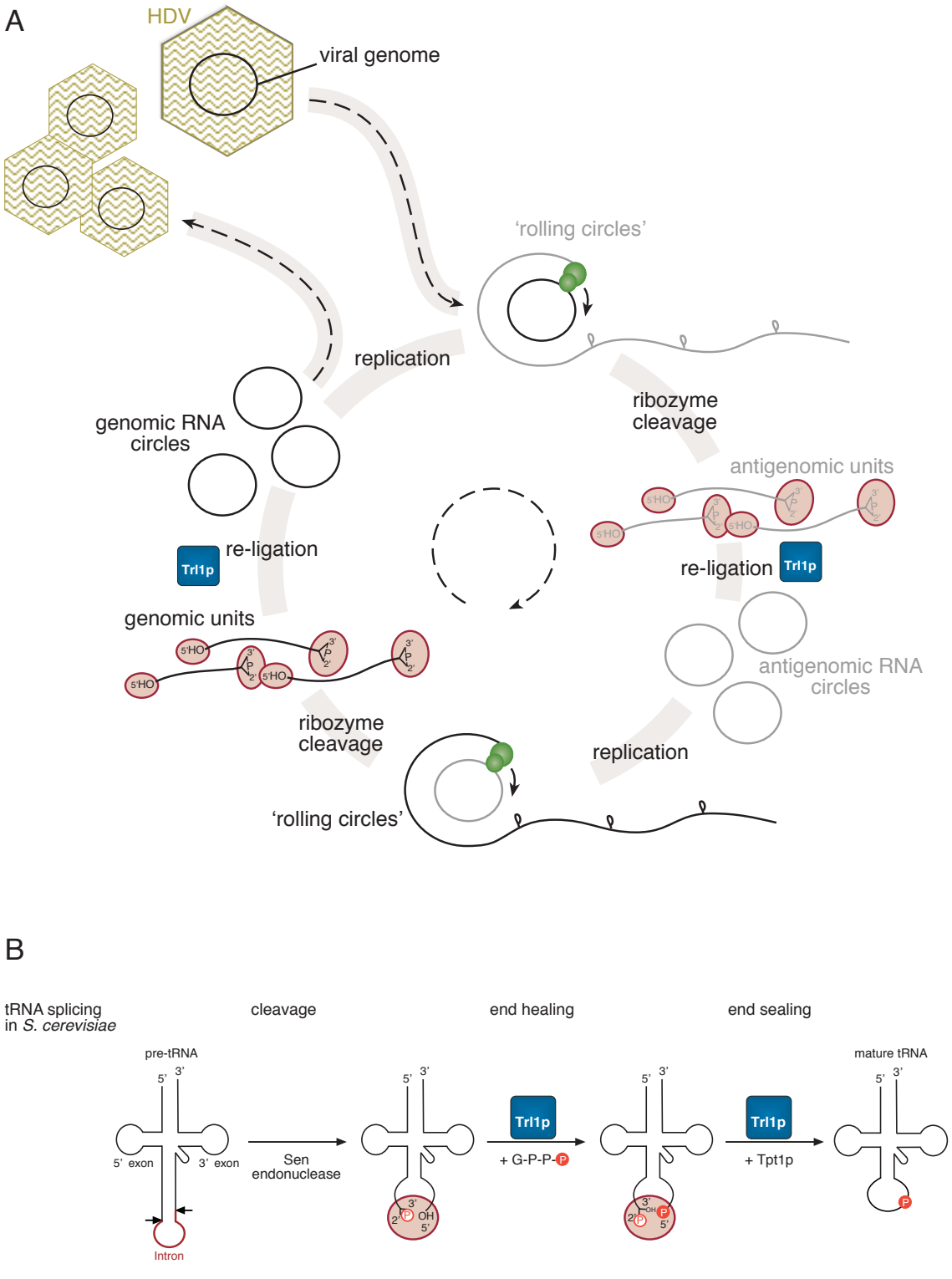

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Figure 11. Similarities between HDV replication and tRNA splicing. A Hepatits Delta Virus (HDV) replicates via rolling circles. The host RNA polymerase I generates a multimeric transcript from the circular RNA genome. Single units are generated by ribozyme-mediated cleavages (depicted as loops), which generate 5' hydroxyl and 2'-3' cyclic phosphate groups. Ligation of single units produces antigenomic circles that are templates for another round of transcription. **B** tRNA splicing involves excision of the intron by the Sen endonuclease, generating 5' hydroxyl and 2'-3' cyclic phosphate groups. Yeast Trl1 'heals' the ends and subsequently ligates the exon halves to produce mature, functional tRNAs.

the three enzymatic activities have been separated onto different polypeptides (Weitzer & Martinez, 2007b). The second ligation mechanism does not involve the addition of an external phosphate but uses the 2'-3' cyclic phosphate created via cleavage for the formation of the phosphodiester bond (Filipowicz & Shatkin, 1983; Laski et al, 1983; Nishikura & De Robertis, 1981).

The spatial proximity of HDV replication and Nol9 proteins is intriguing. Nol9 could be involved in 'end healing' of the 5' hydroxyl termini of the single units produced during rolling circle replication, granted that HDV hijacks the yeast-like tRNA ligation mechanism of the host cell. It would be interesting to investigate whether Nol9-depleted cells show a defect in viral replication upon infection with HDV.

Nol9 in RNA repair?

With the vast amount of RNA trafficking through the nucleolus, it might be necessary to establish on-site quality control and RNA repair competences (Bellacosa & Moss, 2003; Tell et al, 2010). In fact, the human AlkB homologs hAbh2 and hAbh3 partially localize to the nucleolus (Aas et al, 2003). In bacteria, AlkB repairs alkylation-damaged DNA and RNA molecules (Aas et al, 2003; Aravind & Koonin, 2001; Falnes et al, 2002; Trewick et al, 2002). While hAbh2 was only able to demethylate alkylated double stranded DNA substrates, hAbh3 was also active on RNA (Aas et al, 2003).

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Due to its biochemical properties and largely single stranded arrangement, RNAs are most likely more susceptible to oxidative damages than DNA molecules. Lesions like 8-hydroxyguanine, 5-hydroxycytidine, 5-hydroxyuridine and 8-hydroxy-adenosine are common consequences of oxidative stress (Tell et al, 2010). Unless repaired, these damages can interfere with the base-pairing ability of RNAs and, in case of rRNAs, lead to defective translation (Ding et al, 2005).

Recently, oxidatively damaged, abasic RNAs were shown to be detected and 'cleansed' by the endonuclease Ape1 (Vascotto et al, 2009). Ape1 has a well-established role in DNA base excision repair after oxidative damage (Demple et al, 1991), but is also implicated in RNA repair (Barnes et al, 2009; Berquist et al, 2008; Vascotto et al, 2009). After detection of 8-hydroxyguanine sites in RNAs (Hayakawa et al, 2002), YB-1 recruits Ape1 to lesions within RNA molecules (Chattopadhyay et al, 2008). The interaction of Ape1 with Nucleolin in nucleoli suggests a function in ribosomal RNA repair (Vascotto et al, 2009). In accordance, Ape1 depletion leads to impaired protein synthesis (Vascotto et al, 2009).

Since cells rely on the quality of the produced RNAs, especially the highly abundant ribosomal RNAs, it is likely that RNA repair is a general mechanism of the nucleolus. The above examples argue in favor of a nucleolar RNA repair system.
Radiation oftentimes produces strand breaks displaying 3' phosphate and 5' hydroxyl groups (Henner et al, 1982). In this scenario, the 5' kinase activity of Nol9 could act as part of an RNA repair mechanism healing the 5' termini via phosphorylation and thereby enabling re-ligation, in a similar manner as T4 PNK.

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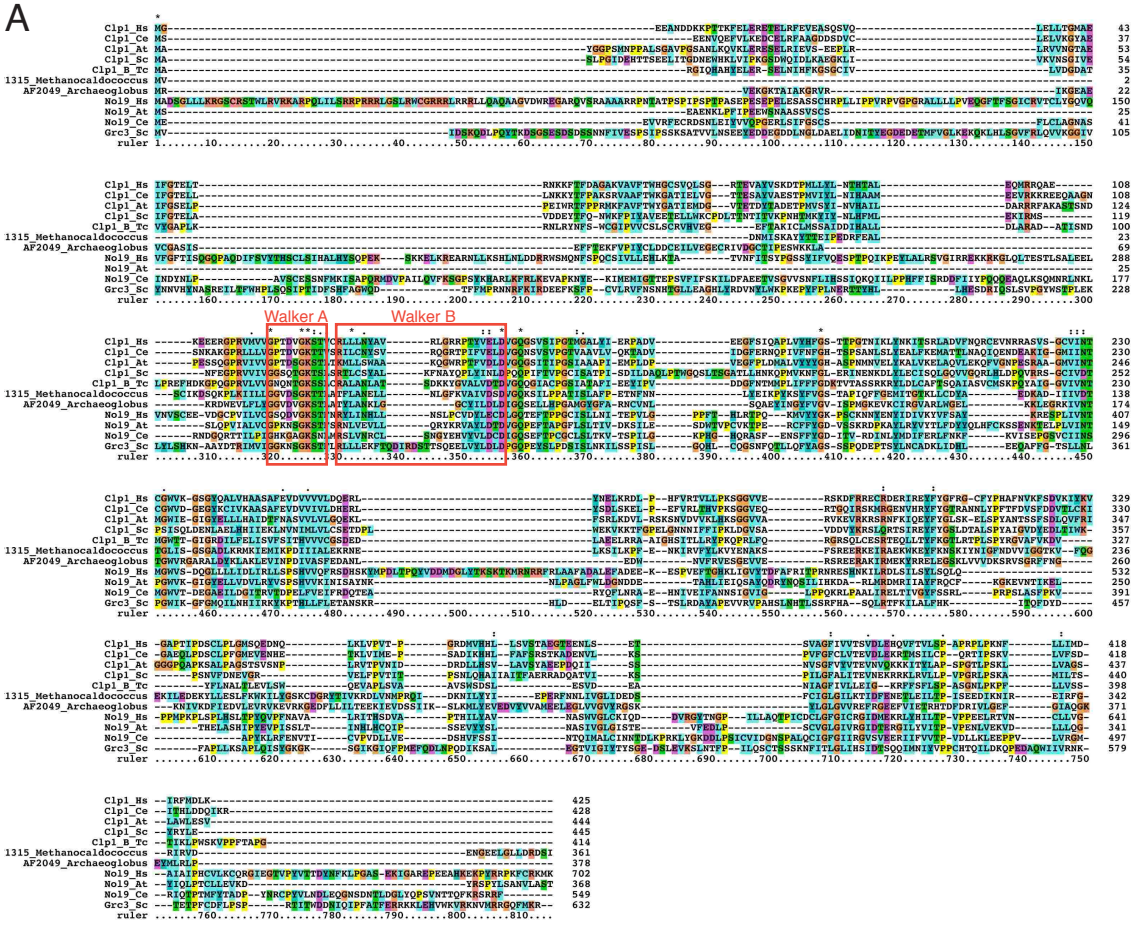
Figure 12. Protein alignments. A Alignment of Clp1 and Nol9 from different species. Hs, *H. sapiens*; Ce, *C. elegans*; At, *A. thaliana*; Sc, *S. cerevisiae*, Tc, *T. cruzi*; Figure was fully done by Alexander Schleiffer. **B** Alignment of human Nol9 and T4 PNK. Amino acids crucial for kinase activity are marked with red circles, those essential for T4 PNK phosphatase activity with pink circles (Zhu et al, 2007). Walker A and B motifs are indicated by red boxes, the motif defining a phosphotransferase superfamily by a pink box. Alignments were carried out using ClustalW (Larkin et al, 2007).

Figure 12. Protein alignments

A



B

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More similarities between Nol9 and T4 PNK?

The well-studied polynucleotide kinase of the bacteriophage T4 (T4 PNK) was the very first example of an RNA repair enzyme (Richardson, 1965). It has evolved as a response to the host defense mechanism, which cleaves its own tRNAs in an effort to impair viral protein production (Amitsur et al, 1987). The homotetrameric T4 PNK heals the cleaved ends of the host tRNAs by phosphorylating the 5' termini and opening 2'-3' cyclic phosphates, which are joined again by a virally encoded ligase. This leads to restoration of host tRNAs and counteracts host defense attempts. As mentioned, T4 PNK contains two separate activities; it acts as 5' kinase and 2'-3' cyclic phosphodiesterase (Becker & Hurwitz, 1967; Cameron & Uhlenbeck, 1977; Richardson, 1965). The domains responsible for kinase and phosphatase activities reside at the N-terminal and C-terminal ends, respectively; The Walker A motif in T4 PNK is spatially separated from the CPD domain and, as for Nol9, confers kinase activity (Figure 12B) (Soltis & Uhlenbeck, 1982a; Soltis & Uhlenbeck, 1982b; Wang et al, 2002a; Wang & Shuman, 2001; Wang & Shuman, 2002). Mammalian polynucleotide kinase / phosphatase (PNKP) is mainly active towards DNA substrates and linked to DNA repair. It was detected, together with other DNA repair proteins like Mre11 or XRCC1, in proteomic analyses of the nucleolus (Andersen et al, 2005).

Human Nol9 is a 78kDa protein as calculated from the amino acid sequence. Similar to its homolog Clp1, Nol9 contains both Walker A and Walker B motifs (Figure 12A), which convey ATP/GTP binding and thus are essential for kinase activity (Walker et al, 1982). Human Nol9 is significantly larger than its *A. thaliana* homolog, the analyzed Clp1 proteins or the 301 amino acids-long T4 PNK. The Walker A and B domains comprise only a short segment in the central part of human Nol9 (Figure 12). While the N-terminus is Nol9-specific and might confer nucleolar localization, the C-terminal part of Nol9 shows similarities to Clp1 and T4 PNK to a certain extent (Figure 12A and B). Although Nol9 does not contain a motif implicated in other enzymatic activities like the DxDxT segment in T4 PNK, it would be interesting to determine if Nol9 also possesses other activities.

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CURRICULUM VITAE

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Personal Data

Full name:	Katrin Heindl
Date of birth:	28.01.1983
Place of birth:	Steyr, Austria
Nationality:	Austrian
Gender:	female

Educational Experience in brief

2009	'EMBO YIP Small Grant' to conduct experiments at Harvard Medical School, Boston, USA
2007-2010	PhD Thesis, Martinez Group, IMBA Vienna. 'Roles of the Novel 5'-Polynucleotide Kinase Nol9 in Ribosome Synthesis'.
2006	Diploma Thesis, Martinez Group, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna (IMBA). 'Cell biological and Biochemical Characterization of the Clp1 Polynucleotide Kinase'.
2005	Erasmus Exchange Program, Universidad Complutense de Madrid, Spain; Laboratory of Margarita Lorenzo
2001 – 2006	Undergraduate Studies of Molecular Biology at the University of Vienna; Graduation "mit ausgezeichnetem Erfolg"
June 2001	Graduation from Bundesgymnasium "mit ausgezeichnetem Erfolg"
1999 – 2000	Exchange Year to Quince Orchard High School, MD, US
1993 – 2001	Bundesgymnasium Werndlpark Steyr, Austria
1989 – 1993	Volkschule Promenade, Steyr, Austria

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