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Processing of preribosomal RNA in *Saccharomyces cerevisiae*

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Most, if not all RNAs, are transcribed as precursors that require processing to gain functionality. Ribosomal RNAs (rRNA) from all organisms undergo both exo- and endonucleolytic processing. Also, in all organisms, rRNA processing occurs inside large preribosomal particles and is coupled to nucleotide modification, folding of the precursor rRNA (pre-rRNA), and assembly of the ribosomal proteins (r-proteins). In this review, we focus on the processing pathway of pre-rRNAs of cytoplasmic ribosomes in the yeast *Saccharomyces cerevisiae*, without doubt, the organism where this pathway is best characterized. We summarize the current understanding of the rRNA maturation process, particularly focusing on the pre-rRNA processing sites, the enzymes responsible for the cleavage or trimming reactions and the different mechanisms that monitor and regulate the pathway. Strikingly, the overall order of the various processing steps is reasonably well conserved in eukaryotes, perhaps reflecting common principles for orchestrating the concomitant events of pre-rRNA processing and ribosome assembly. © 2014 John Wiley & Sons, Ltd.

INTRODUCTION

In practically all eukaryotes, ribosomal DNA (rDNA) is arranged in two transcriptional units: the 5S rRNA gene on one hand and the unit containing the 18S, 5.8S, and 25S rRNA genes on the other hand. To cope with the high demand for cytoplasmic ribosomes, the amplification of the copy number of rDNA genes has been evolutionarily selected. *Saccharomyces cerevisiae* has about 150 rDNA copies per haploid genome on chromosome XII, organized as a tandem array, where, in contrast to higher eukaryotes, the 5S rDNA gene is a part of the rDNA repeat unit (Figure 1(a)). Despite the high copy number, only about half of the rDNA copies are transcribed under exponential growth conditions in yeast. Notably, the active rDNA units are transcribed at high efficiency and it has been calculated that the pre-rRNA transcripts make up more than 60% of all nuclear transcripts.[1]

Figure 1(b) outlines the current knowledge on the diverse steps of the pre-rRNA processing pathways in yeast. In this part, we provide an overall description of these pathways, while detailed discussions of the specific steps will be presented in the following sections. The 5S rRNA is transcribed by RNA polymerase III (RNAPIII) as a precursor whose 5′ end corresponds to the mature 5′ end, while its 3′ end is extended by about 12 nucleotides (nt) (Figure 2). The 3′ end of the pre-5S rRNA is a U-rich sequence as RNAPIII generally uses T-rich sequences contiguous to the mature 3′ ends of RNAs as a termination signal.[2] As discussed later, the 3′ end of mature 5S rRNA is generated exonucleolytically (Figure 2). The 18S, 5.8S, and 25S rRNAs are transcribed by RNAPI as a single polycistronic transcript flanked by external transcribed spacers at both ends (5′ and 3′ external transcribed spacers (ETS)) and containing two internal transcribed spacers (ITS1 and ITS2) that separate the mature rRNA sequences (Figure 1(a)). The longest detectable RNAPI precursor, named 35S pre-rRNA, begins at a transcription start site, which is placed

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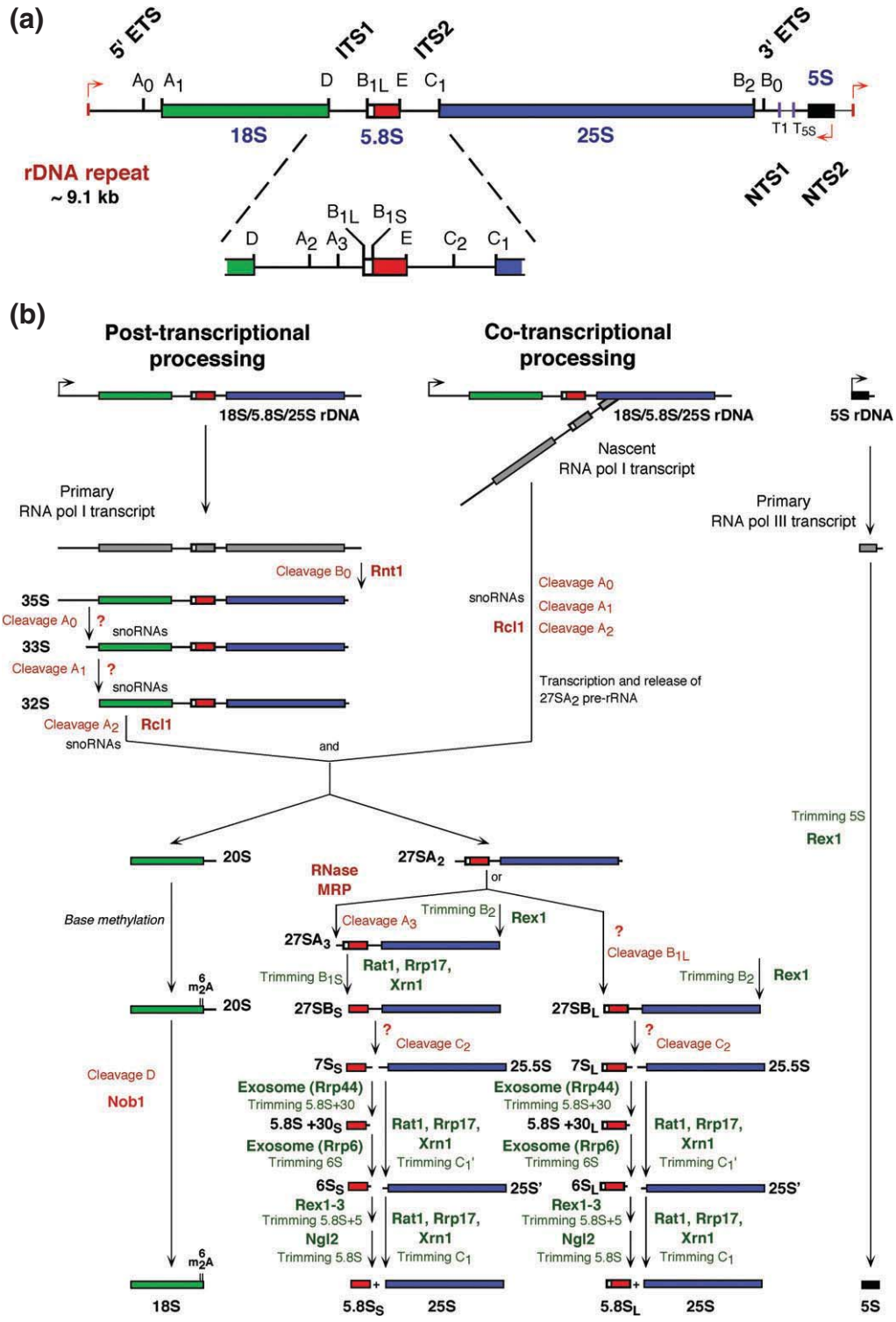

FIGURE 1 | Pre-rRNA processing in *S. cerevisiae*. (a) Structure of an rDNA repeat unit. Each unit contains a large element encoding 18S, 5.8S, and 25S rRNAs, which is transcribed by RNAPI, and a short element encoding 5S rRNA, which is transcribed by RNAPIII. Nontranscribed, external and internal spacers (NTS, ETS, and ITS, respectively) are indicated. The sequences for the mature rRNA species are shown as bars and the spacers as lines (NTSs are shown thinner than ETSs or ITSs). Transcription start sites are shown as red arrows and transcription stop sites (T_1 and T_{5S}) are shown as small purple boxes. The distinct processing sites are indicated. (b) Scheme of the pre-rRNA processing pathway, which is further described in the text. The nucleases responsible of the endonucleolytic cleavages (red) and exonucleolytic trimmings (green) are highlighted. Note that some endonucleases have not yet been identified.

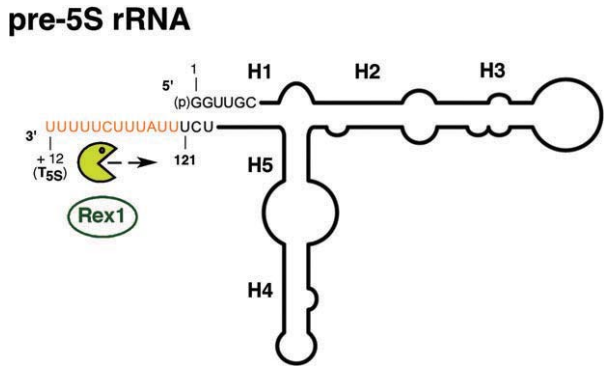

FIGURE 2 | Secondary structure model for *S. cerevisiae* pre-5S rRNA. The secondary structure as well as the residue and helix (H) numbers was taken from the Comparative RNA Web Site (www.rna.ccbb.utexas.edu/). Note that formation of 3′ end of mature 5S rRNA relies on a Rex1-dependent 3′–5′ exonucleolytic step. See text for further details.

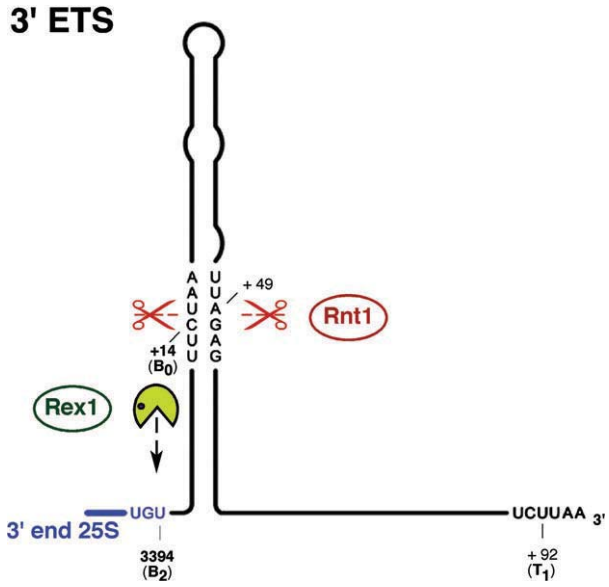

FIGURE 3 | Secondary structure model for the *S. cerevisiae* 3′ ETS. Rnt1 cleavage sites are indicated. The 3′ end of mature 25S rRNA (blue) is generated by Rex1-dependent 3′–5′ exonuclease trimming from the B_0 site. See text for further details.

about 700 nt upstream of the 5′ end of mature 18S rRNA.[3] At its 3′ end, this precursor ends about 14 nt downstream of the 3′ end of mature 25S rRNA.[4] This 3′ end is generated by co-transcriptional cleavage of the nascent RNPAI transcript.[4] It has been shown that termination of RNAPI transcription mostly occurs just upstream of the Reb1-binding site at a T-rich sequence, named T1, which is placed about 90 nt from the 3′ end of mature 25S rRNA.[5] A second termination site, named fail-safe or T2, which is located further downstream at about 250 nt from the 3′ end of mature 25S rRNA, is used when read-throughs occur at T1.[5] Most nascent RNAPI transcripts are co-transcriptionally cleaved by endonuclease Rnt1 at two positions (+14/15 and +49/50 relative to the 3′ end of mature 25S rRNA) lying at opposite sides of a stem-loop structure within the 3′ ETS (Figure 3).[4,6] Co-transcriptional Rnt1-dependent cleavage is important for efficient termination at T1, because this cleavage generates the 5′ end site required for recruitment of the exonuclease Rat1 containing complex, which degrades the remaining nascent transcript to stimulate transcription termination when reaching the paused RNAPI, a process referred to as 'torpedoing' the RNAPI.[5,7,8] When Rnt1 or Rat1 activities are impaired, RNAPI fails to terminate efficiently at T1 and mainly stops at T2.[5,7]

Removal of the pre-rRNA spacers is a multi-step process; the 35S pre-rRNA is initially cleaved at sites A_0 and A_1 in 5′ ETS and at site A_2 in ITS1 (Figure 1(a)), generating the 20S and $27SA_2$ pre-rRNAs and thus separating the pre-40S and pre-60S ribosomal subunits (r-subunits).[9,10] In the late 80s, experimental evidence indicated that removal of the 5′ ETS could take place before 35S pre-rRNA transcription is completed.[11] More recently, co-transcriptional cleavage of nascent pre-rRNA transcripts at site A_2 has been supported by carefully analysing the kinetics of pre-rRNA maturation and by visualization of nascent pre-rRNA transcripts by electron microscopy (EM).[12,13] It has been estimated that about 70% of pre-rRNAs undergo co-transcriptional cleavage in exponentially growing wild-type yeast cells.[12] Co-transcriptional cleavage appears to occur when the RNAPI has traveled into the 25S rDNA around 1.5 kb downstream of site A_2 and requires Rrp5 and specific 60S r-subunit biogenesis factors.[13–15]

Cleavage at site A_2, either co- or post-transcriptional, generates the 20S pre-rRNA, which is then processed to mature 18S rRNA by endonucleolytic removal of the D-A_2 fragment (Figure 1(b)). Interestingly, both in yeast and in higher eukaryotes, this reaction occurs in the cytoplasm.[16,17] Why evolution has selected this compartmentalization of pre-rRNA processing remains unclear. Cleavage at site A_2 also generates the $27SA_2$ pre-rRNA, which is then processed by two alternative pathways that both lead to the formation of mature 5.8S and 25S rRNAs (Figure 1(b)). In the major pathway, about 85% of $27SA_2$ pre-rRNA is first cleaved at site A_3 by endonuclease RNase MRP, which is a ribonucleoprotein (RNP) particle whose large RNA moiety harbors the catalytic activity.[18] The resulting $27SA_3$ pre-rRNA is 5′–3′ exonucleolytically digested up to site B_{1S} to

yield the $27SB_S$ precursor, which contains the mature 5′ end of the $5.8S_S$ rRNA.[19,20] In the minor pathway, about 15% of $27SA_2$ pre-rRNA is processed directly to site B_{1L}, apparently by an endonucleolytic event. This $27SB_L$ precursor harbors the mature 5′ end of the $5.8S_L$ rRNA, which is located about 6 nt upstream of the 5′ end of the $5.8S_S$ rRNA.[21] It has been reported that almost concurrently with B_1 processing, the 3′ end of mature 25S rRNA is generated by 3′–5′ trimming to site B_2 of the remnant B_2–B_0 fragment present in 27SA pre-rRNAs.[4,22]

Processing of ITS2 from both 27SB species appears to be identical (Figure 1(b)). Initially, cleavage at site C_2 separates 7S and 25.5S pre-rRNAs, which are the precursors of mature 5.8S and 25S rRNAs, respectively.[22] Strikingly, the endonuclease that performs this reaction is still unknown. The around 140 nt of ITS2 at the 3′ end of the 7S pre-rRNAs are removed by a stepwise mechanism involving several 3′ to 5′ exonucleases,[23–26] which leads to the formation of at least two discrete and stable pre-rRNA intermediates, 5.8S + 30 and 6S pre-rRNAs. Recently, it has been demonstrated that maturation of 6S pre-rRNAs into mature 5.8S rRNAs occurs in the cytoplasm.[27] Two mature 5.8S rRNA species ($5.8S_L$ and $5.8S_S$ rRNAs) are produced in a ratio of about 1:5. It is unclear if the two forms of 5.8S rRNAs have distinct functions. However, the 25.5S pre-rRNA is 5′–3′ digested to mature 25S rRNA.[20,28] This processing reaction may occur in two discrete steps, as suggested by the detection of a stable 25S′ pre-rRNA intermediate, which is extended by a few nt at the 5′ end of mature 25S rRNA, in wild-type cells.[27,29]

It is important to stress that pre-rRNA processing occurs inside large RNP complexes called preribosomal particles.[30] These particles contain, in addition to the pre-rRNAs and different sets of assembled ribosomal proteins (r-proteins), a multitude of nonribosomal factors including small nucleolar RNAs (snoRNAs) and *trans*-acting proteins. Loss-of-function of most of these factors leads to pre-rRNA processing defects; thus, with the exception of nucleases, it is not obvious to recognize which specific factors are *directly* involved in the pre-rRNA processing reactions. Less than a dozen nucleases or putative nucleases have been described to be involved in pre-rRNA processing, and for only few of these, an enzymatic activity has been demonstrated. Moreover, *bona fide* co-factors for nucleases have only scarcely been reported. Finally, only few steps of yeast pre-rRNA processing could so far be reproduced *in vitro*. This review summarizes our current understanding of yeast pre-rRNA processing, particularly focusing on the role of the *trans*-acting factors that directly participate in the nucleolytic events. For other aspects of pre-rRNA processing (e.g., role of *cis*-acting elements within the pre-rRNA sequences), rRNA modifications or ribosome assembly in yeast and pre-rRNA processing in higher eukaryotes, we refer readers to other reviews.[30–39]

THE INITIAL EVENTS

Co-transcriptional Cleavage at Site B_0

In the post-transcriptional pathway, the earliest pre-rRNA processing event is the endonucleolytic cleavage of the nascent pre-rRNA in the 3′ ETS at site B_0, thereby generating the 35S pre-rRNA. This step is performed by the dsRNA endonuclease Rnt1, which is homologous to bacterial RNase III.[4,6] Rnt1 cleaves both strands of an imperfect stem-loop structure (Figure 3) capped by an (A/U)GNN tetraloop at positions +14 and +49 from the 3′ end of mature 25S rRNA[4]; notably, this reaction could be reproduced *in vitro* with recombinant Rnt1 and a synthetic 3′ ETS substrate.[6,40] Consistently, loss-of-function of Rnt1 leads to reduced synthesis of mature 25S rRNAs and accumulation of 3′ ETS containing pre-rRNAs.[4]

In the co-transcriptional pathway, cleavage at site B_0 appears to release nascent $27SA_2$ or $27SA_3$ pre-rRNAs, as suggested by the observed coupling between B_0 cleavage and ITS1 processing.[41]

Cleavages at Sites A_0, A_1 and A_2

Removal of 5′ ETS is an early event in both the co- and the post-transcriptional pathways of pre-rRNA processing. This involves the cleavages at sites A_0 and A_1, which have been demonstrated to be endonucleolytic processes as the excised 5′–A_0 and A_0–A_1 spacer fragments accumulate in strains defective in components of the nuclear exosome complex and its associated RNA helicase Mtr4/Dob1 and in the 5′–3′ exonucleases Rat1 and Xrn1, respectively.[42–44] Cleavage at site A_0, which is dispensable,[31] was initially identified as a primer stop that specifically decreased on depletion of the U3 snoRNA.[9] Site A_1 is the mature 5′ end of 18S rRNA. It is assumed that, although occurring practically simultaneously, cleavage at site A_0 precedes that at site A_1. However, phenotypic analyses of cleavage-sequence and many *trans*-acting factor mutants clearly indicate that processing at these sites is not coupled. Thus, cleavage at site A_0 is not a prerequisite for cleavage at site A_1 and *vice versa*. In contrast, cleavage at site A_1 is coupled to that at site A_2, and although cleavage at site A_1 could occur without simultaneous cleavage at site A_2, it has so far not been reported that cleavage at site

A_2 could take place without prior cleavage at site A_1. Cleavage at site A_2 is also an endonucleolytic event as the excised D–A_2 and A_2–A_3 fragments are detected in wild-type cells and also accumulate in strains defective in the 5′–3′ exonucleases Rat1 and Xrn1.[43] Among the pre-rRNA processing reactions, cleavage at site A_2 is special since it defines the site for co-transcriptional cleavage and notably separates the intermediates of the large and small r-subunit synthesis pathways.

Processing at sites A_0, A_1 and A_2 occurs within a large preribosome intermediate known as SSU processome or 90S preribosomal particle.[31] The SSU processome is a multi-subunit complex composed of several snoRNPs (U3, U14, snR30 and snR10) and multiple *trans*-acting factors that associate in a hierarchical and stepwise manner onto the nascent pre-rRNA.[45] Mutation or depletion of any of these subunits usually delays or inhibits cleavage at the above sites and results in the accumulation of aberrant 23S, 22S, or 21S pre-rRNAs that extend from +1, A_0, or A_1 to site A_3, respectively.[31] Although some productive processing from all these intermediates may occur in wild-type cells, this does not appear to be the case in processome subunit mutants where a net deficit in 18S rRNA levels is observed.[31] It has been demonstrated that the U3 snoRNP, *via* specific base-pair interactions between the U3 snoRNA and regions of the 5′ ETS and the 5′ end of mature 18S rRNA within pre-rRNAs (Figure 4), plays an essential role in the assembly of the SSU processome and the compaction and folding of the nascent pre-rRNA (i.e., formation of the central 18S rRNA pseudoknot), which is a prerequisite for the cleavage reactions to occur.[47] Interestingly, the U3 snoRNA is unusual in that its loss-of-function leads to a strong inhibition of processing at site A_0, while depletion of other processome subunits only results in a modest to severe kinetic delay of this processing reaction.[31]

The link of cleavages at the sites A_0, A_1, and A_2, especially the tight coupling between cleavages at sites A_1 and A_2, suggests the appealing hypothesis that they might be performed by the same endonuclease. Only few nucleases have been proposed to carry out the cleavages at these sites. For example, the conserved, essential protein Rcl1 is required for *in vivo* 18S rRNA synthesis.[48] As occurs for many components of the SSU processome, inactivation of Rcl1 leads to inhibition of processing at sites A_0, A_1, and A_2, with processing at sites A_0 and A_1 being less affected than that at site A_2.[48,49] More recently, it has been shown that purified recombinant Rcl1 cleaves *in vitro* transcribed pre-rRNA fragments that include the 3′ end of mature 18S rRNA and ITS1, at a site that coincides with the *in vivo* mapped site A_2.[49] Interestingly, point mutations in *RCL1* that impair *in vivo* cleavage at site A_2 also abolish pre-rRNA cleavage *in vitro*.[49] From these studies, it has also been deduced that Rcl1 recognizes a single-stranded substrate, which is consistent with the proposed location of site A_2 in the ITS1 secondary structure[38] (Figure 5). Interestingly, recent data indicate that the *trans*-acting factor Rrp5 binds to an evolutionarily conserved stem-loop located 3′ adjacent to site A_2 in ITS1, suggesting that Rrp5 could define the exact position where cleavage occurs.[15] The SSU processome subunit Utp24, which is predicted to be a nuclease, has been suggested to be the enzyme responsible for pre-rRNA cleavages at sites A_1 and A_2,[51] but, *in vitro* experimental evidence is still missing. However, it is worth mentioning that mutation of residues that are theoretically critical for the putative enzymatic activity of Utp24 impairs cleavage at sites A_1 and A_2 but not at site A_0.[51] No candidate endonuclease for cleavage at site A_0 has so far been identified and it is even possible that distinct snoRNAs, such as U3, could assist in an autocatalytic cleavage reaction at this site.

FORMATION OF 18S rRNAs

Cleavage at Site D

Pre-rRNA processing at sites A_0–A_2 releases the 20S pre-rRNA, which is rapidly exported from the nucle(ol)us to the cytoplasm and subsequently processed at site D, the 3′ end of the mature 18S rRNA.[16] Site D is likely a single-stranded region[52,53] (Figure 5). Processing at site D is endonucleolytic as the D-A_2 fragment strongly accumulates in yeast mutants lacking the 5′–3′ exonuclease Xrn1.[54] Experimental evidence indicates that the evolutionarily conserved Nob1 protein is the D-site endonuclease. First, Nob1 is a component of cytoplasmic pre-40S particles whose loss-of-function leads to a strong accumulation of 20S pre-rRNA and a concomitant depletion of mature 18S rRNA.[55] Moreover, purified recombinant Nob1 binds *in vitro* transcribed pre-rRNA fragments containing site D, possibly as a tetramer, and *in vivo* Nob1 protects a number of pre-rRNA sites, including site D and its adjacent nts.[52,56] Most importantly, Nob1, purified from yeast, cleaves *in vitro* an RNA oligonucleotide containing the RNA sequence surrounding site D precisely at this position.[57] This *in vitro* assay is dependent on the presence of Mn^{2+}, an ion cofactor of PIN-domain nucleases. The enzymatic activity is however very poor and requires elevated concentrations of protein, Mn^{2+} and RNA substrate and long incubation times[57]; this suggests that some important *cis*-elements for cleavage are not present on the minimal RNA substrate and/or that protein

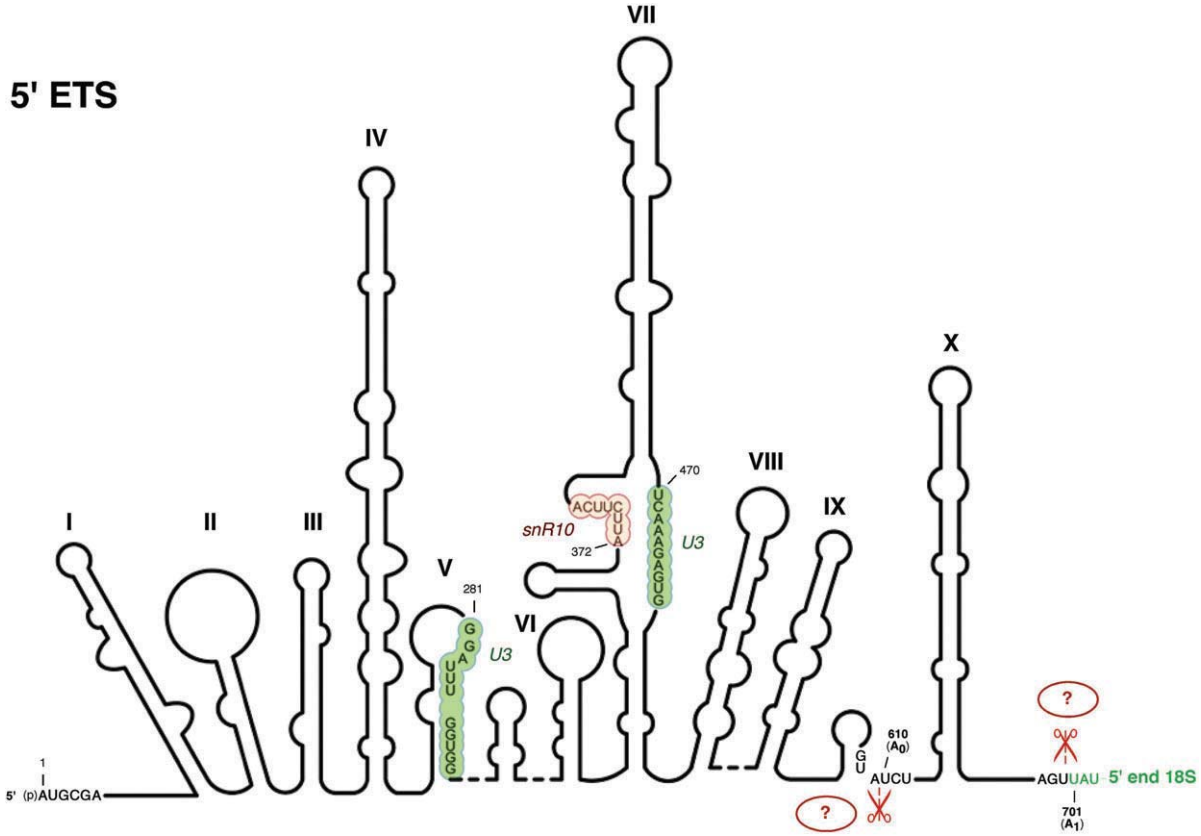

FIGURE 4 | Secondary structure model for the *S. cerevisiae* 5′ ETS. The representation has been adapted from that proposed by Yeh and Lee.[46] The positions of the sites A_0 and A_1, which are cleaved endonucleolytically are indicated. Note that the endonuclease(s) responsible of these cleavages are currently unknown. Sites binding to the U3 snoRNA and suspected to bind to snR10 are also highlighted (shaded encircled nucleotides in green or red, respectively).

co-factors, which may assist Nob1 activity *in vivo*, are lacking in the assay. In line with the latter possibility, it has been shown that Pno1/Dim2 binds directly to Nob1, thereby increasing the RNA binding affinity of Nob1 *in vitro*.[58] In addition, Ltv1, Prp43, and Pfa1 interact genetically and functionally with Nob1,[57] and mutation of several other late *trans*-acting factors and 40S r-subunit proteins impairs cleavage of 20S pre-rRNA.[35]

Nob1 is already present on nucleolar preribosomal particles[55]; therefore, its cleavage activity must be prevented until it reaches the cytoplasm. Several mechanisms have been proposed to explain this regulation of Nob1 activity. First, it has been suggested that D-site cleavage is promoted by a structural rearrangement on preribosomal particles involving a RNA conformational switch that only occurs after cleavage at site A_2 in the nucleolus.[59] This switch would allow the repositioning of Nob1 to the site D context, leading to its catalytic activation. Although not refuted, this model has recently been questioned as it does not sufficiently explain the exclusive activation of Nob1 in the cytoplasm.[60] It has therefore been proposed that yet another kind of structural rearrangement might occur within cytoplasmic pre-40S particles in order to move Nob1 from its primary binding site, apparently located at the base of helix H40 in 18S rRNA, toward site D and unleash its catalytic activity.[60] Other more speculative models for Nob1 activation propose that D-site cleavage is only enabled on restructuring of the long helix H44, which is located close to 3′ end of 18S rRNA, by the RNA helicase Prp43 and its co-factor Pfa1[57] or requires assembly factors to bring Nob1 in proximity of its substrate.[58] Recently, it has been shown that 20S pre-rRNA processing requires cytoplasmic interaction of 60S r-subunits with pre-40S particles and, moreover, depends on the GTPase activity of the translation initiation factor eIF5B and a yet to be identified ATP-binding protein, which is likely the ATPase Rio1.[60–62] This scenario has been further corroborated by the finding that a specific mutation in the 60S r-subunit protein L3 partially impairs 20S pre-rRNA processing by impeding Nob1 stimulation by the GTPase activity of eIF5B.[63]

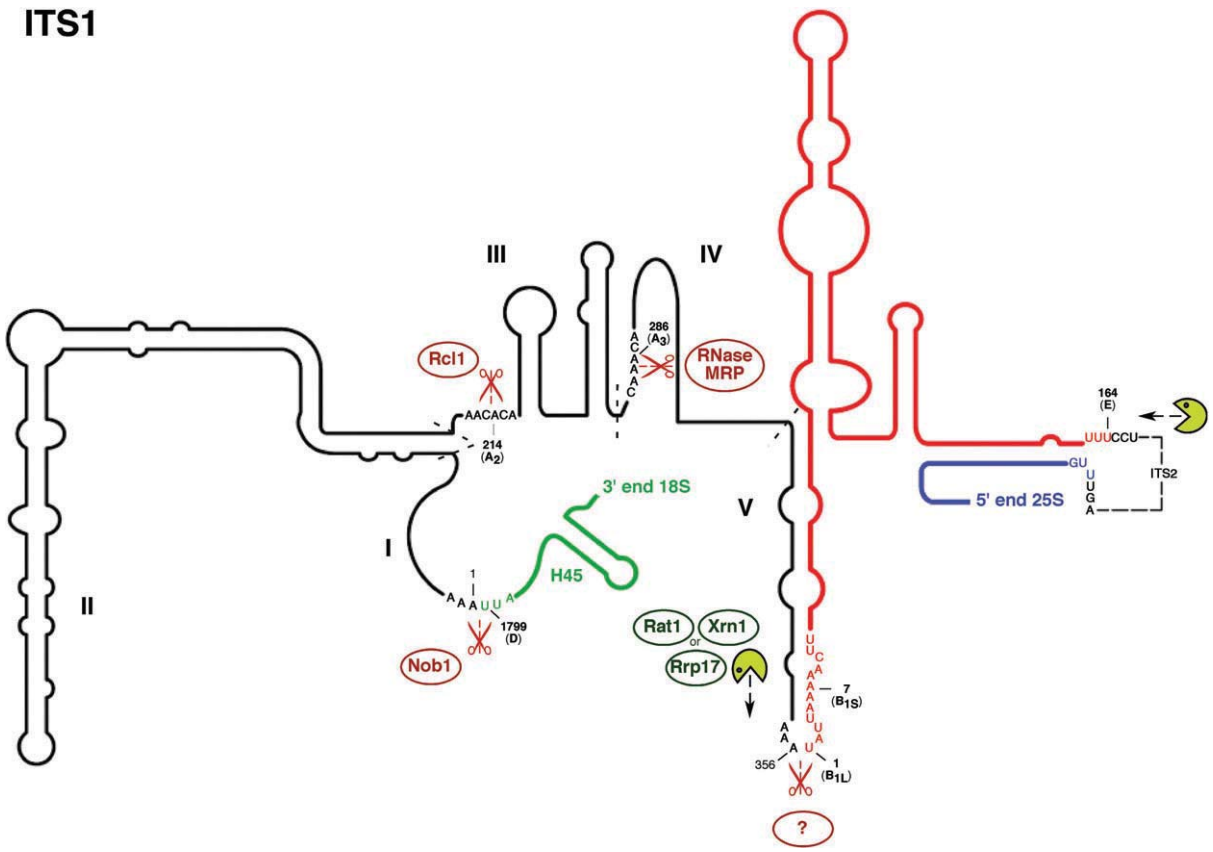

FIGURE 5 | Secondary structure model for the *S. cerevisiae* ITS1. The representation has been adapted from that proposed by Yeh et al.[50] The positions of the sites A_2, A_3, and D are shown, as well as the endonucleases involved in the respective cleavages. The positions of the sites B_{1L} and B_{1S} are also shown. Note that while processing from site A_3 to B_{1S} occurs exonucleolytically by a reaction performed by the redundant exonucleases Rat1, Rrp17, or Xrn1, cleavage at site B_{1L} occurs endonucleolytically by a so-far unknown endonuclease. See text for further details.

FORMATION OF 5.8S AND 25S rRNAs

5′ End Maturation of 5.8S rRNA: Processing at Sites A_3 and B_{1S} *Versus* Processing at Site B_{1L}

The specific processing pathway for synthesis of mature 25S and 5.8S rRNA commences 3′ from site A_2 at two alternative sites, A_3 or B_{1L} (Figure 1(b)) and finally yields two different mature 5.8S rRNA species, the $5.8S_L$ and the $5.8S_S$ rRNAs, which are produced in wild-type cells at a ratio of about 1:5.[31] The existence of heterogeneous 5.8S rRNAs is a constant in the eukaryotic kingdom[19]; however, its biological significance is still unclear.

Processing at the site A_3 is an endonucleolytic event as a discrete excised A_2–A_3 fragment could be detected in yeast mutants defective in the 5′–3′ exonuclease Rat1.[43,64] Cleavage at A_3 likely occurs post-transcriptionally, as suggested by the observation that deletions in the 3′-ETS region, comprising the stem-loop structure where cleavage at site B_0 occurs, specifically impair cleavage at site A_3.[41] How such a long-range coupling of processing sites, which are separated by about 4000 nt, is mechanistically achieved is still unknown. It can, however, be speculated that this coupling could be mediated by bridging *trans*-acting factors such as Rrp5, which is able to bind to pre-rRNA regions in ITS1 and the 3′ terminal part of 25S rRNA,[15] and r-proteins such as L3, which associates early with pre-60S r-particles but predominantly binds to the 3′ terminal part of 25S rRNA. As previously suggested by others,[31,41] coupling between processing at sites A_3 and B_0 could serve as a quality control mechanism that prevents futile processing from aberrant truncated pre-rRNAs.

Cleavage at site A_3 is dependent on the endonuclease RNase MRP *in vivo*.[31,33,65] RNase MRP consists of one RNA molecule and nine protein components, with eight of these being shared with the related endonuclease RNase P.[66] Depletion of the RNA constituent or most of the individual protein components of RNase MRP leads to inhibition of cleavage at site A_3. As a consequence, only the $5.8S_L$ rRNA, but not the $5.8S_S$ rRNA, is synthesized.[18,66,67]

In addition, two other *trans*-acting factors have been described as specifically participating in cleavage at site A_3, the above-mentioned Rrp5 and the RNA helicase Dbp3.[15,68,69] Only the N-terminal S1 RNA-binding domains of Rrp5 are essential for cleavage at site A_3.[70] Interestingly, this N-terminal part binds to the RNA component of RNase MRP and to a short U-rich pre-rRNA region located few nt 3′ to cleavage site A_3,[15] strongly suggesting that Rrp5 is directly required for cleavage at site A_3. Further experiments, however, have shown that this requirement does not involve the recruitment of RNase MRP to pre-60S r-particles, because RNase MRP is apparently still able to bind to pre-60S r-particles on Rrp5 depletion.[15] Deletion of the *DPB3* gene only kinetically delays processing at site A_3 and does not alter the $5.8S_S$:$5.8S_L$ rRNAs ratio.[68] Considering its enzymatic activity, it has been speculated that Dbp3 might optimize the recruitment or the activity of RNase MRP by chaperoning the structure of the ITS1 region in the vicinity of site A_3.[68,71] Unfortunately, this conjecture still requires to be experimentally validated.

Endonucleolytic cleavage at site A_3 has been reproduced *in vitro* using purified RNase MRP from yeast and short *in vitro* transcribed RNA substrates.[72,73] The minimal RNA substrate of RNase MRP is single-stranded, and all information required for substrate recognition and site-specific cleavage *in vitro* is included in a region encompassing few nt upstream and downstream of site A_3, with the presence of a cytosine at position +4 being a prerequisite for the cleavage reaction to take place.[73]

Cleavage at site A_3 provides the entry site for a 5′–3′ exonuclease activity that trims the 76 nt in between sites A_3 and B_{1S} to generate the 5′ end of the $27SB_S$ pre-rRNA and hence that of the mature $5.8S_S$ rRNA. Three *trans*-acting factors have been shown to be involved in this processing step, the homologous Rat1 and Xrn1 as well as the unrelated Rrp17 protein.[19,20] These three proteins exhibit processive hydrolytic 5′–3′ exonuclease activity *in vitro*,[20,74] which is required for the function of these factors in pre-rRNA processing.[7,20] In strains carrying loss-of-function mutations in any of the corresponding *RAT1*, *XRN1*, or *RRP17* genes, longer forms of 5.8S rRNA that extend to site A_3 clearly accumulate.[19,20] The role of Xrn1 and Rat1 in this step is partially redundant, however, the contribution of Rat1 appears to be more relevant than that of Xrn1. While mutation in or depletion of Rat1 leads to a significant accumulation of 5′ extended 5.8S rRNA, only very low levels of these aberrant species can be detected in the *xrn1* null mutant. In agreement with a role for both Rat1 and Xrn1, accumulation of 5′ extended 5.8S rRNA species is further increased in *xrn1*Δ *rat1* double mutants.[7,19,43] As a consequence of impaired of $5.8S_S$ rRNA production, a switch in the ratio of $5.8S_L$:$5.8S_S$ rRNAs is observed (e.g., see[20]). Strikingly, some $5.8S_S$ rRNA is still produced in the *xrn1*Δ *rat1* double mutant, which suggested the involvement of another 5′–3′ exonuclease in 5′ end maturation of $5.8S_S$ rRNA and resulted in the identification of Rrp17.[20] On simultaneous depletion of Rat1 and Rrp17 in an *xrn1*Δ background, practically no $5.8S_S$ rRNA is produced.[20] Rat1 and Rrp17 are predominantly nucleolar proteins while Xrn1 is mainly cytoplasmic.[20,75] Targeting Xrn1 to the nucleus complements the thermo-sensitive growth defect of a *rat1* allele, indicating that Rat1 and Xrn1 are functionally interchangeable proteins that act in different subcellular locations.[75] Whether overexpression of Rat1 or nuclear-targeted Xrn1 is able to complement the function of defective *rrp17* alleles remains to be determined. Moreover, Rat1 interacts physically and functionally with its nonessential co-factor Rai1.[76] The absence of Rai1 strongly enhances the defects of Rat1 depletion in $5.8S_S$ rRNA production[7]; however, it only mildly increases those of Rrp17 depletion,[20], suggesting that Rat1-Rai1 and Rrp17 work independently of each other.[20] At present, it is not clear whether the different exonucleases display distinct sequence and/or structural preferences in the A_3–B_{1S} fragment or whether they act in an entirely independent or coordinated manner.

In addition to these three exonucleases, pre-rRNA processing from site A_3 to site B_{1S} requires several other *trans*-acting factors, generally known as 'A_3 factors', including at least Nop7, Erb1, Ytm1, Rlp7, Nop15, Nsa3/Cic1, Rrp1, Nop12, and Has1.[77–80] Interestingly, most A_3 factors are interdependent for their association with preribosomal particles.[77] Loss of any of these factors leads to accumulation of the $27SA_3$ pre-rRNA and reduced synthesis of $27SB_S$ pre-rRNA and mature $5.8S_S$ rRNA, while levels of the $27SB_L$ pre-rRNA and mature $5.8S_L$ rRNA are not affected.[77,78] Despite their function in A_3–B_{1S} processing in ITS1, most A_3 factors bind to sites around the 3′ end of 5.8S rRNA, the 5′ end of 25S, and the 5′ end of ITS2.[78] Moreover, only the recruitment of Rrp17 to preribosomal particles but not that of Rat1 or Xrn1 appears to be dependent on the presence of A_3 factors.[77] In agreement with this, the analysis of its binding sites in ITS1 revealed that Rat1 is bound to regions immediately 5′ to site A_3 and may therefore already be, prior to cleavage at site A_3, present in early pre-60S r-particles.[78] It is noteworthy that loss of A_3 factors impairs stable assembly of four selected r-proteins L17, L26, L35,

and L37, which are adjacent to each other in mature 60S r-subunits and bind to 5.8S rRNAs.[77,79] It has been suggested that the A_3 factors drive the pre-rRNA rearrangements required to enable 5′ end processing of the $5.8S_S$ rRNA and assembly of r-proteins L17, L26, L35, and L37, which in turn establishes the proper 27SB pre-rRNA conformation for further ITS2 processing.[77–79,81] Notably, L17 binds to helix H2 in domain I of 25S/5.8S rRNA, formed between nt 406-417 of mature 25S and the 5′ end of mature 5.8S rRNA. It has been put forward that binding of L17 to helix H2 may act as a barrier that stops the 5′–3′ exonucleases precisely at the B_{1S} site.[19,77] Formation of helix H2 requires a major pre-rRNA rearrangement where the 5′ end sequence of 5.8S rRNA switches from its base-pairing with the distal part of ITS1 to a new base-pairing with 25S rRNA (Figure 5), which might be stabilized by L17 binding.[39] On depletion of L17, there is a specific accumulation of pre-rRNA species whose 5′ ends are about 10 nt downstream of the B_{1S} site, thus, at the end of helix H2.[77] This shorter pre-rRNA is likely the result of the inability of Rat1 to stop at the B_{1S} site in the absence of L17. Consistently, these aberrant pre-rRNA species accumulate only very mildly on inactivation of Rat1 in a L17-depleted strain.[77] As proposed by others, such a mechanism to block an event of pre-rRNA processing has the advantage that, in conditions of incorrect r-protein assembly, the exonuclease responsible for the pre-rRNA processing would initiate degradation of these, aberrantly processed pre-rRNAs.[19,77]

As $27SA_2$ pre-rRNA molecules can be directly processed at site B_{1L}, thereby generating the $27SB_L$ pre-rRNA,[31] neither processing at sites A_3 and B_{1S} nor RNase MRP or Rat1 are essential for ribosome biogenesis. Indeed, an rDNA construct containing a large distal deletion of ITS1 encompassing site A_3 still supports growth, although at a reduced rate.[19,38] In these circumstances, the $27SA_2$ molecules are only processed at site B_{1L} and the $5.8S_L$:$5.8S_S$ ratio increases dramatically.[19,67] The mechanism by which processing at site B_{1L} takes place has not yet been addressed in detail. However, three facts can be highlighted: (1) As proposed for cleavage at site A_3, processing at site B_{1L} appears to occur post-transcriptionally because deletions in the 3′ ETS also affect this processing reaction.[41] (2) Processing at site B_{1L} is likely endonucleolytic, as deduced by the identification of discrete amounts of an A_2–B_{1L} fragment in strains defective in the RNA component of RNase MRP or Rrp5.[21] As this processing activity involves a single event, formation of $5.8S_L$ rRNA is kinetically faster than synthesis of $5.8S_S$ rRNA.[19] (3) The endonuclease responsible of this cleavage reaction is still unknown. Mutational analysis of the 3′ terminal region of ITS1 show that this endonuclease is not sequence specific and does not depend on sequence elements in the 5′ adjacent region of ITS1.[21,38]

Cleavage at Site C_2

Removal of ITS2 starts by cleavage at site C_2, which separates the specific precursors along the maturation pathway of 5.8S and 25S rRNA. This step is endonucleolytic as an A_2–C_2 fragment clearly accumulates on inactivation of exosome complex subunits or its RNA helicase co-factor Mtr4/Dob1.[82,83] Mutational analysis has demonstrated that cleavage at site C_2 relies on some structural *cis*-elements within ITS2, among them, the so-called ITS2-proximal stem, which is a structure formed by the base-pairing between the 3′ end of 5.8S and the 5′ end of 25S rRNAs (Figure 6), and the nt downstream of the cleavage site.[38] In fact, two base-pairings just contiguous to site C_2 are strictly required for cleavage, perhaps because these are part of the recognition site of the specific C_2-site endonuclease, which still remains to be identified.

In wild-type cells, processing at the site C_2 follows removal of ITS1; however, the identification of an A_2–C_2 fragment indicates that in certain circumstances, $27SA_2$ pre-rRNA could be prematurely cleaved at site C_2.[82] A model to explain this specific order of processing, which is based on the conformational status of ITS2[86] and the sequential association of *trans*-acting factors required for ITS1 and ITS2 processing,[80,87] has been proposed. Accordingly, two alternative, predicted structures appear to exist for yeast ITS2 (Figure 6(a) and (b)), the so-called 'ring' and 'hairpin' structures, which differ primarily in base-pairing of the sequences adjacent to the mature 5.8S and 25S rRNA ends.[84–86] The transition from the ring to the hairpin structure is likely a prerequisite to induce cleavage at site C_2.[86] Apparently, the A_3 factors, which bind at or near ITS2 (see previous section), have different roles in this structural transition. While factors such as Nop15 and Nsa3 have been suggested to hold ITS2 in the ring conformation,[77,78] others, such as Rlp7, Nop12, and Pwp1, appear to be important for the formation of the ITS2-proximal stem.[80,88,89] Moreover, Nop12 and Pwp1 have additional roles in promoting proper folding of the 5.8S rRNA.[80] As discussed above, the concerted action of the A_3 factors promotes, either directly or indirectly, the proper assembly of a set of r-proteins (L17, L26, L35, and L37), which in turn is a prerequisite for the correct association of Nsa2 and Nog2, which belong to the so-called 'B factors'.[81,87,90] The B factors include RNA-binding proteins (Nip7,

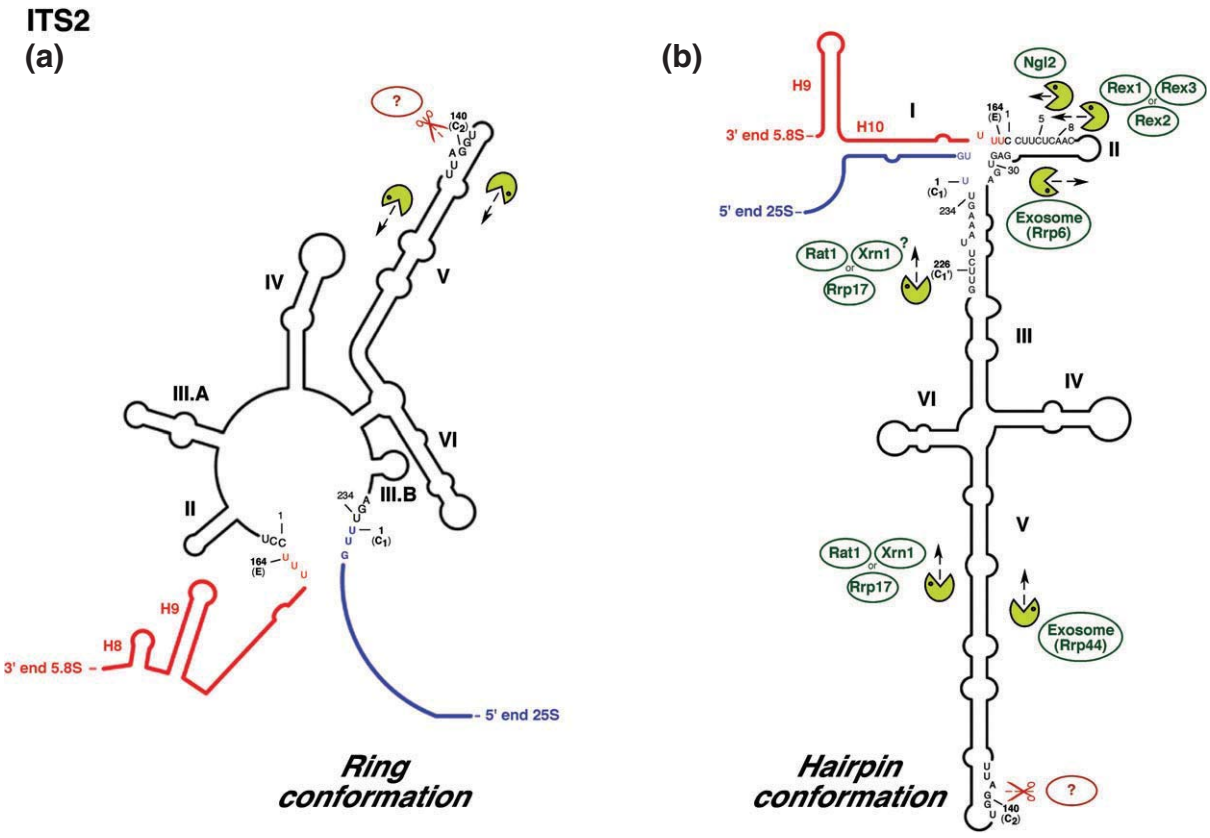

FIGURE 6 | Secondary structure models for the *S. cerevisiae* ITS2. Two conformations have been proposed for ITS2, (a) the ring conformation[84] and (b) the hairpin conformation.[85] The positions of the sites C_1, C_1', C_2, E as well as the 3′ end extended 5.8S + 30, 6S (+8) and 5.8S + 5 species are indicated. Note that the nuclease responsible for endonucleolytic cleavage at site C_2 remains to be identified. Processing from site C_2 to site C_1 occurs by 5′–3′ trimming by either Rat1, Rrp17, or Xrn1. Processing from site C_2 to site E occurs by 3′–5′ trimming by the concerted action of different exonucleases. Note that all these processing reaction are represented only in the hairpin structure. See text for further details.

Nsa2, Rlp24, Rpf2, and Tif6), a scaffolding protein (Mak11), GTPases (Nog1 and Nog2), rRNA methyltransferases (Nop2 and Spb1), and RNA helicases (Drs1 and Has1), but none of these can be suspected to act as the C_2-site endonuclease.[81] Loss-of-function of these B factors leads to a common pre-rRNA processing phenotype, which consists in the accumulation of 27SB pre-rRNA relative to 27SA and 7S pre-rRNAs.[81] It has been shown that B factors associate sequentially with pre-60S r-particles through two converging pathways that result in the final recruitment of Nog2. Whether the GTPase Nog2 promotes the recruitment or facilitates the enzymatic function of the mysterious C_2-site endonuclease is completely unknown.

3′ End Maturation of 5.8S rRNA

Cleavage at site C_2 releases the 7S and 25.5S pre-rRNAs, which are the immediate precursors of mature 5.8S and 25S rRNAs, respectively. Formation of the 3′ end of mature 5.8S rRNAs from the 7S pre-rRNAs is a very complicated 3′–5′ exonucleolytic multi-step process,[91] which is performed by different nucleases that act in a concerted manner. The reason for this complexity is unclear, especially if one considers that aberrant 60S r-subunits containing 3′ end extended forms of 5.8S rRNAs are translationally competent.[23,25]

Four distinct and successive steps during the 3′ end formation of the 5.8S rRNA can be discerned. The initial step consists in the 3′–5′ trimming of the 7S pre-rRNAs, which are extended at their 3′ ends by about 140 nt up to site C_2 in ITS2, to the 5.8S + 30 pre-rRNA species that still retain about 30 nt of ITS2. In the second step, the 5.8S + 30 pre-rRNAs are processed to 6S pre-rRNAs, whose 3′ ends still contain about 8 nt of ITS2. Both steps are carried out by the nuclear exosome. This is a conserved multi-protein complex composed of a core of nine subunits; six of these (Mtr3, Rrp41/Ski6, Rrp42, Rrp43, Rrp45, and Rrp46) harbor an RNase PH fold and form a hexameric barrel-like structure that is stabilized by a cap formed by three proteins (Csl4/Ski4, Rrp4,

and Rrp40), which contain KH and S1 RNA binding domains.[92,93] Biochemical studies indicate that the core functions to bind and thread RNA substrates through the central channel within the barrel-like structure.[94–96] Importantly, the core is catalytically inactive,[92,97,98] and enzymatic activity is only acquired by its association with two nucleases, Rrp44/Dis3 and Rrp6. Rrp44 is a multi-domain protein composed of three RNA binding domains, an N-terminal PIN domain harboring endonucleolytic activity, and a C-terminal RNB domain, which is typical for members of the RNaseII/R family. *In vitro*, Rrp44 exhibits processive hydrolytic 3′–5′ exonuclease activity on both nonstructured and structured RNA substrates with 3′ single-stranded overhangs.[97,99,100] Rrp44 associates with the lower face of the exosome core on the opposite side of the trimeric cap,[93] and, interestingly, its interaction with the core attenuates its exonuclease activity.[95] Rrp6 is a nonessential distributive hydrolytic 3′–5′ exonuclease that belongs to the RNase D family of ribonucleases.[101] Unlike Rrp44, Rrp6 is unable to degrade structured RNAs and its enzymatic activity is not modulated on its interaction with the exosome core.[92] The C-terminal part of Rrp6 is sufficient, by interacting with Csl4, Mtr3, and Rrp43, to mediate the association of Rrp6 with the upper face of the exosome.[93]

In vivo, depletion of any subunits of the exosome core and of Rrp44 is lethal and results in practically similar defects in 3′ maturation of the 5.8S rRNAs, which consist in the accumulation of 3′ extended pre-rRNA intermediates, forming a ladder from the position of the 6S pre-rRNAs up to the position of the 7S pre-rRNAs, and the concomitant deficit in mature 5.8S rRNAs.[24,82,100] Interestingly, a point mutation that abrogates the exonuclease activity of Rrp44 is viable, but entails a similar, albeit slightly less pronounced, 7S pre-rRNA processing phenotype as observed on depletion of exosome components.[97,102] The endonuclease activity of Rrp44 is also dispensable for growth.[99,103] In this case, the inactivation of the endonuclease activity of Rrp44 has practically no effect on 7S pre-rRNA processing.[99,103] In contrast, the simultaneous abrogation of both the endo- and exonuclease activity of Rrp44 does not support growth, and the pre-rRNA processing phenotype of the respective mutant is practically identical to that of the Rrp44-depleted strain.[99,102,103] Taken together, these results indicate that the exonuclease activity of Rrp44 plays an important but not essential role in 3′ end maturation of 5.8S rRNAs. The endonuclease activity likely exerts a minor, but cooperative role by providing access to additional pre-rRNA intermediates. The fact that abrogation of both activities is lethal must be interpreted in terms of redundant roles of these activities in the processing or degradation of different RNA substrates besides 7S pre-rRNAs.[104]

Deletion of *RRP6* is viable and leads to a slow-growth phenotype and a distinct defect in 7S pre-rRNA processing, consisting in a minor accumulation of 7S pre-rRNA, a strong accumulation of 5.8S + 30 species and a deficit in mature 5.8S rRNAs.[25] The growth phenotype of the *rrp6*Δ strain is exacerbated (yeast BY4741 genetic background) or lethal (W303 background) on inactivation of the exonuclease activity of Rrp44.[97,103] In the double mutant, the 5.8S + 30 species are additionally 3′ extended by about 10 nt[103] and, as above, the lethality is unlikely due to the role of the corresponding nucleases in 7S pre-rRNA processing. In contrast, mutation of the endonuclease domain of Rrp44 does practically not enhance the growth defect of the *rrp6*Δ strain and does not lead to 3′ extended forms of 5.8S + 30 pre-rRNAs.[103] It is worth mentioning that a ssRNA stretch of about 30 nt, curiously the same length as the 3′ end extension of 5.8S + 30 species, is threaded through the central channel of the exosome up to the exonuclease active site of Rrp44.[93,95] Thus, the following model for 7S pre-rRNA processing to 6S pre-rRNAs can be envisaged: the 3′ end of 7S pre-rRNAs is pushed through the central pore of the exosome complex and initially processed by the exonuclease activity of Rrp44. As only ssRNA can be accommodated inside the channel, the helical domain II in the ITS2 fragment of 7S pre-rRNAs, likely in association with a bound protein, might block further processing and thereby stabilize the 5.8S + 30 precursors. These species are then released from the channel, handed over to Rrp6 by which they are, in an exosome-dependent or -independent reaction, processed to 6S pre-rRNAs.[105]

Processing of 7S pre-rRNAs requires additional factors, many of them acting as exosome co-factors. The best-characterized exosome co-factor is the RNA helicase Mtr4. On depletion of Mtr4, a strong accumulation of 7S pre-rRNAs, a weaker accumulation of 5.8S + 30 pre-rRNAs and a deficit in mature 5.8S rRNAs are observed.[24,44] This role of Mtr4 is completely independent of the TRAMP complexes, as the loss-of-function of the other components of these complexes (Trf4/5 and Air1/2), has no apparent effect on 3′ end maturation of 5.8S rRNA.[106] Biochemical and structural studies suggest that Mtr4 directs the RNA substrate to either the entry site of the exosome channel or Rrp6.[107] Importantly, the so-called arch domain of Mtr4 is specifically required for processing of 5.8S + 30 species[107]; thus, it has been suggested that Mtr4 could also actively help releasing the 5.8S + 30

pre-rRNAs from the exosome channel to make them accessible to Rrp6.[107] Two other co-factors of the exosome are the nonessential proteins Rrp47/Lrp1[108,109] and Mpp6.[110] Rrp47 forms a stable complex with Rrp6[111] to facilitate binding of the RNA substrate or modulate the enzymatic activity. Deletion of *RRP47* alone or in combination with the deletion of *RRP6* leads to a slow-growth defect and a 7S pre-rRNA processing phenotype identical to those caused by the single *rrp6*Δ mutation.[108,109] Mpp6 is also physically associated with the exosome, and the *mpp6*Δ null mutant only exhibits a very mild accumulation of 5.8S + 30 pre-rRNAs.[110] Another protein that is functionally connected to the exosome is the nonessential Gsp1, which is the yeast homolog of the GTPase Ran. The deletion of *GSP1* leads to 7S pre-rRNA processing defects that are indistinguishable from those observed in exosome mutants. Moreover, Gsp1 directly interacts with Rrp44.[112]

The third and fourth step during 3′ end maturation of 5.8S rRNAs are not carried out by the exosome and, in contrast to the first two steps, they occur in the cytoplasm.[27] The 6S pre-rRNA is not a substrate of Rrp6 because the remaining 8 nt of ITS2 may either be structured and/or protected by a bound protein. In agreement with such a possibility, it has been described that Rrp6 stalls on structured RNAs.[92] Instead, the 6S precursors appear to be a substrate for the nonessential putative 3′–5′ exonucleases Rex1, Rex2, and Rex3. Thus, while each single *rex1–3* null mutant behaves like the wild-type strain, the *rex1*Δ *rex2*Δ double mutant accumulates 6S pre-rRNAs and even higher levels of these species can be detected in the *rex1*Δ *rex2*Δ *rex3*Δ triple mutant.[26] This suggests that the roles of Rex1 and Rex2 in the maturation of 6S pre-rRNAs to 5.8S rRNAs are redundant and that Rex3 has also a minor participation in this step. Trimming of the 6S precursors by the Rex1–3 proteins stops upstream of the 3′ end of mature 5.8S rRNAs, and the shorter intermediate is handed over to the putative nuclease Ngl2, as indicated by the observation that deletion of the nonessential *NGL2* gene leads to the accumulation of 5.8S rRNAs that are 3′ extended by about 5 nt.[23] Whether Ngl2 acts as an endo- or a 3′–5′ exonuclease is unknown.

5′ End Maturation of 25S rRNA

The 25.5S pre-rRNA, also known as 26S pre-rRNA, is extended at the 5′ end by about 94 nt relative to mature 25S rRNA. Maturation of this precursor into mature 25S rRNA occurs by 5′–3′ exonucleolytic trimming from site C_2, which acts as the entry site for the exonucleases.[28] Rat1, Rrp17, and Xrn1 have been reported to be partially redundantly required for this process.[20,28] Consistent with its role in the formation of the 5′ end of mature 25S rRNA, Rat1 has been found to bind to sequences 5′ to site C_2 in 27S pre-rRNAs.[78] Similarly to what occurs during 5′ end processing of 5.8S rRNAs, mutation in or depletion of Rat1 or Rrp17, alone or in combination with the *xrn1* null allele, lead to the accumulation of 5′ extended forms of 25S rRNA up to site C_2.[20,28] Apparently, this reaction takes place very rapidly and proceeds in two steps in wild-type conditions: first, the 25.5S pre-rRNA species is processed to the so-called 25S′ pre-rRNA, which is extended at the 5′ end by about 8 nt (site C1′) compared to the mature 25S rRNA[113]; then, the 25S′ precursor is also 5′–3′ exonucleolytically trimmed to yield the mature 25S rRNA. It is suspected that the latter reaction is also mediated by the above exonucleases as no specific mutants have been found to affect this step without affecting the other. Perhaps site C1′ represents a region of strong secondary structure or the binding site of a protein factor, thus, slowing down the processive exonuclease activity of Rat1 or Rrp17 when they pass through this particular ITS2 region.

Strikingly, processing of 25.5S pre-rRNA is likely coupled to maturation of 7S pre-rRNAs, as suggested by the observation that either the depletion of Rat1 or Rrp17 or the deletion of Rai1 also cause the accumulation of 3′ extended forms of 5.8S rRNAs.[20,114] Moreover, the *trans*-acting factor Las1 is required for pre-rRNA processing at both ends of ITS2.[29] Loss-of-function of Las1 leads to the accumulation of both 3′ extended forms of 5.8S rRNAs and 5′ extended forms of mature 25S pre-rRNA, especially those ending at site C1′ or +15 with respect to the 5′ end of 25S rRNA.[29] The proximity of sites E and C_1 in the context of the hairpin structure of ITS2 has led to the speculation that a common RNA processing complex may exist, which performs processing at both ends of ITS2, following cleavage at site C_2, in a coordinated manner.[29]

3′ End Maturation of 25S rRNA

Final maturation of the 3′ end of the 25S rRNA at site B_2 is perhaps the poorest studied reaction of the yeast pre-rRNA processing pathway. This step consists in the removal of the first 14 nt from the 3′ ETS, which remain at the 3′ end of pre-rRNAs after the endonucleolytic cleavage at site B_0 (Figure 3). Apparently, this reaction occurs concomitantly with the formation of the 5′ end of mature 5.8S rRNAs, as demonstrated by the fact that a 3′-ETS probe hybridizes to the 35S, 32S, and 27SA, but not the

27SB pre-rRNAs.[4] It has been shown that the *rna82-1* mutant accumulates rRNAs that are 3′-extended by 7–10 nt relative to the 3′ end of mature 25S rRNA.[115] Interestingly, the *rna82-1* mutation maps within the *REX1* gene,[26] thus, the final 3′ end formation of 25S rRNA might be the result of a 3′–5 exonucleolytic event. Unfortunately, the contribution of Rex1 to 3′ end processing of the 25S rRNA has so far not been directly assessed by testing the *rex1*Δ mutant alone, or in combination with null mutants of other 3′–5′ exonucleases.

FORMATION OF 5S rRNA

As abovementioned, pre-5S rRNA only requires processing at its 3′ end, which is extended by about 12 nt from the 3′ end of mature 5S rRNA (Figure 2). This step occurs kinetically much faster than formation of 18S, 5.8S, and 25S rRNA, as deduced from pulse-chase labeling analysis of pre-rRNAs. It has been clearly shown that this step relies on a 3′–5′ exonucleolytic activity, which simply trims the pre-5S rRNA to the mature form.[26,116,117] The formation of helix H1 in 5S rRNA, which is due to base-pairing between both mature ends of 5S rRNA (Figure 2), appears to work as a barrier that stops the progressing exonuclease.[116]

It has been shown that, *in vivo*, this trimming reaction is dependent on the 3′–5′ exonuclease Rex1.[26] In the absence of Rex1, there is an accumulation of 3′ extended 5S rRNAs that can assemble into fully functional 60S r-subunits.[26] This indicates that processing of pre-5S rRNA is a nonessential step that neither affects the maturation of 5.8S, 18S, and 25S rRNA nor 60S r-subunit formation.[118,119]

CONSERVATION OF PRE-rRNA PROCESSING THROUGHOUT EUKARYOTES

The study of the primary organization of rDNA and the analysis of pre-rRNA processing in other eukaryotes, including human cells, indicate that the fundamental aspects of this pathway, as well as the stepwise order of the cleavage events, have been fairly well conserved during evolution.[36,37] Two good examples that illustrate this statement are: (1) many structural *cis*-elements within the spacers that are required for processing are conserved in different eukaryotes[84]; and (2) orthologs of most yeast pre-rRNA processing factors are present in other eukaryotes; in many cases these orthologs are able to complement the corresponding yeast mutants.[20,98] Obviously, several remarkable differences are found between selected eukaryotes; and it can be affirmed that, despite the fact that much less is known, pre-rRNA processing has an increased complexity in higher eukaryotes. Thus, when comparing human and yeast, (1) the number of *trans*-acting factors involved in ribosome biogenesis is much higher in humans[120]; (2) in some instances, conserved factors have even additional roles in human cells[121,122]; (3) there are additional cleavage sites in the human pre-rRNAs[36]; and (4) moreover, certain steps of human pre-rRNA processing show significant differences when compared to the presumably analogous steps in yeast.[29,122–124] All these aspects are covered in detail in other, more specialized reviews.[36,37]

CONCLUSIONS AND PERSPECTIVES

Pre-rRNA processing in eukaryotes is a complex multi-step process that has so far most extensively been studied in the yeast *S. cerevisiae*. Yeast research has been essential to characterize many aspects of this process: (1) The use of appropriate yeast strains expressing a plasmid-encoded rRNA unit as the sole source of rRNAs has allowed the characterization of most *cis*-acting elements within the pre-rRNA.[38] After decades of work, we can conclude that most, perhaps even all, cleavage sites on yeast pre-rRNA have been identified. (2) The use of yeast molecular genetics in combination with a vast repertoire of RNA analysis techniques (e.g., polysome profile, Northern, primer extension, pulse-chase analysis, etc.) have facilitated the identification of many factors involved in ribosome biogenesis, among them most of those specifically required for pre-rRNA processing.[31,33,35] (3) More recently, those classical studies have been complemented by the application of protein affinity purification of preribosomal particles[30]; the most refined purification methods have even been useful to establish the timing of action of several nucleases that transiently interact with preribosomal particles.[125] All these approaches have permitted to conclude that the list of *trans*-acting factors involved in yeast ribosome biogenesis is close to saturation. Nevertheless, it is important to remark that some important players in pre-rRNA processing, such as the nucleases required for cleavages at sites A_0, A_1, B_{1L}, and C_2 are still unknown. (4) The probing of RNA structures within the spacers and the identification of RNA–protein interactions have been recently tackled by the development of procedures such as CRAC, CLASH and RNA SHAPE, fast kinetic labeling, or structural probing of pre-rRNAs coupled to affinity purification methods.[56,126,127] Application of these methodologies has already provided information with respect to the interaction sites of the nucleases with

the pre-rRNAs and first insights into the structural changes of pre-rRNAs, which are entailed by certain processing events. In no case, however, do we precisely know the co-factors or understand what specific conformational changes are needed within distinct regions of the pre-rRNAs to activate the respective nucleases for the processing reactions to occur in a defined time window. Of equal importance as the *in vivo* yeast research has been the development of specific *in vitro* nuclease assays performed with purified recombinant wild-type and mutant proteins. However, still not all putative nucleases, described to have a role in the pre-rRNA processing reactions, have been tested in such *in vitro* assays (e.g., Rex1–3 and Ngl2). Moreover, the crystal or cryo-EM structures of most nucleases, except for that of the nuclear RNA exosome, are still missing.[93,96,128] The structural analysis of the exosome complex associated with an RNA substrate is a beautiful example of how these techniques provide valuable insights into how a pre-rRNA precursor is recruited to and processed by an enzyme.

In conclusion, we start getting a rough picture of the complicated process of pre-rRNA processing. Still, one of the most important unsolved questions in the field is why the synthesis of mature rRNAs relies on an evolutionarily conserved series of complex and ordered processing reactions within the transcribed spacers rather than just transcription of the mature forms of rRNAs. Clearly, the spacers have a direct and essential role in pre-rRNA processing. In this review, we have discussed the role of 5′ ETS, snoRNAs (such as U3), and *trans*-acting factors (such as Rrp5) for the folding of a productive structure in the pre-rRNA needed for the early cleavages at the processing sites A_0, A_1, and A_2. We have also highlighted the fact that the spacers provide 'entry' sites for binding pre-rRNA processing factors; in this respect, we have described that the effective removal of ITS1 is dependent on the previous association of the so-called A_3 factors to regions in ITS2. Notably, these A_3 factors might induce complex rearrangements in the ITS1-5.8S-ITS2 region that allow proper assembly of a set of r-proteins, with some of these r-proteins acting as a barrier for the 5′–3′ exonucleases involved in the A_3 to B_1 processing; thereby providing an additional control point for surveillance and rapid degradation of aberrantly processed pre-rRNAs. Moreover, we have discussed that the spacer regions might act as switches to provide directionality to pre-rRNA processing, as proposed for the conformational change from the ring to the hairpin structure that likely occurs in ITS2 and is required for cleavage at site C_2. However, many aspects still remain completely unclear: Why is the maturation of the 3′ end of 5.8S rRNAs so complicated? Is the heterogeneity at the mature 5′ end of 5.8S rRNAs functionally relevant? How are the cleavages at distant sites exactly coupled? And how is pre-rRNA processing integrated into other aspects of ribosome biogenesis, such as assembly of r-proteins into, nucleo-cytoplasmic export of, or acquisition of translation competence of preribosomal particles? Without any doubt, future work will provide us with exciting answers to many of these questions and help establishing the complete picture of the events that lead to the formation of mature ribosomes.

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REFERENCES

1. Warner JR. The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* 1999, 24:437–440.
2. Richard P, Manley JL. Transcription termination by nuclear RNA polymerases. *Genes Dev* 2009, 23: 1247–1269.
3. Klemenz R, Geiduschek EP. The 5′ terminus of the precursor ribosomal RNA of *Saccharomyces cerevisiae*. *Nucleic Acids Res* 1980, 8:2679–2689.
4. Kufel J, Dichtl B, Tollervey D. Yeast Rnt1p is required for cleavage of the pre-ribosomal RNA in the 3′ ETS but not the 5′ ETS. *RNA* 1999, 5:909–917.
5. Reeder RH, Guevara P, Roan JG. *Saccharomyces cerevisiae* RNA polymerase I terminates transcription at the Reb1 terminator in vivo. *Mol Cell Biol* 1999, 19:7369–7376.

6. Abou Elela S, Igel H, Ares M Jr. RNase III cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site. *Cell* 1996, 85:115–124.
7. El Hage A, Koper M, Kufel J, Tollervey D. Efficient termination of transcription by RNA polymerase I requires the 5′ exonuclease Rat1 in yeast. *Genes Dev* 2008, 22:1069–1081.
8. Kawauchi J, Mischo H, Braglia P, Rondon A, Proudfoot NJ. Budding yeast RNA polymerases I and II employ parallel mechanisms of transcriptional termination. *Genes Dev* 2008, 22:1082–1092.
9. Hughes JMX, Ares M Jr. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. *EMBO J* 1991, 10:4231–4239.
10. Veldman GM, Brand RC, Klootwijk J, Planta R. Some characteristics of processing sites in ribosomal precursor RNA of yeast. *Nucleic Acids Res* 1980, 8:2907–2920.
11. Veinot-Drebot LM, Singer RA, Johnston GC. Rapid initial cleavage of nascent pre-rRNA transcripts in yeast. *J Mol Biol* 1988, 199:107–113.
12. Kos M, Tollervey D. Yeast pre-rRNA processing and modification occur cotranscriptionally. *Mol Cell* 2010, 37:809–820.
13. Osheim YN, French SL, Keck KM, Champion EA, Spasov K, Dragon F, Baserga SJ, Beyer AL. Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to being cleaved from nascent transcripts in *Saccharomyces cerevisiae*. *Mol Cell* 2004, 16:943–954.
14. Axt K, French SL, Beyer AL, Tollervey D. Kinetic analysis demonstrates a requirement for the Rat1 exonuclease in cotranscriptional pre-rRNA cleavage. *PLoS One* 2014, 9:e85703.
15. Lebaron S, Segerstolpe A, French SL, Dudnakova T, de Lima AF, Granneman S, Rappsilber J, Beyer AL, Wieslander L, Tollervey D. Rrp5 binding at multiple sites coordinates pre-rRNA processing and assembly. *Mol Cell* 2013, 52:707–719.
16. Udem SA, Warner JR. The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J Biol Chem* 1973, 248:1412–1416.
17. Rouquette J, Choesmel V, Gleizes PE. Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells. *EMBO J* 2005, 24:2862–2872.
18. Chu S, Archer RH, Zengel JM, Lindahl L. The RNA of RNase MRP is required for normal processing of ribosomal RNA. *Proc Natl Acad Sci U S A* 1994, 91:659–663.
19. Henry Y, Wood H, Morrissey JP, Petfalski E, Kearsey S, Tollervey D. The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. *EMBO J* 1994, 13:2452–2463.
20. Oeffinger M, Zenklusen D, Ferguson A, Wei KE, El Hage A, Tollervey D, Chait BT, Singer RH, Rout MP. Rrp17p is a eukaryotic exonuclease required for 5′ end processing of Pre-60S ribosomal RNA. *Mol Cell* 2009, 36:768–781.
21. Faber AW, Vos HR, Vos JC, Raué HA. 5′-end formation of yeast 5.8SL rRNA is an endonucleolytic event. *Biochem Biophys Res Commun* 2006, 345:796–802.
22. Veldman GM, Klootwijk J, van Heerikhuizen H, Planta RJ. The nucleotide sequence of the intergenic region between the 5.8S and 26S rRNA genes of the yeast ribosomal RNA operon. Possible implications for the interaction between 5.8S and 26S rRNA and the processing of the primary transcript. *Nucleic Acids Res* 1981, 9:4847–4863.
23. Faber AW, Van Dijk M, Raué HA, Vos JC. Ngl2p is a Ccr4p-like RNA nuclease essential for the final step in 3′- end processing of 5.8S rRNA in *Saccharomyces cerevisiae*. *RNA* 2002, 8:1095–1101.
24. Allmang C, Kufel J, Chanfreau G, Mitchell P, Petfalski E, Tollervey D. Functions of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO J* 1999, 18:5399–5410.
25. Briggs MW, Burkard KTD, Butler JS. Rrp6p, the yeast homologue of the human PM-Scl 100-kDa autoantigen, is essential for efficient 5.8S rRNA 3′ end formation. *J Biol Chem* 1998, 273:13255–13263.
26. van Hoof A, Lennertz P, Parker R. Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast. *EMBO J* 2000, 19:1357–1365.
27. Thomson E, Tollervey D. The final step in 5.8S rRNA processing is cytoplasmic in *Saccharomyces cerevisiae*. *Mol Cell Biol* 2010, 30:976–984.
28. Geerlings TH, Vos JC, Raué HA. The final step in the formation of 25S rRNA in *Saccharomyces cerevisiae* is performed by 5′–3′ exonucleases. *RNA* 2000, 6:1698–1703.
29. Schillewaert S, Wacheul L, Lhomme F, Lafontaine DL. The evolutionarily conserved protein Las1 is required for pre-rRNA processing at both ends of ITS2. *Mol Cell Biol* 2011, 32:430–444.
30. Kressler D, Hurt E, Bassler J. Driving ribosome assembly. *Biochim Biophys Acta* 2010, 1803:673–683.
31. Venema J, Tollervey D. Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu Rev Genet* 1999, 33:261–311.
32. Venema J, Tollervey D. Processing of pre-ribosomal RNA in *Saccharomyces cerevisiae*. *Yeast* 1995, 11:1629–1650.
33. Kressler D, Linder P, de la Cruz J. Protein *trans*-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1999, 19:7897–7912.

34. Fromont-Racine M, Senger B, Saveanu C, Fasiolo F. Ribosome assembly in eukaryotes. *Gene* 2003, 313:17–42.
35. Henras AK, Soudet J, Gerus M, Lebaron S, Caizergues-Ferrer M, Mougin A, Henry Y. The post-transcriptional steps of eukaryotic ribosome biogenesis. *Cell Mol Life Sci* 2008, 65:2334–2359.
36. Mullineux ST, Lafontaine DL. Mapping the cleavage sites on mammalian pre-rRNAs: where do we stand? *Biochimie* 2012, 94:1521–1532.
37. Gerbi SA, Borovjagin AV. Pre-ribosomal RNA processing in multicellular organisms. In: Olson MOJ, ed. *Nucleolus*. Georgetown, TX: Landes Biosciences/Eurekah.com; 2004, 170–198.
38. van Nues RW, Venema J, Rientjes JMJ, Dirks-Mulder A, Raué HA. Processing of eukaryotic pre-rRNA: the role of the transcribed spacers. *Biochem Cell Biol* 1995, 73:789–801.
39. Woolford JL Jr, Baserga SJ. Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. *Genetics* 2013, 195:643–681.
40. Hartman E, Wang Z, Zhang Q, Roy K, Chanfreau G, Feigon J. Intrinsic dynamics of an extended hydrophobic core in the *S. cerevisiae* RNase III dsRBD contributes to recognition of specific RNA binding sites. *J Mol Biol* 2013, 425:546–562.
41. Allmang C, Tollervey D. The role of the 3′ external transcribed spacer in yeast pre-rRNA processing. *J Mol Biol* 1998, 278:67–78.
42. Venema J, Henry Y, Tollervey D. Two distinct recognition signals define the site of endonucleolytic cleavage at the 5′-end of yeast 18S rRNA. *EMBO J* 1995, 14:4883–4892.
43. Petfalski E, Dandekar T, Henry Y, Tollervey D. Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. *Mol Cell Biol* 1998, 18:1181–1189.
44. de la Cruz J, Kressler D, Tollervey D, Linder P. Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3′ end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J* 1998, 17:1128–1140.
45. Pérez-Fernández J, Roman A, de Las RJ, Bustelo XR, Dosil M. The 90S preribosome is a multimodular structure that is assembled through a hierarchical mechanism. *Mol Cell Biol* 2007, 27:5414–5429.
46. Yeh L-CC, Lee JC. Structure analysis of the 5′ external transcribed spacer of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J Mol Biol* 1992, 228:827–839.
47. Dutca LM, Gallagher JE, Baserga SJ. The initial U3 snoRNA:pre-rRNA base pairing interaction required for pre-18S rRNA folding revealed by *in vivo* chemical probing. *Nucleic Acids Res* 2011, 39:5164–5180.
48. Billy E, Wegierski T, Nasr F, Filipowicz W. Rcl1p, the yeast protein similar to the RNA 3′-phosphate cyclase, associates with U3 snoRNP and is required for 18S rRNA biogenesis. *EMBO J* 2000, 19:2115–2126.
49. Horn DM, Mason SL, Karbstein K. Rcl1 protein, a novel nuclease for 18 S ribosomal RNA production. *J Biol Chem* 2011, 286:34082–34087.
50. Yeh L-CC, Thweatt R, Lee JC. Internal transcribed spacer 1 of the yeast precursor ribosomal RNA. Higher order structure and common structural motifs. *Biochemistry* 1990, 29:5911–5918.
51. Bleichert F, Granneman S, Osheim YN, Beyer AL, Baserga SJ. The PINc domain protein Utp24, a putative nuclease, is required for the early cleavage steps in 18S rRNA maturation. *Proc Natl Acad Sci U S A* 2006, 103:9464–9469.
52. Lamanna AC, Karbstein K. Nob1 binds the single-stranded cleavage site D at the 3′-end of 18S rRNA with its PIN domain. *Proc Natl Acad Sci U S A* 2009, 106:14259–14264.
53. van Beekvelt CA, Jeeninga RE, van't Riet J, Venema J, Raué HA. Identification of cis-acting elements involved in 3′-end formation of *Saccharomyces cerevisiae* 18S rRNA. *RNA* 2001, 7:896–903.
54. Stevens A, Hsu CL, Isham KR, Larimer FW. Fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in *Saccharomyces cerevisiae* lacking 5′Æ3′ exoribonuclease 1. *J Bacteriol* 1991, 173:7024–7028.
55. Fatica A, Oeffinger M, Dlakic M, Tollervey D. Nob1p is required for cleavage of the 3′ end of 18S rRNA. *Mol Cell Biol* 2003, 23:1798–1807.
56. Granneman S, Petfalski E, Swiatkowska A, Tollervey D. Cracking pre-40S ribosomal subunit structure by systematic analyses of RNA-protein cross-linking. *EMBO J* 2010, 29:2026–2036.
57. Pertschy B, Schneider C, Gnadig M, Schafer T, Tollervey D, Hurt E. RNA helicase Prp43 and its co-factor Pfa1 promote 20 to 18 S rRNA processing catalyzed by the endonuclease Nob1. *J Biol Chem* 2009, 284:35079–35091.
58. Woolls HA, Lamanna AC, Karbstein K. Roles of Dim2 in ribosome assembly. *J Biol Chem* 2011, 286:2578–2586.
59. Lamanna AC, Karbstein K. An RNA conformational switch regulates pre-18S rRNA cleavage. *J Mol Biol* 2011, 405:3–17.
60. Lebaron S, Schneider C, van Nues RW, Swiatkowska A, Walsh D, Bottcher B, Granneman S, Watkins NJ, Tollervey D. Proofreading of pre-40S ribosome maturation by a translation initiation factor and 60S subunits. *Nat Struct Mol Biol* 2012, 19:744–753.
61. Strunk BS, Loucks CR, Su M, Vashisth H, Cheng S, Schilling J, Brooks CL III, Karbstein K, Skiniotis G. Ribosome assembly factors prevent premature translation initiation by 40S assembly intermediates. *Science* 2011, 333:1449–1453.
62. Ferreira-Cerca S, Kiburu I, Thomson E, LaRonde N, Hurt E. Dominant Rio1 kinase/ATPase catalytic

mutant induces trapping of late pre-40S biogenesis factors in 80S-like ribosomes. *Nucleic Acids Res* 2014, 42:8635–8647.

63. García-Gómez JJ, Fernández-Pevida A, Lebaron S, Rosado IV, Tollervey D, Kressler D, de la Cruz J. Final Pre-40S maturation depends on the functional integrity of the 60S subunit ribosomal protein L3. *PLoS Genet* 2014, 10:e1004205.
64. Dichtl B, Stevens A, Tollervey D. Lithium toxicity in yeast is due the inhibition of RNA processing enzymes. *EMBO J* 1997, 16:7184–7195.
65. Esakova O, Krasilnikov AS. Of proteins and RNA: the RNase P/MRP family. *RNA* 2010, 16:1725–1747.
66. Chamberlain JR, Lee Y, Lane WS, Engelke DR. Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. *Genes Dev* 1998, 12:1678–1690.
67. Schmitt ME, Clayton DA. Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1993, 13:7935–7941.
68. Weaver PL, Sun C, Chang T-H. Dbp3p, a putative RNA helicase in *Saccharomyces cerevisiae*, is required for efficient pre-rRNA processing predominantly at site A_3. *Mol Cell Biol* 1997, 17:1354–1365.
69. Venema J, Tollervey D. *RRP5* is required for formation of both 18S and 5.8S rRNA in yeast. *EMBO J* 1996, 15:5701–5714.
70. Eppens NA, Rensen S, Granneman S, Raué HA, Venema J. The roles of Rrp5p in the synthesis of yeast 18S and 5.8S rRNA can be functionally and physically separated. *RNA* 1999, 5:779–793.
71. Rodríguez-Galán O, García-Gómez JJ, de la Cruz J. Yeast and human RNA helicases involved in ribosome biogenesis: current status and perspectives. *Biochim Biophys Acta* 2013, 1829:775–790.
72. Lygerou Z, Allmang C, Tollervey D, Séraphin B. Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP in vitro. *Science* 1996, 272:268–270.
73. Esakova O, Perederina A, Quan C, Berezin I, Krasilnikov AS. Substrate recognition by ribonucleoprotein ribonuclease MRP. *RNA* 2011, 17:356–364.
74. Poole SJ, Stevens A. Structural modifications of RNA influence the 5′ exoribonucleolytic hydrolysis by XRN1 and HKE1 of *Saccharomyces cerevisiae*. *Biochem Byophys Res Commun* 1997, 235:799–805.
75. Johnson AW. Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. *Mol Cell Biol* 1997, 17:6122–6130.
76. Xue Y, Bai X, Lee I, Kallstrom G, Ho J, Brown J, Stevens A, Johnson AW. *Saccharomyces cerevisiae RAI1* (YGL246c) is homologous to human DOM3Z and encodes a protein that binds the nuclear exoribonuclease Rat1p. *Mol Cell Biol* 2000, 20:4006–4015.
77. Sahasranaman A, Dembowski J, Strahler J, Andrews P, Maddock J, Woolford JL Jr. Assembly of *Saccharomyces cerevisiae* 60S ribosomal subunits: role of factors required for 27S pre-rRNA processing. *EMBO J* 2011, 30:4020–4032.
78. Granneman S, Petfalski E, Tollervey D. A cluster of ribosome synthesis factors regulate pre-rRNA folding and 5.8S rRNA maturation by the Rat1 exonuclease. *EMBO J* 2011, 30:4006–4019.
79. Dembowski JA, Kuo B, Woolford JL Jr. Has1 regulates consecutive maturation and processing steps for assembly of 60S ribosomal subunits. *Nucleic Acids Res* 2013, 41:7889–7904.
80. Talkish J, Campbell IW, Sahasranaman A, Jakovljevic J, Woolford JL Jr. Ribosome assembly factors Pwp1 and Nop12 are important for folding of 5.8S rRNA during ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* 2014, 34:1863–1877.
81. Talkish J, Zhang J, Jakovljevic J, Horsey EW, Woolford JL Jr. Hierarchical recruitment into nascent ribosomes of assembly factors required for 27SB pre-rRNA processing in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 2012, 40:8646–8661.
82. Allmang C, Mitchell P, Petfalski E, Tollervey D. Degradation of ribosomal RNA precursors by the exosome. *Nucleic Acids Res* 2000, 28:1684–1691.
83. Fang F, Hoskins J, Butler JS. 5-Fluorouracil enhances exosome-dependent accumulation of polyadenylated rRNAs. *Mol Cell Biol* 2004, 24:10766–10776.
84. Joseph N, Krauskopf E, Vera MI, Michot B. Ribosomal internal transcribed spacer 2 (ITS2) exhibits a common core of secondary structure in vertebrates and yeast. *Nucleic Acids Res* 1999, 27:4533–4540.
85. Yeh LC, Lee JC. Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J Mol Biol* 1990, 211:699–712.
86. Côté CA, Greer CL, Peculis BA. Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast. *RNA* 2002, 8:786–797.
87. Gamalinda M, Jakovljevic J, Babiano R, Talkish J, de la Cruz J, Woolford JL Jr. Yeast polypeptide exit tunnel ribosomal proteins L17, L35 and L37 are necessary to recruit late-assembling factors required for 27SB pre-rRNA processing. *Nucleic Acids Res* 2013, 41:1965–1983.
88. Dembowski JA, Ramesh M, McManus CJ, Woolford JL Jr. Identification of the binding site of Rlp7 on assembling 60S ribosomal subunits in *Saccharomyces cerevisiae*. *RNA* 2013, 19:1639–1647.
89. Babiano R, Badis G, Saveanu C, Namane A, Doyen A, Diaz-Quintana A, Jacquier A, Fromont-Racine M, de la Cruz J. Yeast ribosomal protein L7 and its homologue Rlp7 are simultaneously present at distinct sites on pre-60S ribosomal particles. *Nucleic Acids Res* 2013, 41:9461–9470.

90. Babiano R, Gamalinda M, Woolford JL Jr, de la Cruz J. *Saccharomyces cerevisiae* ribosomal protein L26 is not essential for ribosome assembly and function. *Mol Cell Biol* 2012, 32:3228–3241.
91. Mitchell P, Petfalski E, Tollervey D. The 3′ end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism. *Genes Dev* 1996, 10:502–513.
92. Liu Q, Greimann JC, Lima CD. Reconstitution, activities, and structure of the eukaryotic RNA exosome. *Cell* 2006, 127:1223–1237.
93. Makino DL, Baumgartner M, Conti E. Crystal structure of an RNA-bound 11-subunit eukaryotic exosome complex. *Nature* 2013, 495:70–75.
94. Malet H, Topf M, Clare DK, Ebert J, Bonneau F, Basquin J, Drazkowska K, Tomecki R, Dziembowski A, Conti E, et al. RNA channelling by the eukaryotic exosome. *EMBO Rep* 2010, 11:936–942.
95. Bonneau F, Basquin J, Ebert J, Lorentzen E, Conti E. The yeast exosome functions as a macromolecular cage to channel RNA substrates for degradation. *Cell* 2009, 139:547–559.
96. Liu JJ, Bratkowski MA, Liu X, Niu CY, Ke A, Wang HW. Visualization of distinct substrate-recruitment pathways in the yeast exosome by EM. *Nat Struct Mol Biol* 2014, 21:95–102.
97. Dziembowski A, Lorentzen E, Conti E, Séraphin B. A single subunit, Dis3, is essentially responsible for yeast exosome core activity. *Nat Struct Mol Biol* 2007, 14:15–22.
98. Chlebowski A, Tomecki R, Gas Lopez ME, Seraphin B, Dziembowski A. Catalytic properties of the eukaryotic exosome. *Adv Exp Med Biol* 2011, 702:63–78.
99. Lebreton A, Tomecki R, Dziembowski A, Seraphin B. Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature* 2008, 456:993–996.
100. Mitchell P, Petfalski E, Shevchenko A, Mann M, Tollervey D. The exosome: a conserved eukaryotic RNA processing complex containing multiple 3′–5′ exoribonucleases. *Cell* 1997, 91:457–466.
101. Burkard KTD, Butler JS. A nuclear 3′–5′ exonuclease involved in mRNA degradation interacts with poly(A) polymerase and the hnRNA proteim Npl3p. *Mol Cell Biol* 2000, 20:604–616.
102. Schaeffer D, Tsanova B, Barbas A, Reis FP, Dastidar EG, Sanchez-Rotunno M, Arraiano CM, van Hoof A. The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol* 2009, 16:56–62.
103. Schneider C, Leung E, Brown J, Tollervey D. The N-terminal PIN domain of the exosome subunit Rrp44 harbors endonuclease activity and tethers Rrp44 to the yeast core exosome. *Nucleic Acids Res* 2009, 37:1127–1140.
104. Houseley J, Tollervey D. The many pathways of RNA degradation. *Cell* 2009, 136:763–776.
105. Callahan KP, Butler JS. Evidence for core exosome independent function of the nuclear exoribonuclease Rrp6p. *Nucleic Acids Res* 2008, 36:6645–6655.
106. LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A, Tollervey D. RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* 2005, 121:713–724.
107. Jackson RN, Klauer AA, Hintze BJ, Robinson H, van Hoof A, Johnson SJ. The crystal structure of Mtr4 reveals a novel arch domain required for rRNA processing. *EMBO J* 2010, 29:2205–2216.
108. Mitchell P, Petfalski E, Houalla R, Podtelejnikov A, Mann M, Tollervey D. Rrp47p is an exosome-associated protein required for the 3′ processing of stable RNAs. *Mol Cell Biol* 2003, 23:6982–6992.
109. Peng WT, Robinson MD, Mnaimneh S, Krogan NJ, Cagney G, Morris Q, Davierwala AP, Grigull J, Yang X, Zhang W, et al. A panoramic view of yeast noncoding RNA processing. *Cell* 2003, 113:919–933.
110. Milligan L, Decourty L, Saveanu C, Rappsilber J, Ceulemans H, Jacquier A, Tollervey D. A yeast exosome cofactor, Mpp6, functions in RNA surveillance and in the degradation of noncoding RNA transcripts. *Mol Cell Biol* 2008, 28:5446–5457.
111. Feigenbutz M, Jones R, Besong TM, Harding SE, Mitchell P. Assembly of the yeast exoribonuclease Rrp6 with its associated cofactor Rrp47 occurs in the nucleus and is critical for the controlled expression of Rrp47. *J Biol Chem* 2013, 288:15959–15970.
112. Suzuki N, Noguchi E, Nakashima N, Oki M, Ohba T, Tartakoff A, Ohishi M, Nishimoto T. The *Saccharomyces cerevisiae* small GTPase, Gsp1p/Ran, is involved in 3′ processing of 7S-to-5.8S rRNA and in degradation of the excised 5′-A_0 fragment of 35S pre-rRNA, both of which are carried out by the exosome. *Genetics* 2001, 158:613–625.
113. Gadal O, Strauss D, Petfalski E, Gleizes PE, Gas N, Tollervey D, Hurt E. Rlp7p is associated with 60S preribosomes, restricted to the granular component of the nucleolus, and required for pre-rRNA processing. *J Cell Biol* 2002, 157:941–951.
114. Fang F, Phillips S, Butler JS. Rat1p and Rai1p function with the nuclear exosome in the processing and degradation of rRNA precursors. *RNA* 2005, 11:1571–1778.
115. Kempers-Veenstra AE, Oliemans J, Offenberg H, Dekker AF, Piper PW, Planta RJ, Klootwijk J. 3′-end formation of transcripts from the yeast rRNA operon. *EMBO J* 1986, 5:2703–2710.
116. Lee Y, Nazar RN. Ribosomal 5S rRNA maturation in *Saccharomyces cerevisiae*. *J Biol Chem* 1997, 272:15206–15212.
117. Piper PW, Bellatin JA, Lockheart A. Altered maturation of sequences at the 3′ terminus of 5S gene transcripts in a *Saccharomyces cerevisiae* mutant that

lacks a RNA processing endonuclease. *EMBO J* 1983, 2:353–359.

118. Dechampesme A-M, Koroleva O, Léger-Silvestre I, Gas N, Camier S. Assembly of 5S ribosomal RNA is required at a specific step of the pre-rRNA processing pathway. *J Cell Biol* 1999, 145:1369–1380.
119. Nariai M, Tanaka T, Okada T, Shirai C, Horigome C, Mizuta K. Synergistic defect in 60S ribosomal subunit assembly caused by a mutation of Rrs1p, a ribosomal protein L11-binding protein, and 3′-extension of 5S rRNA in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 2005, 33:4553–4562.
120. Tafforeau L, Zorbas C, Langhendries JL, Mullineux ST, Stamatopoulou V, Mullier R, Wacheul L, Lafontaine DL. The complexity of human ribosome biogenesis revealed by systematic nucleolar screening of Pre-rRNA processing factors. *Mol Cell* 2013, 51: 539–551.
121. Alexandrov A, Colognori D, Steitz JA. Human eIF4AIII interacts with an eIF4G-like partner, NOM1, revealing an evolutionarily conserved function outside the exon junction complex. *Genes Dev* 2011, 25: 1078–1090.
122. Sloan KE, Mattijssen S, Lebaron S, Tollervey D, Pruijn GJ, Watkins NJ. Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing. *J Cell Biol* 2013, 200:577–588.
123. Ansel KM, Pastor WA, Rath N, Lapan AD, Glasmacher E, Wolf C, Smith LC, Papadopoulou N, Lamperti ED, Tahiliani M, et al. Mouse Eri1 interacts with the ribosome and catalyzes 5.8S rRNA processing. *Nat Struct Mol Biol* 2008, 15:523–530.
124. Srivastava L, Lapik YR, Wang M, Pestov DG. Mammalian DEAD box protein Ddx51 acts in 3′ end maturation of 28S rRNA by promoting the release of U8 snoRNA. *Mol Cell Biol* 2010, 30:2947–2956.
125. Oeffinger M, Wei KE, Rogers R, DeGrasse JA, Chait BT, Aitchison JD, Rout MP. Comprehensive analysis of diverse ribonucleoprotein complexes. *Nat Methods* 2007, 4:951–956.
126. Spitale RC, Crisalli P, Flynn RA, Torre EA, Kool ET, Chang HY. RNA SHAPE analysis in living cells. *Nat Chem Biol* 2013, 9:18–20.
127. Swiatkowska A, Wlotzka W, Tuck A, Barrass JD, Beggs JD, Tollervey D. Kinetic analysis of pre-ribosome structure *in vivo*. *RNA* 2012, 18: 2187–2200.
128. Schneider C, Tollervey D. Looking into the barrel of the RNA exosome. *Nat Struct Mol Biol* 2014, 21: 17–18.