

# Uncovering the assembly pathway of human ribosomes and its emerging links to disease

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## Abstract

The essential cellular process of ribosome biogenesis is at the nexus of various signalling pathways that coordinate protein synthesis with cellular growth and proliferation. The fact that numerous diseases are caused by defects in ribosome assembly underscores the importance of obtaining a detailed understanding of this pathway. Studies in yeast have provided a wealth of information about the fundamental principles of ribosome assembly, and although many features are conserved throughout eukaryotes, the larger size of human (pre-)ribosomes, as well as the evolution of additional regulatory networks that can modulate ribosome assembly and function, have resulted in a more complex assembly pathway in humans. Notably, many ribosome biogenesis factors conserved from yeast appear to have subtly different or additional functions in humans. In addition, recent genome-wide, RNAi-based screens have identified a plethora of novel factors required for human ribosome biogenesis. In this review, we discuss key aspects of human ribosome production, highlighting differences to yeast, links to disease, as well as emerging concepts such as extra-ribosomal functions of ribosomal proteins and ribosome heterogeneity.

**Keywords** ribosomal protein; ribosome; ribosomopathy; RNA modification; RNA processing

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See the Glossary for abbreviations used in this article.

## Ribosome production: going beyond the yeast model system

Production of proteins is an essential cellular process carried out by ribosomes, which are large ribonucleoprotein complexes (RNPs) composed of four ribosomal RNAs (rRNAs; 18S, 5S, 5.8S and 25S (yeast)/28S (humans)) and approximately 80 ribosomal proteins (RPs) (Ben-Shem *et al*, 2010; Anger *et al*, 2013). Ribosome assembly requires the coordinated action of all three RNA polymerases and is one of the most energy-consuming cellular

processes, with approximately 7,500 new ribosomal subunits synthesised per minute in actively growing HeLa cells (Warner, 1999; Lewis & Tollervey, 2000). In eukaryotes, this process begins in the nucleolus with the RNA polymerase I-mediated synthesis of three of the four rRNAs as a single precursor rRNA (pre-rRNA) transcript. Recruitment of numerous RPs and *trans*-acting ribosome biogenesis factors (RBFs) to this nascent transcript leads to formation of large, early pre-ribosomal particles (90S). Concurrent with protein assembly, the pre-rRNA transcript is extensively processed and numerous rRNA modifications take place. A central pre-rRNA cleavage event gives rise to precursors of the small (SSU; 40S) and large (LSU; 60S) subunits. The pre-40S and pre-60S particles undergo extensive structural remodelling, involving the dynamic association and dissociation of numerous RBFs as well as the establishment of key ribosomal structures. After the immature subunits are independently exported to the cytoplasm, final maturation and quality control steps occur before they engage in translation.

Eukaryotic ribosome synthesis is best characterised in the model organism *Saccharomyces cerevisiae* (budding yeast; Woolford & Baserga, 2013). While the basic features of the pathway are conserved among all eukaryotes, the biogenesis pathway for human ribosomes has evolved to be considerably more complex due to the increased size of human ribosomes and pre-ribosomes, and the evolution of regulatory networks that modulate ribosome assembly and function. Proteomic analyses of human nucleoli and several recent RNAi-based screens (Andersen *et al*, 2002; Scherl *et al*, 2002; Wild *et al*, 2010; Tafforeau *et al*, 2013; Badertscher *et al*, 2015; Farley-Barnes *et al*, 2018) have facilitated identification of human RBFs, and characterisation of some of these factors has already highlighted interesting differences between the assembly pathway of yeast and human ribosomes. Furthermore, these screens have revealed the extent to which ribosome biogenesis is coordinated with other cellular processes. The importance of uncovering such differences and gaining a comprehensive understanding of ribosome assembly in humans is underscored by the increasing number of genetic diseases (ribosomopathies) that have been found to be caused by mutations in genes encoding ribosomal proteins or RBFs (Narla & Ebert, 2010; Mills & Green, 2017). Furthermore, an expanding body of evidence demonstrates that ribosome assembly is regulated by several oncogenic signalling pathways, and defects in

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**Glossary**

<b>cryo-EM</b>	cryo-electron microscopy
<b>DBA</b>	Diamond-Blackfan anemia
<b>ETS</b>	external transcribed spacer
<b>IRES</b>	internal ribosome entry site
<b>ITS</b>	internal transcribed spacer
<b>LSU</b>	large ribosomal subunit
<b>Nm</b>	ribose 2'-O-methylation
<b>NTP</b>	nucleoside triphosphate
<b>RBF</b>	ribosome biogenesis factor
<b>RNP</b>	ribonucleoprotein complex
<b>RP</b>	ribosomal protein
<b>snoRNP</b>	small nucleolar ribonucleoprotein complex
<b>SSU</b>	small ribosomal subunit
<b>UTR</b>	untranslated region
<b>Ψ</b>	pseudouridine

ribosome biogenesis have in turn been linked to the activation of tumour suppressors including p53 (Pelletier *et al*, 2018).

Here, we review the current knowledge of the ribosome biogenesis pathway in humans, focusing on aspects of the process that have been characterised in detail, and highlighting key differences to the pathway in yeast. We further highlight emerging topics such as ribosome heterogeneity, describe the regulation of ribosome assembly and discuss how defects in ribosome synthesis are linked to disease.

### Identification and properties of human ribosome biogenesis factors

In yeast, a list of approximately 200 RBFs has been compiled over many years based on mass spectrometric analyses of isolated pre-ribosomal complexes as well as examination of the effects of depletion of nucleolar proteins on ribosomal subunit production (Woolford & Baserga, 2013). While the inventory of yeast RBFs provides an excellent basis for analysing human ribosome assembly, more than 4,500 proteins have been detected in human nucleoli (Andersen *et al*, 2002; Scherl *et al*, 2002), implying that many additional factors may contribute to ribosome maturation in human cells. To identify novel factors required for ribosome assembly in human cells, several RNAi-based, high-throughput screens have recently been performed (Wild *et al*, 2010; Tafforeau *et al*, 2013; Badertscher *et al*, 2015; Farley-Barnes *et al*, 2018). A pioneering microscopy-based screen monitoring the mis-localisation of pre-ribosomal subunits confirmed the involvement of 153 proteins in human ribosome assembly (Wild *et al*, 2010), and this approach was subsequently extended to a genome-wide level, revealing approximately 300 factors required for 40S maturation (Badertscher *et al*, 2015). Concurrently, 286 human RBFs, including 74 without yeast homologues, were identified in a screen for nucleolar proteins whose depletion affects pre-rRNA processing (Tafforeau *et al*, 2013). Furthermore, 139 potential RBFs were recently discovered by a screen for factors affecting the number or morphology of nucleoli (Farley-Barnes *et al*, 2018). These screens, together with numerous studies characterising individual or subsets of human RBFs, have not only confirmed the involvement of many of the homologues of yeast RBFs in human ribosome assembly, but also highlighted many additional proteins required for this process in human cells. A

significant number of the factors identified have links to disease, emphasising the importance of correct ribosome production for cellular homeostasis. Some of the proteins found in these screens have by now been analysed in detail, but many of the newly discovered human RBFs still lack functional characterisation, and their precise roles in subunit assembly are still unknown. Although depletion of the factors identified in these screens affects ribosome biogenesis, it is unlikely that they are all *bona fide* components of pre-ribosomal complexes, and many of the observed effects may rather point to mechanisms by which ribosome assembly can be regulated. For example, alongside the anticipated RPs and RBFs, proteins involved in transcription, pre-mRNA splicing and translation were found to be required for pre-40S maturation (Badertscher *et al*, 2015), probably indicating crosstalk between subunit assembly and other aspects of the gene expression pathway. Furthermore, various metabolic enzymes and signalling factors were identified as human RBFs (Badertscher *et al*, 2015; Farley-Barnes *et al*, 2018), suggesting the co-ordination of ribosome production with nutrient availability and cell growth; a model that is in line with the high energy demands of ribosome production. Depleting components of protein degradation machineries also affects pre-40S maturation or nucleolar number (Badertscher *et al*, 2015; Farley-Barnes *et al*, 2018), suggesting that maintaining the correct cellular levels of RPs and RBFs may be important for ongoing ribosome production. In the future, it will therefore be important to clearly differentiate between *bona fide* RBFs that are involved directly in subunit assembly, and proteins/pathways that modulate ribosome production.

The inventories of human RBFs highlight the diversity of proteins that contribute to ribosome maturation. As in yeast, some RBFs primarily mediate protein-protein interactions and act as structural scaffolds, while others are RNA-binding proteins, and many possess catalytic activity. The spectrum of enzymatic RBFs includes many nucleases, modification enzymes and kinases (discussed in detail below), as well as a plethora of nucleoside triphosphate (NTP)-dependent proteins such as GTPases, AAA-ATPases and RNA helicases that mediate structural remodelling events. Six active GTPases (Nog1, Nog2, Nug1, Bms1, Lsg1, Efl1) are involved in yeast ribosome biogenesis, and although human homologues for each of these proteins have been found in screens for human RBFs, none of those proteins have been analysed in detail in human cells so far. Three yeast AAA-ATPases (Rea1/Mdn1, Rix7, Drg1) are implicated in triggering the release of specific pre-60S biogenesis factors (reviewed in Kressler *et al*, 2012), and human homologues of each of these proteins (MDN1, NVL2 and AFGH2, respectively) are known. In the case of MDN1, association with pre-60S complexes and the PELP1-TEX10-WDR18 complex (Rix1 complex in yeast) suggest that the function of this enzyme is conserved from yeast to humans (Raman *et al*, 2016). Human NVL2 has been linked to the nuclear exosome and to telomerase (Her & Chung, 2012; Yoshikatsu *et al*, 2015), suggesting that it may be a multifunctional enzyme involved not only in ribosome assembly but also in other aspects of RNA biology. Although the roles of some RNA helicases are common to yeast and humans (e.g. yeast Dhr1 and human DHX37 both mediate release of the U3 snoRNA; Martin *et al*, 2013; Sardana *et al*, 2015; Choudhury *et al*, 2019), additional pre-ribosomal functions have been described for several human RNA helicases. Examples include the requirement for DDX51 for release of the metazoan-specific snoRNA U8 from pre-LSU complexes (Srivastava *et al*, 2010), as well as the roles of

DDX21 in coupling pre-rRNA transcription and processing, and facilitating the access of late-acting small nucleolar RNP (snoRNPs) to pre-40S complexes (Calo *et al*, 2015; Sloan *et al*, 2015). The remodelling events catalysed by such enzymatic RBFs often serve as important checkpoints during ribosome assembly and thereby help maintain the directionality of the pathway.

While many RBFs associate with pre-ribosomal particles independently, a hallmark of eukaryotic ribosome assembly is the modular assembly of pre-ribosomal complexes. In both yeast and humans, the UTP-A, UTP-B and UTP-C complexes, as well as the U3 snoRNP, the RCL1-BMS1 heterodimer and the IMP3-IMP4-MPP10 and EMG1 complexes, are all recruited to the nascent pre-rRNA transcript and form the core of the so-called “SSU processome” (see Table 1 and references therein). Similarly, several sub-complexes, such as the PeBoW (Nop7-Erb1-Ytm1 in yeast) and PELP1-TEX10-WDR18 (Rix1-Ipi3-Ipi1 in yeast) complexes, act during pre-60S biogenesis. Despite the evolutionary conservation of these complexes, their composition varies between species, and several additional components have been identified in human cells (Table 1). DDX21 and DDX27, additional components of the human UTP-B and PeBoW complexes, respectively (Kellner *et al*, 2015; Sloan *et al*, 2015), are both RNA helicases, possibly indicating the need for additional remodelling steps during the early stages of human pre-ribosome assembly compared to yeast. Furthermore, several novel pre-ribosomal sub-complexes have been identified in human cells. For example, apoptosis-antagonising transcription factor AATF, neuroguidin (NGDN) and NOL10 form a nucleolar sub-complex (ANN) in which each of the components co-stabilise the other proteins (Bammert *et al*, 2016). These proteins associate with early pre-ribosomal complexes, and depletion of any of the ANN components leads to defects in early pre-rRNA cleavage events. XND, another nucleolar complex composed of the G-patch protein NF- $\kappa$ B-repressing factor (NKRF), the RNA helicase DHX15 and the 5'-3' exonuclease XRN2, is also involved in early aspects of human ribosome assembly (Memet *et al*, 2017). XRN2 is recruited by NKRF to pre-ribosomal complexes, where it fulfils its various roles in pre-rRNA processing and turnover of excised pre-rRNA fragments. NKRF also stimulates the ATPase and unwinding activity of DHX15 (Memet *et al*, 2017), and it has been suggested that these proteins function together in a pre-rRNA remodelling step that facilitates initial pre-rRNA cleavage. The fact that DHX15 is required for processing of the 5' external transcribed spacer (ETS) in human cells contrasts the roles of its yeast homologue Prp43, which is involved in release of snoRNAs from pre-60S particles and facilitates cleavage of the 3' end of the 18S rRNA in the cytoplasm (Bohnsack *et al*, 2009; Pertschy *et al*, 2009). Furthermore, the NF45-NF90 heterodimer, which was initially characterised for its binding to the interleukin-2 promoter, was found to be a component of human pre-60S complexes (Wandrey *et al*, 2015). The interaction between NF45-NF90 and pre-60S particles was shown to be mediated by the double-stranded RNA-binding domains (dsRBDs) of NF90, implying a direct association with the rRNA. Although no clear defect in pre-rRNA processing was observed upon depletion of either NF45 or NF90, lack of these factors causes changes in nucleolar morphology and the accumulation of pre-60S complexes in the nucleoplasm, confirming the requirement for this complex for biogenesis of the LSU (Wandrey *et al*, 2015).

## Specialisation of the pre-rRNA processing pathway in higher eukaryotes

An aspect of human ribosome assembly that has been studied extensively and where several differences to yeast have emerged is the processing of the pre-rRNA transcript. Maturation of the rRNAs requires removal of the external and internal transcribed spacers (ETS and ITS, respectively), which are up to five times longer in humans than yeast, by a combination of endonucleolytic and exonucleolytic processing (Henras *et al*, 2015; Tomecki *et al*, 2017). While removal of the 5' ETS and cleavage in ITS1 largely occur co-transcriptionally in yeast, the majority of human pre-rRNA processing appears to occur post-transcriptionally (Lazdins *et al*, 1997). Analogous endonucleolytic cleavage sites can be identified in yeast and human pre-rRNAs. Several recent studies indicate that the corresponding endonucleases (Utp24/UTP24, Nob1/NOB1, RNase MRP, Las1/LAS1) are largely conserved (Figure 1), but there are notable exceptions (Schmitt & Clayton, 1993; Fatica *et al*, 2003; Preti *et al*, 2013; Sloan *et al*, 2013a; Gasse *et al*, 2015; Tomecki *et al*, 2015; Wells *et al*, 2016; Goldfarb & Cech, 2017). For example, in yeast, site B0 at the 3' end of the 25S rRNA is cleaved by Rnt1, but depletion of the homologous RNase III enzyme does not support a role for this enzyme in the analogous O2 site cleavage in humans. The endonuclease(s) responsible for cleavage at A<sub>0</sub> (yeast) and A0 (human) cleavage sites in the 5' ETS remain unknown, but processing of the A0 site in humans has been linked to the putative PIN-domain endonuclease UTP23, which unlike its degenerate yeast counterpart retains three of the four amino acids typically required for catalytic activity (Wells *et al*, 2017). Furthermore, an additional, metazoan-specific cleavage site in the 5' ETS, termed O1 or A', has been identified (Lazdins *et al*, 1997). Studies so far suggest that depletion of any of the human orthologues of yeast pre-rRNA processing enzymes does not prevent cleavage at this site. Therefore, it appears that an additional, hitherto unidentified endonuclease is involved in 5' ETS processing in humans. The functional importance of this additional 5' ETS processing event is not yet clear, but it has been suggested to serve as a quality control mechanism for the early stages of pre-ribosome assembly (Wang & Pestov, 2011; Sloan *et al*, 2014). Serial endonucleolytic cleavages within the ETS and ITS release fragments that are degraded by exonucleases (Schillewaert *et al*, 2012; Sloan *et al*, 2013a, 2014; Memet *et al*, 2017; Kobylecki *et al*, 2018), and it has been suggested that turnover of these fragments is important for recycling of bound RBFs. In some cases, mature ends of the rRNAs are directly formed by endonucleolytic cleavages (e.g. mature 5' and 3' ends of 18S rRNA cleaved by UTP24 and NOB1, respectively; Preti *et al*, 2013; Sloan *et al*, 2013a; Tomecki *et al*, 2015; Bai *et al*, 2016; Wells *et al*, 2016), but cleavage events can also occur within the spacer sequences and be followed by exonucleolytic processing. For example, in both yeast and humans, the 3' end of the 5.8S rRNA is formed by the step-wise action of several 3'-5' exonucleases (reviewed in Henras *et al*, 2015). Different from yeast, an additional exonuclease, ISG2OL2, is implicated in this process in human cells (Coute *et al*, 2008). Also, while the final trimming of the 3' end of the 5.8S rRNA is mediated by Ngl2 in yeast, this step is performed by the multifunctional enzyme ERI1 in humans (Faber *et al*, 2002; Ansel *et al*, 2008). In striking contrast to human pre-rRNA processing pathways, the central pre-rRNA cleavage that separates precursors of the SSU and LSU rRNAs is

**Table 1. Composition of pre-ribosomal sub-complexes in yeast and humans.**

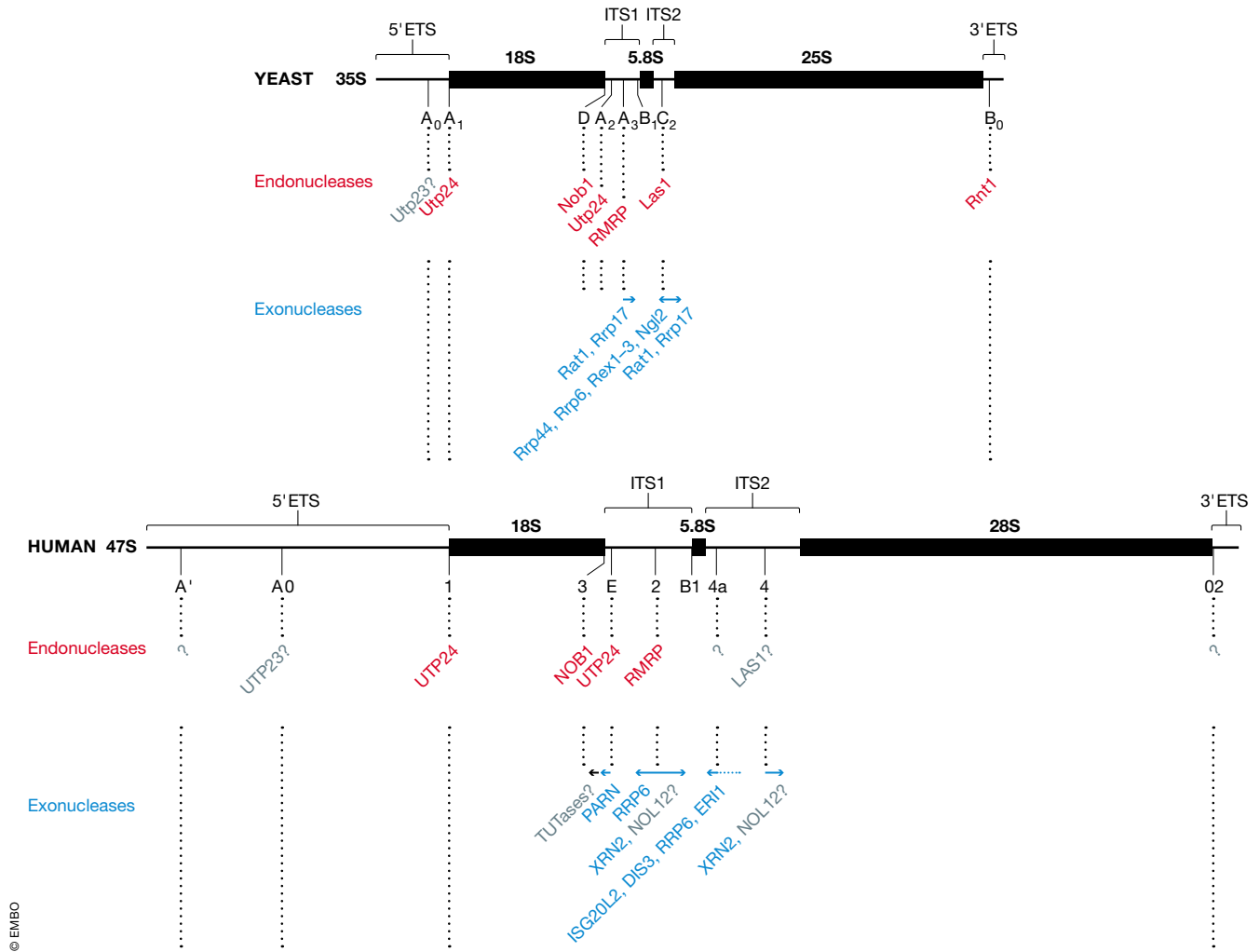
Complex	Composition		Refs.
	Yeast	Human	
UTP-A	Utp4	UTP4 (Cirhin)	Krogan <i>et al</i> (2004), Prieto and McStay (2007), Freed <i>et al</i> (2012)
	Utp5	UTP5 (WDR43)	
	Utp8	–	
	Utp9	–	
	Utp10	UTP10 (BAP28)	
	Utp15	UTP15	
	Utp17	UTP17 (WDR75)	
	–	NOL11	
UTP-B	Utp1	PWP2	Krogan <i>et al</i> (2004), Sloan <i>et al</i> (2015)
	Utp6	UTP6	
	Utp12	UTP12	
	Utp13	TBL3	
	Utp18	UTP18	
	Utp21	WDR36	
	–	DDX21	
	–	NOP2	
UTP-C	Rrp7	RRP7A	Krogan <i>et al</i> (2004), G�erus <i>et al</i> (2010), Baudin-Baillieu <i>et al</i> (1997)
	Utp22	NOL6	
	Cka1	CSNK2A1	
	Cka2	–	
	Ckb1	CSNK2B	
	Ckb2	–	
	Rrp36	RRP36	
U3 snoRNP	U3 snoRNA	U3 snoRNA	Grandi <i>et al</i> (2002), Turner <i>et al</i> (2012)
	Nop56	NOP56	
	Nop58	NOP58	
	Snu13	15.5K	
	Nop1	Fibrillarlin	
	Rrp9	U3-55K	
IMP3-IMP4	Imp3	IMP3	Granneman <i>et al</i> (2003)
	Imp4	IMP4	
	Mpp10	MPHOSPH10	
RCL1-BMS1	Rcl1	RCL1	Wegierski <i>et al</i> (2001), Wang <i>et al</i> (2016)
	Bms1	BMS1	
EMG1	Emg1	EMG1	Liu and Thiele (2001), K�uhn <i>et al</i> (2009), Warda <i>et al</i> (2016)
	Nop14	NOP14	
	Noc4	NOC4L	
	–	UTP14A	
PeBoW	Nop7	PES1	Holzel <i>et al</i> (2005), Rohrmoser <i>et al</i> (2007), Kellner <i>et al</i> (2015)
	Erb1	BOP1	
	Ytm1	WDR12	
	–	DDX27	

**Table 1 (continued)**

Complex	Composition		Refs.
	Yeast	Human	
Rix1	Rix1	PELP1	Finkbeiner <i>et al</i> (2011a,b)
	–	TEX10	
	–	WDR18	
XND	–	NKRF	Memet <i>et al</i> (2017)
	–	XRN2	
	–	DHX15	
ANN	–	AATF	Bammert <i>et al</i> (2016)
	–	NGDN	
	–	NOL10	
NF45-NF90	–	NF45	Wandrey <i>et al</i> (2015)
	–	NF90	

in yeast followed directly by cleavage of the 3'-end of the 18S rRNA by Nob1, while exonucleases play key roles in removing ITS1 sequences in human cells (Figure 1). Site 2 cleavage by human RNase MRP is followed by 3'-5' trimming by the sequential action of the nuclear exosome containing RRP6 and the poly(A)-specific ribonuclease PARN (Sloan *et al*, 2013a; Goldfarb & Cech, 2017; Ishikawa *et al*, 2017; Montellese *et al*, 2017). Moreover, oligouridylated forms of 3'-extended 18S rRNAs have been detected in the cytoplasm, implying that both terminal uridylyltransferases (TUTases) and additional 3'-5' exonucleases likely also contribute to ITS1 removal in humans (Montellese *et al*, 2017). Pre-rRNA processing events can serve as checkpoints during ribosome assembly, and the utilisation of exo- as well as endonucleases for rRNA maturation likely provides mechanisms for coupling of pre-rRNA processing with quality control and turnover of aberrant pre-ribosomes. It remains unclear how the activity of processive exonucleases is regulated to ensure precise trimming of specific pre-rRNA regions. However, it is tempting to speculate that the presence of proteins assembled on the pre-rRNA or the establishment of pre-rRNA secondary structures normally arrests the exonucleases at an appropriate position, while they may be poised to efficiently degrade incorrectly assembled pre-ribosomes upon defects in pre-ribosome assembly.

Analyses of the pre-rRNA processing pathways in yeast and humans indicate that, in contrast to other aspects of ribosome assembly that occur in strict hierarchical order, alternative pre-rRNA processing routes may be used to generate the mature rRNAs (reviewed in detail in Henras *et al*, 2015; Tomecki *et al*, 2017). In yeast, pre-rRNA processing is initiated by removal of the 5' ETS but subsequent cleavage in ITS1 can take place at either site A2 (approximately 85% of pre-rRNAs) or at site A3 (approximately 15% of pre-rRNAs; Schmitt & Clayton, 1993). The utilisation of alternative pre-rRNA processing pathways appears more prominent in human cells, where step-wise removal of the 5' ETS and cleavage at either site E or site 2 in ITS1 can occur in different orders. This flexibility in the order of pre-rRNA cleavages, together with differing kinetics of processing events, leads to variations in the steady-state levels of pre-rRNA intermediates between different organisms and cell types.



**Figure 1. Pre-rRNA processing in yeast and human cells.** Schematic views of the primary pre-rRNA transcripts from yeast (35S, upper panel) and human cells (47S, lower panel) are shown to scale. Mature 18S, 5.8S and 25S/28S rRNA sequences are indicated by black rectangles, external transcribed spacers (5'-ETS and 3'-ETS) and internal transcribed spacers (ITS1 and ITS2) are represented by black lines. Relative cleavage site positions are indicated, and sites are specified below the pre-rRNA transcripts. Enzymes responsible for individual processing steps are indicated (Endonucleases—red; exonucleases—blue; putative enzymes—grey; unknown enzymes—?).

The functional relevance of such alternative pre-rRNA processing pathways remains unclear. While it is possible that having diverse mechanisms to generate the mature rRNAs simply ensures the robustness of ribosome production, the greater flexibility in cleavage order observed in higher eukaryotes may suggest that the alternative pre-rRNA processing pathways serve as a means to regulate subunit assembly.

**Comparison of the yeast and human rRNA modification machineries**

A universally conserved feature of rRNAs is the presence of chemically modified residues in sequences that form the functional centres of the ribosome, such as the peptidyl transfer centre, decoding site and intersubunit interface (Decatur & Fournier, 2002; Polikanov

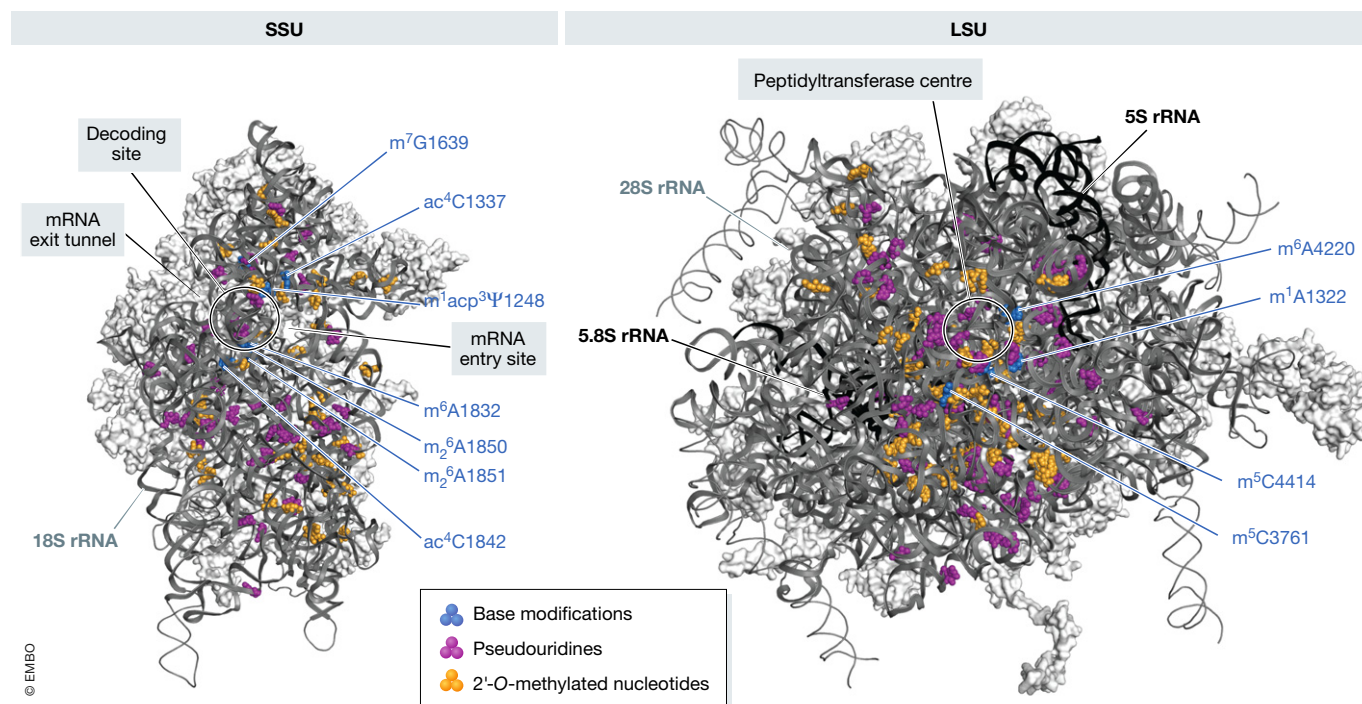
*et al*, 2015; Sloan *et al*, 2017). Although the precise functions of many individual rRNA modifications remain unknown, they are collectively implicated in maintaining the stability of ribosome structure and regulating ribosome function. Mapping the sites of rRNA modifications in the yeast and human rRNAs has revealed 112 and 228 modified sites, respectively, and recent structural analysis of human ribosomes has enabled visualisation of many of these modifications *in situ* (Figure 2; Ofengand & Bakin, 1997; Piekna-Przybylska *et al*, 2008; Carlile *et al*, 2014; Krogh *et al*, 2016; Taoka *et al*, 2016, 2018; Natchiar *et al*, 2017). The majority of modifications present in eukaryotic rRNAs are 2'-O-methylations of ribose moieties (Nm) and pseudouridylations (Ψ), which are introduced by box C/D and box H/ACA snoRNPs, respectively (Watkins & Bohnsack, 2012). In many cases, the snoRNAs responsible for guiding individual modifications have been computationally predicted (Lestrade & Weber, 2006; Gumienny *et al*, 2017), but most of these

predictions still lack experimental confirmation in humans. The single non-snoRNP-dependent 2'-O-methylation in yeast 25S rRNA (Gm2922) is mediated by Sbp1 (Lapeyre & Purushothaman, 2004), but notably, depletion of its human homologue FTSJ3 causes defects in SSU maturation, suggesting that this enzyme has distinct or additional functions in human cells (Morello *et al*, 2011). Due to the extensive basepairing interactions formed between snoRNAs and their substrates, the majority of snoRNA-guided modifications are expected to be installed during the early stages of ribosome biogenesis when the pre-ribosome structure is likely to be more open, but whether the majority of human rRNA modifications are introduced co-transcriptionally as in yeast remains to be determined. The total number of modified bases Nm and  $\Psi$  in the human rRNAs exceeds the number in yeast rRNAs by more than twofold (Piekna-Przybylska *et al*, 2008; Taoka *et al*, 2016, 2018). The higher density of human rRNA modifications probably not only arises due to the larger size of human ribosomes, but also likely reflects a role for rRNA modifications in fine-tuning gene expression and the greater need for such regulation in human cells (reviewed in Sloan *et al*, 2017; see also below).

Of note, the number of identified human snoRNAs far exceeds the number of rRNA Nm and  $\Psi$  (Jorjani *et al*, 2016). Some human snoRNAs guide Nm and  $\Psi$  modifications in different RNA species such as U6 snRNA or likely mRNAs (Aw *et al*, 2016; Sharma *et al*, 2016; Bohnsack & Sloan, 2018), while others guide different types of modification (e.g. 18S-ac<sup>4</sup>C1842 by SNORD13; Cavaille *et al*, 1996; Ito *et al*, 2014; Sharma *et al*, 2015; 2017b), and some, including U3 (SNORD3), U14A/B (SNORD14A/B), U17A/B

(SNORA73A/B) and the metazoan-specific snoRNAs U8 (SNORD118) and U22 (SNORD22), coordinate pre-rRNA folding by forming basepairing interactions with distant regions of the pre-rRNA transcript (Savino & Gerbi, 1990; Peculis & Steitz, 1993; Tycowski *et al*, 1994; Mishra & Eliceiri, 1997; Dunbar & Baserga, 1998). Furthermore, extra-ribosomal functions of some human snoRNAs in the regulation of alternative pre-mRNA splicing have been uncovered; for example, HBII-52 (SNORD115) regulates alternative splicing of the serotonin receptor 2C pre-mRNA, while SNORD86 modulates expression of the snoRNP protein NOP56 by dictating usage of alternative splice sites (Kishore & Stamm, 2006; Lykke-Andersen *et al*, 2018).

Other human rRNA base modifications and their cognate methyltransferases are largely conserved between yeast and humans, except for two N<sup>3</sup>-methyluridines (m<sup>3</sup>Us) at positions 25S-U2634 and 25S-U2843 of yeast 25S rRNA, neither of which are directly analogous to the single one at position 4,500 of the human 28S rRNA (Figure 2; Table 2) (Piekna-Przybylska *et al*, 2008; Taoka *et al*, 2016, 2018). Furthermore, human 18S and 28S rRNAs both carry a single N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) nucleotide not present in yeast, at positions A1832 and A4220, respectively; while the enzyme for A1832 modification remains still unknown, ZCCHC4 has recently been identified as the m<sup>6</sup>A methyltransferase responsible for the latter modification (Ma *et al*, 2019). N<sup>6</sup>-methylation of 28S-A4420 increases base-stacking with A4219 and G4222, thereby likely contributing to stability of this region of the 60S subunit. Consistent with this, lack of ZCCHC4 leads to decreased 60S subunit levels and a global reduction in protein synthesis (Ma *et al*, 2019).



**Figure 2. Distribution of modifications in human rRNAs.**

Tertiary structures of the human small ribosomal subunit (SSU) and large ribosomal subunit (LSU; PDB 4V6X) are shown, with rRNA sequences depicted as ribbons and ribosomal proteins in surface view. The type and nucleotide position of base modifications, as well as the positions of key functional regions of the ribosome, are indicated.

## Insights into pre-ribosome export and cytoplasmic maturation steps

During their maturation, pre-ribosomal subunits undergo extensive remodelling involving establishment of the rRNA folds present in mature ribosomes, incorporation of RPs and dynamic recruitment and dissociation of RBFs. The compartmentalisation of eukaryotic cells into nucleus and cytoplasm allows the physical separation of immature pre-ribosomes and components of the translation machinery, and achieving export competence therefore represents a key milestone for pre-ribosomal subunits. Pre-ribosomal subunits are some of the largest RNPs that translocate through the permeability barrier of nuclear pore complexes, and their transport is facilitated by multiple nuclear transport factors and adaptors (reviewed in Sloan *et al*, 2016). As in yeast, the exportin CRM1 (XPO1) bound to RanGTP contributes to the nuclear export of both the pre-40S and pre-60S subunits in humans, and several putative adaptor proteins containing classical leucine-rich nuclear export signals (NES; Guttler & Gorlich, 2011) have been identified. These include the LSU biogenesis factor NMD3 (Bai *et al*, 2013) and the SSU component RIOK2 (Zemp *et al*, 2009), both of which have been shown to bind to CRM1 in a RanGTP-dependent manner. Upon RIOK2 depletion, the kinetics of pre-SSU export are slowed, confirming a contribution of RIOK2 to pre-SSU export (Zemp *et al*, 2009). However, the finding that RIOK2 is not essential for pre-40S export suggests that additional export adaptors may exist in human cells and that there may be redundancy between them. Indeed, PDCD2L, which lacks a

yeast homologue, was recently identified as a CRM1 adaptor for pre-40S subunits in human cells (Landry-Voyer *et al*, 2016). Furthermore, RAE1, the human homologue of yeast pre-60S export factor Gle2, has been suggested to contribute not only to pre-60S export, but also to transporting pre-40S subunits to the cytoplasm (Wild *et al*, 2010). Moreover, while the Mex67-Mtr2 heterodimer plays key roles in pre-40S and pre-60S export in yeast (Faza *et al*, 2012), its human counterpart NXF1-NXT1 was not found in export-competent pre-ribosomal subunits purified from human cells (Wild *et al*, 2010), questioning whether these functions are conserved in metazoans. Finally, the well-characterised pre-microRNA- and tRNA-export factor EXP5 (Leisegang *et al*, 2012) was identified as export adaptor for human pre-60S complexes (Wild *et al*, 2010); while its pre-ribosomal binding site remains to be determined, it is tempting to speculate that EXP5 may recognise a pre-miRNA-like hairpin structure within the pre-rRNA.

Exciting insights into the composition and dynamics of late nuclear and cytoplasmic human pre-40S complexes have recently been obtained by cryo-electron microscopy (cryo-EM; Larburu *et al*, 2016; Ameismeier *et al*, 2018). While these studies confirmed that the binding sites of most late-acting pre-40S biogenesis factors are largely conserved between yeast and humans, a striking compositional difference between yeast and human cytoplasmic pre-40S complexes is the absence of DIMT1L (Dim1) in the latter. In yeast,  $N^6,N^6$ -dimethylation of two adenosines close to the 3' end of 18S rRNA by Dim1 occurs in the cytoplasm (Lafontaine *et al*, 1998), but the lack of DIMT1L in human cytoplasmic pre-40S complexes is in

**Table 2. The yeast and human rRNA modification enzymes.**

Modification (human)	Modification (yeast)	Enzyme (human)	Enzyme (yeast)	References
18S- $m^1acp^3\Psi$ 1240	18S- $m^1acp^3\Psi$ 1191	SNORA14, EMG1, TSR3	snR35, Emg1, Tsr3	Wurm <i>et al</i> (2010), Meyer <i>et al</i> (2011, 2016), Warda <i>et al</i> (2016)
18S- $ac^4C$ 1337	18S- $ac^4C$ 1280	NAT10	Kre33	Ito <i>et al</i> (2014), Sharma <i>et al</i> (2015)
18S- $m^7G$ 1639	18S- $m^7G$ 1575	WBSCR22	Bud23	White <i>et al</i> (2008), Haag <i>et al</i> (2015)
18S- $m^6A$ 1832	–	Unknown	–	Piekna-Przybylska <i>et al</i> (2008)
18S- $ac^4C$ 1842	18S- $ac^4C$ 1773	NAT10	Kre33	Ito <i>et al</i> (2014), Sharma <i>et al</i> (2015)
18S- $m_2^6A$ 1850/1	18S- $m_2^6A$ 1781/2	DIMT1L	Dim1	Lafontaine <i>et al</i> (1998), Zorbas <i>et al</i> (2015)
28S- $m^1A$ 1332	25S- $m^1A$ 645	NML	Rrp8	Peifer <i>et al</i> (2013), Waku <i>et al</i> (2016)
–	25S- $m^1A$ 2142	–	Bmt2	Sharma <i>et al</i> (2013a)
28S- $m^5C$ 3761	25S- $m^5C$ 2278	NSUN5	Rcm1	Schossere <i>et al</i> (2015)
–	25S- $m^3U$ 2634	–	Bmt5	Sharma <i>et al</i> (2014)
28S- $m^6A$ 4220	–	ZCCHC4	–	Ma <i>et al</i> (2019)
28S- $m^5C$ 4414	25S- $m^5C$ 2870	NSUN1	Nop2	Sharma <i>et al</i> (2013b), Bourgeois <i>et al</i> (2015)
–	25S- $m^3U$ 2843	–	Bmt6	Sharma <i>et al</i> (2014)
28S- $m^3U$ 4500	–	Unknown	–	Piekna-Przybylska <i>et al</i> (2008)
28S-Gm4469	25S-Gm2922	Unknown	Spb1	Lapeyre and Purushothaman (2004)
Nm – (x112)	Nm – (x55)	Fibrillar in box C/D snoRNP	Nop1 in box C/D snoRNP	Kiss-Laszlo <i>et al</i> (1996)
$\Psi$ – (x105)	$\Psi$ – (x46)	Dyskerin in box H/ACA snoRNP	Cbf5 in box H/ACA snoRNP	Ganot <i>et al</i> (1997)

$m^1acp^3\Psi$ — $N^1$ -methyl- $N^3$ -aminocarboxypropylpseudouridine,  $ac^4C$ — $N^4$ -acetylcytidine,  $m^7G$ — $N^7$ -methylguanosine,  $m^6A$ — $N^6$ -methyladenosine,  $m_2^6A$ — $N^6,N^6$ -dimethyladenosine,  $m^1A$ — $N^1$ -methyladenosine,  $m^3U$ — $N^3$ -methyluridine,  $m^5C$ —5-methylcytosine, Nm—ribose 2'-*O*-methylation,  $\Psi$ —pseudouridine. Individual Nm and  $\Psi$  sites are not listed; instead, the total number present in the rRNAs is given.

line with observations that this event takes place prior to pre-40S export in humans (Zorbas *et al.*, 2015; Sloan *et al.*, 2019). The significance of this temporal difference still remains to be uncovered.

A series of structural snapshots of different pre-40S complexes has recently highlighted structural transitions that occur before nuclear export, and provided new information on how the formation of key ribosomal features is regulated (Ameismeier *et al.*, 2018). These data have revealed that the export adaptor RRP12, which binds to the 3' major domain of the 18S rRNA sequence, coordinates substantial rRNA rearrangements within the "head" and "beak" regions of nuclear pre-40S subunits. Coupling maturation of the head region with nuclear export via RRP12 has therefore been suggested to serve as a quality control mechanism ensuring that only correctly assembled pre-40S subunits reach the cytoplasm. Structural rearrangements that occur upon dissociation of RRP12 enable recruitment of RACK1, a protein that is not present in yeast pre-40S particles (Larburu *et al.*, 2016; Ameismeier *et al.*, 2018). As RACK1 has previously been characterised as a translation initiation factor, its requirement for pre-40S maturation (Larburu *et al.*, 2016) raises the possibility that in human cells, the final stages of pre-40S maturation are coupled to translation initiation. Maturation of the decoding centre, containing the ribosomal A and P sites, is one of the final pre-40S biogenesis events to take place. This structure is formed by interconnections between helices h28, h44 and h45 of the 18S rRNA sequence, and the presence of various RBFs (such as TSR1, LTV1, RIO2K, PNO1) in pre-40S particles stabilises h44 in an immature conformation. In this regard, PNO1 not only helps maintain the rRNA sequences that form the decoding centre in an immature state, but also forms close interactions with NOB1 that ensure that the catalytic centre of the endonuclease remains spatially separate from its target site at the 3'-end of the 18S rRNA. It has been suggested that PNO1 release not only enables final assembly of the decoding centre, but also triggers a conformational change that allows NOB1 to contact the 3'-end of the 18S rRNA, thereby enabling these two critical events to be coordinated (Ameismeier *et al.*, 2018); a similar mechanism has also been proposed to operate in yeast.

Less is known about the late nuclear and cytoplasmic phases of human pre-60S maturation; however, aspects that have been studied in detail are the assembly of the P-stalk and the release mechanism of the pre-60S maturation factor eIF6. In both yeast and humans, the RBF Mrt4/MRTO4 acts as a nuclear placeholder for the RP P0, which forms the base of the stalk structure of mature 60S subunits. Release of Mrt4/MRTO4 is triggered by the dual-specificity phosphatase Yvh1/DUSP12 (Lo *et al.*, 2010), allowing P0 recruitment and stalk assembly. Stalk assembly is a pre-requisite for release of eIF6 (Tif6 in yeast), which plays an important role in preventing premature translation by impeding the association of pre-60S particles with 40S subunits (Gartmann *et al.*, 2010; Klinge *et al.*, 2011). Detailed mechanistic insights into how this is achieved in human cells come from an elegant series of structural and biochemical analyses (Finch *et al.*, 2011; Weis *et al.*, 2015; Warren, 2018). SBDS, a protein mutated in Shwachman–Diamond syndrome (Table 3), initially binds to the ribosomal P-site, the peptidyltransferase centre and the P-stalk base, and proof-reads the integrity of these features. Upon its recruitment to pre-60S particles, the GTPase EFL1 competes with SBDS for binding to the P-stalk and triggers a conformational change in SBDS that causes it to alternatively interact with h69 of the 28S rRNA. Rearrangement of EFL1 then leads to competition between EFL1 and eIF6 for binding to the sarcin–ricin loop of the 28S

rRNA sequence. This not only induces release of eIF6 from the pre-60S particle, but also activates GTP hydrolysis by EFL1, triggering concomitant dissociation of EFL1. Together, these structural studies provide insights into the mechanistic basis of the hierarchical release of RBFs during the final stages of subunit maturation. Furthermore, they highlight the extent to which the late stages of subunit assembly are closely coupled with quality control mechanisms to ensure the fidelity of the mature ribosomal subunits. Common themes that emerge are the roles of RBFs as placeholders for late-associating ribosomal proteins, the proof-reading of key ribosomal structures by RBFs and the positioning of RBFs to prevent premature subunit joining.

Alongside the regulation of pre-ribosome maturation events by spatial constraints or steric hinderance (e.g. as described above for 18S rRNA 3'-end cleavage by NOB1), post-translational modifications represent another mechanism that likely contributes to the regulation of subunit assembly. In both yeast and human cells, late-stage pre-40S particles contain several (putative) kinases (Rio1, Rio2 and Hrr25 in yeast; RIO1K, RIO2K, RIO3K and CK1 $\delta/\epsilon$  in humans). Phosphorylation of Ltv1/LTV1 and Enp1/ENP1 by Hrr25/CK1 $\delta/\epsilon$  appears to be a conserved event linked to dissociation of Ltv1/LTV1 from pre-40S particles and to assembly of Rps3/RPS3 onto the beak structure (Schäfer *et al.*, 2006; Zemp *et al.*, 2014). While yeast pre-40S particles contain two putative kinases of the RIO family (Rio1 and Rio2), human cells express three RIO kinases (RIOK1, RIOK2 and RIOK3) that are all required for pre-40S maturation (Vanrobays *et al.*, 2003; Zemp *et al.*, 2009; Baumas *et al.*, 2012; Widmann *et al.*, 2012). Phosphorylation substrates of the RIO family kinases have long remained elusive, but human RIOK2 was recently demonstrated to phosphorylate DIMT1L *in vitro* (Sloan *et al.*, 2019). Furthermore, both Rio1 and RIOK1 were recently found to orchestrate a network of interactions that may couple pre-40S maturation with metabolism and cell proliferation (Iacovella *et al.*, 2018).

### Fundamental principles of ribosomal protein recruitment and assembly

Although RPs do not directly participate in peptide bond formation during protein synthesis, they play essential structural roles in ensuring correct rRNA folding to enable catalysis. As nascent RPs are synthesised on pre-existing ribosomes, ribosomes contribute to their own production (Figure 3). The fundamental advantage of assembling these macromolecular complexes from numerous small RPs was recently demonstrated by mathematical modelling, which showed that this can be accomplished much more efficiently than through the expression of a minimal number of larger proteins (Reuveni *et al.*, 2017). Most human RPs are expressed from single genes, whereas many yeast RPs exist in two isoforms. Approximately 50% of human RNA polymerase II transcription is dedicated to production of mRNAs encoding RPs (Warner, 1999), and the expression of these 80 RPs is carefully coordinated to ensure their equal availability for ribosome assembly. Most RP genes share a common promoter element to synchronise their transcription, as well as 5'-terminal oligopyrimidine (TOP) sequences that allow RP mRNA translation to also be co-regulated. Notable exceptions include the mRNAs encoding RPS27a and RPL40, which are expressed as ubiquitin-fusion proteins (Webb *et al.*, 1994). Most RPs are produced in excess, and as depletion of the CUL4 E3 ubiquitin ligase complex



impairs both pre-40S and pre-60S maturation, it is likely that ubiquitination may target excess RPs for degradation (Lam *et al*, 2007; Badertscher *et al*, 2015). RPs are typically highly basic proteins, a property that renders them inherently prone to aggregation and degradation. Consequently, a common feature of yeast and human ribosome assembly is the employment of dedicated chaperones that bind (often co-translationally) to nascent RPs to facilitate their nuclear import and recruitment to pre-ribosomal complexes (reviewed in Pillet *et al*, 2017). While human homologues have been identified for several of the known yeast RP chaperones (Bcp1/BCCIP, Syo1/HEATR3, Rrb1/GRWD1, Sgt1/AMMP and Tsr2/TSR2), others (Acl4 and Yar1) do not appear to be conserved in metazoans (Schütz *et al*, 2014; Wyler *et al*, 2014; Calviño *et al*, 2015; Pausch *et al*, 2015; Pillet *et al*, 2015, 2017), implying the existence of additional mechanisms for facilitating recruitment of some human RPs. Notably, the ribosomal proteins RPL5 (uL18) and RPL11 (uL5) are recruited to pre-ribosomes as a preformed sub-complex together with the 5S rRNA. Pre-5S rRNA is synthesised by RNA polymerase III, and maturation of its 3'-end requires the REX1, REX2 and REX3 exonucleases as well as the presence of RPL5 (Figure 4A; van Hoof *et al*, 2000; Ciganda & Williams, 2011; Sloan *et al*, 2013b). In both yeast and humans, Rrs1/RRS1 and Rpf2/BXDC1 are required for integration of the 5S RNP into pre-60S complexes, and the PICT1/GLTSCR2 tumour suppressor protein is an additional factor important for this step in human cells (Zhang *et al*, 2007; Sloan *et al*, 2013b).

Many RPs are first weakly tethered to pre-ribosomal complexes, where they chaperone correct rRNA folding and establishment of tertiary structures, in turn resulting in their stable incorporation into ribosomal complexes. A fundamental feature of ribosome assembly that is conserved not only throughout eukaryotes but also occurs during prokaryotic ribosome production (Chen & Williamson, 2013) is the hierarchical assembly of RPs, leading to the sequential assembly of particular subunit domains (Figure 3). Screens analysing pre-rRNA processing defects arising from depletion of individual human RPs showed that the 5'-, central and 3'-minor domains of the 18S rRNA that form the "body" of the SSU assemble first, followed by the so-called head and beak structures (O'Donohue *et al*, 2010). Similarly, the RPs that form the solvent-exposed face of the LSU are positioned first, with those binding to the intersubunit interface and to the central protuberance recruited subsequently (Nicolas *et al*, 2016). In yeast, the 5S RNP is first tethered to pre-60S complexes in an immature conformation, but undergoes a 180° rotation to establish its final position as the central protuberance of the LSU (Bassler *et al*, 2014; Leidig *et al*, 2014). Given the conservation of the temporal assembly of RPs, it is likely that a similar structural rearrangement also takes place during the late stages of human ribosome assembly, but this has not yet been confirmed. The universal nature of the hierarchical recruitment of RPs suggests that step-wise assembly, stabilisation and compaction of different domains of the ribosomal subunits is an important mechanism that helps ensure the fidelity of the assembly pathway.

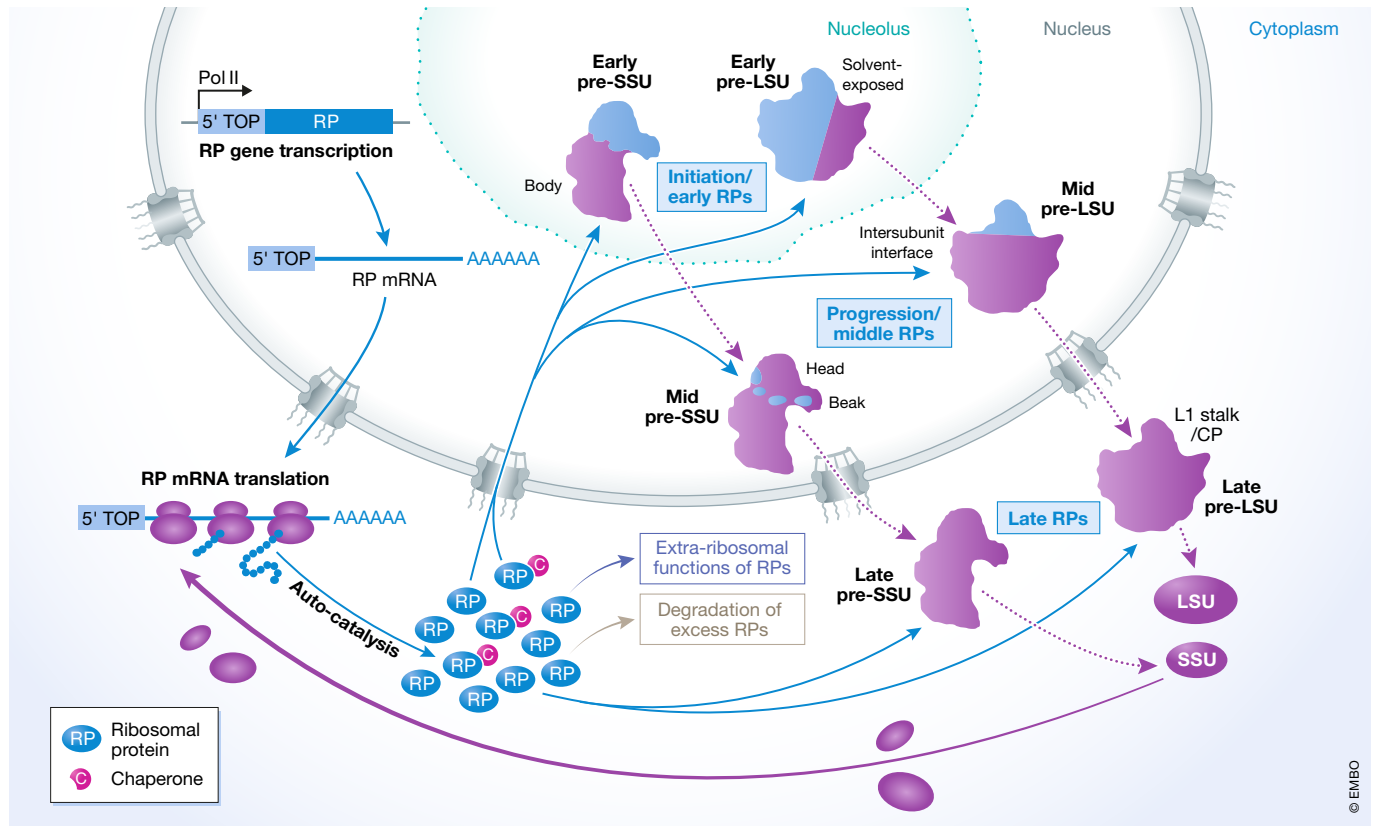
### Extra-ribosomal functions of human ribosomal proteins

A growing body of evidence indicates that several human RPs have additional functions beyond their roles as structural components of ribosomes (Figure 3). For example, extra-ribosomal RPL26 (uL24) and RPL13 (eL13) have been found to modulate translation of

selected mRNAs by binding to their 5'- and 3'-UTRs, respectively (Takagi *et al*, 2005; Mukhopadhyay *et al*, 2008), and RPS3 (uS3) and RPS14 (uS11) have been suggested regulate transcription (Wan *et al*, 2007; Zhou *et al*, 2013). Furthermore, in addition to proposed roles for RPL22 (eL22) and RPL22L1 in the regulation of splicing of specific pre-mRNAs (Zhang *et al*, 2017), several ribosomal proteins (e.g. RPS13 and RPL10a (uL16)) modulate (alternative) splicing of their own pre-mRNAs (Malygin *et al*, 2007; Takei *et al*, 2016). However, the most prominent example of an extra-ribosomal function of RPs is the role of the 5S RNP in a nucleolar stress response pathway that regulates the tumour suppressor p53 (Pelava *et al*, 2016). During homeostasis, the E3 ubiquitin ligase HDM2 (together with its partner HDMX) binds to p53, both inhibiting its transcriptional activity and leading to its ubiquitination-induced proteasomal degradation, with a positive feedback loop in which p53 induces HDM2 expression ensuring that the levels of the tumour suppressor remain low in unstressed cells (Wu *et al*, 1993). Defects in ribosome assembly lead to increased levels of non-ribosome-associated 5S RNP in the nucleoplasm, where it binds to HDM2, inhibits p53 ubiquitination and thereby causes increased cellular p53 levels (Figure 4B) (Bursac *et al*, 2012; Donati *et al*, 2013; Sloan *et al*, 2013b). More specifically, it has been suggested that binding of the 5S RNP to HDM2 impedes its interaction with HDMX and that disruption of this interaction reduces ubiquitination of p53 by HDM2 (Li & Gu, 2011). This 5S RNP/HDM2 pathway of p53 regulation is implicated in the cellular response to many forms of stress, including hypoxia, nutrient stress, growth factor deprivation, UV/gamma radiation and oxidation stress. Furthermore, the tumour suppressor p14<sup>ARF</sup>, which is induced by oncogenic signals such as the expression of RAS or c-MYC, is also implicated in this pathway, as it directly binds RPL11 (Dai *et al*, 2012) and as depletion of RPL5 or RPL11 suppresses the p53 accumulation normally observed in cells overexpressing p14<sup>ARF</sup> (Sloan *et al*, 2013b). Other ribosomal proteins, including RPS27a and RPL23, have also been shown to bind to HDM2, suggesting the presence of alternative mechanisms to regulate p53 levels in response to defects in ribosome assembly (Dai *et al*, 2004; Sun *et al*, 2011). In addition to p53 regulation, components of the 5S RNP have recently been shown to bind to the pro-apoptotic tumour suppressor p73 and to prevent its interaction with HDM2, and RPL11 is implicated in regulating the transcriptional activity of c-MYC (Riley & Lozano, 2012; Liao *et al*, 2014). The 5S RNP therefore constitutes a hub for coordinating ribosome assembly with multiple cell signalling pathways.

### Regulation of ribosome production by cell signalling pathways

The pathway of ribosome assembly is closely coupled with cell cycle progression and modulated by various signalling pathways that control cell growth. Such co-regulation of the ribosome synthesis pathway is unsurprising in light of the essential nature of protein synthesis and the high energy demand of their assembly pathway. Several oncogenes that drive cellular proliferation, such as c-MYC, mTOR, ERK and AKT, and tumour suppressors that help maintain cellular homeostasis, including p53, ARF and PTEN, have been shown to regulate ribosome biogenesis. For example, a screen for c-MYC target genes identified numerous RBFs and several RPs, and



**Figure 3. Production, recruitment and functions of human ribosomal proteins.**

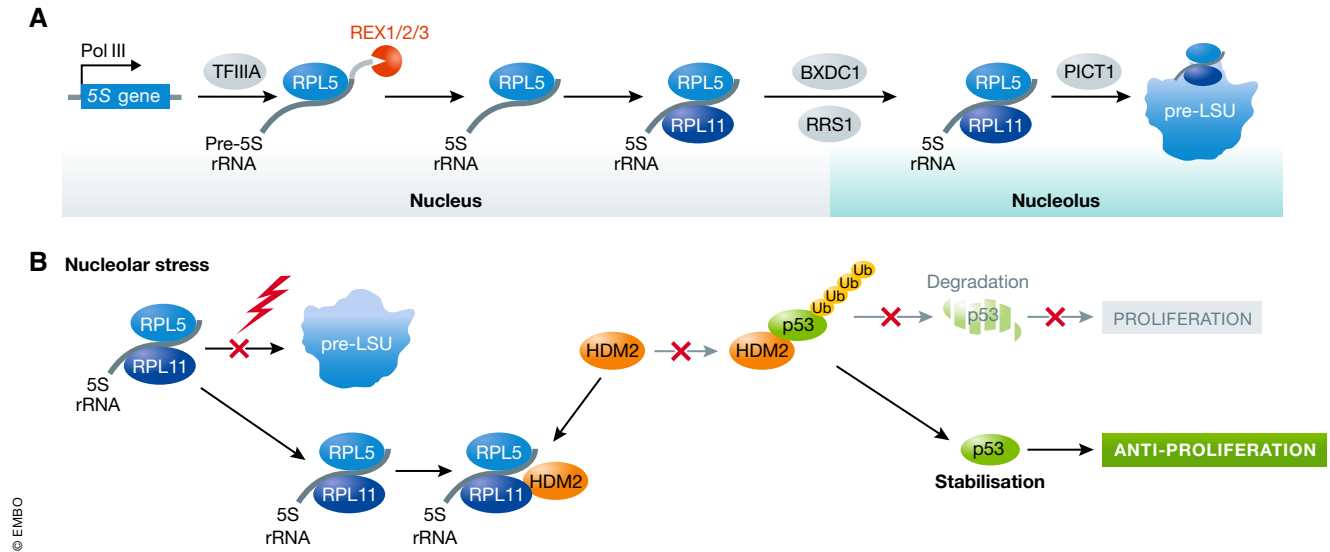
Schematic model showing the expression and assembly of ribosomal proteins (RP). Red arrows indicated sequential steps in the process, and dashed black lines indicate maturation of pre-ribosomal particles. Pink circles containing a white "C" depict dedicated ribosomal protein chaperones. CP, central protuberance.

consistent with this, c-MYC was found to regulate efficient rRNA maturation (Schlosser *et al.*, 2003). Similarly, the mTOR-containing mTORC1 complex, which connects cell growth to nutrient availability on various levels such as transcription, translation, protein degradation and membrane trafficking, promotes rDNA transcription by RNA polymerase I and 5S rRNA synthesis by RNA polymerase III, and also regulates translation of most RP-encoding mRNAs (Mayer & Grummt, 2006; Gentilella *et al.*, 2015). Furthermore, mTORC-dependent S6 kinase activation triggers phosphorylation of RPS6, and the expression of a large proportion of RBFs is altered in cells lacking S6 kinases (Chauvin *et al.*, 2014). Notably, the mTORC2 complex component RICTOR and the L-glutamine synthetase GLUL were recently identified in a screen for factors required for human ribosome biogenesis (Badertscher *et al.*, 2015). As the effect of GLUL on 40S biogenesis was independent of mTORC1, intracellular glutamine synthesis may be important for efficient ribosome synthesis (Badertscher *et al.*, 2015). Finally, in addition to the global up- and down-regulation of ribosome biogenesis observed during tumorigenesis and senescence, respectively, the rate of ribosome biogenesis is also controlled by the circadian clock. Fluctuations in RP gene transcription and mRNA translation rates have been observed, and diurnal changes in mouse liver mass are accompanied by variations in the levels of newly synthesised rRNAs (Jouffe *et al.*, 2013; Sinturel *et al.*, 2017). A recent model integrating these two observations proposes that newly synthesised rRNAs not

assembled with RPs are polyadenylated by PAPD5 and degraded by the nuclear exosome (Sinturel *et al.*, 2017).

### Ribosome heterogeneity

Given the precise molecular interactions and movements required of the ribosome during translation, the essential nature of many RBFs and the extensive monitoring that pre-ribosomal subunits undergo during their maturation, it was long assumed that cells contain a uniform population of ribosomes. However, several lines of evidence suggest that this is not the case and different sources of ribosome heterogeneity have now been identified. On the rRNA level, alternative pre-rRNA processing pathways generate two different (long and short) forms of the 5.8S rRNA. Recent quantitative analyses of rRNA modifications have further revealed that numerous Nm and Ψ sites are substoichiometrically modified, suggesting that subsets of ribosomes likely carry different combinations of modifications (Krogh *et al.*, 2016; Sharma *et al.*, 2017a). Variations in the extent or the sites of modification have been found in cancer cell lines of different origins, or depending on expression status of the tumour suppressor p53 (Krogh *et al.*, 2016; Sharma *et al.*, 2017a; Taoka *et al.*, 2018). At the protein level, there is not only variation in the post-translation modifications present on RPs in different contexts (Simsek & Barna, 2017), but tissue-specific differences in



**Figure 4. Assembly of the 5S RNP and its role in regulation of the tumour suppressor p53.**

(A) Model of synthesis and assembly of the 5S RNP from 5S rRNA and ribosomal proteins RPL5 (uL18) and RPL11 (uL1), and 5S RNP integration into the pre-LSU. (B) Upon nucleolar stress (e.g. in ribosomopathies), 5S RNP integration into the pre-LSU is decreased and 5S RNP accumulates in the nucleoplasm, where it binds the E3 ubiquitin ligase HDM2. This impedes association of HDM2 with p53, promoting p53 stabilisation and inhibiting cell proliferation.

RP mRNA levels have also been observed (Bortoluzzi *et al.*, 2001), as well as differences in the RP composition of translating ribosomes (Shi *et al.*, 2017). The existence of such ribosome heterogeneity raises the question of whether these compositional variations are merely biogenesis artefacts tolerated by (pre-)ribosome quality control pathways, or whether they reflect active fine-tuning of ribosome structure for specialised functions. Supporting the latter proposal, differences in rRNA 2'-O-methylation and pseudouridylation have been shown to influence the translational capacity of human ribosomes. For example, pathogenic mutations identified in the genetic disease dyskeratosis congenita cause changes in rRNA pseudouridylation that impair translation of the mRNAs encoding the tumour suppressors p53 and p27 and the anti-apoptotic factors Bcl-xL and XIAP, thereby promoting tumorigenesis (Yoon *et al.*, 2006; Bellodi *et al.*, 2010a,b). Similarly, changes in rRNA 2'-O-methylation have been observed to specifically affect translation of various IRES-containing mRNAs (Erales *et al.*, 2017). Moreover, human ribosomes containing RPS25 (eS25) or RPL10 (uL1) were observed to preferentially translate specific subsets of mRNAs involved in metabolism, cell cycle and development, indicating that ribosome heterogeneity can serve as a mechanism to fine-tune translation (Shi *et al.*, 2017). It is therefore likely that ribosome heterogeneity not only contributes to the adaptation of gene expression in different conditions, but also to development, disease and tissue identity.

### Elucidating the molecular basis of ribosomopathies

The push for a comprehensive understanding of human ribosome assembly has primarily been driven by the recognition of a family of diseases, collectively termed ribosomopathies, that are caused by

mutations in genes encoding RPs, RBFs or components of the rDNA transcription machinery (Table 3). The new inventories of human RBFs generated by RNAi-based screens include numerous disease-associated proteins and biomarkers, suggesting that the prevalence of disorders arising from ribosome dysfunction may be much greater than previously thought. While most ribosomopathies are relatively rare, the most prominent and best understood example is Diamond-Blackfan anemia (DBA), which is characterised by bone marrow failure due to inhibition of hematopoietic stem cell differentiation along the erythroid lineage. DBA-associated mutations have been identified in eleven different genes encoding RPs of both the small and large subunit (Table 3; Boria *et al.*, 2010). Ribosomopathies manifest with a diverse set of phenotypes, but many share common characteristics or overlapping features such as haematopoietic dysfunction (e.g. DBA, 5q- syndrome, dyskeratosis congenita, Shwachman–Diamond syndrome) and/or skeletal (especially craniofacial) defects (e.g. Treacher Collins syndrome, Bowen-Conradi syndrome and RPS23-related ribosomopathy; Table 3).

Understanding how defects in a fundamental and ubiquitous cellular process such as ribosome synthesis can cause tissue-specific human pathologies remains a major challenge, and several models to explain these phenomena have recently been proposed (Mills & Green, 2017; Emmott *et al.*, 2019). As mutations in the genes encoding RPs or RBFs disrupt ribosome assembly, they lead to stabilisation of p53 via the 5S RNP/HDM2 pathway described above. Indeed, many of the tissue-specific defects observed in ribosomopathies appear to be p53-dependent, with symptoms of ribosomopathies such as Treacher Collins syndrome and 5q- syndrome rescuable by inhibition of p53 function (Jones *et al.*, 2008; Barlow *et al.*, 2010). Elevation of the cellular p53 level leads to cell cycle arrest and apoptosis, and it has been suggested that variations in the threshold or extent of p53 activation in different

**Table 3. Inventory of genetic diseases caused by defects in ribosomal proteins or biogenesis factors.**

Ribosomopathy	OMIM	Affected gene(s)	Mutation(s)	Clinical features	Key references
Diamond-Blackfan anemia	105650	<i>RPL5, RPL11, RPL27, RPL35A, RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, RPS27, RPS28, GATA1, TSR2</i>	Various	Anaemia, microcephaly, hypertelorism, ptosis, micrognathia, cleft palate, short, webbed neck, malformed or absent thumbs, cataracts, glaucoma, strabismus	Boria <i>et al</i> (2010), Ellis and Gleizes (2011)
5q-myelodysplastic syndrome	153550	<i>RPS14</i>	Deletion of ~1.5 Mb region of chr. 5	Severe anaemia, thrombocytosis, dysmegakaryopoiesis	Ebert <i>et al</i> (2008)
Isolated congenital asplenia	271400	<i>RPSA</i>	pP199Sfs*25, pQ9*, p.T54N, p.L58F, p.R180W, p.R180G, p.R186C	Lack of spleen, immunodeficiency	Bolze <i>et al</i> (2013)
RPS23-related ribosomopathy	617412	<i>RPS23</i>	p.R67K	Microcephaly, hearing loss, simian palmar creases, epicanthic folds in the eyes, foetal finger pads, extra front teeth, low back hairline, facial asymmetry and high palate, intellectual disability, autism spectrum disorder	Paolini <i>et al</i> (2017)
Treacher Collins syndrome	154500, 613717, 248390	<i>TCOF1, POLR1C, POLR1D</i>	Various	Craniofacial defects; midface hypoplasia, micrognathia, microtia, conductive hearing loss and cleft palate	Valdez <i>et al</i> (2004), Dauwerse <i>et al</i> (2011), Vincent <i>et al</i> (2016)
Postaxial acrofacial dysostosis (POADS)	263750	<i>DHODH</i>	p.R135C, p.R346W, p.D392G, p.E52G, p.A357T, p.R326X,	Craniofacial defects (micrognathia, orofacial clefts, malar hypoplasia, cup-shaped ears), postaxial limb deformities (lack of fourth/fifth rays of hands/feet; ulner/fibular hypoplasid)	Rainger <i>et al</i> (2012)
Roberts syndrome	268300	<i>ESCO2</i>	p.E251fsX30, p.R169X, p.W539G, p.V84fsX7, p.K486fsX20, p.K369fsX34, p.R293fsX7, p.K138fsX10	Prenatal growth retardation, craniofacial defects, e.g. cleft lip/palate or microcephaly, limb malformations	Vega <i>et al</i> (2005)
Scleroderma	181750	<i>UTP14A</i>	Gene hypermethylation – decreased expression	Hardened/thickened skin, ulcers/sores, swollen joints, fingers or toes, muscle weakness	Selmi <i>et al</i> (2012)
Dyskeratosis congenita	305000	<i>DKC1, TERC, TERT, NOP10, NHP2, TINF2</i>	Various (prevalent Dyskerin p.A353V)	Mucocutaneous abnormalities, pulmonary fibrosis, bone marrow failure, immunodeficiency	Heiss <i>et al</i> (1998), Knight <i>et al</i> (2001), Vulliamy <i>et al</i> (2001, 2008)
Bowen-Conradi syndrome	211180	<i>EMG1</i>	p.D86G	Prenatal and postnatal growth retardation, microcephaly, prominent nose with an absent glabellar angle, micrognathia, joint abnormalities, camptodactyly, rocker-bottom feet, severe psychomotor delay	Armistead <i>et al</i> (2009)
Cartilage-hair hypoplasia	250250	<i>RMRP</i>	Various	Short stature, bone deformities, hair growth abnormalities	Ridanpää <i>et al</i> (2001)

Table 3 (continued)

Ribosomopathy	OMIM	Affected gene(s)	Mutation(s)	Clinical features	Key references
North American Indian childhood cirrhosis	604901	<i>UTP4, NOL11</i>	p.R565W	Biliary cirrhosis, portal hypertension	Freed and Baserga (2010), Freed <i>et al</i> (2012)
Shwachman–Diamond syndrome	260400	<i>SBDS</i>	Various	Exocrine pancreatic insufficiency, bone marrow dysfunction, leukaemia predisposition and skeletal abnormalities	Boocock <i>et al</i> (2003)
Alopecia, neurological and endocrinopathy syndrome (ANE)	612079	<i>RBM28</i>	p.L351P	Alopecia, progressive neurological defects and endocrinopathy	Nousbeck <i>et al</i> (2008), Spiegel <i>et al</i> (2010)
Aplasia cutis congenita	107600	<i>BMS1</i>	p.R930H	Skin defects (especially scalp vertex)	Marneros (2013)
Leukoencephalopathy, intercranial calcifications and cysts	614561	<i>SNORD118</i>	Various	Leukoencephalopathy, intercranial calcifications and cysts	Jenkinson <i>et al</i> (2016)
Cancer-prone bone marrow failure syndrome	617052	<i>DNAJC21</i>	p.R173*, p. P32A, p.E265*	Bone marrow failure, short stature, microcephaly, decreased bone density, predisposition to acute myeloid leukaemia	Tummala <i>et al</i> (2016)
X-linked intellectual disability, cerebellar hypoplasia and spondyloepiphyseal dysplasia	300847	<i>RPL10</i>	p.A64V	Intellectual disability, cerebellar hypoplasia and spondyloepiphyseal dysplasia	Zanni <i>et al</i> (2015)

cell types may underlie tissue specificity in many ribosomopathies. An alternative proposal posits that variations in ribosome composition between different cell types and/or in different tissues (see above section on “ribosome heterogeneity”) lead to specialised translation; mutations that decrease the levels of specific RPs or impair the rRNA modification machinery may therefore reduce or alter production of proteins particularly required in certain cell types, thereby inducing the disease phenotype. Another possible molecular basis of the tissue-specific effects observed in ribosomopathies may be alterations in translation caused by the high demand for ribosomes in fast-proliferating tissues. Supporting this, DBA-associated mutations have been found to reduce ribosome levels in haematopoietic cells such that the number of ribosomes becomes limiting, and translation of a specific subset of mRNAs is affected, which in turn impairs erythroid lineage commitment (Khajuria *et al*, 2018). The differential effects on mRNA translation caused by the lack of available ribosomes were suggested to be caused by inherent properties of specific mRNAs (e.g. highly structured 5'-UTRs) that render these transcripts especially sensitive to reduced ribosome concentration. It is likely that differential p53 responses to ribosome assembly defects and alterations in the translation profile caused by limited ribosome production, and/or the assembly of specialised ribosomes, all contribute to different extents to ribosomopathy phenotypes. Nevertheless, it has also been suggested that some of the tissue-specific effects observed may instead arise from varying abilities of different cell types to overcome such defects, for example *via* ribosome quality control pathways or recovery of RP levels by allelic compensation (Mills & Green, 2017).

## Conclusions and outlook

For many years, the complex pathway of eukaryotic ribosome biogenesis was studied almost exclusively in the yeast model system, where the ease of genetic manipulation coupled with the possibility to isolate large amounts of pre-ribosomal complexes for compositional and structural analysis has provided a wealth of knowledge about fundamental aspects of ribosome assembly. Motivated primarily by the importance of elucidating the molecular basis of ribosomopathies, analyses of the human ribosome assembly pathway have increased exponentially in recent years. While this work has confirmed that many features of the yeast and human ribosome assembly pathways are similar, the emerging differences provide important insights into particular aspects of the pathway that have adapted during evolution. Although a plethora of factors required for human ribosome biogenesis have now been uncovered, it is likely that future research will extend the inventory of human RBFs even further. Major challenges for the next years will be to determine which of the factors required for ribosome synthesis are directly associated with pre-ribosomal complexes, and to dissect the individual roles of such proteins during subunit assembly. The recent cryo-EM structures of yeast pre-ribosomes have provided a vast amount of information on the temporal order, distribution and molecular functions of many RBFs. We eagerly anticipate a similar increase in structural analyses of human pre-ribosomes, which should significantly advance our understanding of human ribosome assembly by enabling visualisation of novel RBFs *in situ* and revealing the dynamics of the ribosome assembly process. The recognition of ribosome biogenesis as a central hub

coordinating protein synthesis and cell proliferation necessitates not only understanding of the assembly pathway itself, but also deeper insight into the complex regulatory networks modulating it. This should extend the scope of ribosome biogenesis research to a cellular and organismal level, and it will be exciting to discover the full extent of crosstalk between ribosome production and other aspects of cellular function.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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