RNA BIOLOGY
2016, VOL. 0, NO. 0, 1–16
http://dx.doi.org/10.1080/15476286.2016.1259781

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REVIEW

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Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function

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ABSTRACT

rRNAs are extensively modified during their transcription and subsequent maturation in the nucleolus, nucleus and cytoplasm. RNA modifications, which are installed either by snoRNA-guided or by stand-alone enzymes, generally stabilize the structure of the ribosome. However, they also cluster at functionally important sites of the ribosome, such as the peptidyltransferase center and the decoding site, where they facilitate efficient and accurate protein synthesis. The recent identification of sites of substoichiometric 2′-O-methylation and pseudouridylation has overturned the notion that all rRNA modifications are constitutively present on ribosomes, highlighting nucleotide modifications as an important source of ribosomal heterogeneity. While the mechanisms regulating partial modification and the functions of specialized ribosomes are largely unknown, changes in the rRNA modification pattern have been observed in response to environmental changes, during development, and in disease. This suggests that rRNA modifications may contribute to the translational control of gene expression.

Abbreviations: A-site, aminoacylated tRNA site; ac^4C, N^4-acetylcytidine; CMC, N-cyclohexyl-N′-(2-morpholinoethyl) carbodiimidemetho-p-toluenesulfonate; DMS, dimethyl sulfate; E-site, exit site; LSU, large ribosomal subunit; m_2^6A, N^6,N^6-dimethyladenosine; m^1A, N^1-methyladenosine; $m^1acp^3\psi$, N^1-methyl-N^3-(3-amino-3-carboxypropyl) pseudouridine; m^5C, N^5-methylcytosine; m^7G, N^7-methylguanosine; m^3U, N^3-methyluridine; mRNA, messenger RNA; P-site, Peptidyl tRNA site; PTC, peptidyltransferase center; RNAi, RNA interference; RNP, ribonucleoprotein complex; rRNA, ribosomal RNA; SAM, S-adenosylmethionine; snoRNA, small nucleolar RNA; snoRNP, small nucleolar RNP; snRNA, small nuclear RNA; SSU, small ribosomal subunit; tRNA, transfer RNA; Ψ, pseudouridine; X-DC, X-linked Dyskeratosis congenita

ARTICLE HISTORY
Received 30 September 2016
Revised 6 November 2016
Accepted 8 November 2016

Keywords
RNA modification; ribosome; snoRNA; RNA methyltransferase; methylation; ribosomopathy; acetylation; pseudouridylation; translation

Introduction

Nucleotide modifications are found in all 3 kingdoms of life and so far more than 140 different types of modification have been identified in cellular RNA.[1-3] Such modifications expand the chemical and topological properties of the 4 standard nucleosides, thereby influencing RNA structure and regulating the biological functions of the RNAs that carry them. Recent transcriptome-wide mapping approaches have shown that RNA modifications occur on all major classes of RNA, including messenger RNAs (mRNAs).[4] After transfer RNAs (tRNAs), which contain up to 17% modified nucleotides,[5] ribosomal RNAs (rRNAs) are the most highly modified class of RNAs with approximately 2% of rRNA nucleotides being modified.

Ribosomes are required for the translation of all cellular proteins and in eukaryotes, cytoplasmic ribosomes are composed of 4 rRNAs and approximately 80 ribosomal proteins arranged into a small subunit (SSU) and a large subunit (LSU).[6,7] During translation, the SSU mediates decoding by monitoring codon-anticodon base-pairing between the mRNA and tRNAs, while the LSU, which harbours the catalytic peptidyltransferase center, is responsible for synthesis of the nascent polypeptide chain. Ribosome production is initiated in the nucleolus by the RNA polymerase I-mediated transcription of a single rRNA precursor that contains the sequences of 3 of the 4 rRNAs (18S, 5.8S and 25S (yeast)/28S (human) rRNAs).[8,9] Assembly of the ribosomal proteins to form key structural features of the mature ribosomal subunits occurs concomitantly with maturation and folding of the pre-rRNA (for a review see ref. 10). After early pre-rRNA cleavage events, the precursor (pre-)SSU and pre-LSU complexes follow separate biogenesis pathways in the nucleolus and nucleus before nuclear export and final maturation steps in the cytoplasm.[9,11–13] In yeast and human cells, more than 200 *trans*-acting cofactors are required to orchestrate this complex and hierarchical process.[9,14] These include numerous enzymatic proteins and ribonucleoprotein complexes (RNPs) that are responsible for the introduction of rRNA modifications.[15,16]

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Despite the relatively high abundance of modified nucleotides in rRNA and the diversity of the RNA modifications observed in nature, only a limited set of different chemical modifications is found in rRNA. While the proteins that modify bacterial rRNA and many non-rRNA species in eukaryotes act mostly as stand-alone modification enzymes, some eukaryotic rRNA-modifying enzymes use RNA-guided mechanisms for recognition of their target nucleotides. In general, the rRNA modifications, which are introduced at different stages during ribosome biogenesis, serve to stabilize the secondary and tertiary structure of the rRNA scaffold,[17] thereby ensuring the efficiency and accuracy of translation. It has recently emerged, however, that rRNA modifications can also represent an important source of ribosome heterogeneity that may alter ribosome function in response to environmental and developmental cues and in disease.

Modifications guided by small nucleolar RNAs

In eukaryotes, the most abundant rRNA modifications are 2′-O-methylation of the ribose, which can occur on any nucleotide, and the isomerisation of uridine to pseudouridine (Ψ). To date, 55 2′-O-methylation sites and 45 Ψ sites have been identified in the rRNAs of the yeast *Saccharomyces cerevisiae* and approximately 100 of each modification are reported in human rRNAs.[18-21] Together these modifications outnumber base modifications almost 10-fold, which is in stark contrast to the distribution of modifications in *Escherichia coli*, where twice as many base methylations as ribose methylations and pseudouridylations are present. The extent of 2′-O-methylation and pseudouridylation in yeast rRNA was first estimated almost 30 years ago by 2D thin layer chromatography.[22-24] Later, the use of primer extension-based assays, either under limiting dNTP conditions for the detection of 2′-O-methylation[25] or after derivatization using CMC in the case of pseudouridylation,[26] provided evidence for 99 specific sites of modification (Table 1).[27,28] More recently, sequencing-based profiling approaches that monitor reverse transcriptase drop-off rates (Ψ) or exploit the resistance of 2′-O-methylated RNA to alkaline lysis, mass spectrometry, and nuclease protections assays combined with reversed phase high-performance liquid chromatography (RP-HPLC) have confirmed the presence of these ribose methylations and pseudouridines and have clarified the presence of a 2′-O-methylation at position G562 of the 18S rRNA (note, the numbering of residues in yeast rRNAs in this review is based on the sequences annotated in the *Saccharomyces Genome Database* and may therefore differ from numbers given in some of the original literature cited).[18,21,29-31] Such systematic mapping methods have also been applied to human rRNA, generating an updated inventory of 2′-O-methylation sites; no evidence was found for the previously reported 18S-Gm1536 and 18S-Um1602, but modifications at 28S-A1868 and 28S-G3771 were newly identified.[32] Similarly, a recent quantitative mass spectrometry-based approach has led to the identification of an additional pseudouridylation site at position U2347 of the yeast 25S rRNA (Table 1).[21]

These 2′-O-methylations and pseudouridines are mostly introduced by 2 classes of small nucleolar (sno)RNPs, termed box C/D and box H/ACA snoRNPs respectively.[16,33–35] The rare exceptions in yeast are a pseudouridine (Ψ50) in the 5S rRNA, which is introduced by the pseudouridine synthetase Pus7 that specifically modifies targets with the RSUNΨAR consensus motif (where R is a purine, S is guanine or cytosine and N represents any nucleotide), and the stand-alone 2′-O-methyltransferase Sbp1 that modifies G2922 of the 25S rRNA.[36,37] Eukaryotic snoRNPs, which are structurally and functionally conserved from archaeal sRNPs, are ribonucleoprotein complexes containing a snoRNA, which base-pairs with the pre-rRNA and directs the catalytic protein component of the snoRNP to modify a specific target residue. H/ACA box snoRNAs, so called due to the presence of a conserved H box (5′-ANANNA-3′) and an ACA sequence, form a double hairpin structure. Base-pairing with the pre-rRNA takes place in the "pseudouridylation pocket" within a hairpin, leaving the target uridine non-base-paired and hence accessible for isomerisation by the pseudouridine synthetase Cbf5 (dyskerin; Fig. 1A).[38] The other protein components of H/ACA box snoRNPs, Nop10, Nhp2 and Gar1 largely perform structural functions, stabilizing the tertiary fold of the RNA and ensuring correct positioning of the target nucleotide in the Cbf5 active site.

Box C/D snoRNAs form a single hairpin structure through the base-pairing of conserved sequence motifs (C box, 5′-RUGAUGA-3′ and D box, 5′-CUGA-3′) at the 5′ and 3′ ends of the transcript respectively, as well as through the interaction of internal degenerate C′ and D′ boxes. Base-pairing of the pre-rRNA with the snoRNA adjacent to the D/D′ box is facilitated by the protein components of the snoRNP, Nop56, Nop58 and Snu13 (15.5K in humans). This positions the catalytic site of the methyltransferase Nop1 (fibrillarin)[39] to modify its target, 5 nucleotides upstream of the D/D′ box (Fig. 1B). Box C/D snoRNAs form extensive interactions with the pre-rRNA, with guide sequences ranging from 10 to 21 nucleotides in length, and in several cases additional regions of snoRNA-rRNA complementarity close to the methylated residue have been shown to enhance methylation.[40] Interestingly, recent structural analysis of archaeal sRNPs indicate that only 10 basepaired nucleotides can be accommodated during catalysis, leading to the suggestion that the additional basepairing interactions facilitate accurate and efficient snoRNP recruitment and that the subsequent unwinding, which is necessary for modification, extends the residence time of the snoRNA on the pre-rRNA to prevent premature or mis-folding of the target site.[41]

Although the majority of box C/D and H/ACA snoRNAs guide modification of only a single rRNA nucleotide, this is not always the case and several snoRNAs have been shown to direct modification of multiple positions using distinct mechanisms. Due to the presence of both D and D′ motifs, box C/D snoRNAs have the potential to base-pair with and modify 2 alternative sites in the pre-rRNA.[34,42] Some such snoRNAs introduce 2 modifications into the same rRNA (e.g. snR60 for 25S-Am817 and 25S Gm908) while others act on both the small and the large ribosomal subunits (e.g., snR52 for 18S-Am420 and 25S-Um2921). Interestingly, snR60 guides 2 ribose methylations that are on opposite sides of a hairpin, suggesting that in addition to its catalytic function, the snoRNP may expedite formation of this rRNA secondary structure through its base-pairing interactions.[43] Indeed, several other snoRNAs, including U14 and snR10, not only function in rRNA modification but

Table 1. Inventory of rRNA modifications in yeast. The rRNA, position (Posn.) and type (Mod.) of the modification and the enzyme/snoRNP(s) that introduce it are given, with information on whether partial modification was observed (✓; <85%)[21] or not (x), and whether modification occurs early/chromatin-associated (E),[18] in late nucleolar/nuclear (LN) particles or in the cytoplasm (C). A lack of information on the timing is indicated by "–." Enzymes/snoRNPs targeting the same position sequentially are separated by "," and alternative enzymes/snoRNPs that can install a single modification at a given site are separated by "/". The information presented in this table is compiled from various references cited in the text.

rRNA	Posn.	Mod.	Enzyme/snoRNP	Partial	Timing	rRNA	Posn.	Mod.	Enzyme/snoRNP	Partial	Timing
18S	28	Am	snR74	x	E	18S	1415	Ψ	snR83	✓	—
18S	100	Am	snR51	✓	LN	18S	1428	Gm	snR56	x	E
18S	106	Ψ	snR44	x	—	18S	1572	Gm	snR57	x	E
18S	120	Ψ	snR49	x	—	18S	1575	m^7G	Bud23	x	—
18S	211	Ψ	snR49	✓	—	18S	1639	Cm	snR70	✓	E
18S	302	Ψ	snR49	x	—	18S	1773	ac^4C	Kre33	x	—
18S	414	Cm	U14	x	E	18S	1781	m_2^6A	Dim1	x	C
18S	420	Am	snR52	x	E	18S	1782	m_2^6A	Dim1	x	C
18S	436	Am	snR87	✓	E	5.8S	73	Ψ	snR43	✓	—
18S	466	Ψ	snR189	✓	—	25S	645	m^1A	Rrp8 (Bmt1)	x	—
18S	541	Am	snR41	x	E	25S	649	Am	U18	x	E
18S	562	Gm	?	✓	E	25S	650	Cm	U18	x	E
18S	578	Um	snR77	x	E	25S	663	Cm	snR58	✓	E
18S	619	Am	snR47	x	E	25S	776	Ψ	snR80	x	—
18S	632	Ψ	snR161	✓	—	25S	805	Gm	snR39b	x	E
18S	759	Ψ	snR80	x	—	25S	807	Am	snR39/snR59	x	E
18S	766	Ψ	snR161	x	—	25S	817	Am	snR60	x	LN
18S	796	Am	snR53	x	E	25S	867	Gm	snR50	✓	LN
18S	974	Am	snR54	x	E	25S	876	Am	snR72	✓	LN
18S	999	Ψ	snR31	✓	—	25S	898	Um	snR40	x	LN
18S	1007	Cm	snR79	x	E	25S	908	Gm	snR60	x	E
18S	1126	Gm	snR41	x	E	25S	956	m^3U	Bmt5	x	—
18S	1181	Ψ	snR85	x	—	25S	960	Ψ	snR8	x	—
18S	1187	Ψ	snR36	x	—	25S	966	Ψ	snR43	x	—
18S	1191	$m^1acp^3Ψ$	snR35,Emg1,Tsr3	x	E,LN,C	25S	986	Ψ	snR8	x	—
18S	1269	Um	snR55	x	E	25S	990	Ψ	snR49	x	—
18S	1271	Gm	snR40	x	E	25S	1004	Ψ	snR5	✓	—
18S	1280	ac^4C	Kre33	✓	—	25S	1042	Ψ	snR33	x	—
18S	1290	Ψ	snR83	x	—	25S	1052	Ψ	snR81	x	—
25S	1056	Ψ	snR44	x	—	25S	2351	Ψ	snR82	x	—
25S	1110	Ψ	snR82	✓	—	25S	2416	Ψ	snR11	x	—
25S	1124	Ψ	snR5	x	—	25S	2417	Um	snR66	x	E
25S	1133	Am	snR61	x	E	25S	2421	Um	snR78	x	LN
25S	1437	Cm	U24	x	E	25S	2619	Gm	snR67	x	E
25S	1449	Am	U24	x	E	25S	2634	m^3U	Bmt5	x	—
25S	1450	Gm	U24	x	E	25S	2640	Am	snR68	x	LN
25S	1888	Um	snR62	x	—	25S	2724	Um	snR67	x	E
25S	2129	Ψ	snR3	x	—	25S	2729	Um	snR51	✓	E
25S	2133	Ψ	snR3	x	—	25S	2735	Ψ	snR189	x	—
25S	2142	m^1A	Bmt2	x	—	25S	2791	Gm	snR48	x	E
25S	2191	Ψ	snR32	x	—	25S	2793	Gm	snR48	x	E
25S	2197	Cm	snR76	x	E	25S	2815	Gm	snR38	x	E
25S	2220	Am	snR47	x	E	25S	2826	Ψ	snR34	x	—
25S	2256	Am	snR63	x	LN	25S	2843	m^3U	Bmt6	x	—
25S	2258	Ψ	snR191	x	—	25S	2865	Ψ	snR46	x	—
25S	2260	Ψ	snR191	x	—	25S	2870	m^5C	Nop2 (Bmt4)	x	—
25S	2264	Ψ	snR3	x	—	25S	2880	Ψ	snR34	x	—
25S	2266	Ψ	snR84	x	—	25S	2921	Um	snR52/Sbp1	x	E
25S	2278	m^5C	Rcm1 (Bmt3)	x	—	25S	2922	Gm	Sbp1	x	LN
25S	2280	Am	snR13	x	E	25S	2923	Ψ	snR10	x	—
25S	2281	Am	snR13	x	E	25S	2944	Ψ	snR37	✓	—
25S	2288	Gm	snR75	x	E	25S	2946	Am	snR71	x	E
25S	2314	Ψ	snR86	x	—	25S	2948	Cm	snR69	x	E
25S	2337	Cm	snR64	x	—	25S	2959	Cm	snR73	x	E
25S	2340	Ψ	snR9	x	—	25S	2975	Ψ	snR42	x	—
25S	2347	Um/Ψ/Ψm	snR65,snR9	x	—	5S	50	Ψ	Pus7	x	—
25S	2349	Ψ	snR82	x	—						

also have well characterized roles in establishing long-range interactions within pre-ribosomal complexes.[44-46] Notably, in several cases the sites modified by the same snoRNA are adjacent or separated by only a single nucleotide (snR13 for 25S-Am2280/1, snR48 for 25S-Gm2791/3 and U24 for 25S-Am1449/Gm1450). In these cases, an alternative mechanism involving the use of different D/D′ boxes within the same snoRNA is employed. It has been proposed that formation of a bulge within the snoRNA-rRNA duplex may lead to "sliding" of a nucleotide adjacent to the canonical fifth nucleotide into the active site of Nop1.[34,42] More recent data indicates that instead, the presence of non-canonical C′/D′ boxes in snR13, snR48 and U18 allows the formation of alternative snoRNPs that differ subtly in the arrangement of their protein

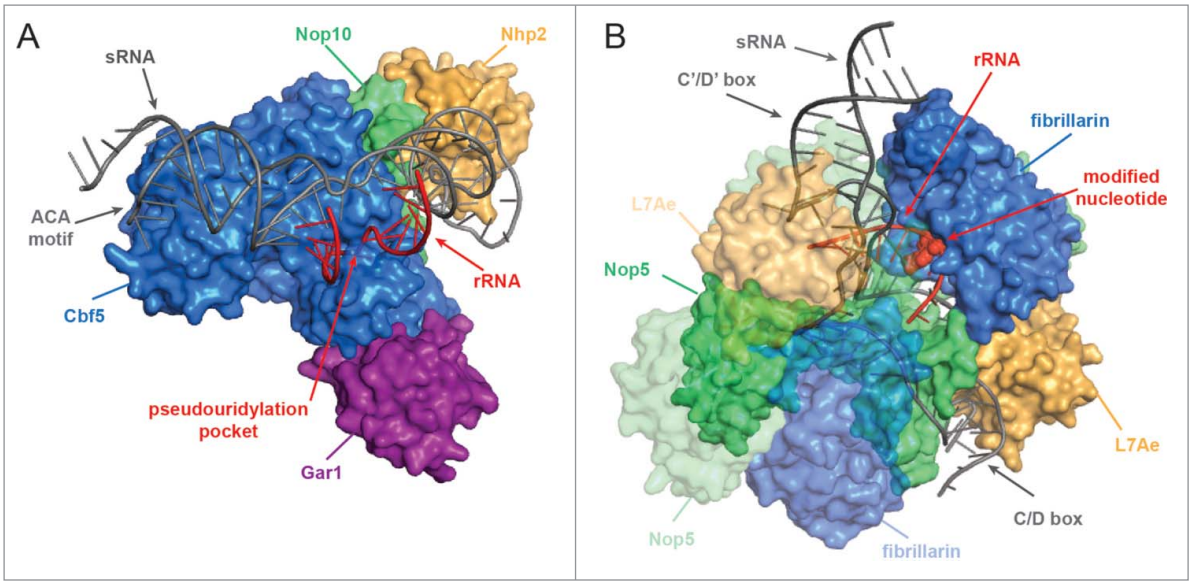

Figure 1. Structures of box C/D and box H/ACA sRNP complexes. (A) Structural model of an H/ACA box sRNP from *Pyrococcus furiosus* (PDB 3HAY)[154] A surface view of the protein components is shown and sRNA and rRNA are indicated in dark gray and red, respectively. (B) Structural model of a *Sulfolobus solfataricus* box C/D sRNP (PDB 3PLA)[155] is shown as in A. The modified nucleotide is shown in surface view. Note, that archaeal L7Ae is an ortholog of eukaryotic Snu13 and in eukaryotes, Nop56 and Nop58 are orthologous to archaeal Nop5.

components and can therefore catalyze additional modifications that do not conform to the classical "5-base-pair rule."[47] The short, discontinuous base-pairing interactions formed between H/ACA box snoRNAs and their pre-rRNA targets allows an even greater degree of target flexibility and the snR3 and snR49 snoRNPs have been linked to 3 and 4 pseudouridylation events respectively.[48] In addition to snoRNPs targeting multiple sites in pre-rRNAs, there are also examples of more than one snoRNP targeting the same nucleotide. This can result in redundancy between snoRNPs of the same class in mediating 2′-O-methylation of a particular site (e.g., snR39/snR59; 25S-Am807), but it has also recently been reported that the box H/ACA snoRNA snR9 and the box C/D snoRNA snR65 both target 25S-U2347 leading to the formation of Ψ, Um or Ψm at this site.[21] Furthermore, 25S-U2921 can be methylated by both the snR52 snoRNP and the stand-alone methyltransferase Spb1, which additionally guides modification of the adjacent nucleotide (Gm2922), where its activity is exclusively required.

Computational predictions, often supported by experimental evidence, have enabled the vast majority of snoRNAs to be assigned to one or more modification target(s) in rRNA or small nuclear (sn)RNA (Table 1).[42,49-52] In addition to the U3 and snR30 snoRNAs that play well established roles in regulating rRNA folding rather than guiding modifications, there are 3 more yeast snoRNAs, snR4, snR45 and snR190, for which no cognate rRNA modifications have been identified.[34,53] In contrast, human cells contain a significant number of "orphan" snoRNAs, i.e. snoRNAs for which modification targets have not been identified (76; representing 17% of known human snoRNAs).[54] It was recently suggested that some of these snoRNAs might guide modifications already attributed to other snoRNPs, implying that functional redundancy may be more prevalent than previously thought.[54] The presence of orphan snoRNAs is, however, also likely to reflect the involvement of human snoRNAs in non-ribosome-associated functions, such as the regulation of alternative pre-mRNA splicing, maintaining chromatin structure, and microRNA-dependent gene silencing.[55-57] Recently, some snoRNAs and snoRNA-derived fragments have also been suggested to directly regulate steady-state mRNA levels.[58] Intriguingly, some of the snoRNAs implicated in such functions (e.g., SNORD27, SNORA64, SNORA75 and SNORA44) are also known to guide rRNA modifications, raising the possibility of crosstalk or co-regulation of rRNA modification with these cellular processes.

Base modifications introduced by stand-alone enzymes

Besides the myriad of 2′-O-methylations and pseudouridylations, eukaryotic ribosomes contain 8 different types of base modifications, which are introduced at sites distributed between the small and large ribosomal subunits (Table 1; for a review see ref. [15]). In yeast, the small subunit contains an N^7-methylguanosine (m^7G1575) residue, 2 highly conserved N^6-dimethyladenosine residues (m^6_2A1781 and m^6_2A1782), 2 acetylated cytosine residues (ac^4C1280 and ac^4C1773) and a complex N^1-methyl-N^3-aminocarboxypropylpseudouridine ($m^1acp^3\psi$) hypermodification at position 1191 of the 18S rRNA. Modifications are less diverse on the large subunit, which carries only 2 N^1-methyladenosine (m^1A645 and m^1A2142), 2 N^3-methyluridine (m^3U2634 and m^3U2843) and 2 C^5-methylcytosine (m^5C2278 and m^5C2870) residues. While all the modifications in the 18S rRNA and both the m^5C modifications in the LSU appear to be conserved in humans, only one m^1A at position A1322 (equivalent to A645 in yeast) is present and a single m^3U modification at position U4500 of the 28S rRNA has also been detected.[20,59] It is suggested, however, that in humans both the 18S and 28S rRNAs carry additional modifications, which are not found in yeast.[20]

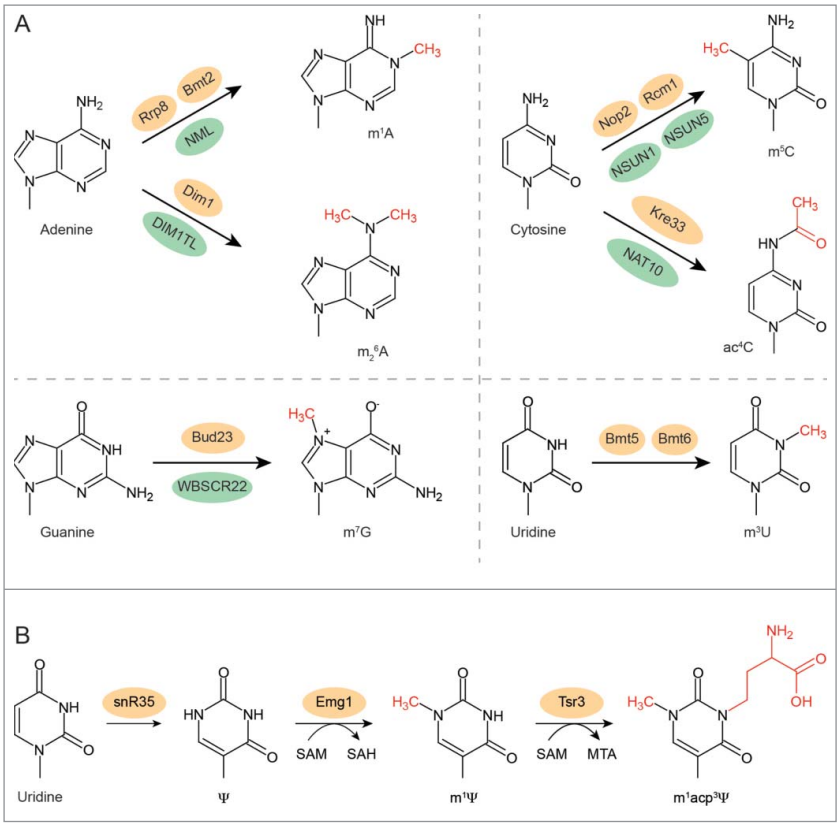

Figure 2. Base modifications in rRNA and the enzymes that install them. (A) Chemical structures of the 4 nucleotides and the modifications that are added in yeast rRNAs. The additional chemical groups are marked in red and the enzymes that introduce them in yeast are indicated above the arrow (yellow) and in humans below the arrow (green). (B) Three-step modification pathway for U1191 of the 18S rRNA in yeast.

The enzymes responsible for introducing the base methylations have been identified in yeast and humans (Table 1; Fig. 2A): Bud23/WBSCR22 (m^7G1575/m^7G1639),[60,61] Dim1/DIMT1L (m^6_2A1781/m^6_2A1850 and m^6_2A1782/m^6_2A1851),[62,63] Rrp8/Bmt1/NML (m^1A645/m^1A1309),[64,65] Bmt2 (m^1A2142),[66] Rcm1/Bmt3/NSUN5/WBSCR20 (m^5C2278/m^5C3761),[67-69] Nop2/Bmt4/NSUN1/NOL1 (m^5C2870/m^5C4413/4),[69,70] Bmt5 (m^3U2634)[71] and Bmt6 (m^3U2843).[71] These RNA methyltransferases belong to the Rossman-like fold family and use S-adenosylmethionine (SAM) as a methyl group donor. In contrast, the multi-step modification of U1191 is initiated by the H/ACA box snoRNP snR35, which catalyzes a pseudouridylation that is an essential pre-requisite for N^1-methylation of this residue by the SPOUT class RNA methyltransferase Emg1/Nep1 (Fig. 2B).[72-76] Recently, it has been discovered that the final step in this modification pathway, the addition of an aminocarboxypropyl group to the N^3 position, is mediated by Tsr3 (Fig. 2B).[77] Analysis of the crystal structure of the archaeal homolog of Tsr3 has indicated that, similar to Emg1, this enzyme has a SPOUT fold and utilises SAM, but that the distinctive mode of SAM binding ensures that the acp moiety rather than a methyl group is transferred to the $m^1\psi$1191 substrate by Tsr3.[77] Furthermore, the classical Gcn5-related acetyltransferase Kre33/Rra1 (NAT10 in humans) has recently been identified as the acetyltransferase responsible for acetylation of the cytosines 1280 and 1773 in helices 34 and 45 of the 18S rRNA.[78,79] Unlike the other base-modifying enzymes, which appear to function exclusively in ribosome biogenesis, Kre33/Rra1/NAT10 also acetylates position 12 of $tRNA^{Ser}$ and $tRNA^{Leu}$, although it requires a specific cofactor, Tan1/THUMPD1 for this function.[79]

Some of the enzymes required for rRNA base modifications in yeast are conserved in higher eukaryotes, while others appear to lack (Bmt2, Bmt5 and Bmt6) human counterparts.[80] Interestingly, the enzymes for which human orthologues are known are essential in yeast, while those that do not have human counterparts are dispensable. This could suggest that the modifications catalyzed by the non-conserved enzymes play less important roles in ribosome function. Several of the enzymes (e.g., Dim1/DIM1TL, Emg1/EMG1, Bud23/WBSCR22) that are conserved in evolution appear, however, to have additional functions in ribosomal subunit assembly as complementation assays using catalytically defective mutants have demonstrated that the presence of these proteins in pre-ribosomal complexes, rather than their catalytic activity, is required for pre-rRNA processing.[60-63,72,74] Nevertheless, this is not always the case as significant defects in pre-60S biogenesis and pre-rRNA processing were observed in yeast strains expressing a catalytically impaired mutant of Nop2.[69] In both yeast and humans, Bud23/WBSCR22 is required for pre-rRNA processing steps that are necessary for nuclear export of pre-SSU complexes,[60,61] while in yeast, Emg1 has been suggested to be required for the recruitment of the ribosomal protein eS19 (Rps19) into pre-SSU complexes.[81] The finding that in addition to catalyzing

modifications, several rRNA methyltransferases are involved in other aspects of ribosomal subunit assembly raises the possibility that the incorporation of the base modifications is coordinated with other steps in the assembly pathway, such as structural remodelling and transport of pre-ribosomal complexes.

Functions of rRNA modifications

Strikingly, neither the snoRNA-guided modifications nor the base modifications introduced by stand-alone enzymes are evenly distributed over the ribosome, but instead they cluster in functionally important regions including the decoding and tRNA binding sites (the A-, P- and E-sites), the peptidyltransferase center and the intersubunit interface (Fig. 3).[6,82] This distinctive spatial distribution, which has been conserved throughout evolution,[17,19] suggests that rRNA modifications play important roles in regulating ribosome function.[82]

Although early studies using catalytically inactive Nop1 and Cbf5 mutants demonstrated the global importance of 2′-O-methylation and pseudouridylation for cell growth and ribosome production,[39,83] loss of only a few individual modifications was found to significantly impact cell viability or ribosome function. A screen for phenotypes in cells depleted of each box C/D snoRNA individually revealed only subtle defects,[84] while the presence of the H/ACA box snoRNA snR191 that guides the modification of 2 highly conserved pseudouridylations (Ψ2258 and Ψ2260) in the intersubunit bridge was found to confer a slight growth advantage compare with a deletion strain in a competition assay.[85] In contrast, deletion of snR35, which initiates the hypermodification of 18S-U1191, has been shown to impair SSU biogenesis significantly.[73] Similarly, expression of a catalytically inactive version of Spb1 strongly affects cell growth and ribosome structure, which is likely to reflect the importance of the Gm2922 modification, but may also be due to the redundant function of Sbp1 in formation of Um2921 as the growth defects observed in strains expressing catalytically inactive Sbp1 have been reported to be exacerbated by deletion of snR52.[86]

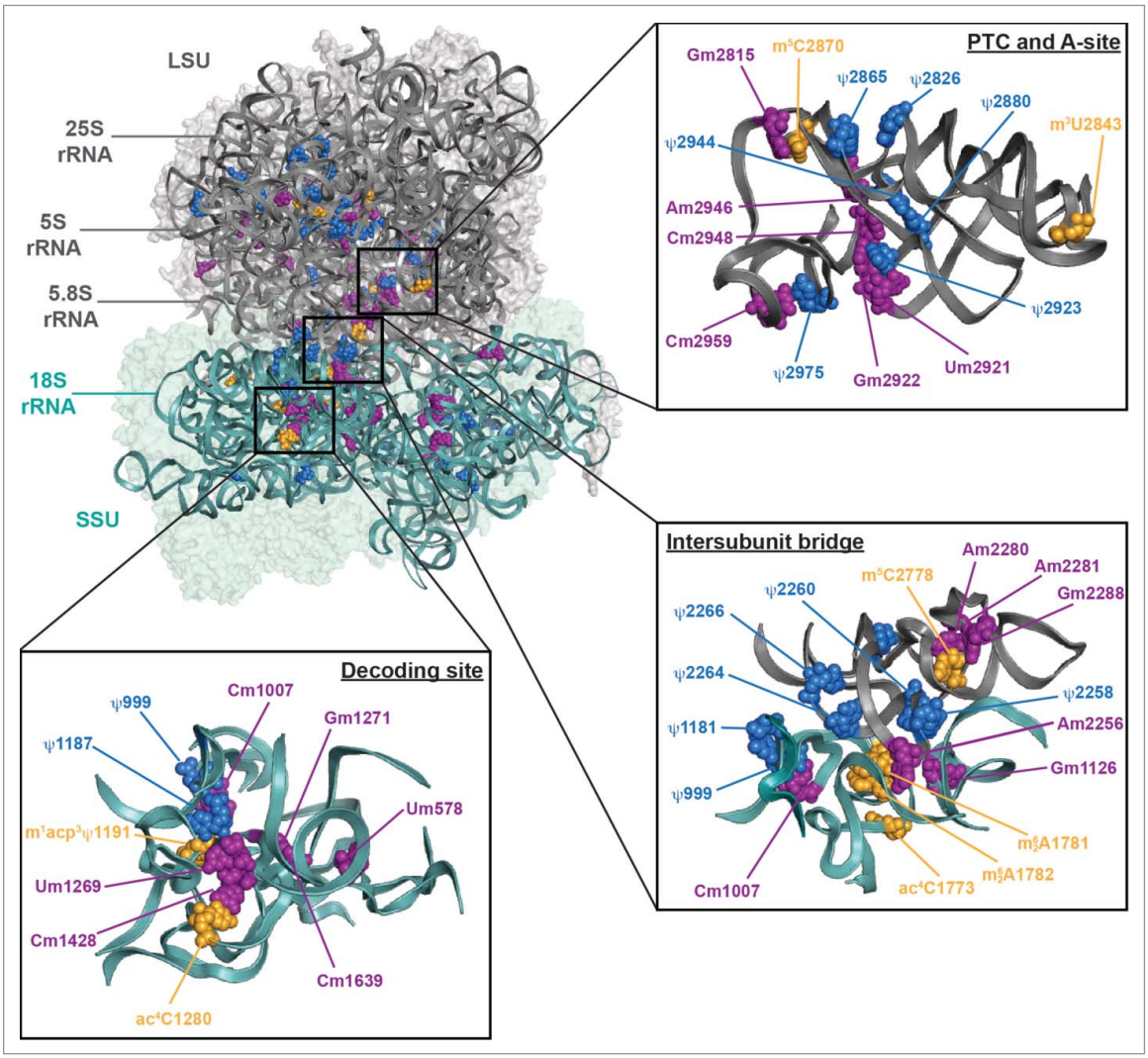

Figure 3. Distribution of rRNA modifications on the yeast ribosome. The *S. cerevisiae* 80S ribosome (PBD 4V88)[6] is shown – 40S in teal and 60S in gray. The positions of 2′-O-methylations (purple), pseudouridines (blue) and base modification installed by stand-alone enzymes (orange) are indicated. Three functionally important regions of the ribosome, the peptidyltransferase center (PTC), the decoding site and the intersubunit bridge eB14, are also shown in a magnified view.

An elegant series of studies conducted by the Fournier lab and others, involving deletions of clusters of modifications in specific functional regions of the ribosome, demonstrated that the modifications instead act in a cumulative manner as significant phenotypes were often only observed upon loss of multiple modifications.[45,73,86-88] Many of the strains in which multiple snoRNAs were deleted showed more severe growth defects and were more sensitivity to ribosome-targeting antibiotics than strains lacking individual snoRNAs. Furthermore, analysis of the global translation rate in these strains by monitoring ^{35}S-labeled amino acid incorporation revealed that 2′-O-methylations and ψ modifications at the P-site and in the vicinity of A-site play an important role in maintaining efficient translation.[73] Similarly, removal of 3 or 4 modifications in helix (H)69 (a part of the intersubunit bridge B2a), or 6 ψs in the PTC caused a significant decrease in the translation rate.[45,88] The importance of these clusters of modifications in translation fidelity has also been investigated and lack of subsets of modifications in the decoding center was shown to impair stop-codon termination and affect reading frame maintenance.[87] Base methylations have also been implicated in translation as defects in protein synthesis were observed in cells expressing a catalytically defective form of Dim1,[89] the loss of Rcm1 was shown to reduce translation fidelity[68] and Emg1 mutants display increased sensitivity to ribosome-targeting antibiotics that influence translation.[90,91]

Excitingly, in addition to maintaining the accuracy and efficiency of global protein synthesis, the functions of rRNA modifications in translation may also extend to include roles in regulating the translation of particular subsets of mRNAs. For example, alterations in rRNA pseudouridylation have been reported to affect translation initiation on specific mRNAs containing internal ribosome entry sites (IRES) by altering the affinity of the ribosome for these mRNA structures.[92-94] Similarly, loss of m^{5}C2278 modification by Rcm1 has been shown to modulate the translation of a subset of mRNAs involved in the oxidative stress response by promoting their recruitment to polysomes.[68] The implications of these findings and their relevance in disease are just beginning to emerge (see below).

The basis of how rRNA modifications affect ribosome function lies in their capacity to expand the topological potential of specific nucleotides. It is generally thought that the chemical modifications present in rRNA serve to stabilize its secondary and tertiary structures.[82,95] In line with this, 2′-O-methylation, which is highly abundant in rRNA, stabilises helices by increasing base-stacking. Similarly, pseudouridylation, which occurs at numerous positions in rRNAs, confers greater hydrogen bonding potential than uridine and also enhances the rigidity of the sugar-phosphate backbone. The base modifications found in rRNA exert similar stabilizing effects on RNA structure but also have distinctive properties. For example, N^7-methylation of guanine generates a quaternary nitrogen that increases the positive charge of the nucleotide thereby promoting ionic interactions between RNAs and proteins, methylation of uridine at the N^3 position promotes hairpin formation and the introduction of m^{5}C increases the stability of base-pairing with guanine.[96-98]

The effect of clusters of modifications on local rRNA structure within the ribosome has been monitored directly by chemical probing techniques. In dimethyl sulfate (DMS) structure probing experiments, lack of a methyl group on U2264 was found to affect the accessibility of A2252 with which U2264 normally base-pairs in H69 of the intersubunit bridge B2a.[88] Importantly, as snoRNAs can also contribute to rRNA folding, this study also included snoRNA mutants that are able to base-pair with the rRNA sequences but which do not guide methylation, enabling the direct influence of the modifications themselves on the RNA structure to be ascertained. Similarly, loss of 6 Ψs in the PTC or the deletion of both snR52 and Spb1 alters the protection pattern observed, indicating structural changes in the corresponding regions of the PTC.[45,86] Structural changes observed in ribosomes lacking both the m^{5}C2278 base modification and the ribose methylation at G2288 have been shown to cause decreased ribosome stability and loss of ribosomal proteins from the LSU.[67] This link between rRNA modification and ribosome integrity is consistent with an earlier report that loss of a cluster of modifications in H69 decreases rRNA levels as a result of increased RNA turnover.[88]

In addition to their functions in modulating local rRNA structure, rRNA modifications are proposed to participate in communication between distant regions of the ribosome. On one hand, this is likely to result from alterations in the dynamics of rRNA folding and ribosome assembly, as loss of an individual snoRNA can affect the extent of modification not only at its target site but also at other positions on the ribosome.[18] On the other hand, long-range effects have also been suggested to arise from interactions between rRNA modifications and ribosomal proteins.[15] For example, the m^{5}C2278 modification in the LSU and the ac^{4}C1773 and m$^6_2$ A1781- m$^6_2$ A1782 modifications in the SSU are directly contacted by the ribosomal protein eL41 (Rpl41), which forms a bridge between the LSU to the decoding center in the SSU and conducts structural information between the subunits.[6] Interestingly, this ribosomal protein also acts as a pivot for 40S subunit rotation during translation, suggesting a mechanism by which monitoring for the presence of rRNA modifications is coupled with translation efficiency via ribosomal protein interactions.[15,99]

In summary, the minimal set of chemical modifications found in rRNA and the specific sites of rRNA modification appear to have been carefully selected during evolution to allow formation of a stable ribosome structure. A core set of modified sites, conserved from prokaryotes, likely render the ribosome capable of efficient and accurate translation, while the additional modifications found in higher organisms might not only reflect the increased size and complexity of the eukaryotic ribosome structure, but might also enable fine-tuning of the eukaryotic translation cycle to modulate gene expression.

Timing and regulation of rRNA modifications

rRNA modifications are introduced at different stages during the maturation of the ribosomal subunits. Kinetic labeling in yeast has revealed that the vast majority of 2′-O-methylations in the 18S rRNA are introduced co-transcriptionally, while such methylations are introduced both co- and post-transcriptionally into the 25S rRNA.[100] A recent study comparing the 2′-O-methylation status of chromatin-associated RNA with that of total RNA supported this conclusion and also provided site-specific information about the timing of individual

modifications.[18] In the 18S rRNA, only one modification, Am100 occurs after release of the nascent transcript from the rDNA, while the extent of 2′-O-methylation of A817, G867, A876, A2256, U2421 and A2640 was significantly higher in the mature 25S rRNA than in the chromatin-associated RNA. Another notable exception is the 2′-O-methylation of G2922 by the stand-alone methyltransferase Sbp1, which has been reported to occur during maturation of the 27S precursor rRNA.[37] Consistent with this, the recent structure of a 90S pre-ribosomal complex from *Chaetomium thermophilum* did not contain any snoRNPs (except the U3 snoRNP, which does not mediate rRNA modification), implying that most snoRNPs have already dissociated from the pre-rRNA transcript when it is released from the rDNA.[101] The finding that snoRNP-mediated modifications are largely introduced during the early stages of ribosome biogenesis, when the pre-ribosomal complexes are thought to have a more open structure, is fitting with the snoRNA-rRNA base-pairing mechanism by which Nop1 finds its target sites and may also reflect the secondary function of snoRNAs in assisting rRNA folding. By analogy, it is assumed that H/ACA box snoRNP-mediated pseudouridylations also occur at an early stage of ribosome biogenesis but this has not been experimentally demonstrated so far. It is also not known if pseudouridylation of the 5S rRNA by Pus7 takes place before or after integration of the 5S RNP (consisting of the 5S rRNA and the ribosomal proteins uL5 (Rpl11) and uL18 (Rpl5)) into the pre-LSU.

In human cells, the majority of snoRNA-guided modifications are also likely to occur on early pre-ribosomal complexes, however, some snoRNAs have been shown to associate with later pre-SSU particles.[102] Remarkably, the modifications guided by these snoRNAs lie within the binding site of the RNA helicase DDX21.[102] This suggests a model in which the activity of the helicase is required for structural rearrangement of pre-ribosomal particles to enable these late-acting snoRNAs to access their pre-rRNA target sites. In yeast, the RNA helicase Prp43 has also been implicated in enabling snoRNA recruitment, as the levels of snR64 and snR67 on pre-ribosomes are reduced when Prp43 is depleted.[103] Furthermore, decreased 2′-O-methylation of C2377, the target nucleotide of snR64, is observed when a catalytically inactive version of Prp43 is expressed.[104]

Taken together, the findings that most 2′-O-methylations and pseudouridylations occur during the short timeframe of pre-rRNA transcription, that these modifications are densely clustered, and that snoRNAs form extensive and often overlapping base-pairing interactions with the rRNA sequences, imply that in many cases individual modifications must be introduced in a stepwise manner. However, whether the association of particular snoRNAs with their pre-rRNA base-pairing sites occurs stochastically or if there is a defined hierarchy for snoRNA recruitment to pre-ribosomal complexes currently remains unclear. It has been shown, however, that knock-in of an artificial snoRNA that guides a 2′-O-methylation at the P-site and which has overlapping base-pairing sites with natural snoRNAs that guide 3 neighboring methylations reduces the adjacent modifications.[18] This suggests that in some cases, snoRNAs may compete for their pre-rRNA basepairing sites and that the presence of one snoRNA can impede the modification of a nearby site guided by another snoRNA. Similarly, snR9 and snR65 have been suggested to compete for access to U2347, leading to the formation of Um, Ψ and Ψm at this site.[21]

In contrast to the 2′-O-methylations and pseudouridylations that are mostly introduced during the early stages of ribosomal subunit maturation, base modifications are generally thought to occur later, but the precise timing of most of them has not been determined so far. In the case of the N^3-acp modification of 18S-$m^1\Psi$1191, however, the exclusively cytoplasmic localization of the Tsr3 enzyme that installs this modification clearly identifies this as a late event in yeast.[77] Furthermore, early cytoplasmic pre-40S complexes (isolated via the shuttling biogenesis factors Enp1 and Ltv1) show low levels of N^3-acp modification of 18S-$m^1\Psi$1191, while in later particles (isolated via Nob1 and Rio1), this position is acp-modified to a similar extent as the mature 18S rRNA, indicating that the final step in the hypermodification pathway of U1191 occurs just before cleavage of the 3′ end of the 18S rRNA by Nob1.[105,106] Similarly, the m^6_2A1781 m^6_2A1782 dimethylation close to the 3′ end of the 18S rRNA is introduced by Dim1 after the export of pre-SSU complexes to the cytoplasm, but in contrast to Tsr3, Dim1 is already bound to early pre-ribosomal complexes in the nucleolus.[62] It is suggested that the association of Dim1 with nucleolar pre-ribosomes acts as a surveillance mechanism in which pre-ribosomes that cannot be dimethylated, and will therefore be impaired in translation, are targeted for degradation.[89] Furthermore, structural analysis of the pre-ribosomal binding site of Dim1 demonstrated that the presence of the RNA methyltransferase on the SSU inhibits its association with the LSU.[107,108] This suggests that the presence of Dim1 in late pre-ribosomal complexes may serve a second quality control function by preventing premature subunit joining and immature ribosomal complexes engaging in translation. Interestingly, modification of the corresponding positions in human cells by DIMTL1 occurs in the nucleus, although the relevance of this difference in timing of the modification is currently unknown.[63] While the 18S-m^7G1575 modification is installed by Bud23 in the nucleus, it was recently demonstrated that this modification occurs on the 20S pre-rRNA during the later stages of pre-SSU biogenesis.[109] So far, Bud23 is the only rRNA methyltransferase that has been shown to require a cofactor (Trm112) to achieve its modification. In the Bud23-Trm112 complex, rather than stimulating the catalytic activity of Bud23, the cofactor is required for stability of the methyltransferase.[109,110] Furthermore, Bud23-Trm112 binds directly to the RNA helicase Dhr1[111] and, similar to the proposed role of RNA helicases in enabling some snoRNAs to access their base-pairing sites, Dhr1 is required for recruitment of Bud23-Trm112 to pre-ribosomal complexes.[109] Efficient acetylation of the 18S rRNA by Kre33 has also been shown to involve helicase action, but in this case the activity is derived from a RecA domain within the acetyltransferase itself.[79]

The alternative mechanisms (snoRNA-guided and protein-only) by which rRNA modifying enzymes recognize their targets are broadly employed to introduce modifications at different stages during ribosome maturation. Although little is known about how stand-alone enzymes identify their sites of action, it is possible that sequence specificity, RNA secondary structures or multiple contacts in the broader context of the pre-ribosome are required for

target identification and/or catalysis. Furthermore, an emerging principle that applies to both snoRNA-guided modifications and those installed by stand-alone enzymes is that late-acting enzymes or snoRNPs often require the coordinated action of an RNA helicase to enable target site access. The highly structured nature of pre-ribosomal complexes, compare with other RNAs/RNPs that are subjected to modification, may make this close co-ordination between modifying enzymes and helicases uniquely important for rRNA modifications.

Partially modified sites in rRNA

An exciting development in the field of RNA modification arose recently with the detection of sites of partial (also termed fractional or substoichiometric) modification in rRNA, challenging the long-held belief that rRNA modifications are constitutively present.[18,21,112] While under normal growth conditions all the positions carrying base modifications (except Ψ) are almost fully modified, sites of partial 2′-O-methylation and pseudouridylation have been identified.[18,21,113] More specifically, it was recently reported that of the 112 known sites of modification in yeast, 18 are modified on less than 85% of ribosomes.[21] Similarly, in human cell lines, approximately one-third of 2′-O-methylations appear to be substoichiometric.[32] These observations highlight rRNA modification as a major source of ribosome heterogeneity and contribute to the mounting evidence for specialized ribosomes. Diversity among ribosomes on the rRNA level has long been known, because alternatively processed forms of the 5.8S rRNA are present in polysome-associated large ribosomal subunits, however, it is suggested that this is a constitutive difference rather than a dynamic one.[114] Differences in ribosome composition also arise through the incorporation of alternative ribosomal protein isoforms (which exist due to ribosomal protein gene duplication) and ribosomal proteins that have undergone differential post-translational modifications such as phosphorylation, ubiquitination and acetylation (for a review see refs. 115–118).

The major question of what determines the extent of modification of these sites still remains open and it is likely that several alternative mechanisms exist. It is known that snoRNA levels can vary in cells, so one possibility is that limiting amounts of certain snoRNAs can determine the extent of 2′-O-methylation or pseudouridiylation at specific positions. Consistent with this model, the substoichiometric 2′-O-methylation of the 18S rRNA at position A100 has been shown to correlate with the low cellular levels of snR51 that guides this modification as overexpression of the snoRNA leads to complete methylation of 18S-A100.[113] Another possibility is that the assembly of specific snoRNAs into functional snoRNPs is restricted as "activation steps," including the removal of a placeholder assembly factor, have been identified in the assembly pathways of both box C/D and box H/ACA snoRNPs.[114,119,120] Also, structural elements within the snoRNAs guiding these modifications may share a common feature that limits the extent of modification. For box C/D snoRNAs in HeLa cells, this possibility was examined in detail considering both the conservation of the D/D′ motifs as well as the "strength" of the guiding sequences and no clear correlations could be detected.[32] As snoRNP-mediated substoichoimetric modifications mostly occur co-transcriptionally, partial modifications might arise due to changes in the accessibility of target or base-pairing sites during the highly dynamic process of ribosome maturation. Alternative rRNA folding pathways might result in modifications not taking place if particular regions of the ribosome have already acquired stable structures or ribosomal proteins have been assembled. This notion is supported by the observation that several partially modified sites are in close proximity (e.g., 18S-Am438 (73%) and 18S-Ψ468 (60%); 25S-Gm867 (78%) and 25S-Am876 (75%))[21] but this finding is also in line with a model where competition between snoRNAs for base-pairing sites might limit the extent of modification in densely modified regions of the rRNA. Comparison to the recently described dynamic and reversible modification of other RNA species including mRNAs by demethylases such as FTO/ALKBH5[121] also raises the possibility that some partial rRNA modifications might be the result of selective removal. To date, however, enzymes capable of "erasing" Ψ or Nm have not been identified and it is very likely that the complex tertiary structure and protein-rich surface of the ribosome would impede the access of such erasers to modified residues that are mostly buried in the functional core of the ribosome.

It is not yet clear how different rRNA modification patterns affect ribosome function. It has been proposed that the constitutively modified positions, which are generally the most conserved, serve to stabilize the core of the ribosome, while the positions that are modified in some species but not others contribute to fine-tuning of ribosome function for optimal translation accuracy.[32] It will also be interesting to see if the subpopulations of differently modified ribosomes perform specific functions in the cell. For example, localized translation at the endoplasmic reticulum might be driven by a subset of ribosomes with a particular rRNA modification pattern.

Intriguingly, as well as variations in the extent of modification of specific sites in rRNAs under normal growth conditions, the degree of modification at some positions has been found to change in response to environmental changes. For example, after diauxic shift a 2-fold change in the extent of 25S-Ψ2314 was detected[122] and heat-shock was observed to alter the fraction of 5S-Ψ50 that is modified by Pus7.[123] The latter observation might, however, reflect a change in the subcellular localization of Pus7 or the distribution of this multifunctional enzyme between its rRNA and mRNA targets, rather than a direct role for 5S-Ψ50 in optimising ribosome function under these conditions. Interestingly, sites of inducible pseudouridylation have been identified in snRNAs upon nutrient stress,[124,125] raising the possibility that additional modifications may likewise be incorporated into some rRNAs under certain physiological conditions. Together, these findings suggest that partial modification of rRNA nucleotides may not only optimise the function of different populations of ribosomes but that these modifications can also be regulated, which could allow adaption of translation and the cellular proteome in response to specific intracellular or environmental cues.

rRNA modifications in development and disease

An increasing body of evidence links defects in components of the rRNA modification machinery and changes in the extent of rRNA modifications to development, genetic diseases and cancer. For example, loss-of-function of any of 3 individual snoRNAs has been found to cause profound developmental defects

in zebrafish.[126] Examples of disease-associated abnormalities that affect stand-alone rRNA-modifying enzymes include a point mutation in the *EMG1* gene that causes Bowen-Conradi syndrome and deletion of a chromosomal region encompassing the *WBSCR22* and *WBSCR20/NSUN5* genes in Williams-Beuren syndrome.[127-129] In addition, NML is associated with high-fat diet-induced obesity and NSUN5 is reported to increase lifespan and stress resistance in some organisms.[68,130] It is, however, unclear whether the lack of the modifications mediated by these enzymes is the cause of pathogenicity or whether these diseases could also be attributed to a general decrease in ribosome levels or deletion of other essential genes in the vicinity. Nevertheless, it is also possible that loss of the modifications introduced by these enzymes affects ribosome function, leading to changes in translation and the cellular proteome, thereby indirectly causing the disease phenotype.

Furthermore, changes in the expression levels and activity of core components of both box H/ACA and box C/D snoRNPs in disease have been reported. For example, the box C/D snoRNP component NOP56 is overexpressed in Burkitt's lymphoma-associated c-Myc mutants[131] and NOP58 mRNA levels are elevated in metastatic melanoma lesions,[132] making these proteins good markers of cancer progression.[133,134] It is possible, however, that these changes in protein expression level might primarily reflect an upregulation of ribosome synthesis during cell proliferation. Similarly, many snoRNAs are dysregulated in cancers (for a review see refs. 135,136), but whether this actively promotes tumorigenesis remains unknown, and if so, it is unclear whether this is due to a change in rRNA modification.

Remarkably, mutations in genes encoding the catalytic snoRNP proteins and changes in their expression level have been linked to widespread changes in the rRNA modification pattern that are reported to influence ribosome function and thereby cause tumorigenesis. For example, mutations in the box H/ACA pseudouridine synthetase dyskerin are found in X-linked Dyskeratosis congenita (X-DC), a severe disorder that is characterized by bone marrow failure, lung fibrosis and increased susceptibility to cancer.[137] These mutations reduce rRNA pseudouridylation[138,139] and although this does not change the overall rate of protein synthesis, it is suggested to selectively influence the translation of a specific subset of mRNAs that contain IRES elements. Interestingly, in X-DC, the translation of mRNAs coding for the tumor suppressor proteins p53 and p27 as well as the anti-apoptotic factors Bcl-xL and XIAP is impaired,[94,140,141] while IRES-dependent translation of the growth factor VEGF mRNA is promoted.[142] Together, these finding suggest that the altered rRNA pseudouridylation profile caused by the dyskerin mutations may reprogram the ribosome to promote tumorigenesis, thereby explaining the increased cancer susceptibility of X-DC patients.

Changes in the rRNA 2′-O-methylation pattern have also been linked to cancer and differences in the extent of rRNA modification at several sites have been observed in different cancer cell lines.[32] Mutations in TCOF1/treacle that are found in Treacher Collins syndrome, a ribosomopathy that predisposes patients to cancer, have been reported to impair 2′-O-methylation of rRNA.[143] Furthermore, in mammary epithelial cells, expression of the box C/D methyltransferase fibrillarin was suggested to be regulated by the tumor suppressor p53 and fibrillarin-dependent defects in translational fidelity were described in cells lacking p53.[144] Reciprocally, depletion of fibrillarin has been proposed to cause an increase in p53 expression levels[145] and surprisingly, this response was suggested to not only be mediated by the nucleolar stress response pathway involving binding of the 5S RNP to HDM2 when ribosome assembly is perturbed,[146,147] but also by an increase in the cap-independent translation of the p53 mRNA.[145] Also, an RNAi-based synthetic interaction screen identified the gene encoding the box C/D snoRNP assembly chaperone NOLC1/NOPP140 as a p53 target gene,[148] indicating that regulation of snoRNP biogenesis might be an alternative mechanism by which p53 could regulate rRNA 2′-O-methylation and cellular proliferation.

Many studies have highlighted the central role of ribosome synthesis and function in disease.[149] Ribosome assembly is regulated by key proto-oncogenes such as $p14^{ARF}$ and *c-MYC*[150] and defects in ribosome assembly, including those found in ribosomopathies, regulate the tumor suppressor p53.[151] Several recent reports indicate, however, that during development and disease, both global and site-specific alterations in rRNA modification may be induced by key cell signalling pathways and also that changes in the rRNA modification pattern might be important regulators of cellular transformation.

Concluding remarks

Since the first mapping of pseudouridines in eukaryotic rRNA more than 20 years ago, much has been revealed about the types of modifications present on the rRNAs, their number and positions, the enzymes that install them and their functions. Interestingly, despite the presence of modified rRNA nucleotides in all 3 phylogenetic kingdoms, the relative amounts of different classes of modification vary considerably throughout evolution. In bacteria, the majority of rRNA modifications are base methylations and a greater diversity of modifications are present in bacterial ribosomes than in ribosomes of any other taxa.[152] In contrast, in thermophillic archaea (such as *Sulfolobus solfataricus*) and in eukarya, 2′-O-methylations exceed base methylations by almost 10-fold.[21,153] This difference is likely to reflect the key role of 2′-O-methylation in stabilization of RNAs, which is important for the structural fidelity of archaeal ribosomes at higher temperatures and the larger eukaryotic ribosomes. Furthermore, the mechanisms by which 2′-O-methylations and pseudouridylations are introduced have diverged during evolution; in bacterial ribosomes they are introduced by stand-alone enzymes, whereas these modifications are mediated by sRNPs and snoRNPs in archaea and eukarya respectively. It is possible that this difference may arise due to the increase in the number of such modifications in these species and therefore the need for a strategy by which a single modifying enzyme can be directed to modify multiple specific sites within the rRNAs. To date, no rRNA modifications have been detected in the spacer fragments that separate the mature rRNA sequences in the pre-rRNA transcripts, but instead, in all species, rRNA modifications are found in the mature rRNAs at positions that form functionally relevant sites in the ribosome. Indeed, the structures of prokaryotic and eukaryotic ribosomes have enabled vizualization of the clustering of modifications in functionally important regions of the ribosome, such as the peptidyltransferase center and decoding site, paving the

way for the identification of the collective and conserved role of these modifications in enabling efficient and accurate translation.

The development of sensitive, unbiased, systematic methods for the identification of rRNA modifications has yielded updated inventories of the sites of rRNA modification in eukaryotes. These methods, combined with the advanced computational predictions of snoRNA base-pairing sites and yeast genetics, have provided a comprehensive picture of the stand-alone enzymes and snoRNP complexes responsible for installing these modifications in yeast. We have also gained substantial insights into the diverse timing of rRNA modifications and an understanding of how some modifying enzymes are regulated. Notably, enzymes that install modifications during the later stages of ribosome assembly often require the coordinated action of an RNA helicase, presumably to facilitate access to their target sites on a compact pre-ribosome in which many complex rRNA folds present in the mature ribosome have already been established.

Most recently, the identification of sites of partial modification has overturned the view that all rRNA modifications are constitutive features and highlighted them as an important source of ribosome diversity, alongside differential protein composition and alternative pre-rRNA processing pathways. The ribosome heterogeneity arising from partial modifications, together with the emerging concept that alterations in the rRNA modification profile may selectively adapt the ribosome to translate specific subsets of mRNAs, could suggest the existence of a new layer of translational control of gene expression. This additional level of regulation of gene expression may be particularly relevant in higher eukaryotes as during evolution there has been relative increase in the number of pseudouridylations and 2′-O-methylations, which can be substoichiometric, compare with base modifications that, at least under optimal growth conditions, appear to be constitutive. In the future, it will be very interesting to see whether the ribosome sub-populations characterized by particular rRNA modification patterns perform specific functions within the cell, for example in localized translation or in promoting/repressing translation of particular subsets of mRNAs. It will also be important to explore potential changes in the rRNA modification pattern in different cell types (in particular in primary cells and tissues), in response to environmental and developmental cues, and in disease, as this will provide a basis for understanding the role of rRNA modifications in regulating translation in order to adapt the cellular proteome.

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (SPP 1784: BO3442/2–1 to M.T.B., EN134/12–1 and EN134/13–1 to K.D.E.), the Alexander von Humboldt Foundation (post-doctoral fellowship to K. E.S. and M.T.B), the European Molecular Biology Organization (post-doctoral fellowship ALTF644–2014 to S.S.), the Université Libre de Bruxelles (ULB), the Fonds National de la Recherche (F.R.S/FNRS), the Walloon Region (DGO6), the Fédération Wallonie-Bruxelles, and the European Research Development Fund (ERDF) (to D.L.J.L.), the State of Hesse and Goethe University Frankfurt (to K.-D.E.) and the Faculty of Medicine, Georg-August–University Göttingen (M.T.B).

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