Review

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# Modifications in small nuclear RNAs and their roles in spliceosome assembly and function

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**Abstract:** Modifications in cellular RNAs have emerged as key regulators of all aspects of gene expression, including pre-mRNA splicing. During spliceosome assembly and function, the small nuclear RNAs (snRNAs) form numerous dynamic RNA-RNA and RNA-protein interactions, which are required for spliceosome assembly, correct positioning of the spliceosome on substrate pre-mRNAs and catalysis. The human snRNAs contain several base methylations as well as a myriad of pseudouridines and 2′-*O*-methylated nucleotides, which are largely introduced by small Cajal body-specific ribonucleoproteins (scaRNPs). Modified nucleotides typically cluster in functionally important regions of the snRNAs, suggesting that their presence could optimise the interactions of snRNAs with each other or with pre-mRNAs, or may affect the binding of spliceosomal proteins. snRNA modifications appear to play important roles in snRNP biogenesis and spliceosome assembly, and have also been proposed to influence the efficiency and fidelity of pre-mRNA splicing. Interestingly, alterations in the modification status of snRNAs have recently been observed in different cellular conditions, implying that some snRNA modifications are dynamic and raising the possibility that these modifications may fine-tune the spliceosome for particular functions. Here, we review the current knowledge on the snRNA modification machinery and discuss the timing, functions and dynamics of modifications in snRNAs.

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## Introduction

In eukaryotes, the removal of intron sequences from pre-mRNAs by splicing is a key step in gene expression that is mediated by a large and dynamic ribonucleoprotein (RNP) complex termed the spliceosome. The major spliceosome, which is composed of five small nuclear RNPs (snRNPs; U1, U2, U4, U5 and U6) and various additional proteins, is responsible for excision of >99% of all pre-mRNA introns (U2-type). A functionally similar, minor spliceosome composed of the U11, U12, U4atac and U6atac snRNPs together with the U5 snRNP mediates removal of a specific subset of atypical introns (U12-type). The step-wise assembly of the major spliceosome on the precursor messenger RNA (pre-mRNA) substrate has previously been described in detail (see Wills and Lührmann, 2011; Hoskins and Moore, 2012; Matera and Wang, 2014, and references therein). In brief, it is initiated by the recruitment of the U1 snRNP and the formation of basepairing interactions between the 5′ end of the U1 small nuclear RNA (snRNA) and the 5′ splice site (5′SS) of the pre-mRNA. This is followed by association of the U2 snRNP, in which the U2 snRNA basepairs with the branch site sequence (BSS), causing extrusion of the branch site nucleotide (typically adenosine). Recruitment of a pre-assembled tri-snRNP complex, composed of the U4/U6.U5 snRNPs, leads to formation of the B complex, which undergoes a series of conformational rearrangements, triggering release of the U1 and U4 snRNPs. Further remodelling events generate a catalytically active complex (B*) that mediates the first transesterification step of splicing involving nucleophilic attack of the 2′ hydroxyl group of the branch site adenosine on the 5′SS. This leads to formation of complex C and the second splicing reaction in which the 3′ hydroxyl of the free 5′ exon attacks the phosphodiester bond at the intron-lariat to 3′ exon boundary to produce the spliced mRNA, the excised intron lariat and

the post-spliceosomal complex, which is finally disassembled enabling the U2, U5 and U6 snRNPs to be recycled.

The snRNAs play critical roles in positioning the spliceosome on the substrate pre-mRNA and in some cases, also contribute to the catalytic reactions. During their maturation, the snRNAs undergo numerous processing and folding steps as well as associating with many proteins to form functional snRNPs. Spliceosome assembly and function involves dynamic basepairing interactions between snRNAs, (e.g. U4 and U6 within the tri-snRNP), and also between snRNAs and the pre-mRNA (e.g. U1 and the 5′SS, and U2 and the BSS), as well as a myriad of RNA-protein interactions. Fine-tuning these interactions is likely important for maintaining the efficiency and fidelity of pre-mRNA splicing. The snRNAs contain numerous modified nucleotides that are proposed to contribute to such optimisation (Karijolich and Yu, 2010). Here, we will review the knowledge on the sites and functions of modifications in snRNAs, and the enzymes that install them. We focus primarily on modifications in human snRNAs, but also discuss examples from other species to highlight conceptually interesting features of snRNA modifications.

## snRNA modifications and the enzymes that install them

Synthesis of U6 and U6atac is mediated by RNA polymerase III (Pol III) and these transcripts are substrates of the capping enzyme MEPCE, which installs a monomethylated guanosine triphosphate cap (Singh and Reddy, 1989). In contrast, all other snRNAs are produced by RNA polymerase II (Pol II) and are initially capped at their 5′ ends with an $N^7$-methylguanosine ($m^7G$) installed by the coordinated action of RNGTT (RNA 5′ triphosphatase and RNA guanylyltransferase; Yue et al., 1997) and the RNMT-RAM complex (RNA methyltransferase; Tsukamoto et al., 1998), which is subsequently converted to a 2,2,7-trimethylguanosine (TMG; $m_3G$) cap by the action of trimethylguanosine synthetase TGS1 (Mouaikel et al., 2002). Analogous to many pre-mRNAs, these snRNAs are also subjected to ribose 2′-*O*-methylation of the cap proximal nucleotides (cap+1 and cap+2) by the stand-alone RNA methyltransferases CMTR1 and CMTR2, respectively (Werner et al., 2011; Smietanski et al., 2014).

The most abundant internal snRNAs modifications are 2′-*O*-methylations (Nm) of the ribose moieties of any

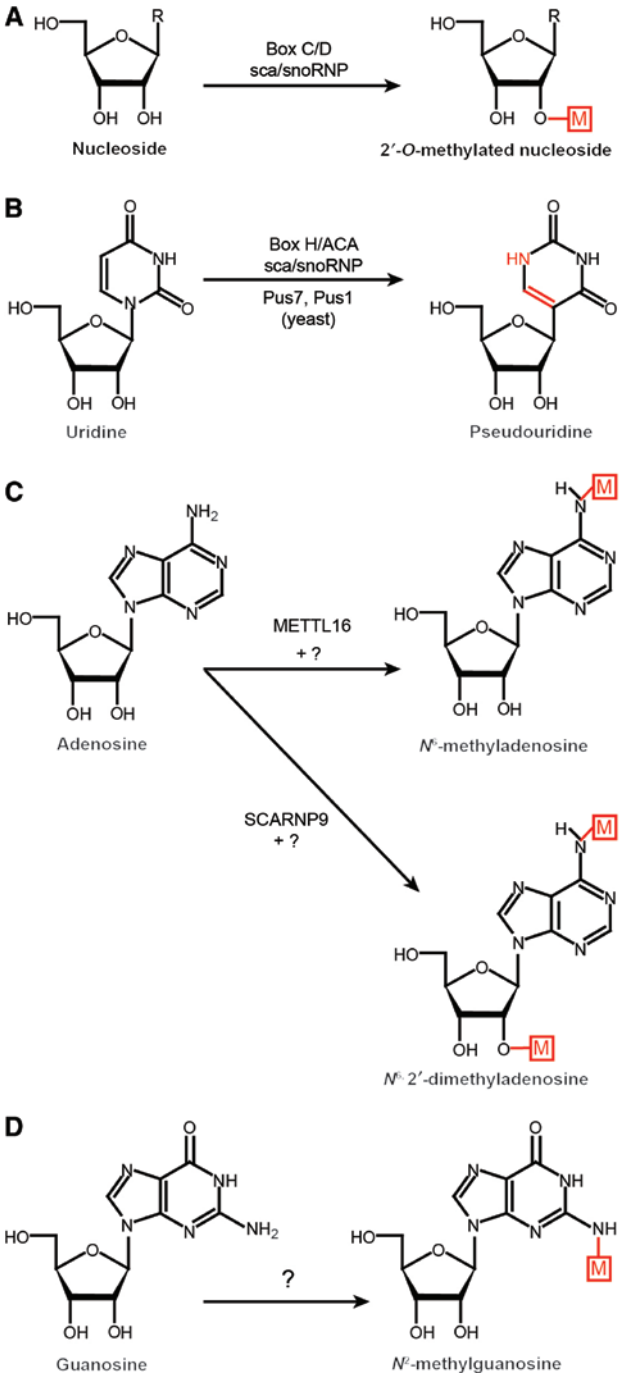


**Figure 1:** Modification pathways for nucleosides present in human snRNAs.
(A) The isomerisation of uridine (U) to pseudouridine (Ψ). (B) Methylation (boxed M) of the 2′ hydroxyl group of the ribose moiety of a nucleoside (R = any base). (C) $N^6$-methylation and $N^6$,2′-*O*-dimethylation of adenosine. (D) $N^2$-methylation of guanosine. Enzymes known to be responsible for installing these types of modification in human or yeast snRNAs are indicated above or below the arrows and unknown enzymes are indicated by '?'.

nucleotide [guanosine (G), cytidine (C), adenosine (A), uridine (U); Figure 1A] and the isomerisation of uridine to pseudouridine (Ψ; Figure 1B). To date, excluding the cap-proximal modifications, 22 2′-*O*-methylations and 24 pseudouridines have been detected in the human snRNAs of the major spliceosome, while only four pseudouridines and two 2′-*O*-methylations are present in the U4atac, U6atac and U12 snRNAs of the human minor spliceosome (Table 1; Massenet and Branlant, 1999; Karijolich and Yu, 2010; Krogh et al., 2017a). In human cells, these modifications, like the vast majority of 2′-*O*-methylations and pseudouridines in the ribosomal RNAs (rRNAs; Sloan et al., 2017), are installed by RNA-guided modification enzymes. 2′-*O*-methylations are introduced by fibrillarin (Nop1 in the yeast *Saccharomyces cerevisiae*), which assembles with the scaffold proteins NOP56, NOP58 and 15.5 K (Snu13 in yeast) and a box C/D guide RNA that base-pairs with the snRNA to direct modification of a specific target nucleotide (Figure 2A–D). Similarly, pseudouridylation is mediated by the pseudouridine synthase dyskerin (Cbf5 in yeast) within of a complex composed of NOP10, NHP2, GAR1 and a box H/ACA guide RNA (Watkins and Bohnsack, 2012). Such RNPs predominantly localise to either the nucleolus or to nuclear foci termed Cajal bodies (CBs) and are therefore designated as small nucleolar RNPs (snoRNPs; Figure 2A and B) or small Cajal body-specific RNPs (scaRNPs; Figure 2C and D) respectively. Both snoRNAs and scaRNAs share common features, such as the C/C′, D/D′, H and ACA boxes, but additional conserved sequence motifs (5′-UGAC-3′ (CAB box) in H/ACA scaRNPs and G•U/U•G wobble stem in C/D scaRNPs; Figure 2C and D) are present in scaRNPs (Richard et al., 2003; Marnef et al., 2014). These sequences are bound by the WD40 repeat protein WDR79 (albeit with low affinity in the case of the C/D scaRNPs) and this contributes to the localisation of these complexes to Cajal bodies (Tycowski et al., 2009).

**Table 1:** Internal modifications in human snRNAs.

| snRNA | Mod. | Enzyme | Guide RNA | snRNA | Mod. | Enzyme | Guide RNA |
|---|---|---|---|---|---|---|---|
| U1 | Ψ5 | Dyskerin | SCARNA16 | U4 | Ψ79 | ? | ? |
| | Ψ6 | Dyskerin | SCARNA18 | | $m^6A100$ | ? | ? |
| | Am70 | Fibrillarin | SCARNA7 | U5 | Gm37 | ? | ? |
| U2 | Ψ6 | ? | ? | | Um41 | Fibrillarin | SCARNA5/6 |
| | Ψ7 | Dyskerin | SCARNA14 | | Ψ43 | Dyskerin | SCARNA11 |
| | Ψ15 | ? | ? | | Cm45 | Fibrillarin | SCARNA10 |
| | Gm11 | Fibrillarin | SCARNA2 | | Ψ46 | Dyskerin | SCARNA10/12 |
| | Gm12 | ? | ? | | Ψ53 | Dyskerin | SCARNA13 |
| | Gm19 | Fibrillarin | SCARNA9 | U6 | Ψ31 | Dyskerin | SNORA7 |
| | Gm25 | Fibrillarin | SCARNA2 | | Ψ40 | Dyskerin | SCARNA23/3 |
| | $m^6Am30$ | Fibrillarin/? | SCARNA9/- | | $m^6A43$ | METTL16 | – |
| | Ψ34 | Dyskerin | SCARNA8 | | Am47 | Fibrillarin | SNORD7 |
| | Ψ37 | Dyskerin | SCARNA15 | | Am53 | Fibrillarin | SNORD8/9 |
| | Ψ39 | Dyskerin | SCARNA4 | | Gm54 | ? | ? |
| | Cm40 | ? | ? | | Cm60 | Fibrillarin | SNORD67 |
| | Ψ41 | Dyskerin | SCARNA4 | | Cm62 | Fibrillarin | SNORD94 |
| | Ψ43 | ? | ? | | Cm63 | ? | ? |
| | Ψ44 | Dyskerin | SCARNA8 | | Am70 | ? | ? |
| | Um47 | Fibrillarin | SCARNA28 | | $m^2G72$ | ? | ? |
| | Ψ54 | Dyskerin | SCARNA13 | | Cm77 | Fibrillarin | SNORD10 |
| | Cm61 | | SCARNA2 | | Ψ86 | Dyskerin | SNORA79 |
| | Ψ88 | ? | ? | U4atac | Ψ12 | ? | ? |
| | Ψ89 | Dyskerin | SCARNA1 | | Gm19 | ? | ? |
| | Ψ91 | ? | ? | | Ψ83 | ? | ? |
| U4 | Ψ4 | ? | ? | U6atac | Ψ19 | Dyskerin | SCARNA21 |
| | Cm8 | Fibrillarin | SCARNA17 | | Gm22 | Fibrillarin | SCARNA17 |
| | Am65 | Fibrillarin | SCARNA5 | U12 | Ψ28 | Dyskerin | SCARNA20 |
| | Ψ72 | ? | ? | | | | |

Am/Um/Gm/Cm, 2′-*O*-Methylation of the ribose of adenosine, uridine, guanosine or cytidine, respectively; Ψ, pseudouridine; $m^6A$, $N^6$-methyladenosine; $m^6Am$, $N^6$,2′-*O*-dimethyladenosine; $m^2G$, 2-methylguanosine. Numbers indicate the position of the modification within the human snRNA. Listed modification sites are as reported in Massenet and Branlant (1999); Karijolich and Yu (2010) and Krogh et al. (2017a).

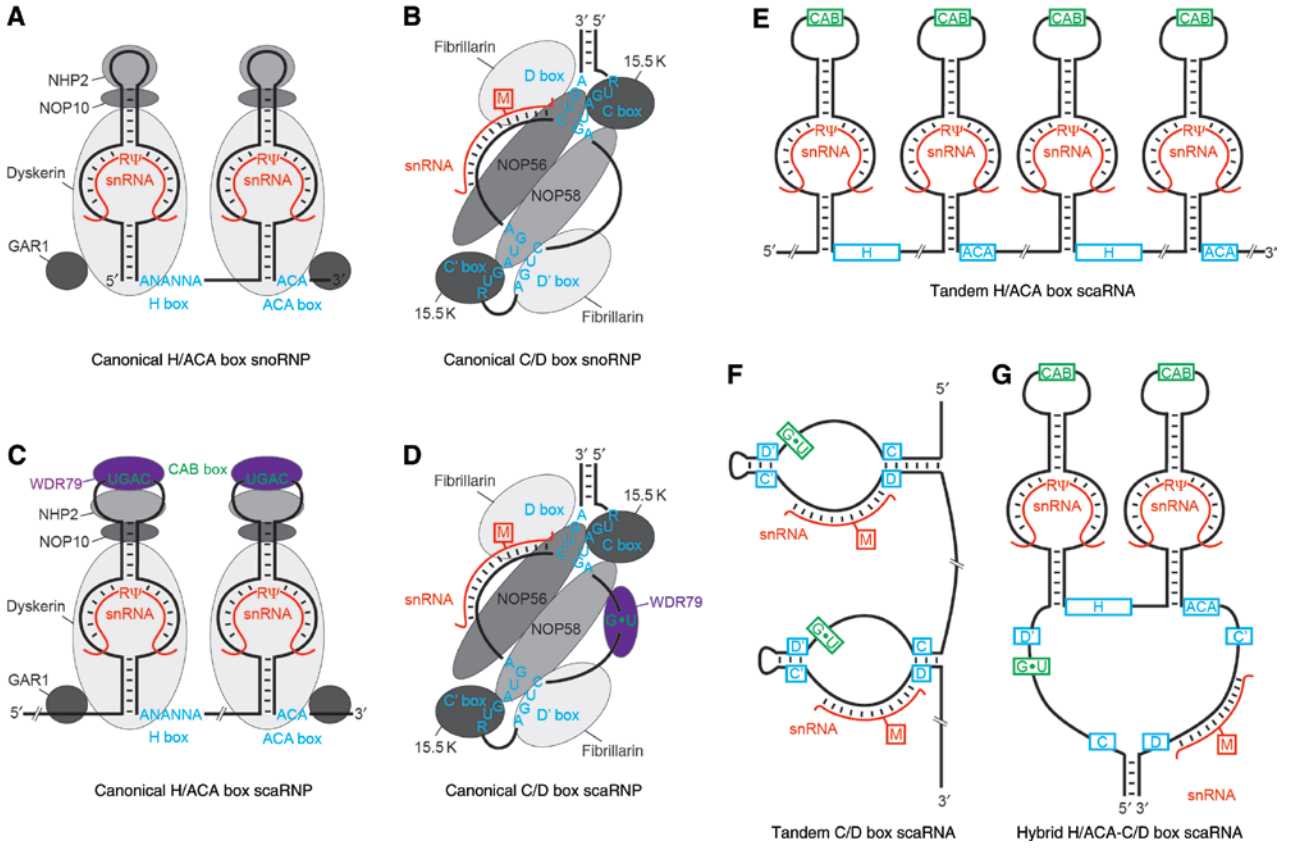


**Figure 2:** snoRNP and scaRNP structure and composition.
(A–D) Schematic views of the structures of canonical H/ACA box snoRNPs (A), C/D box snoRNPs (B), H/ACA small scaRNPs (C) or C/D box scaRNPs (D) are depicted. Conserved sequence motifs present in both snoRNAs and scaRNAs are highlighted in blue and scaRNA-specific motifs are marked in green. Proteins present in both snoRNPs and scaRNPs are shown in shades of grey and the scaRNP-specific protein WDR79 in purple. For C/D box snoRNPs and scaRNPs, only basepairing with an snRNA in proximity to box D is shown, but methylation can also be guided by the D′ box. Boxed M and Ψ represent the methylated and pseudouridylated snRNA nucleotides respectively. (E–G) Schematic views of the secondary structures of tandem H/ACA box scaRNAs (E), tandem C/D box scaRNAs (F) and a hybrid H/ACA-C/D box scaRNA (G) are shown as in (A–D).

Bioinformatics analyses based on the complementarity of sno/scaRNA sequences to sequences adjacent to known snRNA modification sites have enabled the sca/snoRNPs responsible for most modifications to be predicted in humans (Table 1), but many still require experimental confirmation. Notably, several scaRNAs contain either tandem H/ACA motifs (SCARNA13; Figure 2E) or tandem C/D motifs (SCARNA2, SCARNA9, SCARNA9L, SCARNA17; Figure 2F) or exist as hybrid H/ACA and C/D RNAs (SCARNA5, SCARNA6, SCARNA10, SCARNA12, SCARNA21; Figure 2G), enabling a single scaRNA to target more than one snRNA or to guide multiple modifications within a particular snRNA (Jády and Kiss, 2001; Darzacq et al., 2002; Kiss et al., 2002). While it is possible that this RNA-guided mechanism is employed to reduce the number of different modification enzymes required, it is also likely that the extensive basepairing interactions sno/scaRNAs form with snRNAs influence the dynamics of snRNA folding and thereby contribute to the regulation of snRNP assembly.

Interestingly, in yeast, Cbf5 (in the context of H/ACA box sno/scaRNAs) is not the only enzyme responsible for snRNA pseudouridylation as the U2-Ψ44 and U2-Ψ35 modifications are catalysed by the stand-alone pseudouridine synthetases Pus1 and Pus7 respectively (Massenet et al., 1999; Ma et al., 2003). While human PUS7 is also capable of pseudouridylating the equivalent residue (U34) in human U2 *in vitro*, *in vivo* evidence for this activity is lacking and the U2-Ψ34 modification can also be installed by SCARNP8, suggesting that either human U2 is not a physiological substrate of PUS7 or that redundant modification mechanisms exist. In yeast, U2-Ψ35 lies within a 'UGUAG' sequence context that is common for Pus7 targets (Safra et al., 2017) and interestingly, the

stem-loops IIa and IIb of the U2 snRNA are also required for pseudouridylation of U35 by Pus7 (Ma et al., 2003), implying that either the primary sequence or the secondary structure of these regions are also recognised by Pus7. In contrast, while Pus1 is a well-characterised tRNA and mRNA methyltransferase, it is not yet clear how its substrate specificity is achieved.

Alongside 2′-*O* methylations and pseudouridines, the human U6, U4 and U2 snRNAs also contain several base methylations, U2-m$^6$Am30 ($N^6$,2′-*O*-dimethyladenosine; Figure 1C), U4-m$^6$A100 ($N^6$-methyladenosine; Figure 1D), U6-m$^6$A43 and U6-m$^2$G72 ($N^2$-methylguanosine; Figure 1E) (Table 1). Although SCARNP9 is anticipated to 2′-*O*-methylate the ribose of U6-A30, the enzyme responsible for base methylation of this nucleotide as well as those that mediate U4-m$^6$A100 and U6-m$^2$G72, remain unknown. However, METTL16 was recently identified as an $N^6$-methyltransferase that targets A43 of U6 (Pendleton et al., 2017; Warda et al., 2017). Interestingly, this enzyme does not bind to either the U2 or U4 snRNAs (Warda et al., 2017) and neither U2-A30 nor U4-A100 lie within the DRACH (D = A, G or U; R = A or G; H = A, C or U) motif that is specifically recognised by the other known human m$^6$A methyltransferase complex containing METTL3 and METTL14 (Liu et al., 2014).

## Timing and sub-cellular localisation of snRNA modifications

During their maturation, the snRNAs are trafficked through various cellular compartments where different maturation steps take place. The Pol II-transcribed snRNAs are produced in the nucleus and m$^7$G-capping can take place already co-transcriptionally. Binding of the m$^7$G cap by the snRNA export adaptor PHAX1 and the nuclear transport receptor CRM1 promotes snRNP translocation to the cytoplasm where cap hypermethylation by TSG1 occurs. The TMG/m$_3$G cap is recognised by snurportin, leading to importin β-mediated re-import of snRNPs into the nucleus, where they are directed to CBs (Fischer et al., 2011). Localisation studies have revealed that while the 2′-*O*-methyltransferase CMTR1 is present exclusively in the nucleoplasm, CMTR2 is distributed throughout the nucleoplasm and cytoplasm (Werner et al., 2011). Neither $N^7$-methylation of the guanosine cap nor cap + 1 methylation are strict pre-requisites for cap + 2 methylation but the catalytic activity of CMTR2 has been suggested to be higher on cap + 1 methylated RNA substrates, implying that the action of CMTR1 may typically precede that of CMTR2.

It is widely accepted that the majority of scaRNP-mediated modifications take place in CBs following the re-import of snRNAs into the nucleus. This model is supported by the findings that neither a mutant of U2 that cannot undergo re-import, nor U5 that is re-directed to nucleoli, are modified *in vivo* (Jády et al., 2003). However, it appears that the CB structure itself is not essential for snRNA modification as in cells lacking CBs (e.g. SMN or coilin knockout cells) or cells in which the scaRNPs are not directed to CBs (e.g. WDR79 knockout cells), fully modified snRNAs are detected (Jády et al., 2003; Deryusheva et al., 2012). This suggests that among other functions, CBs serve to concentrate scaRNPs and snRNPs, thereby promoting efficient snRNA modification (Meier, 2017). Given the close proximity of 2′-*O*-methylations and pseudouridines in the snRNAs, and the extensive base-pairing interactions that scaRNAs form with their substrates, it is likely that such modifications take place sequentially; however, whether the association of scaRNAs takes places stochastically or if they are recruited in a strict hierarchal order remains to be determined. The modification of snRNAs occurs during the latter stages of snRNP biogenesis, which is in contrast to the rRNAs where the vast majority of RNA-guided modifications take place co-transcriptionally or during early biogenesis prior to significant rRNA folding and RNP assembly. As the presence of RNA-binding proteins would impede the access of scaRNAs to their target sites, it must be assumed that the interactions of scaRNAs with their cognate snRNAs are carefully co-ordinated with snRNA folding and recruitment of snRNP proteins. Little is known about factors that regulate the dynamics of scaRNP-association with snRNAs but, by analogy to snoRNA-rRNA interactions, it is possible that RNA helicases are required for the association or release of scaRNAs from their snRNA basepairing sites. In line with this hypothesis, the DEAH-box RNA helicase DHX15, which is the human homologue of yeast Prp43, a protein implicated in modulating snoRNA dynamics on pre-ribosomes (Bohnsack et al., 2009), and its cofactor ZIP (also known as ZGPAT) were recently found to localise in CBs and associate with the U4/U6.U5 tri-snRNP (Chen et al., 2017).

The U6 snRNA follows a different maturation pathway and in contrast to the other snRNAs, does not leave the nucleus (reviewed in Mroczek and Dziembowski, 2013; Didychuk et al., 2018). Capping of the nascent U6 transcript by MEPCE may take place during U6 synthesis as MEPCE has been shown to co-transcriptionally target other substrates (e.g. 7SK), and it is likely that MEPCE also remains associated with U6 following capping. The initial U6 transcript contains a short oligouridine tail with a terminal 2′,3′ *cis* diol that is specifically recognised and bound by

the chaperone-like protein La. Recent work indicates that $N^6$-methylation of A43 of the human U6 snRNA by METTL16 occurs during the early stages of U6 biogenesis as METTL16, which is localised in both the nucleoplasm and nucleolus, associates with a 3′ oligouridylated precursor form of U6 and co-precipitates La, LARP7 and MEPCE (Warda et al., 2017). It is likely therefore that U6-$m^6$A43 is one of the first internal modifications installed in U6, but whether this modification is a pre-requisite for the introduction of any other modifications remains to be determined. The 3′ end of U6 is further oligouridylated by TUT1 in the nucleolus, where the various snoRNP-guided 2′-*O*-methylations and pseudouridylations also take place (Table 1). Interestingly, based on sequence complementarity, the scaRNAs SCARNA23 and SCARNA3 are predicted to direct pseudouridylation of human U6-U40 (Kiss et al., 2004), suggesting that either these scaRNAs do not localise to CBs and that this modification takes place in the nucleoplasm or that the U6 snRNA also briefly transits through CBs during its maturation. Following translocation of U6 to the nucleoplasm, its 3′ end is trimmed by the 3′–5′ exoribonuclease Usb1 to leave a characteristic phosphate group (2′,3′-cyclic phosphate in humans, 3′ noncyclic phosphate in yeast; Lund and Dahlberg, 1992; Didychuk et al., 2017). Formation of the mature 3′ end of the U6 snRNA reduces the binding of La to the U6 snRNA and is required for recruitment of the Lsm2-8 complex (Didychuk et al., 2017; Montemayor et al., 2018).

## Functions of modifications in snRNAs

On a molecular level, RNA modifications alter the properties of the four basic nucleotides, thereby influencing the inter- and intra-molecular interactions of the RNAs that carry them. 2′-*O*-methylation typically stabilises RNA helices by increasing base-stacking whereas pseudouridine has a higher hydrogen bonding capacity than uridine and also increases the rigidity of the sugar-phosphate backbone (Prusiner, 1974; Charette and Gray, 2000). These chemical and topological properties explain the prevalence of these particular modifications in the highly structured snRNAs that are required to form numerous critical RNA-RNA interactions. In contrast, $m^6$A can have diverse effects on RNA secondary structure by either destabilising RNA duplexes or, when present in single-stranded RNAs, promoting base stacking, thereby enhancing RNA stability (Roost et al., 2015). $m^2$G has only a minimally stabilising effect on RNA structure but is proposed to alter the base-pairing interactions compared to guanosine.

It is possible that modifications in snRNAs arise due to the high concentration of modification enzymes in the nucleus and the accessibility of particular snRNA sequences. However, the presence of a dedicated snRNA modification machinery, together with the clustering of snRNA modifications in functionally important sequences that form key RNA-RNA or RNA-protein interactions, instead implies that snRNA modifications likely serve to fine-tune these interactions to optimise the efficiency and fidelity of pre-mRNA splicing. For example, in the context of the di- and tri-snRNPs, the U4 and U6 snRNAs are extensively basepaired, and approximately half of the modified nucleotides in these snRNAs are present in sequences involved in establishing these interactions (Figure 3A). Dissociation of the U4-U6 basepairing by the RNA helicase Brr2 is a key event that is proposed to serve as a proof-reading step during formation of the catalytically active spliceosome (Wills and Lührmann, 2011) and it is tempting to speculate that modifications within the base-paired sequences influence the kinetics of RNA unwinding and re-assembly. Likewise, within pre-catalytic and catalytic spliceosomes, the U6 snRNA forms extensive and dynamic interactions with the U2 snRNA, and a myriad of modified nucleotides lie within the sequences involved in basepairing (Figure 3B). In pre-catalytic spliceosomes, U6 and U2 are proposed to form three- and a four-helix junctions and single-molecule Förster resonance energy transfer (smFRET) analyses indicate a dynamic equilibrium between different conformations in human cells (Karunatilaka and Rueda, 2014). Post-transcriptional modifications (for example, Ψ15, Gm11, Gm12 and Gm19 in humans) within the human U2 stem I are suggested to regulate these conformational changes and to stabilise the four-helix structure (shown in Figure 3B; Karunatilaka and Rueda, 2014). However, the effects observed in this *in vitro* reconstituted system are relatively mild, leading to the suggestion that these modifications may not only modulate snRNA-snRNA interactions but may also influence snRNA-protein binding *in vivo*.

Alongside potentially modulating snRNA-snRNA interactions, RNA modifications may also influence base-pairing interactions between snRNAs and their pre-mRNA substrates. The high density of modifications within such sequences is exemplified by the 5′ region of the U1 snRNA that contains two cap-proximal 2′-*O*-methylations (U1-Am1 and U1-Um2 in humans) and two evolutionarily conserved Ψ (U1-Ψ5 and U1-Ψ6 in humans) and which basepairs with the 5′SS in the spliceosomal E and A complexes (Figure 3C). Although 5′SS selection does not solely rely

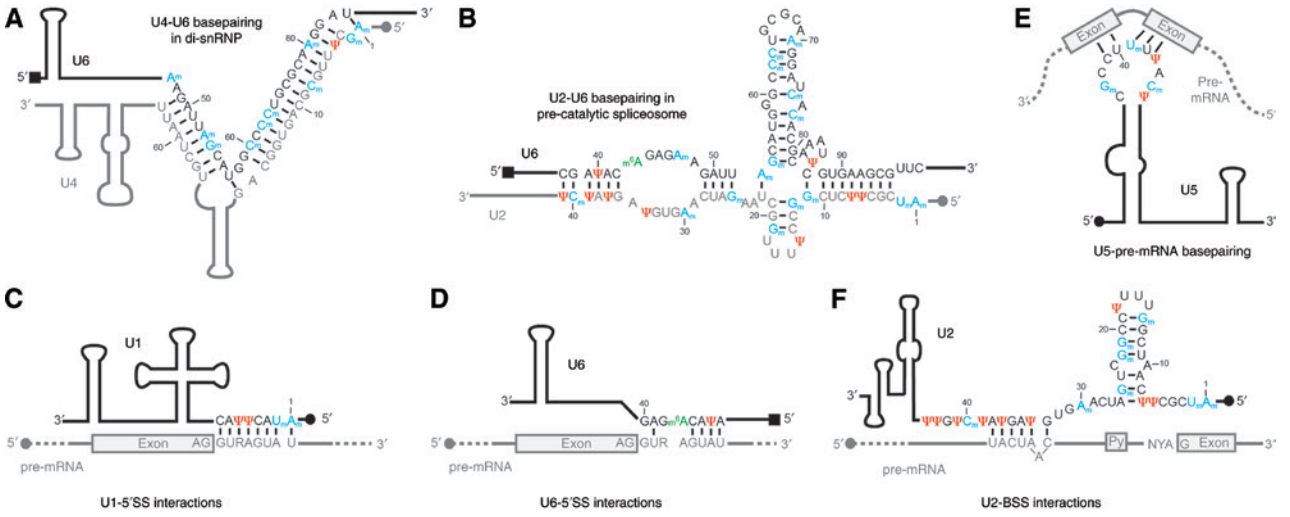


**Figure 3:** Many modified nucleotides in snRNAs lie within sequences that basepair with pre-mRNAs or other snRNAs.
(A–F) Secondary structure models of the indicated human snRNA-snRNA (A and B) and snRNA-pre-mRNA (C–F) interactions are shown. 2′-*O*-methylated nucleotides are indicated by blue $N_m$, pseudouridine by red Ψ and base methylations are marked in green. The $m_3G$ cap structure is depicted by a filled black/grey circle and the monomethylated guanosine triphosphate cap of U6 by a filled black square. Numbers indicate the nucleotide positions with the snRNA.

on complementarity to the 5′ end of the U1 snRNA, the strength of U1-5′SS basepairing is suggested to correlate to some extent with 5′SS usage. The presence of the two U1 Ψ's leads to formation of A-Ψ and R-Ψ wobble basepairs and the effects of modifications/mutations within these sequences have been extensively studied. Early work indicated that 'suppressor U1 snRNAs', which restore perfect basepairing with strong 5′SSs, had little or no effect on 5′SS usage, questioning the importance of Ψs at these sites (Zhuang and Weiner, 1986; Siliciano and Guthrie, 1988). Furthermore, *in vitro* thermodynamic analyses demonstrated that the substitution of pseudouridine for uridine does not influence the free energy of duplex formation between the sequence at the 5′ end of U1 and a complementary RNA strand (Hall and McLaughlin, 1991). More recently, however, positive effects of 'suppressor U1 snRNAs' were reported (Freund et al., 2003; Sorek et al., 2004) and *in vitro* competition assays suggested that the presence of G-Ψ basepairs at the 5′ end of U1 can be advantageous for 5′SS selection (Roca et al., 2005). While the precise role of the U1 5′ end Ψs therefore remains unclear, it is possible that they have minimal influence on the interactions between the U1 snRNA and strong 5′SS but that they may effect basepairing of U1 with weaker 5′SS.

Similarly, a human U6 sequence containing three Nm, one Ψ (U6-Ψ40) and 1 $m^6A$ (U6-$m^6$A43) binds the 5′SS during the subsequent catalytic phase of splicing (Figure 3D). In yeast, mutations within the U6 sequence that binds the 5′SS are lethal (see for example, Fabrizio and Abelson, 1990; Madhani et al., 1990; Lesser and Guthrie, 1993), highlighting the critical nature of this sequence for pre-mRNA splicing and raising the possibility that modifications within this region could play important roles in regulating the formation or stability of U6-5′SS basepairing. Interestingly, the ACm$^6$AGAGA sequence of U6 is contacted by U5 snRNP protein PRP8, which plays a crucial role in the formation of the catalytic core of the spliceosome, and it is possible that the presence of U6-m$^6$A43 modulates this interaction. This model is supported by the finding that the expression levels of the m$^6$A methyltransferase METTL16 and PRP8 are co-regulated in human cells. Interestingly, PRP8 also binds to an evolutionarily conserved 11 nucleotide loop in the U5 snRNA, which contains three Nm (U5-Gm37, U5-Um41 and U5-Cm45) and two Ψ (U5-Ψ43 and U5-Ψ46) in humans, and directly contacts the 5′ exon and 3′ exon of pre-mRNAs during the first and second catalytic steps of splicing respectively (Figure 3E). While experimental evidence demonstrating the importance of these modifications for splicing is still lacking, it is possible that they either influence directly the U5 snRNA-pre-mRNA basepairing or that they are required for the PRP8-mediated stabilisation of exon-U5 loop1 interactions during splicing (Karijolich and Yu, 2010).

U2 sequences that basepairs with the pre-mRNA BSS are also highly modified and modified nucleotides within such sequences have been suggested to be important for U2 snRNP biogenesis, spliceosome assembly, pre-mRNA interaction and also the catalytic activity of the spliceosome (Figure 3F). Yeast U2 lacking Ψ within the branch

site recognition region (BSRR) can assemble to form a non-functional 12S pre-U2 snRNP particle but cannot progress to the functional 17S complex and consequently, pre-mRNA splicing is abolished leading to growth defects (Yu et al., 1998). In humans, modifications at the 5′ end of U2 are required for formation of the spliceosomal E complex and while U2-Am1, U2-Um2, U2-Gm12 and U2-Gm19 are individually essential for pre-mRNA splicing, the Ψs within this region are only collectively required (Dönmez et al., 2004). It has further been shown in yeast that the RNA helicase Prp5 binds to U2 lacking Ψ42 and Ψ44 within the BSRR with significantly lower affinity than the modified form and that consequently, the RNA-dependent ATPase activity of Prp5 is reduced (Wu et al., 2016). This implies that the progression of spliceosome assembly from the early (E) complex to complex A is impeded in the absence of U2 pseudouridylation. When U2 is basepaired with the pre-mRNA, Ψ34 (human) or Ψ35 (yeast) directly opposes the branch point nucleotide, which mediates nucleophilic attack on the 5′SS during the first catalytic step. Excitingly, structural data from yeast have revealed that presence of the U2 Ψ plays an important role in precisely positioning the nucleophile to allow the catalytic reaction to take place (Newby and Greenbaum, 2002; Lin and Kielkopf, 2008).

There is a marked contrast between the number of modifications reported in the snRNAs of the major and minor spliceosomes, with no modifications so far detected in the human U11 snRNA, and only very few identified in the U4atac, U6atac and U12 snRNAs (Krogh et al., 2017b). As the snRNAs of the minor splicoeosome are present in the cell at much lower levels than those of the major spliceosome (Patel and Steitz, 2003), it is possible that this apparent difference may arise from the technical challenges of detecting, potentially sub-stoichiometric, modifications in low abundance RNAs. However, the recent application of modification mapping approaches such as RiboMeth-seq (Birkedal et al., 2015; Dai et al., 2017; Krogh et al., 2017a), Pseudo-seq (Carlile et al., 2014; Schwartz et al., 2014) and $m^6$A-seq (see for example, Dominissini et al., 2012; Chen et al., 2015; Linder et al., 2015), which are coupled to next generation sequencing and therefore highly sensitive, have not provided new evidence for additional modifications in these RNA species, suggesting a *bona fide* difference in the extent of modification of the major and minor snRNAs. Compared to major U2-type introns, the 5′SS and BSS of U12-type introns removed by the minor spliceosome have significantly lower sequence diversity (Sharp and Burge, 1997; Turunen et al., 2013) and consequently, it is possible that a lower degree of modification is required in the snRNAs of the minor spliceosome. This hypothesis is supported by the observation that in yeast, where there is less variation in the sequences of the pre-mRNA splice sites, the snRNAs are less modified than in humans.

## The stoichiometry and dynamics of snRNA modifications

The development of quantitative techniques for the detection of RNA modifications have recently provided the first insights into the stoichiometry of modifications in abundant RNAs and together with the discovery of demethylases that can act as modification ‘erasers’, these studies have highlighted the dynamic nature of RNA modifications and emphasised their potential as important regulators of gene expression. A recent analysis of snRNA 2′-*O*-methylation demonstrated that all canonical sites are almost fully modified in diverse human tissues (Krogh et al., 2017b) and it is anticipated that the vast majority of other known snRNA modifications are also constitutively present. However, alterations in the modification status of snRNAs have also been reported, further supporting the model that modifications at specific snRNA positions can have important physiological roles.

In yeast, two additional Ψ at positions 56 and 93 of the U2 snRNA are detected in nutrient-deprived cells, and Ψ56 is also observed upon exposure of cells to heat shock (Wu et al., 2011). These inducible modifications are installed by the action of the stand-alone pseudouridine synthetase Pus7 (U2-Ψ56) and the H/ACA box snoRNP snR81 that normally modifies U1051 in the 25S ribosomal RNA (U2-Ψ93). Interestingly, U2-U56 lies within a sequence context that is similar to the typical Pus7 consensus motif and the sequences proximal to U2-U93 have similar, but imperfect, complementarity to the snR81 guide sequences; it is therefore suggested that under stress conditions, the recognition criteria of Pus7 and snR81 become less stringent enabling these additional sites to be targeted. Using reporter systems, it has been shown that the presence of U2-Ψ93 impedes pre-mRNA splicing and mechanistically, it is suggested that Ψ56 and Ψ93, which lie within stem IIa and stem IIc of U2, may influence the kinetics of U2 switching between its optimal substrate-interaction conformation and its catalytic conformation, thereby negatively affecting pre-mRNA splicing (Wu et al., 2011). Recently, smFRET experiments demonstrate that, compared to U93-containing RNAs, the presence of Ψ93 increases the conformational flexibility of the stem IIc sequence, whereas Ψ56 reduces the conformational dynamics of stem II and stabilises stem IIc (van der Feltz et al., 2018). A further example of inducible

snRNA modification is that during filamentous growth of *S. cerevisiae*, U6-U28 is pseudouridylated by Pus1. Interestingly, it has been shown that installation of this additional modification is an important aspect of the filamentous growth programme (Basak and Query, 2014). It is proposed that U6-Ψ28 may influence the recruitment or binding of Cwc2, which is a critical step during catalytic activation of the spliceosome, and that this may in turn affect the splicing of particular pre-mRNAs that encode proteins required for filament formation. Likewise, changes in the extent of 2′-*O*-methylation of specific snRNA nucleotides (cap + 2 of U4 and U5 and various internal sites in U2 and U6) have also been observed during human T cell activation (Krogh et al., 2017a). Compared to primary T cells, a generally lower level of snRNA 2′-*O*-methylation was detected in Jurkat cells, a common T cell leukaemia model, and it is suggested that due to the very high growth and RNA synthesis rates in these cells, the modification machinery becomes limiting, leading to substoichoimetric modification of diverse RNA species. In contrast to most human tissues, the U4 snRNA in Jurkat cells lacks the Cm8 modification (Krogh et al., 2017b). In the free U4 snRNP, Cm8 likely contributes to stabilising a short intra-molecular helix whereas in the context of the U4/U6.U5 tri-snRNP, U4-Cm8 lies within a long stretch of basepairing with the U6 snRNA. It is possible, therefore, that the absence of U4-Cm8 promotes incorporation of U4 into the tri-RNP, thereby increasing the efficiency of spliceosome assembly and pre-mRNA splicing in Jurkat cells (Krogh et al., 2017a).

The presence of both constitutive and inducible modifications in snRNAs raises the intriguing possibility that the constitutive modifications represent a core subset that are involved in fundamental aspects of spliceosome assembly or function, whereas inducible modifications likely subtly alter the stability, kinetics or dynamics of interactions within spliceosomal complexes to promote or impede splicing of particular target pre-mRNAs. Interestingly, a recent report indicates that changes in snRNA modifications may not only arise at the level of modification installation (Mauer et al., 2018). It is proposed that in addition to 2′-*O*-methylation by CMTR1, the cap + 1 nucleotides of the RNA pol II synthesised snRNAs also undergo $N^6$-methylation, leading to the formation of $m^6Am$, but that in some cell lines and tissues, these base modifications are efficiently removed by the demethylase FTO. Cap proximal $m^6Am$ is suggested to promote snRNA stability and increased exon inclusion is observed in cells lacking FTO. Interestingly, FTO-mediated demethylation of snRNAs can be inhibited by specific intracellular metabolites, suggesting a model in which changes in metabolism may affect pre-mRNA splicing by altering the cap proximal methylation status of snRNAs. To date, no demethylases are known to target internal snRNA modifications, possibly indicating that the protein-rich environment of (partially) assembled snRNPs largely impedes access of demethylation enzymes to potential target sites.

## Conclusions and outlook

Since the first detection of modified nucleotides in snRNAs approximately 40 years ago, significant insights have been gained into the sites, functions and dynamics of snRNA modifications and the diverse mechanisms by which they are installed. Knowledge about the snRNA modification machinery has recently been expanded by the discovery of the first snRNA base methyltransferase and while the inventory of human snRNA modification enzymes remains incomplete, recent advances in methodologies for the detection of RNA modifications (see for example, Dominissini et al., 2012; Carlile et al., 2014; Schwartz et al., 2014; Chen et al., 2015; Linder et al., 2015; Dai et al., 2017; Krogh et al., 2017b) and RNA-RNA interactions *in vivo* (see for example, Kudla et al., 2011; Aw et al., 2016) as well as the transcriptome-wide mapping of modification enzyme target sites (see for example, Haag et al., 2017) will likely soon enable the missing enzymes to be identified. It will be interesting to see if the non-RNA-guided 2′-*O*-methyltransferases or pseudouridine synthetases also target human snRNAs as in other organisms. A complete inventory of snRNA modification enzymes will facilitate a comprehensive analysis of the functions of each snRNA modification *in vivo*. Recent high-resolution cryo-electron microscopy structures of various human spliceosomal complexes also provide exciting new insights into the architecture of these complexes, and interactions that the snRNAs make with each other and with spliceosomal proteins. Although the currently available structures lack the resolution to directly visualise snRNA modifications, mapping of known modifications on these structures will also likely drive new hypotheses about the precise functions of snRNAs modifications and help advance our understanding of their importance in regulating gene expression.

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