

Biology and applications of small nucleolar RNAs

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Abstract Small nucleolar RNAs (snoRNAs) constitute a group of non-coding RNAs principally involved in post-transcriptional modification of ubiquitously expressed ribosomal and small nuclear RNAs. However, a number of tissue-specific snoRNAs have recently been identified that apparently do not target conventional substrates and are presumed to guide processing of primary transcripts of protein-coding genes, potentially expanding the diapason of regulatory RNAs that control translation of mRNA to proteins. Here, we review biogenesis of snoRNAs and redefine their function in light of recent exciting discoveries. We also discuss the potential of recombinant snoRNAs to be used in modulation of gene expression.

Keywords Small nucleolar RNAs · Biogenesis · Alternative splicing · RNA editing · Gene silencing

Introduction

Small nucleolar RNAs (snoRNAs) are a group of single-stranded non-coding RNAs generally of ~60–300 nt in length, primarily functioning in posttranscriptional modification of ribosomal RNAs (rRNAs) and small nuclear

RNAs (snRNAs). Located in the nucleolus, most snoRNAs guide 2'-*O*-ribose methylation or pseudouridylation (isomerization of uridine) of specific RNA nucleotides, while a few are required for pre-rRNA endonucleolytic processing. Intriguingly, a number of orphan snoRNAs have been identified that have no sequence complementarity to conventional substrates (rRNAs or snRNAs) and are assumed to target other RNAs. For example, recent reports demonstrated the involvement of the snoRNA SNORD115 (previously called HBII-52) in the regulation of alternative splicing [1, 2] and/or RNA-editing [3] of the serotonin receptor subtype 2C mRNA in the brain. An additional observation indicates that some snoRNAs could serve as narrowly specialized biochemical mediators (in contrast to the ones that aid in universal processes such as rRNA biogenesis). Namely, several snoRNAs are expressed in a tissue-specific manner [4], potentially implying their role in distinct physiological processes. However, the exact function of such orphan snoRNAs has yet to be identified. Here, we review the biogenesis and functions of endogenous snoRNAs and discuss potential applications of recombinant snoRNAs for modulating gene expression.

Biogenesis

snoRNAs are evolutionarily very conserved and a number of homologues of eukaryotic snoRNA have been identified in prokaryotes (denoted sRNAs for sno-like RNAs) [5, 6]. snoRNA-encoding genes display diverse organization patterns in different organisms (reviewed in [7]). snoRNAs can either be transcribed from independent RNA polymerase (Pol) II (less commonly Pol III) promoters or arise from introns excised from protein- or non-protein-coding transcripts. In any case, snoRNA genes can either exist

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individually or form polycistronic clusters. Polycistronic precursor snoRNAs are processed by RNase III-like activity and subsequently trimmed by exonucleases (in yeast 5' and 3' trimming is performed by Rat1 and Xrn1 proteins, and exosome, respectively) [8]. In the course of maturation, snoRNAs transit through Cajal bodies (nuclear sub-organelles of ribonucleoprotein nature) where they are chemically modified before being transported to the nucleolus [9].

In mammals and the nematode *Caenorhabditis elegans*, in contrast to flies and plants, snoRNAs are almost exclusively monocistronic [7]. Vertebrate snoRNAs are mostly of intronic origin with the exception of autonomously transcribed essential snoRNAs that direct pre-rRNA cleavage. Notably, many intronic snoRNAs are associated with genes coding for ribosomal and nucleolar proteins. Mature snoRNAs are usually trafficked to the nucleolus. This process requires the presence of conserved structural elements within the nucleotide sequence of snoRNA [10] and relies on a number of transport factors, such as the cap-binding complex (CBC), the phosphorylated export adapter (PHAX), and the exportin CRM1 [11, 12].

Based on conserved nucleotide motifs, snoRNAs are classified into two distinct families (Fig. 1): box C/D (denoted SNORD followed by a consecutive number) and box H/ACA snoRNAs (denoted SNORA followed by a consecutive number). Each snoRNA family recruits a specific set of effector proteins (see next section) to form functional snoRNA ribonucleoproteins (snoRNPs), and this process is initiated co-transcriptionally, likely to protect the snoRNAs from degradation by RNases. Moreover, diverse auxiliary factors transiently interact with snoRNP core proteins to guide snoRNP assembly through complex mechanisms that are still poorly understood [13–19]. For example, McKeegan et al. [14] found relatively few interactions between core box C/D proteins but demonstrated that a number of biogenesis factors associate with one or more core C/D proteins, indicating their potential role as molecular chaperones or recruiting factors in snoRNP assembly. Indeed, two biogenesis factors, human protein Nufip [14, 15] and its yeast homologue Rsa1 [15], were shown to promote interactions between the core proteins during box C/D snoRNP assembly. Furthermore, Nufip and Rsa1 link the nascent snoRNP complex to a ubiquitous chaperone Hsp90 (heat shock protein 90) via the R2TP complex and evidence suggests that Hsp90 controls folding of core proteins during formation of mature RNP [15]. The R2TP complex, consisting of an Hsp90 co-chaperone Tah1, assembly factor Nop17 (also called Pih1), and two AAA + helicases Rvb1/Rvb2 (also termed Tip49/Tip48), in addition to providing Hsp90 anchorage, actively participates in snoRNP maturation [19, 20]. In fact, it has recently been proposed that Hsp90 primarily acts to

stabilize Nop17 thereby maintaining R2TP activity [21]. Allegedly, the R2TP complex plays a major role in restructuring snoRNPs by either displacing the improperly bound core proteins or unwinding the mispaired snoRNA bases [19, 20, 22] before RNPs are shuttled to nucleolus for final maturation steps. While R2TP complex, Hsp90, and Nufip/Rsa1 appear to be general assembly factors that promote box C/D and box H/ACA snoRNP as well as selenoprotein mRNP assembly [15], nuclear assembly factor 1 (Naf1) and Shq1, a protein chaperone homologous to Hsp90 co-chaperones, seem to be box H/ACA snoRNP-specific. Both interact with the same box H/ACA core protein (dyskerin/Cbf5, see next section) and are required for stability and proper subcellular localization of nascent RNP complex, whereby Shq1 acts earlier in the assembly process than Naf1 [23, 24]. Interestingly, Naf1 was observed to interact with the phosphorylated C-terminal domain of Pol II, indicating that it may act as a recruiting factor for box H/ACA core proteins, facilitating their binding to the pre-snoRNA transcript [25]. Altogether, a multitude of trans protein factors are involved in snoRNP biogenesis, coordinating concomitant processes of snoRNA transcription and processing, recruitment of core proteins, and trafficking, assembly and restructuring of snoRNP complexes (reviewed in [20, 24, 26, 27]).

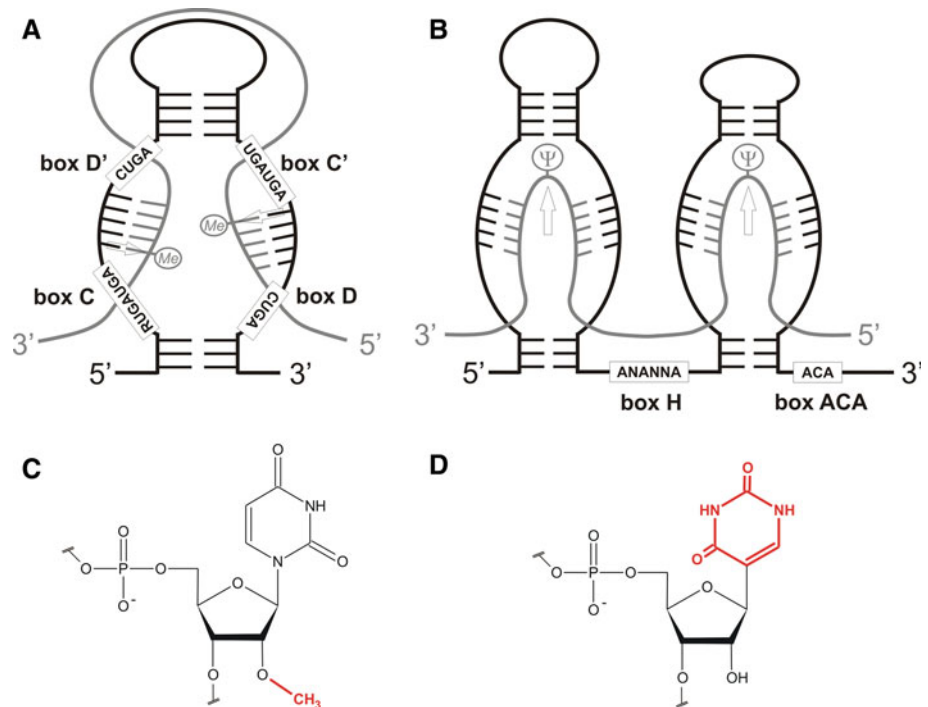
A subset of snoRNAs termed small Cajal RNAs (scaRNAs) specifically localizes to Cajal bodies, foci presumed to be the center of covalent modification of small nuclear RNAs (snRNAs), as well as snoRNAs and their assembly into mature snoRNPs [28]. scaRNAs conform to the C/D-H/ACA classification, but some contain structural motifs characteristic of both snoRNA families. Such chimeric scaRNAs associate with partner proteins of both families of canonical snoRNAs. A specific feature of scaRNAs is the Cajal-body specific localization signal (CAB box) required for retention in Cajal bodies [9].

All published human snoRNA sequences and annotating data are stored in the snoRNABase sequence database (<http://www-snomna.biotoul.fr/>) [29]. The current version 3 contains 402 experimentally confirmed human snoRNAs. Additionally, there are many computationally predicted snoRNA-like sequences within the human genome that have been identified using several computational approaches [4, 30, 31] and still remain to be experimentally verified. Taken together, it is projected that the total number of snoRNA-like sequences in the human genome may be greater than 1,000 [32].

Mode of action

Most snoRNAs serve as guides specifying the nucleotides in target RNAs to be chemically modified by snoRNA-

Fig. 1 Structural features of the modification guide snoRNA families and types of posttranscriptional modifications they promote. Schematic secondary structures of **a** box C/D snoRNAs and **b** box H/ACA snoRNA are shown on top. Substrate RNA is depicted in gray. Sites targeted for nucleotide modification are indicated with block arrows. *Me* stands for 2'-*O*-methyl group introduced onto the ribose ring (c) and ψ represents pseudouridine (d)



associated enzymes. Besides target recognition sequences (the antisense elements), snoRNAs contain conserved stretches of nucleotides and tridimensional architectural elements which are largely responsible for assembly of snoRNPs and their cellular localization.

The box C/D family of snoRNAs is characterized by a kink-turn (stem-bulge-stem) structure containing two consensus motifs (Fig. 1a). Box C sequence (RUGAUGA, where R stands for A or G) is typically located close to the 5' terminus, whereas box D sequence (CUGA) is situated near to the 3' end. In the folded snoRNA, the two boxes are brought together by base pairing of the 5' and 3' termini. Many C/D snoRNAs contain a duplication of boxes C and D (denoted as box C' and box D', respectively) in the central RNA region. C' and D' boxes of some snoRNAs are highly divergent, yet they appear to be functional [33]. C/D snoRNAs base pair with substrate RNAs with a short (i.e. 10–20 nts) stretch of nucleotides positioned upstream of box D and/or box D'. Recently, van Nues et al. [33] noted that additional conserved elements bearing sequence complementarity to substrate RNA may be present within box C/D snoRNAs, and demonstrated that the target recognition sequence can be longer than originally proposed [34]. This provides an explanation on how snoRNAs get access to highly structured target sites. C/D snoRNAs typically promote 2'-*O*-ribose methylation (Fig. 1c) on sites located five nucleotides across from the CUGA motif in the upstream direction [34]. The enzyme responsible for substrate methylation is fibrillarlin [known as nucleolar protein 1 (Nop1) in yeast]. In addition, three other proteins are

required to form functional C/D snoRNPs in eukaryotes: Nop56, Nop58 and the 15.5-kDa protein [known as small nuclear ribonucleoprotein 13 (Snu13) in yeast] [10]. Partner proteins contribute to snoRNA maturation and stability and are requisite for proper nuclear localization [10, 20].

The box H/ACA snoRNAs are somewhat larger than box C/D snoRNAs and adopt a typical secondary structure consisting of two stem-bulge-stem domains separated by a hinge region (Fig. 1b) containing the consensus motif ANANNA (where N denotes any nucleotide) known as box H. The second conserved motif, termed box ACA, is located three nucleotides upstream of the 3' terminus. One of the hairpins (or less often both) contains an internal loop with 9- to 13-nucleotide antisense elements on each strand that base pair with the substrate RNA and form the so-called pseudouridylation pocket. The site destined for pseudouridylation (Fig. 1d) is bound 14–16 nucleotides upstream of the H and/or ACA box motifs. The enzyme carrying out uridine isomerization is termed dyskerin (known as Cbf5 in yeast). Additional core proteins (Gar1, Nhp2 and Nop10) associate with H/ACA snoRNAs to form mature snoRNPs. All except Gar1 are essential for snoRNP stability, while Gar1 is required for snoRNP function (reviewed in [10, 24]).

Although a number of scaRNAs with a C/D-H/ACA composite structure have been identified, only SCARNA10 (previously called U85) was experimentally confirmed to direct both 2'-*O*-methylation and pseudouridylation [35]. It targets two consecutive nucleotides in the invariant loop 1 of the human U5 spliceosomal RNA, a member of the

snRNAs. This is achieved through alternative base pairing of targeted sequence with the two antisense elements.

With respect to prokaryotes, homologues of C/D and H/ACA snoRNPs are present in archaea but not in bacteria. Pseudouridylation and 2'-O-methylation of RNA are also intrinsic to bacteria (although they occur at much lower frequency compared to eukaryotes) and are brought about by single-polypeptide enzymes requiring no snoRNA guides for substrate recognition [10]. Similarly, uridines in both prokaryotic and eukaryotic transfer RNAs (tRNAs) are isomerized by stand-alone pseudouridine synthases. The separation of catalytic function from target selection in snoRNPs was evolutionally favorable as it allowed for significant expansion of the number of sites that can be specifically modified [10].

A number of RNAs structurally related to canonical snoRNAs do not direct 2'-O-methylation or pseudouridylation but perform other diverse functions. Members of the SNORD3 family, for example, act as chaperones inducing proper pre-rRNA conformation for subsequent endonucleolytic processing by trans-acting endonucleases not associated with snoRNPs [27, 36, 37]. A similar function was attributed to SNORD118 (snoRNA U8) [38], SNORD14 (U14) [39] and SNORD22 (U22) [40]. This contrast with the active role that the mitochondrial RNA processing (MRP) RNA plays in rRNA precursor cleavage. MRP RNA, a ribozyme found in the RNP complex called MRP RNase, is structurally unrelated to snoRNAs but also predominantly localizes to nucleus [41, 42].

Another non-coding RNA functionally distinct from canonical snoRNAs, the telomerase RNA component (TERC), possesses snoRNA-like architectural features. TERC serves as a template for reverse transcription of telomeres [43, 44]. The 3' H/ACA domain and CAB box of TERC direct telomerase RNP assembly and processing of precursor RNA, as well as provide for proper compartmentalization within nucleus [44].

Function

Role of snoRNAs in rRNA and snRNA modification

The exact reasons as to why nucleotide sequences of tRNA, rRNA and snRNA molecules are so heavily modified remain under debate. Nevertheless, the fact that methylation and pseudouridylation reactions occur on primary rRNA transcripts (i.e. before they are cleaved), yet are limited only to regions preserved in ribosomes, strongly indicates the biological importance of covalent alterations. Moreover, the modified nucleotides were found to accumulate at functional rRNA centers, most notably the peptidyl transferase region of 28S rRNA (23S in

prokaryotes) [45, 46]. Studies on prokaryotic and yeast systems showed that most individual modified nucleotides are not required for cell viability and show no strong effects on cell growth, demonstrating the collective function of modifications in ribosome biogenesis and/or protein translation [47]. Conversely, global perturbation of rRNA modification leads to severe reduction of growth rate [27, 48, 49].

Both types of modification have distinct effects on the properties of nucleotides and affect the diversity of RNA molecule. Not only do they change the interacting potential of the RNA chain but they also profoundly influence its structural stability. Uridine isomerization and addition of methyl groups to the ribose ring both introduce conformational constraints into an RNA sequence. Markedly, pseudouridines are found only in those RNA species whose tertiary structure is essential to their biological function (i.e. tRNAs, rRNAs and snRNAs) [49]. Through pseudouridylation, RNA gains functional groups that can serve as additional hydrogen bond donors. The potential to form new intramolecular interactions is expected to result in new RNA folds and to influence interactions with partner proteins [48]. Methylation, on the other hand, decreases ribose's hydrophilic character (i.e. masks its hydrogen-bonding potential) and protects phosphodiester bonds against degradation by nucleases [27, 48].

snoRNA in regulation of gene expression

Recently, a number of snoRNAs with tissue-specific localization and no apparent sequence complementarity to rRNA or snRNAs have been identified. These orphan snoRNA are predicted to direct mRNA modification as a part of still poorly understood gene regulatory mechanism. To date, a single orphan snoRNA, the brain-specific SNORD115 (previously called HBII-52), was studied in detail with respect to posttranscriptional regulation of its putative target, the serotonin receptor 2C (5-HT_{2C}R) mRNA. Human SNORD115 genes are located on the imprinted locus 15q11-q13, in a region observed to be frequently deleted in the Prader–Willi syndrome (PWS). PWS is a rare neurological disorder, characterized by developmental, behavioral, and mental abnormalities. In the locus, there are 47 repeats of *SNORD115* genes along with 27 repeats of *SNORD116* (*HBII-85*), and single copies of *SNORD64* (*HBII-13*), *SNORD107* (*HBII-436*), *SNORD9A* and *B* (*HBII-438A* and *B*). SNORD115 has initially attracted the attention of researchers because it has an 18-nt-long conserved antisense element complementary to a segment of 5-HT_{2C}R pre-mRNA, and the notion that PWS can be linked to abnormal serotonin metabolism (PWS patients respond to treatment with selective serotonin reuptake inhibitors). Moreover, both SNORD115 and

5-HT_{2C}R mRNA are abundantly expressed in the chorioid plexus, and the region of serotonin receptor pre-mRNA (more explicitly a part of the Vb alternatively spliced exon), presumed to be targeted by SNORD115, was known to undergo extensive site-specific adenosine-to-inosine (A-to-I) RNA editing. In turn, such a change of encoded genetic information has been previously shown to affect serotonergic signal transduction [50]. Also of importance, the first intron of 5-HT_{2C}R pre-mRNA contains an H/ACA snoRNA, potentially indicating that the receptor primary transcript might be subjected to nucleolar trafficking. Consistent with the above observations, Vitali et al. [3] demonstrated SNORD115-RNP to inhibit nucleolar ADAR2 (adenosine deaminase acting on RNA 2)-mediated A-to-I editing of 5-HT_{2C}R pre-mRNA in vitro. Later, Kishore and Stamm [1] showed that the same snoRNA participates in regulation of alternative splicing of 5-HT_{2C}R, probably by binding to a splicing silencing element in alternative exon Vb to promote its inclusion, thereby inducing production of full-length serotonin receptor. Intriguingly, the mechanism of 5-HT_{2C}R expression regulation by SNORD115 proposed by Kishore and Stamm [1] is inherently editing-independent, while Vitali et al. [3] suggested ribose methylation to be responsible for hindrance of A-to-I editing. Still, both mechanisms are consistent with observed PWS behavioral phenotypic traits that can be explained by reduced

5-HT_{2C}R sensitivity to serotonin, presumably as a direct consequence of SNORD115 deletion [51]. While it is true that exon Vb editing decreases the chance of producing the truncated non-functional receptor isoform, it is accompanied by changes of amino acid sequence of the receptor intracellular loop responsible for G-protein binding. Alterations of the loop sequence negatively affect signal transduction. A recent study [51] only found evidence for an increase of A-to-I editing but not alternative splicing of the pre-mRNA 5-HT_{2C}R in a mouse model of PWS lacking SNORD115 expression. On the other hand, substantial evidence indicates that in fact deletion of the *SNORD116* gene cluster might be responsible for most of the phenotypic traits of PWS [52–55], although the mechanism behind it has not yet been addressed. Nevertheless, as expression of SNORD115 is lost in the majority of PWS cases, it is believed it contributes to the complex behavioral phenotype seen in this disorder [51].

An astonishing finding implicating SNORD115 in regulation of alternative splicing of at least five pre-mRNAs was recently reported by Kishore et al. [2]. They presented evidence that a variant of mouse SNORD115 undergoes cleavage to smaller RNAs. These fragments [termed processed small nucleolar RNAs (psnoRNAs)] lack the sequences that form the snoRNA stem but retain the C/D box motifs and the antisense element. In the pull-down assay using a probe complementary to the antisense

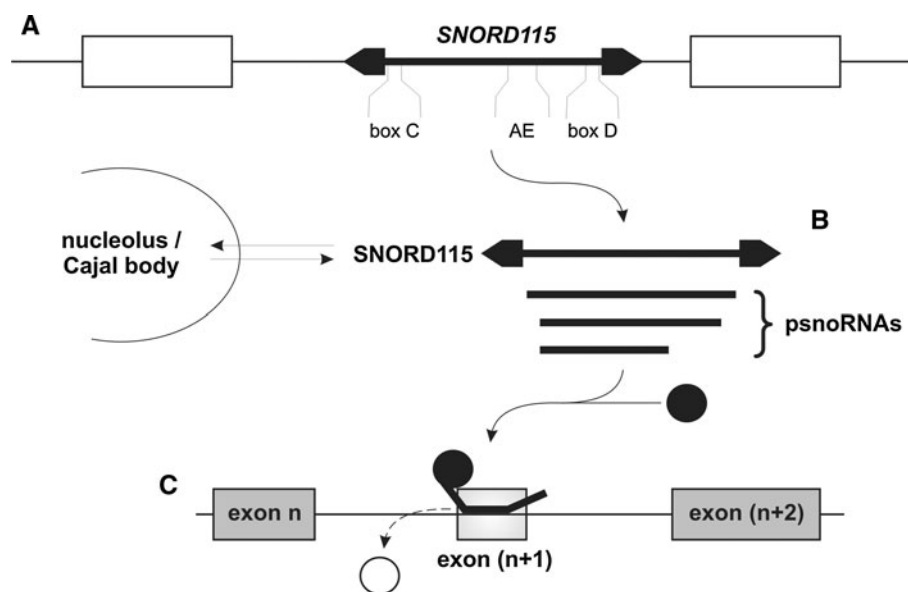


Fig. 2 Model describing SNORD115 processing and the action of resulting psnoRNAs (adapted from [2]). **a** The snoRNA-rich imprinted locus 15q11-q13 contains 47 copies of *SNORD115* (thick line; a single copy is shown for simplicity) located in introns between non-coding exons (open boxes). The snoRNA is characterized by stem-forming sequences (arrowheads), box C, box D, and an antisense element (AE). **b** This unit generates the canonical SNORD115, which localizes to nucleolus and Cajal bodies (possibly

serving as storage sites), as well as a number of shorter nucleoplasmic RNAs, called processed snoRNAs (*psnoRNAs*), which associate with heterogeneous nuclear ribonucleoproteins (hnRNPs). **c** psnoRNAs can change splice-site selection by binding to complementary sequences. psnoRNA either displaces regulatory proteins (open circle) from pre-mRNA targets or acts as guides for hnRNPs (black circles), functioning as exon recognition complexes

element of SNORD115, heterogenous nuclear ribonucleoproteins (hnRNP) were co-purified with psnoRNAs, but not proteins associated to canonical snoRNAs. Notably, hnRNPs are known to be involved in splice-site selection [56]. Furthermore, in five of the potential targets predicted by a computational screen, SNORD115-dependent splicing events were observed. The researchers speculated that this was caused by competition of psnoRNAs with splicing regulatory factors on the pre-mRNA or by psnoRNAs acting as guides for hnRNPs (Fig. 2).

snoRNAs might exert their action on regulation of gene expression through additional mechanisms as recently exemplified by Ono et al. [57]. They noticed that SNORD88A-C isolated from HeLa cell nucleoli contain a 19–21-nt region (named box M; located downstream of the methylation guide sequence and including box C') that was (partially) complementary to intronic and exonic sequences of several pre-mRNAs. Although the effects of the SNORD88 family of snoRNAs on the expression of endogenous genes were not reported, several model protein targets were successfully knocked-down using chimeric SNORD88C in which M boxes were specifically tailored to be complementary to targeted pre-mRNAs. The exact silencing mechanism is still unknown but was shown to be distinct from siRNA/miRNA-mediated repression of gene expression as it apparently takes place in the nucleus [57, 58]. Thus, the SNORD88 family (and perhaps other snoRNAs), in addition to guiding 2'-O-methylation, likely mediate regulation of gene expression with an antisense-type mechanism that encompasses either induction of pre-mRNA degradation or inhibition of pre-mRNA splicing and/or trafficking.

A growing body of evidence suggests that many snoRNAs from both box C/D and box H/ACA families can give rise to other regulatory RNA species, most notably microRNA (miRNA)- and piwi-interacting RNA (piRNA)-like short RNAs, in a wide variety of organisms [58–64]. These sno-derived RNAs (sdRNAs) are produced in a regulated fashion from double-stranded snoRNA structures by the components of an RNA interference pathway [59–61, 64]. At least some sdRNA precursors bind to snoRNP core proteins [58, 62], which implies they still possess some snoRNA functionality. sdRNAs interact with Argonaute proteins [59, 61, 64], the principal components of the RNA-induced silencing complex (RISC) which mediates gene silencing by canonical miRNAs and piRNAs, and Brameier et al. [64] showed that 11 snoRNA-derived miRNAs effectively repress recombinant reporter genes. Taken together, this indicates an ancient link between snoRNAs and RNA silencing, and reasons in favor of orphan snoRNAs as sdRNA precursors [61, 62]. Since mammalian snoRNA genes are believed to exhibit genetic mobility [65], the duplicate copies may have evolved into other classes of

short regulatory RNAs without any selection pressure [58]. Interestingly, recent data indicate that putative short gene silencing RNAs also arise from other ncRNA species, such as tRNA, snRNA and vault RNA (vRNA) [66].

The implication of tissue-specific snoRNAs in gene expression regulation may well be a part of a widespread mechanism. Rogelj [67] suggested that orphan snoRNAs may act as RNA-editing and/or -splicing switches in response to learning. The premise was based on the proposed SNORD115 functions described above as well as the notion that expression levels of two brain-specific snoRNAs change during learning of a complex behavior. Specifically, Rogelj et al. [68] demonstrated that SNORD115 and MBII-48, two snoRNAs that are abundantly expressed in mouse hippocampus and amygdala but to a lesser extent in other areas of the brain, show a transient up- and downregulation, respectively, during contextual memory consolidation. These changes were restricted to a hippocampus-dependent learning task (contextual fear conditioning) and were not induced by control treatments. Thus, snoRNAs likely present yet another layer of what seem to be infinitely complex mechanisms of control of gene expression, contributing to a complexity of organisms that surpasses the one that might be inferred from the relatively small number of genes.

Applications

To date, applications of snoRNAs were mostly limited to rRNA functional mapping. By engineering new antisense elements into the snoRNA scaffolds, specific methylation and pseudouridylation guides can be designed [69, 70], facilitating analysis of functional importance of individual sites within the RNA sequence [27]. Of note, far more success has been achieved with designing methylation guides compared to the ones directing pseudouridylation, which can be attributed to a more complex architecture of H/ACA snoRNAs, requiring two guide sequences to specify modification site [27].

Evidence implicating snoRNAs in regulation of gene expression was only found fairly recently, and the mechanisms by which snoRNAs accomplish this remain largely unknown. What is agreed on is that snoRNAs use base complementarity for recognition of pre-mRNA targets to bring in associated protein partners that catalyze chemical modifications of the RNA substrates. Thus, it is tempting to imagine designed snoRNA guides to be used as switches for fine-tuning targeted gene expression, such as induction of gene knockdown or preferential generation of certain splice variants. However, this field is still in its infancy and reports of projects tackling this issue are scarce. Perhaps the biggest achievement in the field is the development of a mammalian

vector system (called snoMEN for snoRNA modulator of gene expression) based on designed snoRNA expression for targeted knockdown of one or multiple genes simultaneously [57]. It has been demonstrated that chimeric snoRNAs (derived from the SNORD88 family which was previously shown to guide methylation of 28S rRNA) can repress genes via base pairing between an integral stretch of 19–21 nt of the snoRNA, termed box M, and the (partially) complementary targeted site of pre-mRNA located either in exonic or in intronic regions. Increasing the length of the M box sequence for up to 8 nt enhanced the silencing activity. Chimeric snoRNAs modulated both RNA and protein expression levels and the process was distinct from the classical RNA interference pathway. Knockdown was dependent on correct snoRNA processing as shown by the lack of silencing activity of snoRNAs harboring mutations in the essential structural regions, but at the same time was not coupled to methylating activity as 28S-antisense element mutants retained silencing activity. In fact, silencing activity was enhanced upon introducing mismatches into a methylation guide sequence, indicating dual functionality of chimeric snoRNAs (shift from rRNA methylation provided greater levels of snoRNA entering the gene silencing pathway). Additionally, complex expression cassettes were constructed enabling expression of multiple silencing snoRNAs (targeting different regions of a single pre-mRNA or multiple pre-mRNAs) along with a protein-coding RNA as part of a single transcript, providing means for simultaneous knockdown and protein replacement (“knockin”). The ability to target intronic sequences in the endogenous gene to be replaced avoids the need to create substitute cDNA with altered codons in order to prevent it from being knocked-down along with the cellular mRNA. The use of snoMEN vectors holds promise for basic gene expression research, in drug screening and target validation studies, and for gene therapy.

Outlook

In the last decade, snoRNAs have gone a long way from boring decorators of rRNAs and snRNAs to mysterious regulators of gene expression. At least one snoRNA has been linked to complex posttranscriptional processing of a protein-coding gene [1–3] and others (especially the ones with tissue-specific expression and as yet unidentified targets [67, 68]) are expected to perform similar tasks. Functional studies of snoRNAs have been hampered by gene redundancy, rendering gene knockout technology difficult to implement [47], and inaccessibility (tight packing) of snoRNAs in RNP complexes, resulting in only modestly successful gene silencing [71]. The importance of individual snoRNAs for normal physiology was thus mostly inferred from forward genetic screens. Recently, a method allowing

efficient knockdown of endogenous snoRNAs (and other ncRNAs) has been developed [72] that relies on the use of 2'-methoxyethyl/phosphorothioate backbone-containing RNA-DNA chimeric antisense oligonucleotides (ASO) to trigger RNaseH1-mediated cleavage of target RNAs. The entire length of a snoRNA is initially probed with an array of ASOs to determine the accessible sites. The most active ASO is then used for the specific depletion of targeted snoRNA. Chimeric ASOs reportedly reduced cellular levels of targeted RNAs by as much as 95% and effectively depleted snoRNAs in vitro (in cell culture) as well as in vivo following systemic administration to mice. Importantly, expression of genes that harbor intronic snoRNAs does not seem to be affected by the treatment. Thus, the method should allow the study of phenotypes resulting from selective attenuation of snoRNA function.

Bioinformatics is also likely to play an important role in the functional analysis of orphan snoRNAs. Computational tools for prediction of targets of orphan snoRNAs, such as snoTARGET [73], PLEXY [74], and RNAsnoop [75], are expected to aid in deciphering the physiological roles of these intriguing regulatory molecules. Provided that the snoRNA and their putative targets show similar expression profiles (i.e. are expressed simultaneously in the same tissue), these genes make excellent candidates to be analyzed at molecular level for potential impact of the snoRNA on the processing of cognate transcripts in cell lines. Also of note, the SnoReport software has been recently designed by Hertel et al. [76] to identify novel snoRNA genes in genomic sequences based solely on RNA secondary structure prediction combined with a machine-learning algorithm. As the approach does not rely on target sequence information to locate snoRNA-encoding genes, it should enable unbiased annotation of snoRNAs (i.e. even the orphan snoRNAs).

Interestingly, using their snoTARGET software, Bazeley et al. [73] have predicted that a number of SNORD116 family members preferentially bind to exonic rather than intronic sequences. Moreover, most SNORD116 supposedly target alternatively spliced pre-mRNAs, indicating that SNORD115 might not be a lone example of a snoRNA molecule regulating a differential splicing process. When it comes to ncRNAs, even “old acquaintances” such as snoRNAs, it seems we have only scratched the surface, and the future will bring many exciting surprises.

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