The emerging landscape of small nucleolar RNAs in cell biology

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Small nucleolar RNAs (snoRNAs) are a large class of small noncoding RNAs present in all eukaryotes sequenced thus far. As a family, they have been well characterized as playing a central role in ribosome biogenesis, guiding either the sequence-specific chemical modification of pre-rRNA (ribosomal RNA) or its processing. However, in higher eukaryotes, numerous orphan snoRNAs were described over a decade ago, with no known target or ascribed function, suggesting the possibility of alternative cellular functionality. In recent years, thanks in great part to advances in sequencing methodologies, we have seen many examples of the diversity that exists in the snoRNA family on multiple levels. In this review, we discuss the identification of novel snoRNA members, of unexpected binding partners, as well as the clarification and extension of the snoRNA target space and the characterization of diverse new noncanonical functions, painting a new and extended picture of the snoRNA landscape. Under the deluge of novel features and functions that have recently come to light, snoRNAs emerge as a central, dynamic, and highly versatile group of small regulatory RNAs. © 2015 The Authors. *WIREs RNA* published by John Wiley & Sons, Ltd.

INTRODUCTION

Small nucleolar RNAs (snoRNAs) form a large and abundant family of small noncoding RNA playing a conserved role in ribosome biogenesis. Present in all eukaryotes, snoRNAs are components of well-characterized ribonucleoprotein complexes referred to as snoRNPs.[1] They can be divided in two main classes, the box C/D and the box H/ACA snoRNAs, which differ in terms of their characteristic motifs and structure as well as in their protein binding preferences and the chemical modifications they

catalyze.[1–3] The first snoRNAs to be characterized functionally, the essential and highly conserved box C/D snoRNAs U3 and U8, are encoded in their own independent transcription units and play a role in the endonucleolytic cleavage and folding of pre-rRNA (ribosomal RNA).[4–6] While a small number of additional snoRNAs were subsequently also found to function in rRNA cleavage and processing, a much larger proportion of snoRNAs serve as guides for site-specific chemical modifications, mainly in rRNAs and also in the Pol-III-transcribed U6 small nuclear RNA (snRNA).[7,8] Related molecules, the small Cajal body RNAs (scaRNAs), guide the modification of the remaining snRNAs, which are Pol-II transcribed.[9] In vertebrates, guide snoRNAs are encoded in introns of host genes and their expression is thus tightly coupled. Tens to hundreds of residues of vertebrate rRNA are modified by snoRNAs, box C/D snoRNAs catalyzing 2′-O-ribose methylation and box H/ACA snoRNAs guiding pseudouridylation[2,10] (as described

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TABLE 1 | Classification of Human snoRNA According to Data from snoRNAbase[11]

	Box C/D	Box H/ACA
Member count	267	108
Endonucleolytic cleavage and folding of pre-rRNA	6 snoRNAs	0
Modifications in rRNA	118 snoRNAs (guiding 110 2′ O-methylated positions)	86 snoRNAs (guiding 100 pseudouridylated positions)
Modifications in U6 snRNA	6 snoRNAs (guiding 5 2′ O-methylated positions)	1 snoRNA (guiding 2 pseudouridylated positions)
Orphans	137 snoRNAs	21 snoRNAs

snoRNA, small nucleolar RNA; rRNA, ribosomal RNA.

in Table 1 for human). However, in several organisms, experimental and computational efforts to identify the whole snoRNA complement also led to the identification of snoRNAs for which no targets could be found (e.g., Refs 12–14) as listed in Table 2 for human. In addition to orphan snoRNAs, diverse recent studies describe snoRNAs displaying noncanonical characteristics and expression patterns, as well as interacting with unexpected protein partners and performing various noncanonical functions, suggesting a broader snoRNA reality than previously thought. In this review, we contrast canonical features, biogenesis, and function of snoRNAs with newly emerging aspects of the snoRNA landscape.

BOX C/D AND H/ACA snoRNAS: CHARACTERISTICS AND TARGETS

Canonical Boxes, Structures, and Guide Sequences

Typical box C/D snoRNAs (Figure 1(a)) are between 60 and 90 nucleotides in length and are characterized by the presence of conserved boxes C (consensus sequence RUGAUGA) and D (consensus sequence CUGA) near their 5′ and 3′ termini, respectively.[2,19,20] The boxes C and D align and fold into a kink-turn (k-turn) motif involving noncanonical G–A base pairing resulting in a sharp bend in the axis of the double-stranded RNA molecule.[3,21] The box C/D k-turn is essential for biogenesis and proper localization, serving as a binding site for core box C/D snoRNP proteins.[3,19,21,22] Additional motifs, the boxes C′ and D′, with the same consensus sequences as the boxes C and D, respectively, but typically less well conserved and often degenerate, are found toward the middle of the molecule[2,20] (Figure 1(a)). The guide regions with complementarity to the targets are located immediately upstream from the boxes D′ and/or D, the modified residues always base

pairing with the fifth residue upstream from these motifs.[3,19,20,17]

Box H/ACA snoRNAs are longer than typical box C/D snoRNAs, measuring between 120 and 140 nucleotides in length, and display a characteristic secondary structure consisting of two hairpins (Figure 1(b)). The hinge region connecting the two hairpins is formed of the H box (ANANNA where N can be any nucleotide). The highly conserved ACA box is found exactly three nucleotides upstream of the 3′ end of the molecule, immediately after the second hairpin.[20,23] The guide regions targeting positions in rRNA run through bulges in the middle of the hairpins, specifying by complementarity the exact position to be pseudouridylated in the target. The modified uridine is typically located 14–15 nucleotides upstream from the H or ACA box.[3,20,18] As for box C/D snoRNAs, box H/ACA snoRNAs can recognize up to two different substrates.[2,20]

Revised Definitions: Clarifying Canonical Targets and the Extent of the snoRNA Family

Updated Guide Sequences and Modulated Modifications

Canonical snoRNA guide regions have been well described to interact by complementarity with their targets, forming an RNA duplex of 6–20 base pairs, with no bulges and few mismatches.[23,24] While snoRNA antisense elements form a continuous segment immediately upstream from the box D′ and/or D in box C/D snoRNAs, the guide sequence is bipartite in the case of box H/ACA snoRNAs, passing through a bulge in one of the hairpins and involving residues on both sides of the hairpin[23,24] (Figure 1).

The identification and extent of canonical snoRNA antisense elements has been re-examined in recent years, providing a nuanced view of target

TABLE 2 | Description of Human Orphan snoRNAs

snoRNA Family[1]	Type	Member Count	Position in Genome (hg19)[2]	Host Gene	Predicted Target in rRNA[3]
SNORD112 (14q(0))	C/D	1	chr14:101364182-101364408	MEG8	–
SNORD113 (14q(I))	C/D	9	chr14:101391083-101412131	MEG8	–
SNORD114 (14q(II))	C/D	31	chr14:101416095-101459721	MEG8	–
SCARNA22 (ACA11)	Cajal	1	chr4:1976288-1976562	WHSC1	–
SNORA18 (ACA18)	H/ACA	1	chr11:93466557-93466838	TAF1D	–
SNORA29 (ACA29)	H/ACA	1	chr6:160206551-160206840	TCP1	–
SNORA38 (ACA38)	H/ACA	1	chr6:31590781-31591062	BAT2	–
SNORA71E/SNORA39 (ACA39)	H/ACA	1	chr20:37076651-37076936	SNHG11	–
SNORA49 (ACA49)	H/ACA	1	chr12:132515694-132515980	EP400	–
SNORA51 (ACA51)	H/ACA	1	chr20:2635638-2635919	NOP56	28S-1849
SNORA53 (ACA53)	H/ACA	1	chr12:98993338-98993737	SLC25A3	–
SNORA59A (ACA59)	H/ACA	1	chr1:12567225-12567526	VPS13D	–
SNORA59B (ACA59B)	H/ACA	1	chr17:19460798-19461099	FLJ10847	–
SNORA35 (HBI-36)	H/ACA	1	chrX:113865184-113865461	HTR2C	–
SNORD23 (HBII-115)	C/D	1	chr19:48259035-48259294	GLTSCR2	–
SNORD64 (HBII-13)	C/D	1	chr15:25230172-25230388	SNURF-SNRNP	–
SNORD89 (HBII-289)	C/D	1	chr2:101889323-101889586	RNF149	–
SNORD90 (HBII-295)	C/D	1	chr9:125642417-125642673	RC3H2	–
SNORD107 (HBII-436)	C/D	1	chr15:25227066-25227290	SNURF-SNRNP	–
SNORD108 (HBII-437)	C/D	1	chr15:25231997-25232217	SNURF-SNRNP	–
SNORD109A,B (HBII-438A,B)	C/D	2	chr15:25287046-25523631	SNURF-SNRNP	28S-4414
SNORD115 (HBII-52)	C/D	42	chr15:25417707-25515080	SNURF-SNRNP	–
SNORD116 (HBII-85)	C/D	29	chr15:25296548-25351826	SNURF-SNRNP	–
SNORA11B	H/ACA	1	chr14:91592694-91592972	C14orf159	–
SNORA11C	H/ACA	1	chrX:47247973-47248251	ZNF157	–
SNORA11D,E	H/ACA	2	ChrX:51806368-51933919	MAGED4	–
SNORA38B	H/ACA	1	chr17:65736710-65736990	NOL11	–
SNORA84	H/ACA	1	chr9:95054668-95054950	IARS	28S-3863
SNORD123	C/D	1	chr5:9548873-9549092	LOC100505806	–
SNORD124	C/D	1	chr17:38183720-38183973	MED24	–
SNORD125	C/D	1	chr22:29729077-29729322	AP1B1	–
SNORD126	C/D	1	chr14:20794534-20794760	CCNB1IP1	–
SNORD101 (U101)	C/D	1	chr6:133136371-133136593	RPS12	–
SNORD12C (U106)	C/D	1	chr20:47895407-47895635	ZFAS1	–
SNORA11 (U107)	H/ACA	1	chrX:54840728-54841008	MAGED2	–
SNORA12 (U108)	H/ACA	1	chr10:101996838-101997134	CWF19L1	–
SNORA73A,B (U17a,b)	H/ACA	2	chr1:28833802-28835349	RCC1	–
SNORD22 (U22)	C/D	1	chr11:62620307-62620582	SNHG1	–
SNORD50B (U50B)	C/D	1	chr6:86387232-86387452	SNHG5	–
SNORD73A,B (U73a,b)	C/D	2	chr4:152023134-152025118	RPS3A	–
SNORD118 (U8)	C/D	1	chr17:8076696-8076981	TMEM107	–
SNORD117 (U83)	C/D	1	chr6:31504076-31504301	DDX39B	–

TABLE 2 | Continued

snoRNA Family[1]	Type	Member Count	Position in Genome (hg19)[2]	Host Gene	Predicted Target in rRNA[3]
SNORD83A,B (U83A,B)	C/D	2	chr22:39709749-39711387	RPL3	18S-468
SNORD84 (U84)	C/D	1	chr6:31508803-31509030	DDX39B	–
SNORD86 (U86)	C/D	1	chr20:2636668-2636903	NOP56	–
SNORD97 (U97)	C/D	1	chr11:10822939-10823230	EIF4G2	–
SNORA16B (U98b)	H/ACA	1	chr1:212526085-212526367	PPP2R5A	–

snoRNA, small nucleolar RNA.
[1]Names of orphan snoRNAs were obtained from snoRNAbase[11] and HGNC.[15]
[2]For families with more than one member (member count > 1), the genomic interval indicated encompasses all members of the family (unless they are encoded in another region in the genome, in which case they appear on another line). The positions were obtained from snoRNAbase.[11]
[3]Targets of orphan snoRNAs in rRNA were predicted by comparative genomics analysis.[16] Predicted targets known to be modified are indicated here. Readers are referred to the supplementary data of Ref 16 for predictions of orphan snoRNA targets not known to be modified.

identification, modification, and regulation. In 2011, a subset of both *Saccharomyces cerevisiae* and human box C/D snoRNAs were shown to have extended complementary sequences that are not contiguous with the guide sequence and in some cases, are at the other end of the snoRNA.[25] The novel interactions involve between 4 and 10 additional snoRNA–target base-pairing residues that had not been described previously. The extra base pairing likely promotes the snoRNA–target interaction or increases its stability, possibly by facilitating access to highly structured regions in rRNA.[25] The extra base pairing was shown to stimulate methylation by up to fivefold.[25]

In a subsequent study, extensive comparative genomics analyses led to the prediction of targets for orphan snoRNAs. Functions of homologous snoRNAs were found to be evolutionarily stable, allowing the assignment of targets in rRNA for four human orphan snoRNAs (see Table 2) and the prediction of guiding snoRNAs for eight known modified positions in rRNA and two known modified positions in snRNA, for which no guide had been previously assigned.[16] In addition, complementarities were predicted between many orphan snoRNAs and regions in rRNA not known to be modified, raising the possibility that these modifications might be tissue- or condition-specific and might contribute to the specialization of ribosomes.[16,26] Experimental validation will be important to confirm these proposed functional relationships.

These studies collectively suggest that rRNA modification by snoRNAs can be modulated or dosed by other regions of the snoRNA and that the level or likelihood of modification of many snoRNA-guided positions in rRNA can be regulated. Supporting the latter concept, the mammalian target of rapamycin (mTOR) pathway was recently found to regulate the abundance of the box H/ACA snoRNA U19, responsible for the highly conserved pseudouridylation of 28S rRNA on two residues.[27] Rapamycin treatment, which inhibits the mTOR pathway, resulted in an increase of 28S rRNA pseudouridylation, suggesting that mTOR pathway can conditionally regulate rRNA modification.[27] It can reasonably be expected that an increasing number of such examples will emerge over the next few years.

snoRNA Redundancy

Related to the variability in target modification levels, redundancy also adds a level of complexity in the study of the regulation of snoRNA targets. Redundancy in snoRNA members and targets has been noticed in numerous organisms, with many snoRNAs being present as small families of two or three near identical members, but some present as tens of copies, such as the 42 copies of HBII-52 in human,[28] and even up to tens of thousands of copies for a snoRNA retroposon in the platypus.[29] When they are not orphans, such paralogs typically guide modification of the same targets and are encoded in different introns of the same host gene although this is not always the case.[30,31] In human, according to snoRNAbase,[11] 22 of 120 (18%) positions modified by snoRNAs in the large ribosomal subunit are identified by more than one snoRNA, while for the small ribosomal subunit, this proportion of residues modified by redundant snoRNAs goes up to 29% (22 of 76).

snoRNAs have been described as mobile genetic elements that can both migrate intragenomically to different locations and copy themselves using retrotransposition mechanisms as witnessed by their numerous copies in mammalian genomes and close relatives.[29,32,33] Many snoRNA families are ancient and can be traced back to the last eukaryotic common ancestor (LECA), but while many of their host genes are believed to have existed in the LECA, comparative
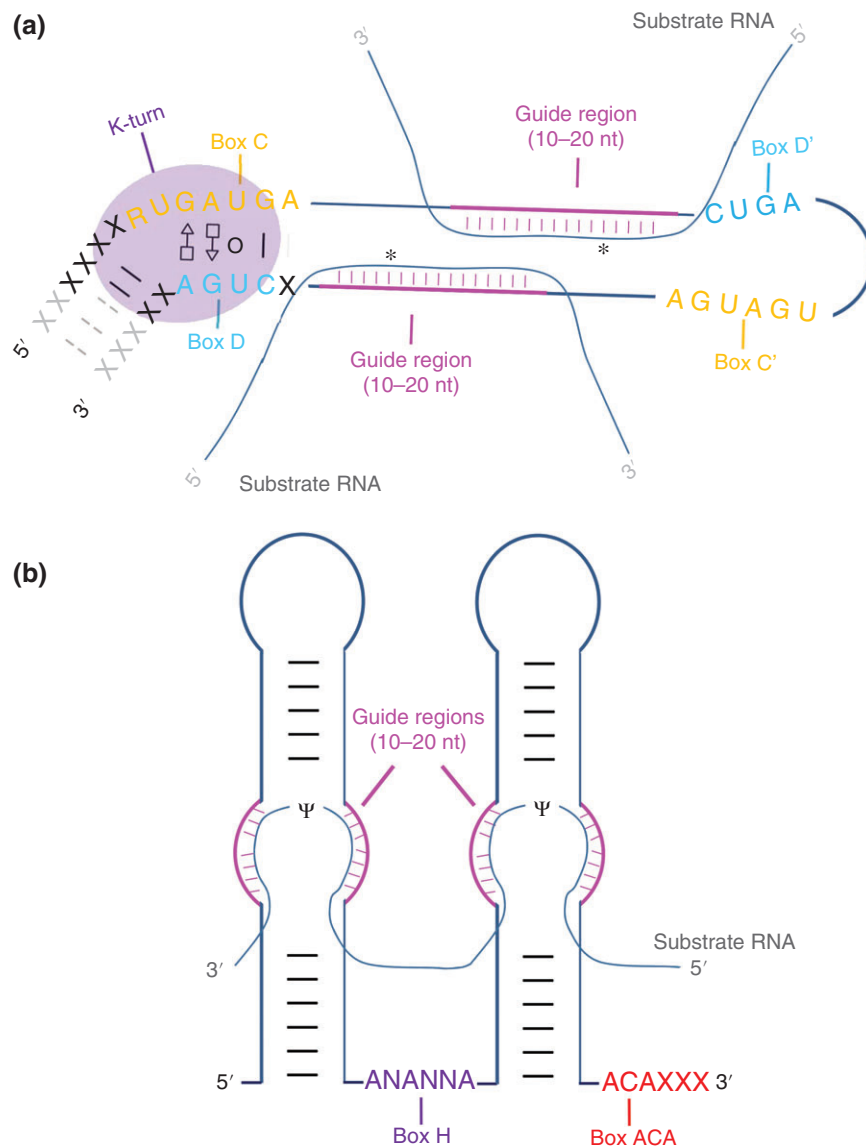
FIGURE 1 | Features of small nucleolar RNAs (snoRNAs). (a) Box C/D snoRNAs are characterized by the presence of boxes C/C' and D/D' sequence motifs, represented in orange and cyan, respectively. Boxes C and D can interact forming noncanonical G–A pairings resulting in a structural motif called a k-turn (highlighted in purple). The k-turn is stabilized by the presence of canonical base pairing in proximity. Guide regions of box C/D snoRNAs, specifying the residue that will be methylated in the target, are found immediately upstream from the boxes D and/or D' (shown in pink). The modified residue in the target is base paired with the snoRNA residue located exactly five nucleotides upstream from the box D or D' (indicated by an asterisk).[17] (b) Box H/ACA snoRNAs consist of two hairpins separated by a box H sequence motif (shown in purple) and followed by a box ACA found three nucleotides from the 3' end (shown in red). The guide regions (shown in pink) specifying the residue that will be pseudouridylated (ψ) in the target are found in bulges in the hairpins and the modified residue is located 14–15 residues upstream from the box H or ACA.[18]

genomic studies by Hoeppner and Poole suggest that the intronic location of snoRNAs is not ancestral.[31] They have recently proposed that snoRNA evolution follows a constrained drift model whereby snoRNAs migrate intragenomically to different host genes based primarily on their expression profiles.[31] It is proposed that the high mobility and redundancy of snoRNAs likely favored the acquisition of new targets and new functions.[33]

New Members

While the mammalian complement of both box C/D and box H/ACA snoRNAs has been extensively probed using both experimental and computational approaches (see, e.g., Refs 12–14, 34, and 35), deep sequencing is still identifying potential new members of these classes. Recent photoreactive nucleotide-enhanced cross-linking and immunoprecipitation (PAR-CLIP) of core snoRNA-associated
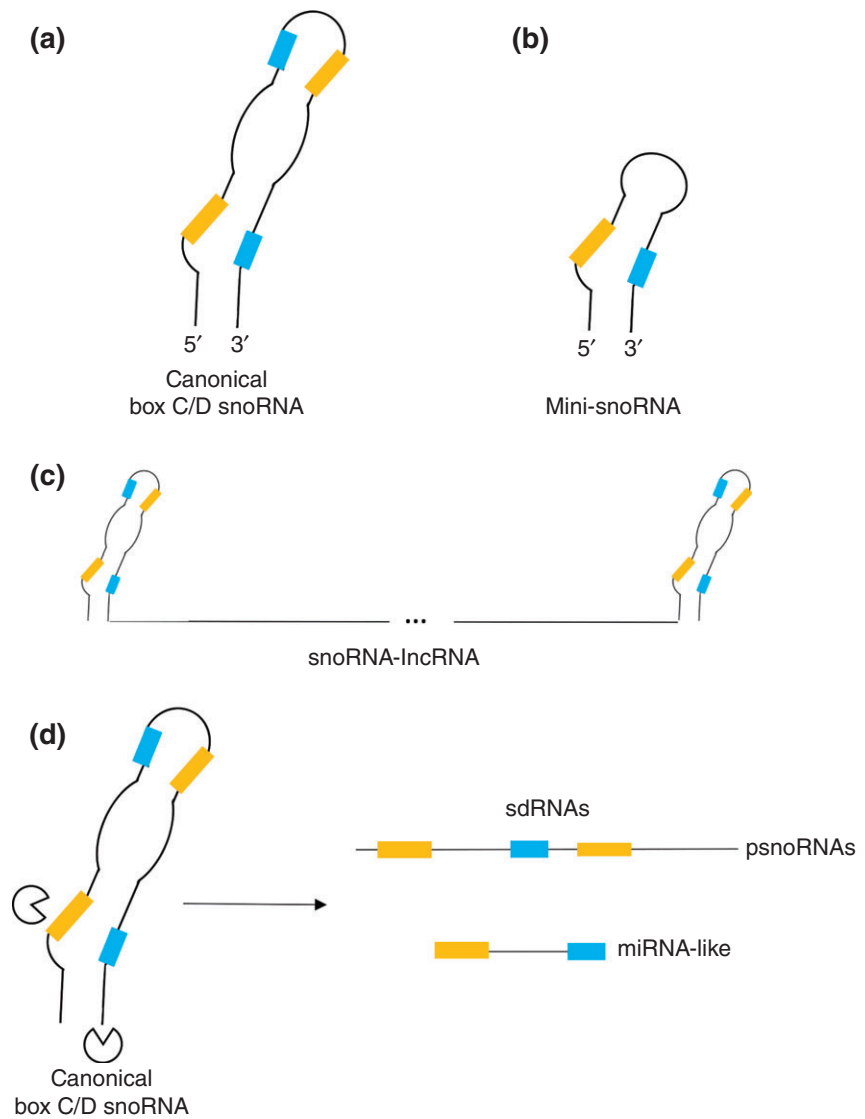
FIGURE 2 | Extent and diversity of box C/D small nucleolar RNA (snoRNA)-like molecules. While box C/D snoRNAs typically range between 60 and 90 nucleotides in length (a), mini-snoRNAs, binding the same interaction partners as classical snoRNAs, but lacking boxes C' and D' have been detected by sequencing (b). At the other end of the spectrum, snoRNA-lncRNA (long noncoding RNA) consisting of two box C/D snoRNAs and the intronic sequence separating them have also been reported (c). Many studies have also detected stable snoRNA-derived RNAs (sdRNAs) likely generated by the action of endonucleases or exonucleases. Some sdRNAs are miRNA-like[37–39] while others are longer products[40] (d). Boxes C/C' and D/D' are represented, respectively, by orange and cyan colored boxes.

proteins followed by sequencing of the bound RNA has allowed the detection of dozens of novel human snoRNAs, some of which have also been detected by northern blot and/or are likely copies or homologs of known snoRNA identified in human or in other organisms.[36] Deep sequencing is also revealing the existence of very small and very large snoRNA-like molecules (Figure 2). Mini-snoRNAs of type box C/D with boxes C and D but lacking boxes C' and D' were detected by deep sequencing of HEK293 cells and were detected bound to core-associated proteins.[36] Such mini-snoRNAs can be as short as 27 nucleotides in length and would not be long enough to hold two guide sequences.[36] In parallel, long noncoding RNAs with snoRNA ends (sno-lncRNAs) were identified by deep sequencing of the nonpolyadenylated transcriptomes of HeLa and human embryonic stem cells and, in a later study, in rhesus and mouse embryonic stem cells.[41,42] Sno-lncRNAs are produced from loci containing two snoRNAs within the same intron. While they are believed to be processed by the canonical snoRNP machinery, they also bind the splicing factor RBFOX2 and have been proposed to act as molecular sinks, titrating RBFOX2 in specific cell types, resulting in splicing alteration.[41] The small number of pairs of snoRNAs encoded in the same host intron appears to limit the number of possible sno-lncRNAs; although one example of a sno-lncRNA produced from snoRNAs in neighboring introns has been described in mouse, its expression proposed to be regulated by alternative splicing.[42] Overall, the snoRNA count is likely to continue to increase over the next few years, although newly discovered members are expected to be of decreasing abundance, highly cell-type specific, or highly noncanonical.

BIOGENESIS AND INTERACTORS OF snoRNA

In vertebrates, the great majority of snoRNAs (>98% in human) are encoded in introns of host genes.[1] The biogenesis of most intronic snoRNAs is believed to involve co-transcription with the host gene, splicing, debranching of the intron lariat, and exonucleolytic digestion of both the 5′ and 3′ sequences flanking the snoRNA.[22,43,44] Binding of proteins near the snoRNA termini likely blocks exonucleolytic trimming ensuring production of mature snoRNAs with conserved ends.[45]

Box H/ACA snoRNP assembly involves the preformation of a protein-only complex containing assembly factors and core-binding proteins including NAP57/dyskerin.[46,47] During remodeling of the complex, the early and late assembly factors Shq1 and Naf1 are believed to prevent nonspecific RNA binding and inhibit the activity of the immature RNP, respectively.[46,47] Following binding to the RNA, Naf1 is replaced by the core protein Gar1, resulting in a fully active RNP.[48] The exact termini of H/ACA snoRNAs have been known for many years, all members ending exactly three nucleotides after the ACA box.[11] Recent PAR-CLIP experiments show strong preferential binding of NAP57/dyskerin to the box H and the 5′ portion of the first hairpin.[36]

In contrast to box H/ACA snoRNPs, the assembly of box C/D snoRNAs was hypothesized to involve the sequential binding of core proteins on the snoRNA. The core protein 15.5K was proposed to initiate the assembly by binding the k-turn secondary structure motif resulting from the noncanonical base pairing of residues in the boxes C and D.[49] The binding of 15.5K, believed to occur at the C1 splicing complex stage, was proposed to serve as a scaffold for the subsequent assembly of the mature snoRNP complex, including the binding of core proteins NOP58 and fibrillarin (FBL).[22,44] However, extensive proteomic analyses reported recently lead to the conclusion that as for box H/ACA snoRNPs, box C/D snoRNP formation also involves preassembly of a protein-only complex involving core proteins 15.5K and NOP58.[50] The binding of the assembly factor NUFIP to 15.5K during the early maturation steps of the complex, before binding to the snoRNA, is proposed to prevent premature activation of the complex, similar to the role of Naf1 for box H/ACA snoRNPs.[50] NUFIP serves as an adaptor protein allowing the recruitment of the large HSP90–R2TP chaperone complex required for the accumulation of snoRNPs and the proper folding of its core proteins (reviewed in Ref 51). Fibrillarin and then NOP56 would subsequently bind the complex with release

of assembly factors including NUFIP, resulting in an active box C/D snoRNP.[50] *In vivo* cross-linking experiments had suggested that the core-binding protein NOP58 and one copy of the methyltransferase FBL bind the C and D box portions involved in the internal stem of the k-turn (Figure 1(a)), while NOP56 and another copy of FBL bind boxes C′ and D′.[22,44] However, recent PAR-CLIP experiments showed very similar binding patterns for FBL, NOP58, and NOP56 across all detectable box C/D snoRNAs, with no significant difference between the NOP58 and NOP56 patterns with respect to the characteristic boxes.[36] The discrepancies between these studies might be explained by snoRNA-specific differences or by the different resolution of the methods and will require further experiments to clarify.

Two independent recent deep-sequencing studies of small RNAs have shown that human box C/D snoRNAs have very precise termini determined by the position of the boxes C and D. With very few exceptions, box C/D snoRNAs start exactly four to six nucleotides upstream from the box C (Figure 1(a)) and end between two and five nucleotides downstream from the box D,[36,52] supporting the idea of protection from exonucleolytic cleavage by the presence of proteins bound to the k-turn.[36,45,52] Interestingly, box C/D snoRNAs can be further classified with respect to their exact ends by considering their dependency on the core-binding protein NOP58.[52] Although NOP58 is seen as a core component of all box C/D snoRNPs, a large subpopulation of box C/D snoRNAs is not affected by a knockdown of this protein in human. snoRNAs significantly affected by the depletion of NOP58 typically have 'long' ends, starting five nucleotides upstream from the box C and ending four or five nucleotides downstream from the box D. The extra terminal residues are complementary, and should support the formation of a terminal stem that is likely to increase the stability of the k-turn.[52] In contrast, box C/D snoRNAs that are not affected by the depletion of NOP58 typically have 'short' ends, many with only two nucleotides after the box D, making k-turn formation unlikely. These data suggest that a subset of different proteins could bind the ends of these snoRNAs, resulting in differential trimming. In support of this possibility, a subset of human snoRNAs with short ends are strongly affected by the depletion of the splicing factor RBFOX2,[52] for which previously determined consensus binding sites include a partial box C (GUGAUG) as determined by CLIP-seq in human embryonic stem cells.[53] This CLIP-seq dataset included many reads mapping to snoRNAs,[53] suggesting that RBFOX2 could be a

component of a subset of box C/D snoRNPs, could compete with core-binding proteins, or could be important for splicing during snoRNA biogenesis. As mentioned above, RBFOX2 has already been shown to bind sno-lncRNAs.[41]

NONCANONICAL TARGETS AND FUNCTIONS FOR snoRNAS

During the last decade, a multitude of independent studies have reported diverse noncanonical functions for subsets of snoRNAs (summarized in Table 3). In particular, regions of complementarity between snoRNAs and nonclassical RNA targets, and diverse innovative flavors of deep sequencing prompted investigations that have led to significant discoveries extending our view of snoRNA functions.

Methylation and Pseudouridylation of Noncanonical Targets

Canonical targets of snoRNAs have been extensively characterized and hundreds of 2′-O-methylated or pseudouridylated residues in human rRNA have been identified as modified by snoRNAs (Table 1).[2,10,11] However, these two types of noncoding RNA might just be the tip of the iceberg in terms of snoRNA targets. In archaea, in addition to modifying rRNA, small RNAs are also known to modify tRNAs.[2] It was shown that snoRNA guide elements could be altered to target the modification of selected non-rRNAs, albeit less efficiently than rRNA, presumably because the substrate was not in the nucleolus.[65] Recent PAR-CLIP experiments in HEK293 cells described above for the identification of new snoRNA members also led to the identification of new potential targets. Among the RNAs repeatedly identified as bound by core box C/D snoRNPs, vault RNA 1-2, 7SK RNA, and 7SL RNA were detected, and subsequently validated as carrying 2′-O-methyl sites, although the targeting snoRNAs are unknown.[36] In addition, box C/D snoRNAs were identified cross-linked to box H/ACA snoRNPs and vice versa, suggesting that snoRNAs can guide modifications on each other. Indeed, primer extension experiments confirmed that the box H/ACA snoRNA SNORA61 is 2′-O-methylated and box C/D snoRNAs SNORD16 and SNORD35A are pseudouridylated although the guiding snoRNAs were not identified.[36] Finally, numerous mRNAs were also detected cross-linked to snoRNPs in these experiments. A statistically significant number of guiding snoRNAs were identified by complementarity

for a substantial number of these mRNAs, suggesting that snoRNA might play a role in mRNA maturation.[36]

More recently, two groups performed pseudouridine profiling using deep-sequencing approaches in yeast and human.[66,67] In both organisms, two classes of pseudouridylation sites can be described: those guided by snoRNAs and those produced by pseudouridine synthases recognizing their targets through consensus sequences rather than with an RNA guide. Both studies detected known pseudouridylated positions in rRNA and snRNA, as well as in both box C/D and H/ACA snoRNAs, supporting the idea of snoRNA cross-modifying each other.[36,66,67] Hundreds of pseudouridylated residues in mRNA were also detected by both groups.[66,67] Genetic perturbation experiments of different pseudouridine synthases and statistical analyses comparing the extent of complementarity between targets and guide snoRNAs to shuffled sequences suggest that many pseudouridylation sites in mRNA are likely snoRNA-guided.[67] As discussed in the next section, it was not the first time snoRNAs were proposed to modify mRNA, and although further experiments will be required to match pseudouridylated mRNA targets with their guiding box H/ACA snoRNAs, these studies suggest a much broader landscape of chemically modified snoRNA targets than previously described. Interestingly, as reported previously for modifications in rRNA and snRNA, Schwartz et al. note that pseudouridylation seems to occur in regions critical for interactions, suggesting that such studies could also help determine sites involved in intramolecular and intermolecular interactions.[67]

Noncanonical Functions of snoRNAs
Regulation of Editing, Splicing, or Both?

The most comprehensively studied snoRNAs involved in noncanonical functions are the orphan box C/D snoRNA families SNORD115 (M/HBII-52) and SNORD116 (M/HBII-85) encoded in the imprinted SNURF-SNRPN locus on human chromosome 15.[40,68] Loss of expression of regions of the SNURF-SNRPN locus is believed to be the main cause of the Prader–Willi syndrome (PWS), a neurodevelopmental congenital disease representing the most common genetic cause for marked obesity in humans (reviewed in Ref 69). SNORD115 and SNORD116 are large families of box C/D snoRNAs consisting of 42 and 29 members, respectively, in human,[11] and which are encoded in introns of very large nuclear-retained noncoding transcripts.[70] While SNORD116 has been shown to produce both

TABLE 3 | Reported Noncanonical Functions of Human snoRNAs

Function	Box C/D snoRNAs	Box H/ACA snoRNAs	References
Regulation of chromatin structure[1]	SNORD97/U97 (F)	SNORA64/U64 (F)	54
	SNORD15A/U15A (F)	SNORA75/U23 (F)	
	SNORD105B/U105B (F)	SNORA44/ACA44 (F)	
	SNORD15B/U15B (F)	SNORA73B/U17b (F)	
	SNORD13/U13 (H)	SNORA43/ACA43 (F)	
	SNORD50B/U50B (H)	SNORA73A/U17a/E1 (F)	
	SNORD50A/U50 (H)	SNORA40/ACA40 (F)	
		SNORA81/HBI-61 (F)	
		SNORA74A/U19 (H)	
		SNORA72/U72 (H)	
		SNORA38/ACA38 (H)	
		SNORA62/E2 (H)	
		SNORA74/U19 (H)	
		SCARNA4/ACA26 (H,F)	
Regulation of splicing	SNORD115/HBII-52		40,55
	SNORD116/HBII-85		41
	SNORD88C/HBII-180C		56
Modification of noncanonical target		SNORA70/U70 (RN7SK[2])	36
		SNORA31/ACA31 (RN7SL[2])	
Mediators of oxidative stress	SNORD32A/U32A	SCARNA22/ACA11	57,58
	SNORD33/U33		
	SNORD35A/U35A		
	SNORD3A/U3-2		59
	SNORD60/U60		60
sdRNAs with miRNA-like capabilities[3]	SNORD2/snR39B	SCARNA15/ACA45	38,39
	SNORD3/U3		
	SNORD78/U78		
	SNORD93/HBII-336		
	SNORD66/HBII-142		
	SNORD27/U27		
	SNORD100/HBII-429		
	SNORD83A/U83A		
	SNORD15A/U15A		
	SNORD74/U74		
snoRNAs overlapping annotated miRNA-producing loci[4]	SNORD12b/HBII-99B (miR-1259)	SNORA34/ACA34 (miR-1291)	39,61,62
		SNORA81/HBI-61 (miR-1248)	
	SNORD126 (miR-1201)	SNORA36B/ACA36B (miR-664)	

snoRNA, small nucleolar RNA; caRNA, chromatin-associated RNA; sdRNA, sno-derived RNA.
[1]snoRNAs displaying an enrichment of at least 10-fold in caRNA in human fibroblasts (F) or HeLa cells (H) as reported in Ref 54 using datasets from Refs 63 and 64.
[2]Predicted noncanonical target experimentally shown to be pseudouridylated indicated in parentheses.
[3]sdRNAs that display the same level of activity as ACA45 as evaluated in Ref 39.
[4]Overlapping miRNA indicated in parentheses.

snoRNAs and sno-lncRNAs, and to interact with the splicing factor RBFOX2 as discussed above,[41] numerous reports suggest noncanonical functions and interaction partners for SNORD115.

In place of a region of complementarity to rRNA upstream of their box D, members of the SNORD115 family display a conserved region of 18 nucleotides complementary to an alternatively spliced portion of exon V of the serotonin 2C receptor (5-HT2CR).[34] 5-HT2CR transcripts are known to undergo adenosine-to-inosine editing on five closely spaced sites on exon V, resulting in altered translation products and reduced activity owing to decreased efficiency of G-protein coupling. Mutant mice

expressing only the fully edited version of 5-HT2CR display many characteristics of PWS, demonstrating the importance of tight regulation of editing of these transcripts.[71] The antisense element of SNORD115 is complementary to a region encompassing three of the five edited sites, and one of these three sites, the C-site, is predicted to pair with the fifth nucleotide upstream of the SNORD115 box D.[72] Mouse SNORD115 base pairing with 5-HT2CR was shown by Vitali et al. to decrease the efficiency of RNA editing on the C-site, suggesting a role for SNORD115 in the regulation of editing of 5-HT2CR transcripts.[72] The exact mechanism of regulation is still however unclear, as it was then proposed by Kishore and Stamm that the regulation of alternative splicing of exon V did not depend on methylation of the C-site but rather resulted from masking of a splicing silencer by SNORD115 binding.[55] Supporting a role in the regulation of editing, the loss of SNORD115 in a mouse model of PWS was found to lead to increased editing but not splicing of 5-HT2CR pre-mRNAs.[73] Thus, the biological significance of the SNORD115:5-HT2C base paring requires further validation and characterization.

To complicate things further, Kishore et al. then reported mouse SNORD115 snoRNAs processed into smaller fragments referred to as processed snoR-NAs (psnoRNAs), which associate with heterogeneous nuclear ribonucleoproteins (hnRNPs) rather than canonical interactors.[40] In the same study, the alternative splicing of several pre-mRNAs, including pre-5-HT2CR, was shown to depend on the abundance of SNORD115 snoRNAs, and it was proposed that the longest SNORD115 psnoRNA, which retains its boxes C and D but not the terminal stem, might associate with hnRNPs to regulate the splicing of these events.[40] As found for 5-HT2CR, regions of complementarity to the SNORD115 psnoRNAs were identified in the other target pre-mRNAs, in close proximity to the regulated alternative splicing events, suggesting direct binding.[40] The importance of SNORD115 psnoRNAs in human and mouse brain was however disputed by Bortolin-Cavaillé and Cavaillé who failed to detect the reported variants despite extensive experimentation.[74] Following re-analysis of the original SNORD115 psnoRNA data, they propose that the canonical form is the most abundant species.[74]

Confusion also surrounds the identity of the main protein interactors of SNORD115 members. Supporting previous findings by Kishore and Stamm discussed above,[40] an antisense RNA affinity purification approach followed by mass spectrometry, to purify SNORD115 complexes from mouse brain, revealed several protein interactors including nucleolin and ELAVL1, both of which were confirmed by

immunoprecipitation, as well as RNA helicases and hnRNPs but not canonical box C/D snoRNA-binding proteins.[75] In contrast, Bortolin-Cavaillé and Cavaillé identify SNORD115 snoRNAs as canonical box C/D snoRNAs associated with fibrillarin, as was previously reported.[72,74]

Thus, while the importance and relevance to human health of the SNURF/SNRPN snoRNAs is not disputed,[69,76] and their involvement in noncanonical functions is clear, further studies are required to determine the exact RNP composition and regulatory mechanisms guided by these intriguing snoRNAs.

Regulators of Pre-mRNA Splicing and Maturation

In addition to the SNORD115 family proposed to regulate the alternative splicing of a handful of pre-mRNA including the serotonin 2C receptor,[40,55] and the SNORD116 family proposed to regulate the availability of the splicing factor RBFOX2,[41] it has been suggested that other snoRNAs participate in pre-mRNA maturation (Table 3). SNORD88C (HBII-180C), a box C/D snoRNA with a box D′ guide sequence complementary to 28S rRNA, was also shown to harbor a region of complementarity to intronic portions of several pre-mRNA including the fibroblast growth factor receptor 3 (FGFR3), immediately downstream from its box D′, termed the M-box[56] (Figure 3). The overexpression of a mini-gene containing part of the FGFR3 pre-mRNA including the target region resulted in the appearance of a splicing isoform of endogenous FGFR3 that was recapitulated in a cell line that naturally expresses SNORD88C at a low level.[56] The long region of complementarity to several different pre-mRNAs and its unusual position (immediately downstream from the box D′ rather than upstream, as shown in Figure 3) prompted further investigation, and it was found that artificial SNORD88C constructs with a modified M-box could mediate the knockdown of complementary targets.[78] Such snoMEN were thus proposed as a novel technology for the modulation of gene expression, which can easily be used for the generation of stable cell lines with targeted protein replacement.[78,79] Supporting these findings, other artificial snoRNAs were also shown to regulate the splicing and maturation of specific targets. Analogs of the box C/D snoRNA U24 were designed with altered box D guide sequences, introducing complementarity to key positions in the second intron of the chaperone HSPA8.[80] Antisense elements to the branch point, splice donor and acceptor sites, as well as the first and last nucleotides of the intron all resulted in increased alternative splicing and increased production of a known minor isoform of HSPA8.[80] In a
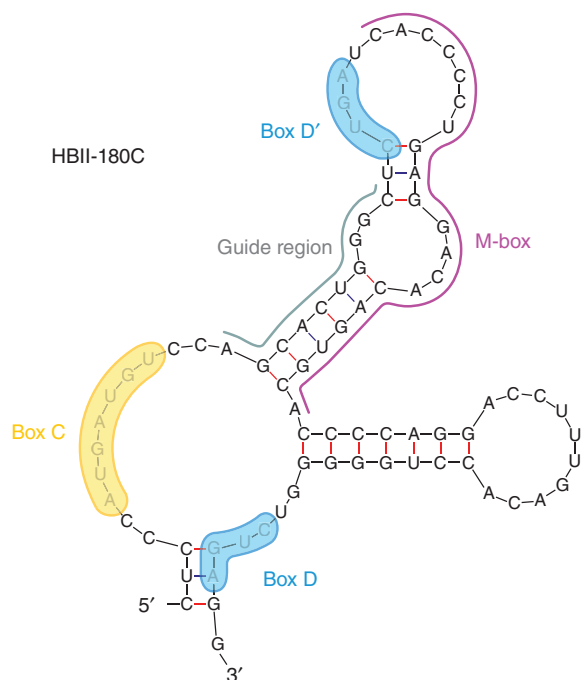
FIGURE 3 | Sequence features of HBII-180C (SNORD88C). HBII-180C is a box C/D small nucleolar RNA (snoRNA) displaying boxes C (highlighted in orange), D′ and D (highlighted in cyan), as well as a guide region complementary to 28S rRNA specifying its methylation on position C3680.[11] HBII-180C also has a region called the M-box (highlighted in pink) complementary to regions in specific pre-mRNA including FGFR3.[56] The sequence shown for HBII-180C is the most abundant form detected by deep sequencing in multiple human cell lines[52] and the structure was predicted using RNAfold.[77]

follow-up study, a subset of these artificial U24 snoR-NAs directed at HSPA8 pre-mRNA were also shown to cause a significant decrease in mRNA levels.[81] As for the snoMEN constructs,[78] this knockdown effect was dependent on the complementary interaction between snoRNA and target, but independent of the ability of the snoRNA to guide methylation of the target.[81]

Chromatin-Associated Factors

In Drosophila, snoRNAs have been found to make up a significant proportion of the chromatin-associated RNA (caRNA), which is stably linked to chromatin and is essential for the establishment of open chromatin structures within euchromatic regions.[54] The strong enrichment of snoRNAs in caRNA had previously been reported in human.[63,64] Chromatin condensation was found to be a reversible process, as incubation of compacted RNaseA-treated chromatin with nuclear RNA led to chromatin reopening, in both Drosophila and human. The RNA-dependent decondensation effect is specific and does not occur following the addition of tRNA, poly(U), or a DNA

oligonucleotide.[54] Among the most enriched caRNA in both Drosophila and human, a small subset of low abundance snoRNA (Table 3), predominantly of type H/ACA, were identified, suggesting a specific role for these snoRNAs in chromatin structure regulation.[54,63,64] The chromatin opening is dependent on the decondensation factor Df31, detected by mass spectrometry among the proteins bound to chromatin. Df31 was shown to specifically bind to chromatin-associated snoRNAs, but intriguingly, none of the core C/D or H/ACA snoRNP proteins were detected, raising the possibility of a strong noncanonical protein composition for chromatin-associated snoRNPs.[54]

Mediators of Stress and Regulators of Lipid Traffic

In 2011, a genetic screen to identify genes critical for survival under lipotoxic growth conditions, a metabolic stress implicated in the pathogenesis of diabetes complications, unexpectedly turned up three highly conserved box C/D snoRNAs.[57] U32A (SNORD32A), U33 (SNORD33), and U35A (SNORD35A), encoded in the introns of ribosomal protein gene RPL13A, were found to accumulate in the cytoplasm of Chinese hamster ovary cells following palmitate treatment, which were used to model a lipotoxic pathophysiological state. In addition, the directed knockdown of the RPL13A snoRNAs led to protection against lipotoxicity.[57] Further experiments demonstrated that these snoRNAs are mediators of general oxidative stress, responding in the same manner to H_2O_2 treatment as to lipotoxicity. It was thus concluded that the RPL13A snoRNAs U32A, U33, and U35A carry out a noncanonical role in the regulation of oxidative stress response pathways, perhaps through the regulation of mRNA translation or the sequestration of specific RNAs.[57]

More recently, two other studies identified additional box C/D snoRNAs possibly involved in the regulation of endoplasmic reticulum function and stress response. The box C/D snoRNA SNORD3A was shown to display elevated expression in Creutzfeldt–Jacob patients, in a mouse model of the disease and in scrapie-infected mice, compared with normal controls.[59] SNORD3A expression was also shown to correlate with the activation of ATF6, a central event in the unfolded protein response.[59] Then, a study to delineate genes involved in the internalization of cholesterol identified the SNORD60 box C/D snoRNA as involved in cholesterol transport to the endoplasmic reticulum.[60] Reduced expression of SNORD60 results in a cholesterol trafficking defect that is abrogated by complementation with

SNORD60, with a requirement for an intact box D′ and antisense element.[60] In addition, SNORD60 was found to bind canonical snoRNP proteins.[60] Interestingly, as for U32A, U33, and U35A, the downregulation of SNORD60 does not result in reduced methylation of its predicted 28S rRNA target, indicating that the observed phenotypes are not caused by aberrant rRNA modification.[57,60] Although the mechanism by which these snoRNAs are regulating these processes is not clear and multiple hypotheses have been proposed, it involves base pairing of the guide region in the case of SNORD60 and cytoplasmic localization of the RPL13A snoRNAs. The next few years should provide welcome clarification of these noncanonical mechanisms for these unusualsnoRNAs.

In addition to box C/D snoRNAs reported to modulate the cell's response to stress, ACA11, an orphan box H/ACA snoRNA, was reported as frequently overexpressed in multiple cancers and as involved in the suppression of oxidative stress.[58] ACA11 was initially investigated owing to its high expression in multiple myelomas and other cancers displaying a t(4; 14) chromosomal translocation affecting the expression of the histone methyltransferase WHSC1. However, it was found that the increased expression of ACA11, encoded in an intron of WHSC1, but not the overexpression of the WHSC1 cDNA, was responsible for the phenotypic effects observed, including resistance to chemotherapy and increased proliferation of multiple myeloma cells.[58] ACA11 was detected binding proteins involved in RNA processing including hnRNPs, splicing factors, and an RNA helicase, but not canonical box H/ACA snoRNA interactors, by both RNA affinity purification followed by mass spectrometry, and cross-linking immunoprecipitation followed by real-time polymerase chain reaction (RT-PCR).[58] As ACA11 was originally cloned from a HeLa cell extract immunoprecipitated with an antibody against GAR1, a canonical interactor of box H/ACA snoRNAs,[82] it will be important to further investigate whether ACA11 can form more than one type of snoRNP and whether these complexes are regulated in a tissue-specific manner. Interestingly, an immunoprecipitation of ILF3, one of the protein interactors of ACA11, pulls down snoRNA intermediates including U33 and its host gene RPL13A,[58] providing a possible link with the regulation of oxidative stress pathways discussed above.[57] The authors investigated this link, finding that ACA11 can suppress oxidative stress, increase resistance to chemotherapy, and contribute to proliferation of multiple myeloma cells, suggesting a possible oncogene role for ACA11.[58] It will be important to further characterize the relationship between ACA11 and the other snoRNAs involved in oxidative stress regulation in future studies.

Precursors to Smaller RNAs

Numerous small RNA sequencing datasets from diverse organisms, generated predominantly for the study of miRNAs, have revealed the presence of small fragments mapping to both box C/D and box H/ACA snoRNAs (see, e.g., Refs 37, 56, and 83) (Figure 2(d)). Such sno-derived RNAs were abbreviated sdRNAs for snoRNA-derived small RNAs, and were found to measure predominantly between 17 and 30 nucleotides in length,[37] although as discussed above, longer psnoRNAs have also been detected.[40] In 2008, as these sdRNAs were first being reported, a study of RNAs co-immunoprecipitated by antibodies against human Argonaute proteins in HEK293 cells identified several sdRNAs originating from H/ACA snoRNAs.[38] Argonaute proteins have been well characterized as cellular binding partners of miRNAs, which together form the core of the RNA-induced silencing complex (RISC).[84] One such sdRNA co-immunoprecipitated with AGO2 originated from the H/ACA snoRNA ACA45. The ACA45 sdRNAs display miRNA-like functionality in luciferase assays and require the miRNA-processing enzyme Dicer, but not Drosha/DGCR8, for their biogenesis.[38] In a subsequent report, other sdRNAs, this time derived mostly from box C/D snoRNAs, were also found to have miRNA capabilities displaying efficient gene silencing in four human cell lines.[39] In parallel to sdRNA displaying miRNA characteristics, reported miRNAs with precursors resembling snoRNAs were also identified.[61,62] Several well-characterized miRNAs with validated gene silencing targets originate from precursors displaying box C/D or box H/ACA features including antisense regions with complementarity to rRNA, nucleolar localization, and binding to core snoRNA interactors fibrillarin or dyskerin. Such sno-miRNAs include the precursors of let-7g, mir-16-1, mir-140, and mir-151.[61,62] The mir-605 and mir-140 miRNA precursors are predicted to target positions in rRNA known to be modified but for which no guides were previously proposed.[62] While a subset of these miRNAs represent mis-annotated snoRNAs, another such group displays evidence for both miRNA and snoRNA functionality,[61,62] suggesting an evolutionary relationship from snoRNAs to miRNAs.[85] SnoRNAs with reported connections with miRNA are listed in Table 3.

Because of their size distribution and the emerging evidence that some snoRNAs can serve as precursors for miRNAs, several studies since 2009

were undertaken to investigate whether sdRNAs follow the same biogenesis pathway as miRNAs and function in the same way. Using mouse embryonic stem (ES) knockout cells of DGCR8 and Dicer1, both enzymes essential for canonical miRNA processing, the biogenesis of a subset of H/ACA sdRNAs was shown to depend on Dicer but not DGCR8/Drosha,[37] as seen by Ender et al. for ACA45.[38] In contrast, box C/D sdRNAs were found to be independent of these factors.[37] The dependency of a subset of box H/ACA but not box C/D sdRNAs on Dicer was subsequently confirmed by considering a Dicer knockdown in the human breast cancer cell line MCF-7.[86] Surprisingly, high-throughput sequencing following DGCR8 cross-linking immunoprecipitation (HIT-CLIP) in HEK293T cells identified many snoRNAs as substrates of DGCR8.[87] The cleavage of snoRNAs by DGCR8 was found to affect a subset of both box C/D and box H/ACA snoRNAs and to be independent of Drosha, suggesting the involvement of other endonucleases.[87] However, whether the fragments generated are functional or whether this cleavage is on the contrary involved in the degradation of snoRNAs remains to be seen.[87] Recent studies involving AGO2 immunoprecipitations followed by sequencing report different levels of interaction of snoRNAs and AGO2, with one study indicating a very low proportion of AGO2-bound RNAs originating from snoRNAs[36] and a second reporting no enrichment for reads mapping to snoRNAs[88] while a third identifies a significant minority of reads overlapping with snoRNAs.[89] Although further experiments will be required to clarify the relationship between snoRNAs/sdRNAs and AGO2, only a small subset of sdRNAs have been shown to be processed by Dicer to form functional complexes with AGO2 and to have miRNA-like capabilities. The majority of sdRNAs do not appear to function like canonical miRNAs.

Thus, despite the validated miRNA-like functionality of a small subset of sdRNAs, the question of the functionality of most sdRNAs still remains. While some do not display strong abundance and conserved processing, thus likely representing transient degradation products, many display strong and conserved accumulation profiles, suggesting that they are protected from degradation.[56] In addition, many display asymmetric processing favoring either the 5′ or the 3′ fragment, as displayed by miRNAs and other noncoding RNAs, but not mRNAs.[90] It is thus tempting to speculate that in addition to miRNA-like sdRNAs, another group of sdRNAs carry out alternate cellular functions in complex with different proteins, as has been found for a subset of psnoRNAs (reviewed in Ref 91).

CONCLUSION

The rapid emergence of affordable high-throughput, and in particular deep-sequencing, technologies has significantly widened our view of snoRNA characteristics and functionality. These technologies allowed the identification of additional members and more importantly, the simultaneous analysis of a much larger proportion of the snoRNA class than previously, revealing striking diversity in snoRNA abundance, targets, interaction patterns, processing profiles, and response to specific conditions. Given the recent explosion of independent reports describing noncanonical aspects of snoRNA biology, it is reasonable to expect the emergence of other novel characteristics, targets, and functions over the next few years. The seminal snoRNA studies of the 1990s and of the beginning of the 21st century provided extensive characterization of the canonical biogenesis and function of snoRNAs, but were performed mostly on a small subset of canonical snoRNAs, projecting the image of a generally homogeneous class of RNAs. It will thus be important over the next few years to correctly classify snoRNAs according to their interactors and functions, and to determine which features are common to all members and which characteristics define the different specialized snoRNPs.

The protein composition of snoRNPs will be of particular interest. Among the snoRNAs displaying noncanonical functionality, a subset including sno-lncRNAs[41] were found to interact with canonical protein partners. However, many noncanonical snoRNA interactors were identified, and in several cases, including snoRNAs associated with chromatin and ACA11 regulating oxidative stress,[54,58] core snoRNA binders were absent from their lists of interactors, suggesting that the composition of snoRNPs can vary considerably. Further experiments will be required to confirm these findings in a more widespread manner and to determine whether some snoRNA may be part of multiple independent and mutually exclusive complexes. Several studies mention that the depletion of such snoRNAs does not affect the methylation levels of their complementary rRNA targets.[57,60] It will thus be important to determine whether these are true targets, whether constitutive levels of rRNA modification can be achieved with low guide snoRNA levels, or whether these targets can also be modified by other regulators, snoRNA, or not.

It is also important to keep in mind that much of the evidence currently supporting noncanonical functions for snoRNAs is correlative or results from ectopic expression or knockdown experiments in cell lines. The biological significance of many of these noncanonical functions still remains unclear and will

require further experimentation, including demonstration and characterization *in vivo*. The small number of snoRNA knockout models and *in vivo* studies in mouse offer significant insights and new perspectives into the global role of specific snoRNAs on phenotypic, physiological, and molecular levels, including their dynamic expression and target gene modification/expression (rRNA as well as mRNA targets).[92–95] Such *in vivo* models, combined with the ever increasing number of innovative new flavors of high-throughput sequencing and proteomics methodologies, will undoubtedly contribute to clarifying the cellular roles of this intriguing family of ncRNA over the next few years.

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