C/D-box snoRNAs form methylating and non-methylating ribonucleoprotein complexes: Old dogs show new tricks

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C/D box snoRNAs (SNORDs) are an abundantly expressed class of short, non-coding RNAs that have been long known to perform 2′-O-methylation of rRNAs. However, approximately half of human SNORDs have no predictable rRNA targets, and numerous SNORDs have been associated with diseases that show no defects in rRNAs, among them Prader-Willi syndrome, Duplication 15q syndrome and cancer. This apparent discrepancy has been addressed by recent studies showing that SNORDs can act to regulate pre-mRNA alternative splicing, mRNA abundance, activate enzymes, and be processed into shorter ncRNAs resembling miRNAs and piRNAs. Furthermore, recent biochemical studies have shown that a given SNORD can form both methylating and non-methylating ribonucleoprotein complexes, providing an indication of the likely physical basis for such diverse new functions. Thus, SNORDs are more structurally and functionally diverse than previously thought, and their role in gene expression is underappreciated. The action of SNORDs in non-methylating complexes can be substituted with oligonucleotides, allowing devising therapies for diseases like Prader-Willi syndrome.

Keywords:
alternative splicing; obesity; Prader-Willi syndrome; RNA methylation; RNA processing; RNA therapy; SNORD; snoRNA

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The classic picture of snoRNAs

Small nucleolar RNAs are a highly expressed class of non-coding RNAs

Small nucleolar RNAs (snoRNAs) are an abundantly expressed class of non-coding RNAs (ncRNA) that accumulate in the nucleolus. They are about 60–300 nucleotides (nt) long and based on conserved sequence elements, snoRNAs are classified either as C/D box or H/ACA box. C/D box snoRNAs (SNORDs) contain C (RUGAUGA, R = purine) and D (CUGA) boxes, which are usually present in duplicates (C' and D' boxes) and up to two antisense boxes hybridizing to the RNA target [1]. 5′- and 3′-end of SNORDs base-pair to that form a stem in the final SNORD ribonucleoprotein complex (SNORNP) (Fig. 1A).

snoRNAs are highly expressed. For example, a typical mammalian cell contains an estimated 200,000 copies of SNORD3, and 20,000 copies of SNORD13/14 [2, 3], which compares to estimated 200,000 mRNA molecules in a cell [4]. snoRNAs are one of the longest and best studied ncRNAs, as their high expression allowed their sequencing using RNase-mapping in 1979 [5], shortly after Sanger sequencing became available [6].

Human SNORDs are located in introns and form ribonucleoprotein complexes through a multistep assembly process

Almost all of the currently known 267 human SNORDs are located in introns, with the exception of SNORD3@ (@

DOI 10.1002/bies.201600264

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Abbreviations:
ncRNA, non-coding RNA; **PKR,** protein kinase RNA; **psnoRNA,** processed snoRNA; **PWS,** Prader-Willi syndrome; **SNORD,** C/D box snoRNA; **snoRNA,** small nucleolar RNA; **SNORNP,** SNORD ribonucleoprotein complex.

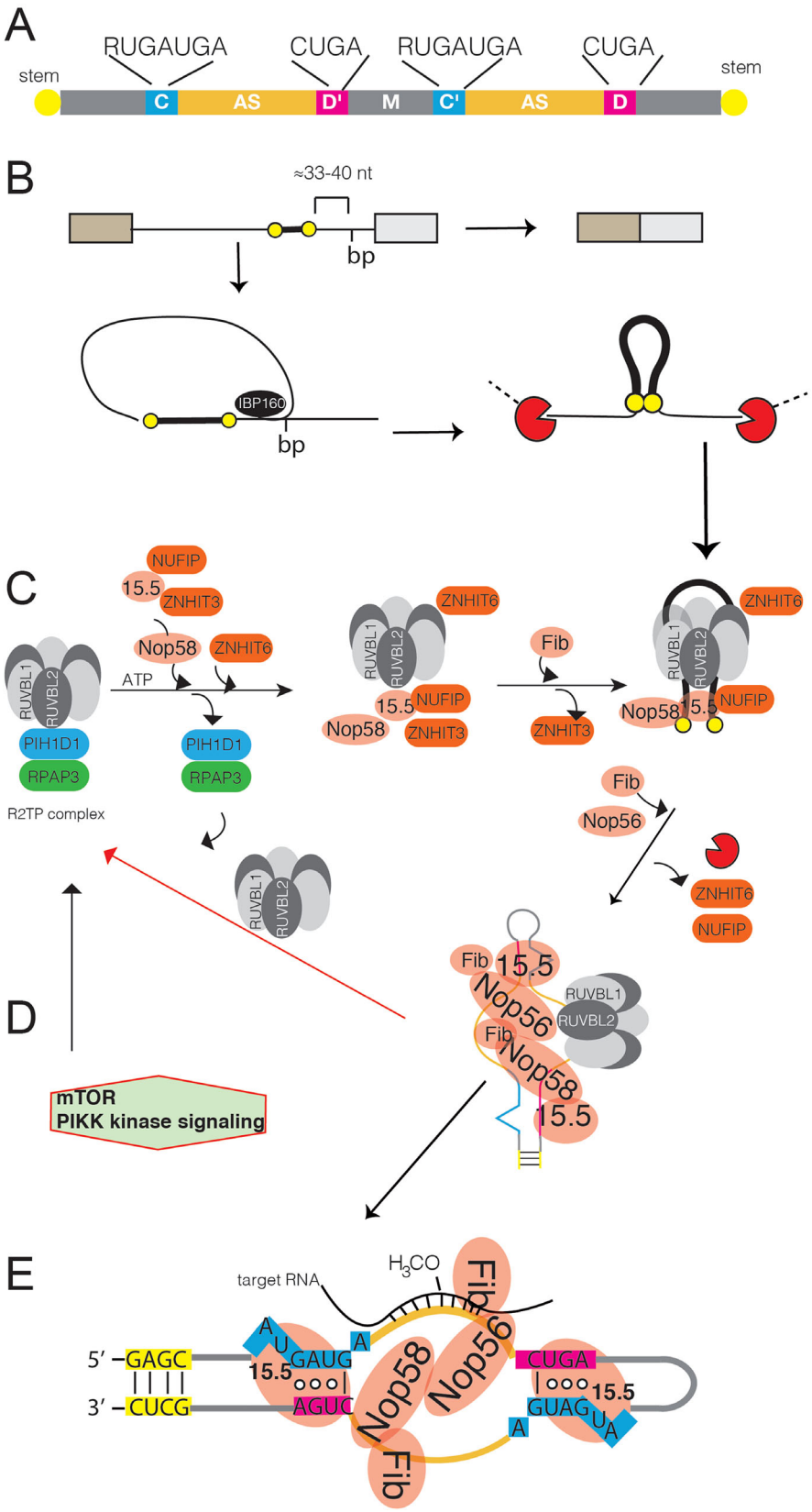

Figure 1. Continued.

indicating all four human SNORD3 genes, see Box 1 for SNORD nomenclature), SNORD8, SNORD13, and SNORD118 (U8) that are controlled by their own promoters [7, 8]. The intronic localization connects SNORD biogenesis with the pre-mRNA processing of their hosting genes. After the splicing reaction, the introns are released as lariats, which are opened up by the debranching enzyme and are subsequently degraded through exonuclease where XRN1/2 acts on the 5′ end and the RNA exosome at the 3′ end [9, 10]. The close connection between pre-mRNA splicing and SNORD biogenesis is reflected by a distance requirement for SNORDs that are located around 33–40 nt upstream of the branch point, which was shown using biochemical studies on a few SNORDs [11, 12]. SNORDs escape degradation by associating with proteins in a snoRNP precursor, with the stem-termini forming a dsRNA stem that protects from exonucleases (Fig. 1B). Proteomic studies deciphered the snoRNP assembly pathway for human U3 snoRNA [13]. (named after the yeast proteins ATPases Rvb1 and Rvb2, which are named after *Escherichia coli* DNA repair enzyme ruvB), Pih1 (protein interacting with Hsp90), and Tah1 (TPR-containing protein associated with Hsp90) [14]. TheR2TP complex acts like a chaperone allowing the formation of complexes between proteins and snoRNA.

The R2TP complex components RUVBL1 and RUVBL2 associate with the SNORD core proteins *NHP2L1* (*15.5k*, *SNU13*) pre-bound to NUFIP and ZNHIT3, and NOP58 in an ATP-dependent manner, which releases PIH1D1 and RPAP3. This protein complex associates with the SNORD and fibrillarin, removing ZNHIT3. In a further maturation step, a second molecule of fibrillarin, as well as NOP56 enters the complex, removing the adaptors ZNHIT6 and NUFIP. Unloading of RuvBL1/2, leads to further rearrangement and generated the final snoRNP complex (Fig. 1C). The components of this system are conserved from human to yeast, where it was shown that the R2TP components PIH1D1 and RPAP3 shuttle between cytosol and nucleus using the Crm1 and Kap121 export/import systems [15], which is regulated by the nutritional status of the cells via the mTOR pathway [15] (Fig. 1D).

The described assembly pathway has been determined for SNORD3@, which are expressed under their own promoters, but given the similarity between snoRNP complexes, it is likely that intronic SNORDs follow a similar pathway. The knockdown of R2TP components in yeast changes expression of several snoRNAs, suggesting that the general features of this pathway are conserved [16].

Intronic SNORDs use additional factors for maturation, for example, IBP160 (intron binding protein 160 kd) that interacts with U2snRNP measures the distance between intron branchpoint, likely allowing for a folding of the SNORD [12]. Another factor is the spliceosome component Prp43p that is associated with SNORDs, possibly helping to recycle SNORDs during rRNA processing [17] and whose loss leads to extended SNORD ends upon deletion [18].

snoRNP structure: The SNORD-ribonucleoprotein complex positions the methylase fibrillarin toward the target RNA

The final snoRNP consists of two molecules *NHP2L1* (*15.5k*, *SNU13*) bound to the RNA kink-turn formed by the C/D box interactions [19], one molecule of NOP56 and NOP58 bridging these structures and two molecules of fibrillarin [13], reviewed in [20] (Fig. 1E). Detailed insight into SNORD complexes came from cryo-EM and NMR-based structural studies from archaeal SNORDs [21, 22], demonstrating that *NHP2L1* (*15.5k*, *SNU13*) binds to the two RNA kinks formed by the interaction between the C and D and C' and D' boxes, forming a bipartite structure. These two domains are connected by NOP56 and NOP58, which is one molecule NOP5 in archaea. NOP5 stabilizes the binding of the methylase fibrillarin in this complex through direct interaction. The assembled SNORNPs form dimers, likely in a

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Figure 1. Structure and formation of methylating snoRNP complexes. **A:** General structure of C/D box snoRNAs (SNORDs). Overall structure of a C/D box snoRNA. C, D, C′ and D′; boxes with consensus sites indicated. Yellow circles indicate the stem termini, short regions that are complementary to each other. M, middle box defined in some SNORDs acting in pre-mRNA processing, AS, antisense box. **B:** Excision of intronic facilitates SNORD's folding. The SNORD (thick line flanked by circles depicting the termini) is located in an intron ≈33–40 nt upstream the branch point (bp). Boxes indicate the flanking exons. In the lariat, the sequence between the branchpoint and the SNORD is occupied by IBP160, which likely facilitates in folding the SNORD. The lariat structure is opened through the debranching enzyme and then degraded by exonucleases (red half circles). This RNA structure forms the SNORDRNP complex by interacting with proteins assembled by the R2TP complex. **C:** Formation of the snoRNP C/D-box snoRNPs or SNORDRNP. Proteins associated with the mature C/D-box snoRNP or SNORDRNP are shown in pink, the R2TP components RuvBL1/2 in gray and auxiliary factors in orange. The R2TP complex assembles with a preformed 15.5/NUFIP/ZNHIT3 complex, and NOP58 and ZNHIT6, which requires ATP bound to RuvBL1/2. This protein complex binds to the nascent SNORD in a step that incorporates fibrillarin (Fib) and releases ZNHIT3. The SNORDRNP further matures due to the addition of the second fibrillarin molecule and NOP56, releasing ZNHIT6 and NUFIP. The RUVBL1/L2 complex catalyzes the final structural arrangements and is recycled as the R2TP complex after release of the mature snoRNA. Note that this pathway has been worked out for SNORD3@, which is not an intronic RNA, but one regulated by its own promoter and due to the sequence conservation, it is likely that intronic SNORDs follow the same maturation pathway. **D:** The activity and intracellular localization of the R2TP complex is regulated by the mTOR pathway sensing nutritional status and possibly other phosphatidyl inositol 3′ kinase-related kinases (PIKK), which was shown in yeast. **E:** Structure of a methylating SNORNP. The SNORD forms a protein complex made of 15.5 (also known as SNU13 and NHP2L1), NOP56/58 and the methyltransferase fibrillarin (Fib) that 2′-O-methylates (H_3CO-) rRNA at a defined position (5 nt upstream of the D- or D′-box). The coloring of the SNORD is similar to (A). Circles indicate the base interaction within the RNA kink-turn motif. Only one antisense box is shown in rRNA targeting, but both antisense boxes can be used. The structure is adopted from an archea C/D-box snoRNP, based on NMR and cryo-EM studies [21, 22]. In the diagram, we used a shorter stem, which is 2–5 nt in humans [8, 119] and substituted the archea single NOP5 protein with the human NOP56 and NOP58 proteins.

Box 1

Nomenclature of SNORDs

SNORDs have a confusing nomenclature, reflecting more than 30 years of research on these RNAs. The HUGO name is SNORD for small nucleolar RNA C/D box, followed by a unique number. Identical SNORDs made from different hosting genes are depicted with an @, such as SNORD3@. SNORDs in gene clusters have their position indicated, such as SNORD115-1. Historically, small nuclear RNAs were termed U for Uracil-rich, and given a number, such as U1, U2, U3, etc. Some of these small nuclear RNAs were later to be recognized as SNORDs, such as U3. Newly discovered snoRNAs were given consecutive numbers, and the "U-nomenclature" did not discriminate between C/D and H/ACA box snoRNAs. Sometimes these numbers have more meaning, for example U27 (now SNORD27) is predicted to guide methylation of A27 of the 18S rRNA. The numbers in SNORD and U names are usually the same, but there are exceptions, for example U8 is now SNORD118. Sequencing projects identified new SNORDs that were given names related to libraries, for example HBII-52, now SNORD115, was named after human brain library II, clone 52 and similarly MBII-52 stands for mouse brain library. Nomenclature questions can be best addressed by referring to the snoRNA database (www-snorna.biotoul.fr) or the genecard database (http://www.genecards.org).

parallel orientation where the stems of the two monomers interact [22].

The SNORD RNA has two functions in this arrangement: first, it provides the scaffold for the RNP complex, by forming two RNA kink structures which locate 15.5/*NHP2L1* and by forming a double stranded terminal stem that protects the RNA from further exonucleic cleavage. Secondly, two elements of the SNORD, known as antisense boxes are not covered with proteins and are thus free to interact with target RNAs. The antisense boxes recognize sequences in target RNAs resulting in the fifth nucleotide upstream of the D or D' box being 2′-O-methylated by the methylase fibrillarin [1], which is properly positioned in the complex. The antisense boxes in eukaryotes vary in length (10–21 nt), but only a maximum of 10 nt can be used for modifications, due to the structure of the snoRNP [23]. With a few exceptions, such as the targeted U6 snRNA [24], SNORDs modify ribosomal RNAs at around 100 sites. Most of these modifications are constitutive and play a role in rRNA folding and translational fidelity [25]. However, some rRNA sites are only partially methylated and some SNORDS exhibit sequence complementarity to rRNAs without a resulting modification, which suggests additional functions for SNORDs [26, 27]. A few SNORDs including SNORD3@ and U8 and U13 direct pre-rRNA cleavage [28]. In SNORD3@, the interaction with rRNA is mediated by SNORD3-specific sequences upstream of the C box [29].

Diseases caused by snoRNA loss indicate new functions

Orphan snoRNAs: Half of the known SNORDs are orphan, as they have no known target

The structure and targeting properties of SNORNPs allowed the identification of their likely targets through sequence comparisons using the known 2′-O-methylation sites of rRNA (Fig. 1E). However, a target could be identified for only half of the known SNORDs. The remaining SNORDs were thus considered "orphan" [8, 30], suggesting that they might have other functions than 2′-O-methylation. The association of changes in SNORD expression with numerous diseases, where there were no obvious defects in ribosomal function, supports the concept that SNORDs mediate additional functions beyond rRNA methylation.

Loss of SNORD expression is critical for Prader-Willi syndrome (PWS)

Genetic defects in the 15q11.2-q13.1 regions provide some of the most striking examples of SNORDs functioning outside rRNA methylation. Due to imprinting, only the paternal allele of the 15q11.2-q13.1 region is expressed. Loss of the paternal allele, either through a deletion of the paternal allele or through uniparental disomy of the mother's allele leads to PWS. PWS is one of the most frequent genetic causes of morbid obesity and intellectual disability in humans. It has a frequency of about 1:10,000–1:30,000 individuals. In early infancy, PWS is characterized by severe hypotonia, which improves in later infancy and is followed by excessive eating (hyperphagia), leading to morbid obesity when untreated. PWS subjects further show low growth hormone levels, short stature, hypogonadism, a characteristic behavior with temper tantrums, manipulative behavior, and obsessive-compulsive symptoms [31].

The imprinted region lost in PWS contains four protein-coding genes and six orphan SNORDs located in the 3′ UTR of the bicistronic SNURF-SNRPN gene. SNURF-SNRPN encodes the SNRPN protein, a member of the SmB protein family that forms spliceosomal snRNPs, and the nuclear SNURF (SNRPN upstream reading frame) protein of unknown function. The 3′ UTR of this transcript hosts six orphan SNORDs, each flanked by two non-coding exons: SNORD107, -64, 108, -109a, -116 (29 copies falling into five distinct classes) and -115 (47 almost identical copies) (Fig. 2A). The SNORDs are predominantly expressed in the brain, but can also be detected at lower levels in peripheral tissues [32]. The brain specificity increases toward 3′ end of the SNRPN-SNURF transcript and SNORD115 can be detected only in neurons.

In addition to SNORDs, the Prader-Willi critical region generates five different snoRNA-related lnc RNAs (sno-lncRNAs) consisting of the introns surrounded by one copy of SNORD116 on both 5′- and 3′-end. The function and generation of these sno-lncRNAs remains to be determined [33].

Microdeletions affecting SNORD116 identified in six individuals recapitulate several features of PWS in a PWS-like phenotype that can be clinically hard to detect. Comparing all microdeletions

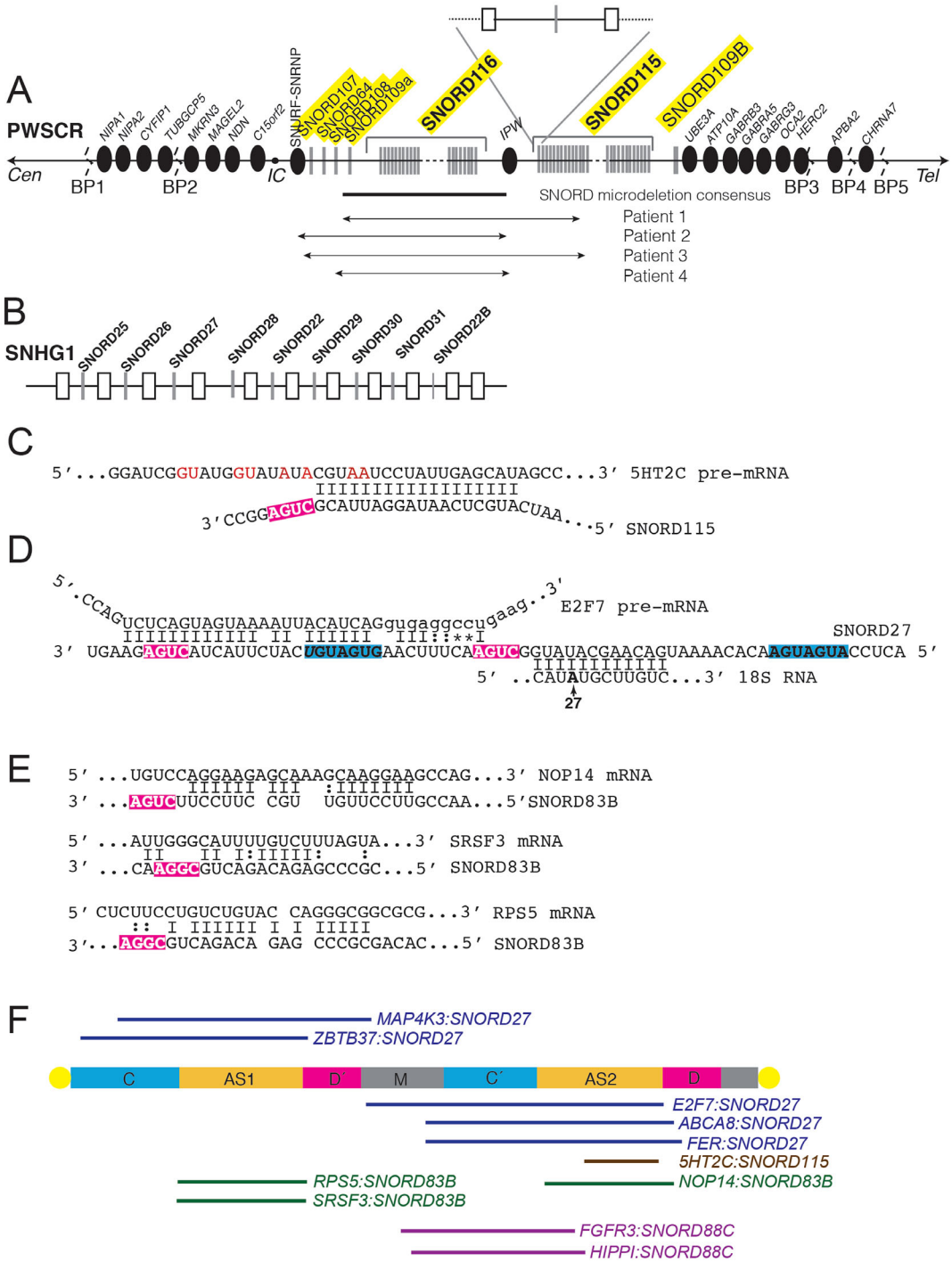

Figure 2. SNORDs involved in human diseases and function of SNORDs outside rRNA methylation. **A:** Schematic structure of Prader-Willi syndrome critical region (PWSCR). Genes are ovals, SNORDs are gray lines. Each SNORD is surrounded by two non-coding exons as shown in the insert above. IC, imprinting center; BP, breakpoint; cen, centromer; tel, telomer. The microdeletions identified in four patients leading to a PWS-like phenotype [34–37] are indicated. **B:** Schematic structure of the SNHG1 (small nucleolar RNA host gene 1) harboring SNORD27, -25, -30, and -31 marking the progression of smoldering multiple myeloma. SNHG1 is a non-protein coding gene, consisting of exon (boxes) that flank the SNORDs (gray lines). **C:** Binding of SNORD115 to the alternative exon Vb of the serotonin receptor 2C, identified by bioinformatic prediction [32] and validated by mutation analysis [75]. The D box is highlighted. **D:** Binding of SNORD27 to the alternative exon in the E2F7 pre-mRNA, experimentally validated through mutagenesis [79]. C-, D-,C′-, and D′-box are highlighted, as well as the binding of the methylating snoRNP to 18S rRNA, leading to the modification of A27 is shown. Small letters indicate the intron. **E:** Interaction between SNORD83B and three mRNA targets which regulates their expression levels as determined by LiGR [80]. **F:** Scheme of interactions between SNORDs and pre-mRNA targets. The schematic structure of a SNORD is shown and the site of interaction with various pre-mRNA and mRNAs indicated by lines. Note that the sites of interaction include the C and D boxes.

identifies a region containing SNORD116 and SNORD 109, suggesting that the loss of these SNORDs plays a central role in PWS disease etiology [34–38] (Fig. 2A).

The gene dosage of SNORDs in this region can not only be modified by deletion, but also doubled through duplications of the 15q11.2-q13.1 regions. Paternal interstitial duplications of 15q11.2-q13.1 double the SNORD gene doses, leading to Duplication 15q syndrome (Dup15q). Paternal duplications show variable penetrance for autism, in which they may be asymptomatic or associated with phenotypes ranging from anxiety and sleep disturbance [39] to developmental delay [40]. Similarly, mice overexpressing SNORD115 show autistic features [41], illustrating that SNORD expression levels must be under tight control to prevent disease.

The complex phenotypes associated with alterations in SNORD expression in the 15q11.2-q13.1 region reveal the physiological importance of SNORDs and raise the question of their molecular function.

Changes of SNORD expression in many types of cancers

Changes in SNORD expression are also observed in several forms of cancer [42, 43]. For example, the chromosomal translocation breakpoint involved in some B-cell lymphomas is located in the non-protein coding gene SNHG5 (small nucleolar RNA host gene 5). SNHG5 harbors SNORD50A which methylates C(2848) of 28S rRNA [44] and is lost in numerous breast and prostate cancer cell lines and primary tumors [45–47]. The ncRNA gene GAS5 (growth arrest specific 5) regulates cell survival, possibly by sequestering glucocorticoid hormone receptors. GAS5 harbors 10 SNORDs, whose expression is highly associated with cancer prognosis [48–52] and SNORD78 from this cluster is a biomarker for lung cancer [53]. Similarly, the non-protein coding gene SNHG1 hosts SNORD-27, -25, -30, and -31 (Fig. 2B). Loss of these SNORDs mark the progression of smoldering multiple myeloma, which cannot be explained through their predicted roles in rRNA methylation [54]. It is known that the localization and function of the R2TP complex that is necessary to form snoRNPs (Fig. 1B and D) is regulated by the mTOR pathway [15] and likely altered in cancer [14, 55], which could affect snoRNP formation, but the molecular role of these SNORDs in cancer cannot be explained by their action on rRNA.

Some SNORDs mediate lipotoxic stress and influence cholesterol trafficking

Another unexpected role of SNORDs was unveiled in a screen aimed at identifying factors that mediate lipotoxicity, the physiological response to lipid overload in non-adipose tissues, which is caused by obesity. When non-adipose cells are stimulated by high lipid concentration, they activate a stress response that involves activation of NADPH oxidase, leading to mitochondrial dysfunction and finally cell death. Surprisingly, deletion of the SNORDs-32a, 33, 34, and 35a prevents this lipotoxicity [56]. These four SNORDs, rather than the ribosomal protein L13A (RPL13A) in which they are located, mediate the blockade of lipotoxicity. Unexpectedly, this study also found that three of these SNORDS accumulate in the cytosol after treatment with fatty acids [56]. A later study using RNAseq analysis found that 94/119 expressed SNORDs can be detected in the cytosol after drug-induced lipotoxicity [57] and that the cytosolic accumulation of SNORDs is mediated by NADPH oxidases through an unknown mechanism [57].

Similar to lipotoxicity, SNORDs participate in other processes, including glucose tolerance and cholesterol trafficking. Mice with a specific knock down of SNORD32A, 33, 34, and 35a that did not alter expression of the protein encoded by the hosting genes RPL13A exhibited an increased systemic glucose tolerance and glucose-mediated insulin secretion [58]. Thus lack of these SNORDs promotes the clearance of circulating glucose possibly caused by decreased production of reactive oxygen species [58]. Using a similar screening system SNORD60 was found to act in intracellular cholesterol trafficking, which is independent of its suggested function in ribosomal RNA methylation [59]. SNORDs 32A, 33, 34, 35a, and 60 are predicted to methylate ribosomal RNAs, but their role in lipotoxic stress and cholesterol trafficking, as well as their cytosolic function suggests additional functions. SNORD33 is also selectively increased in the serum of subjects with non-small cell lung carcinoma [60], but a possible link to lipotoxicity remains to be determined. It is well established that reactive oxidative species activate mTOR signaling [61], which regulates the snoRNP assembly factors of the R2TP complex (Fig. 1D), providing a mechanistic link between reactive oxidative species and snoRNP formation.

The expression of some SNORDs oscillates in circadian rhythm

Deep sequencing (RNA-seq) of *Drosophila* brains collected at multiple timepoints during the day and night identified transcripts that exhibit 24-hour profiles [62]. As well as identifying robust daily oscillations in a large number of genes, similar to previous microarray studies, this study revealed that ~10% of the cycling genes are ncRNAs including snoRNAs. SNORDs in seven dUhgs (drosophila U22 host genes) exhibited oscillations that peaked during the light phase and appeared to be driven by light [62]. The majority of the Uhgs SNORDs are predicted to target rRNAs, although some of them are orphan. Because not all SNORDs exhibited a daily rhythm, those that do may have specialized functions related to daily rest-activity rhythms. If so, such a function might echo the proposed role of alterations in SNORDS in human diseases with the alterations in daily rest-activity rhythms. For example, patients with PWS or Dup15q syndrome exhibit alterations in daily sleep-wake cycles and in sleep architecture [31, 39, 63].

SNORDs as biomarkers for diseases and physiological changes

A subset of SNORDs change their expression in aging in *C. elegans* [64]. In humans, alteration of cardiac SNORD expression is associated with the tetralogy of Fallot, a frequent

congenital malformation of the heart [65]. SNORDs could further be markers of diseases, as SNORD3A is the RNA most strongly overexpressed in blood of Creutzfeldt-Jacob patients, as well as in mouse models of prion disease [66]. SNORD33, SNORD66, and SNORD76 or their fragments can also be detected in blood, where they may serve as biomarkers for non-small cell lung carcinoma [60]. The possible functionality of these SNORDs was tested in a proof of principle experiment, in which the addition of synthetic SNORD fragments to cell culture media led to an increase of several transcription factors and mRNAs involved in the innate immune response [67].

Similarly, SNORD126 is up-regulated in hepatocellular carcinoma and colorectal cancer and upregulates genes involved in the PI3-AKT pathway [68]. Osteoarthritis is the most common degenerative disease of the joints, characterized by joint cartilage breakdown that affects about 4% of the population. SNORD116 is up-regulated in post-traumatic osteoarthritis mouse models and horses with natural developed osteoarthritis, the latter show a 400-fold increase [69]. Smaller changes were observed for SNORD18, -49A, -58, -21, -27, -90, and 11.

Collectively, these reports suggest a role of SNORDs outside rRNA modification, as no defects in translation or general protein synthesis were associated with the diseases.

SNORDs form non-methylating ribonucleic protein complexes and are precursors for shorter RNAs

Understanding the role of SNORDs outside rRNA modification proved to be extremely difficult, predominantly because RNA targets cannot be bioinformatically predicted for most orphan SNORDs. Novel SNORD functions were indicated by genome wide RNA profiling of diseases that showed deregulation of numerous SNORDs, but gave little mechanistic insights [65, 69, 70–73]. A few SNORDs were analyzed in great detail using mostly transfection studies [74–76] and even transgenic mice [58, 77, 78], which gave insight in to physiological processes. Mechanistical insight has come from detailed biochemical analysis [79] and the development of new techniques identifying RNA-RNA interactions [80]. These diverse studies used human and mouse system and support the novel concept of SNORDs acting in non-methylating ribonucleic protein complexes, outlined here.

SnoRNA precursors contain complementary sequences, for example the future stems that inhibit degradation by exonucleases (Fig. 1B). It is thus not surprising that some SNORDs are recognized by proteins binding to dsRNAs.

SNORD expressing units are precursors for miRNAs

miRNAs are generated from nuclear pri-miRNAs characterized by a stem-loop structure that is cut by the dsRNase drosha [81], leading to the export of a pre-miRNA into the cytosol, where it is further cleaved by dicer to 21 nt long miRNAs that are loaded onto argonaute proteins. The sequencing of RNAs associated with argonaute proteins revealed that several snoRNAs, both from the H/ACA and C/D box snoRNAs classes give rise to miRNAs [76, 82–85], previously reviewed in [70, 71]. Several studies showed a correlation between SNORD-derived miRNAs and diseases, for example cancer [55, 86]. Most of the studies rely solely on sequencing data, and thus the underlying biological mechanism and biological relevance of SNORD-derived miRNAs is unclear. However, several SNORD-derived miRNAs are associated with argonaute proteins, suggesting that they are functional in gene regulation [85].

SNORDs activate protein kinase R

The second dsRNA-binding protein identified to bind to SNORDs was protein kinase RNA-activated (PKR). PKR contains a double strand RNA binding domain whose activation of viral dsRNAs is well established. However, lipotoxic stress caused by palmitic acid also activates PKR and unexpectedly, SNORDs and H/ACA snoRNAs were found associated with activated PKR, suggesting that under metabolic stress conditions SNORDs mediate a stress response to PKR [87]. SNORDs associated with PKR included SNORD50A and 50B that are lost in some forms of cancer [45, 46]. SNORD50A/B were also found to directly bind to K-Ras, which decreases G-protein binding and subsequently the activity of K-ras [88]. The loss of SNORD50A/B expression is thus predicted to increase Ras-ERK1/ERK2 signaling which could promote tumor formation. It is possible that in addition, PKR activation contributes to this process.

SNORDs can be precursors of piRNAs

SNORDs can also give rise to piRNAs, abundant 26–31 nt long non-coding RNAs associated with PIWI proteins. The biogenesis of piRNAs is independent of DICER, and likely requires single stranded RNA [89]. One of these snoRNA-derived piRNAs, piR30840, works in nuclear-exosome mediated degradation of interleukin-4 pre-mRNA by recruitment of Ago4/Piwil4 to an intronic site [90].

SNORDs form non-methylating ribonucleoprotein complexes

SNORD115 was the first SNORD shown to regulate alternative splicing [75] and thus proteins associated with SNORD115 were the first to be analyzed, using direct pull down from total nuclear lysates [74] as well as pull downs from nuclear extracts fractionated through a density gradient [91]. Both approaches indicated that SNORD115 associates with proteins different from the canonical SNORD proteins: 15.5/NHP2L1, NOP56, NOP58, and fibrillarin (Fig. 1E). SNORD115 associated with hnRNPs (A1, B1, A3, D0, hnRNPL2, ELAVL1), RNA helicases (DHX9, DDX), structural proteins (nucleolin, matrin3), and transcription factors (PURA). The number of these proteins and the broad distribution of SNORD115 complexes in glycerol gradients [91] suggested the presence of structurally diverse SNORD115-protein complexes.

Further insight into the composition of new SNORD complexes came from the analysis of HeLa nuclear extract fractionated under native conditions [79]. In this method nuclei are extracted using native salt conditions, which preserve higher order structure, and the extracts are subsequently separated on native glycerol gradients. Surprisingly, 29 SNORDs were found in nuclear fractions associated with the spliceosome, representing about 24% of the expressed SNORDs. Although most of these SNORDs have been previously shown to methylate rRNA, the nuclear fraction containing these SNORDs was devoid of fibrillarin, NOP58 and NOP56. A detailed analysis of SNORD27, predicted to perform the known 2′-O-methylation of A27 in 18S RNA [92], revealed that SNORD27 associated with diverse RNA binding proteins (FUBP3, FUBP1, KHSRP, hnRNPQ, L, ELAVL1,) and the RNA helicase DDX5, as well as proteins implicated in splicing (PRPF3, PRPF4, SFPQ), which is reflected in the ability of SNORD27 to change splice site selection. The biochemical separation of SNORDs from fibrillarin-containing fractions was further confirmed using RNase protection for SNORD2, 60 and 78 [79].

SNORDs can be subdivided into two groups based on the number of paired nucleotides downstream of k-turn motif – one group has long ends ($snoRNA_L$) and the other one short ends ($snoRNA_{SH}$) [8]. The processing of $snoRNA_L$ was more sensitive to NOP58 depletion; in contrast the processing of $snoRNA_{SH}$ was affected by depletion of the splicing factor RBFOX2. This additionally suggests that there is more than one processing pathway for SNORDs that could lead to generation of diverse RNPs.

Thus, the protein complexes formed by SNORD can be biochemically separated into complexes that contain fibrillarin (methylating SNORDs) and lacking fibrillarin (non-methylating SNORDs). Together, these data suggest that SNORDs form protein complexes that lack the methylase fibrillarin, which we refer to as non-methylating SNORNPs in addition to the well-known methylating SNORNPs containing 15.5/NHP2L1, NOP58, NOP56, and fibrillarin. Some SNORDs like SNORD27, 2, 60, and 78 form both complexes and thus have dual functions [79]. SNORD3@ is atypical, because it is not an intronic SNORD, showing that SNORDs associated with fibrillarin do not necessarily perform 2′-O-methylation. SNROD3@ cleaves pre-rRNA using an extra 5′ sequence, containing A and B boxes that aid in rRNA cleavage site recognition. SNORD3@ also forms a classic methylating SNORNP that is used for nuclear localization, but not targeting [29].

Some SNORDs form shorter RNAs, processed snoRNAs (psnoRNAs)

Within the methylating SNORD-ribonucleoprotein particle, the RNA is protected from degradation by the associated proteins and a terminal stem. However, such a protection might not be the case in heterogenous and more loosely formed non-methylating SNORDs. Deep sequencing experiment showed the presence of SNORD fragments with a medium length longer than 27 nt, which distinguishes them from miRNAs having a length distribution of 21–22 nt [93, 94]. Using a cloning technique allowing the identification of SNORD-fragments detected by RNase protection analysis, the presence of SNORD fragments, termed psnoRNA for processed snoRNA was further confirmed for mouse SNORD115 and SNORD116 [73, 74]. The analysis of the human transcriptome using RNAseq showed the expression of psnoRNAs for the human 115, 116, 113, 114 families and SNORDs-50, 19, 32B, 123, 111, 72, 93, 23, and 85 [72, 73], listed in Supplemental Fig. S1.

Non-methylation snoRNPs could explain the presence of psnoRNAs, as not the whole SNORD is protected in a well-folded non-methylating complex from the actions of RNases, but only shorter fragments are protected by RNA binding proteins. Inspection of the SNORDs known to generate psnoRNAs shows that with the exception of SNORD 115 none of these SNORDs has all four C, C′, D, and D′ boxes in full consensus (Supplemental Fig. S1), which could impair proper snoRNP folding.

What are the functions of non-methylating SNORDs?

The formation of non-methylating SNORDs can explain mechanistically the influence of SNORDs on gene expression, which could contribute to diseases caused by SNORD loss. As described above, due to the double-stranded nature of SNORD precursors, SNORDs can activate protein kinase R and SNORD fragments can become part of argonaute proteins, acting as miRNAs.

In addition, the function of SNORDs in non-methylating RNA-protein complexes is emerging. Glycerol gradient analysis shows a wide size distribution of non-methylating snoRNA-protein complexes [79, 91], indicating their heterogeneity. The absence of the defined structures indicates that each part of the SNORD, not just the antisense boxes, is free to interact with other RNA molecules, which has been confirmed by detailed studies of SNORD:pre-mRNA interaction (Fig. 2C–F).

SNORDs bind to pre-mRNAs, regulating splice site selection

SNORD115 (previously called HBII-52) is an orphan SNORD expressed in the Prader-Willi critical region (Fig. 2A). It shares an 18 nt sequence complementarity with the alternative exon Vb of the serotonin receptor 2C and promotes the inclusion of this exon [75] due to direct SNORD-mRNA interaction (Fig. 2C). SHAPE assays showed that the serotonin receptor 2C pre-mRNA in this region forms a stable double-stranded structure that sequesters the regulated splice site, causing exon skipping [95] (Fig. 3A). A chemical screen identified pyrvinium pamoate as a substance that binds to this double-stranded region, changes the structure, which de-represses the regulated splice site [95]. Since SNORD115 binds to the dsRNA structure, it likely acts through a similar structural change. The dsRNA of the serotonin receptor 2C pre-mRNA is stable enough to be A->I edited by ADAR2, which leads to exon activation by similarly disrupting the dsRNA area, which activates the regulated splice site [95, 96]. SNORD115 has no influence of RNA editing, but changes the ratio between

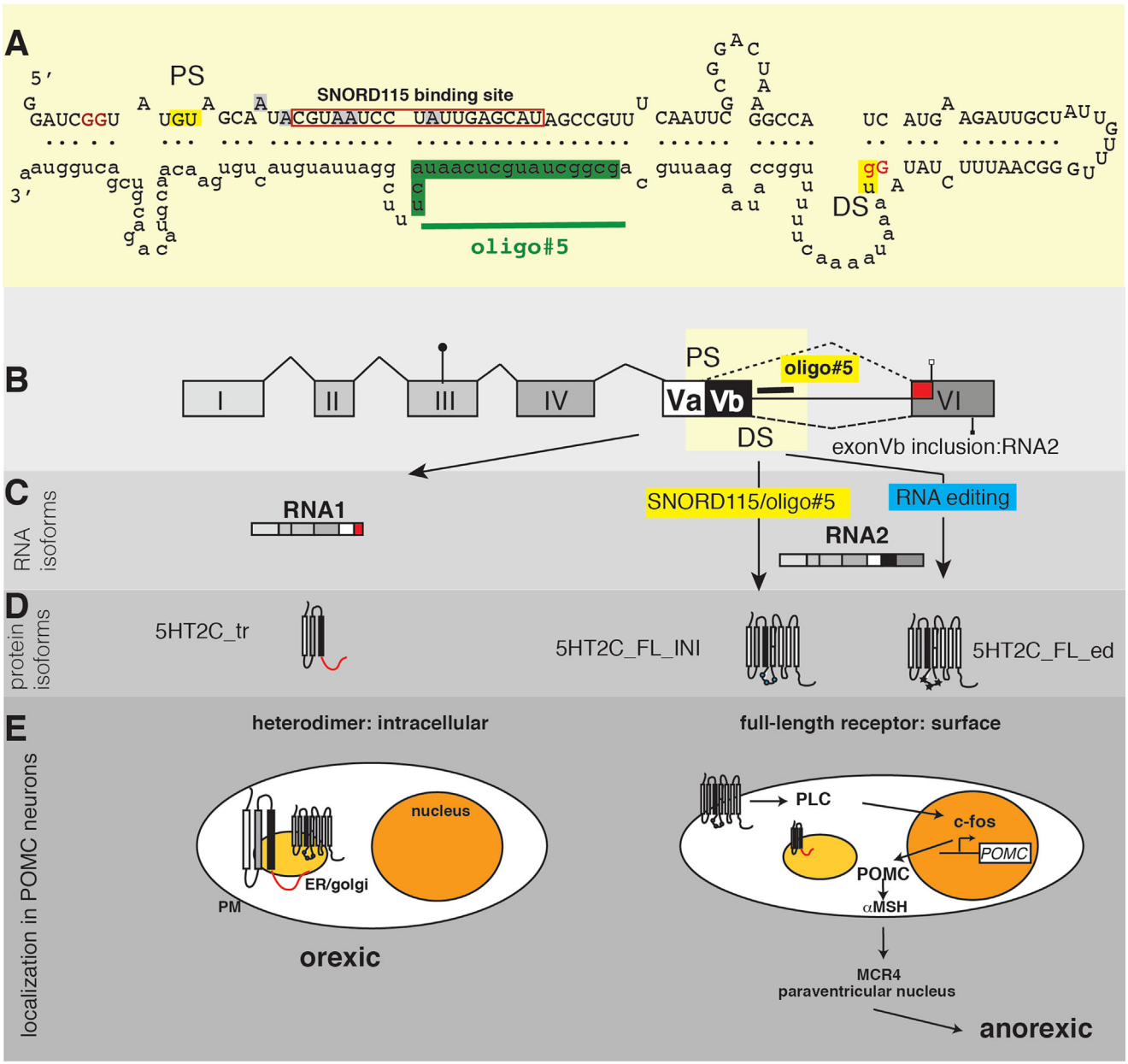

Figure 3. Substituting SNORD115 with an oligonucleotide. **A:** Secondary structure of the serotonin receptor 2C pre-mRNA that encompasses exon Vb between the proximal (PS) and distal (DS) splice sites (yellow). Intron V is shown in small letters. In this structure, the distal splice site is blocked and the proximal site used. Binding of SNORD115 to the boxed site likely causes a structural change, similar to binding of pyrvinium pamoate [95], which activates the distal site. Editing of up to five adenosines (gray boxes) to guanosines similarly activates the distal splice site by weakening the RNA stem. Oligo#5 complementary to the green region strongly activates the distal site [103]. **B:** Gene structure of the 5HT2C. Exons are shown as boxes and the alternative splice patterns indicated by dashed lines. The region in (A) is indicated by a pale yellow box and the location of oligo#5 is shown. The start codon in exon III is indicated by a round arrow. Skipping of exon Vb results in an early stop codon (open square arrow), whereas its inclusion results in a later stop codon (black square arrow). **C:** RNA isoforms generated by the processing events. Exon Vb skipping generates RNA1 and its inclusion generates RNA2. Due to editing combinations, RNA2 represents 32 RNA isoforms. SNORD115 and oligo#5 promote the inclusion of the non-edited exon Vb. **D:** Protein isoforms generated by the processing events. RNA2 encodes 24 isoforms, one non-edited isoform 5HT2C_FL_INI with the amino acids INI in the second intracellular loop and 23 isoforms (5HT2C_FL_ed) that are edited. Circles in the second intracellular loop of the serotonin receptor 2C indicated non-edited amino acids and stars depict the editing events. **E:** Effect of the isoform ratios in on signaling POMC-neurons. Left: 5HT2C_tr is present in intracellular membranes (ER/golgi) and sequesters the full-length receptor, stopping its signaling at the plasma membrane (PM). The lack to signaling does no inhibit food intake, such generating an orexic response. Right: A relative decrease of 5HT2C_tr caused by SNORD115 or oligo#5 leads to an increased occupation the full-length receptors at the plasma membrane surface and its signaling via the phospho lipase C (PLC) pathway. PLC activation induces c-fos, which activates POMC transcription. The resulting POMC peptide is processed into alpha melanocyte-stimulating hormone (αMSH), which activates melanocortin 4 receptors (MCR4) receptors in the paraventricular nucleus, which results in an anorexic behavior response.

inclusion and skipping of a non-edited exon Vb into the serotonin receptor pre-mRNA through direct SNORD-pre-mRNA interaction.

Proof of principle experiments showed that SNORD115 can perform 2′-O-methylation of short artificial RNAs sent to the nucleolus by using reporter constructs driven by an RNA polymerase I promoter. SNORD115 can also influence editing of an RNA fragment corresponding to serotonin receptor 2C pre-mRNA when expressed under a RNA polymerase I promoter [97]. However, physiologically, the serotonin receptor 2C is controlled by a RNA polymerase II promotor and thus the endogenous transcripts are not associated with the nucleolus. SNORD115 knock out mice show a change in serotonin receptor 2C splicing, indicating that physiologically, SNORD115 changes splicing in the nucleoplasma [78].

SNORD27 has been known to methylate rRNA and the finding that it changes splice site selection similar to SNORD115 was thus surprising [79]. Bioinformatic prediction validated by mutational analysis showed that SNORD27 binds to the 5′ splice sites of the E2F7 transcripts and most likely blocks U1 snRNP access, leading to exon skipping (Fig. 2D). A similar effect was found for several exons that are only expressed in SNORD27's absence, suggesting that repression of exons is a general function of SNORD27. Importantly, SNORD27 also regulates 2′-O-methylation of rRNA [44], indicating that the SNORD has a dual function in both rRNA modification, acting in a methylating snoRNP and in alternative splice site selection, acting in a non-methylating RNP [79].

A similar dual function has been reported for SNORD88C, which is predicted to guide 28S rRNA methylation [7], but also regulates the alternative splicing of the *FGFR3* pre-mRNA [98] (binding sites indicated in Fig. 2F).

SNORDs regulate gene expression by direct binding to RNA

A major impediment in understanding the interaction between non-methylating SNORDs and their target RNAs are the difficulties in determining the SNORD: target RNA interactions, as these interactions can occur across the whole SNORD molecule, not just the antisense boxes. Furthermore, RNA-RNA interactions are more promiscuous than DNA-DNA interactions, allowing for example G-U base pairing and tolerating gaps. It is therefore not a surprise that the validation of bioinformatically predicted SNORD:pre-mRNA interaction is poor. This problem has persisted now for over 20 years [99], preventing the understanding of orphan SNORD function. Recently, LiGation of interacting RNA and high-throughput sequencing (LiGR) was developed to detect RNA-RNA interaction [80]. In LiGR, RNAs are cross-linked with the psoralen derivative AMT (4′-aminomethyl-trioxsalen hydrochloride), a molecule selective for dsRNA, single stranded ends are trimmed with RNase R and the ligated RNAs sequenced. Using this method, an interaction between the orphan SNORD83B and three pre-mRNAs (NOP14, SRSF3, and RPS5) was detected. Knock-down of SNORD83B caused an increase of mRNA of these target genes, but not their pre-mRNA, suggesting that SNORD83B could regulate mRNA stability (Fig. 2E) [80]. Since the interaction between SNORD27 and E2F7 can be demonstrated by RNA pull down without crosslink [79] and was not detected using LiGR [80], it is likely that more SNORD-mRNA interaction exist.

A possible role of SNORDs in mRNA stability is also suggested by the overexpression of one copy of mouse SNORD116 in cells not expressing endogenous SNORD116, which changes the expression of about 200 mRNAs, but has no detectable influence on alternative splicing [100]. Thus, regulating mRNA stability, possibly similar to miRNAs [101] by competing with RNA stabilizing proteins, could be a more general function of non-methylating SNORDs.

The comparison of these SNORDs acting in splice site selection and mRNA expression shows an emerging common mechanism: the SNORDs associate with various RNA binding proteins, likely forming diverse non-methylating snoRNPs. The sequence complementarity between target RNA and SNORD includes sequences outside the antisense boxes, which should be taken into account in any prediction programs (Fig. 2F).

Substitution of non-methylating SNORDs to combat human disease

The concept that SNORDs act in non-methylating protein complexes suggested therapeutic approaches for diseases caused by SNORD loss. Whereas a methylating SNORD complex has to be assembled during the splicing reaction from a large intron through several assembly steps (Fig. 1B and C), it might be possible to substitute a non-methylating SNORD using an oligonucleotide that recapitulates the SNORD-mRNA interaction. The use of RNase-resistant modified oligonucleotides makes them independent from the binding of protecting proteins. This concept was first tested to influence alternative splicing of the serotonin receptor 2C, recapitulating the effect of SNORD115, the first SNORD shown to regulate splice site selection [102, 103].

The serotonin receptor 2C regulates food intake

The serotonin receptor 2C (5HT2C) regulates food uptake through its action on pro-opiomelanocortin (POMC) neurons in the hypothalamus. Activation of the 5HT2C receptor leads to an increase of phospholipase C, which activates c-fos leading to POMC synthesis in these neurons. Alpha-melanocyte stimulating hormone (alpha-MSH), which is generated by processing of the POMC precursor, activates neurons in the paraventricular nucleus (PVN) via melanocortin 4 receptors [104, 105]. Activation of PVN neurons induces satiety, i.e. cessation of eating (Fig. 3E). Knock-out mice lacking the 5HT2C receptor thus exhibit hyperphagia and are obese [106], whereas activation of the 5HT2C receptor using an agonist such as the FDA-approved drug, lorcaserin, inhibits food intake [107]. Since patients with PWS do not express SNORD115, they show a dysregulation of 5HT2C alternative splicing, as observed in mouse models of this syndrome [78].

The activity of the serotonin receptor 2C is regulated by its RNA processing

The 5HT2C pre-mRNA undergoes both pre-mRNA editing and splicing of its exon Vb, which generates at least 25 proteins that respond differently to serotonin binding [108, 109] (Fig. 3C and D). Skipping of exon Vb generates "RNA1" encoding a "truncated receptor 5HT2C_tr" that is expressed as a protein [103]. The 5HT2C_tr is localized intracellularly and through heterodimerization sequesters the full-length receptor inside the cell [103, 110], decreasing the receptor's activity. Exon Vb inclusion generates "RNA2." RNA2 formation is promoted by SNORD115 [75], resulting in a "non-edited full-length receptor 5HT2C_FL_INI," with the amino acids INI in the second intracellular loop. Editing through ADAR2 at five sites also promotes exon Vb inclusion, leading to 32 RNA2 isoforms. The editing changes three amino acids, leading to

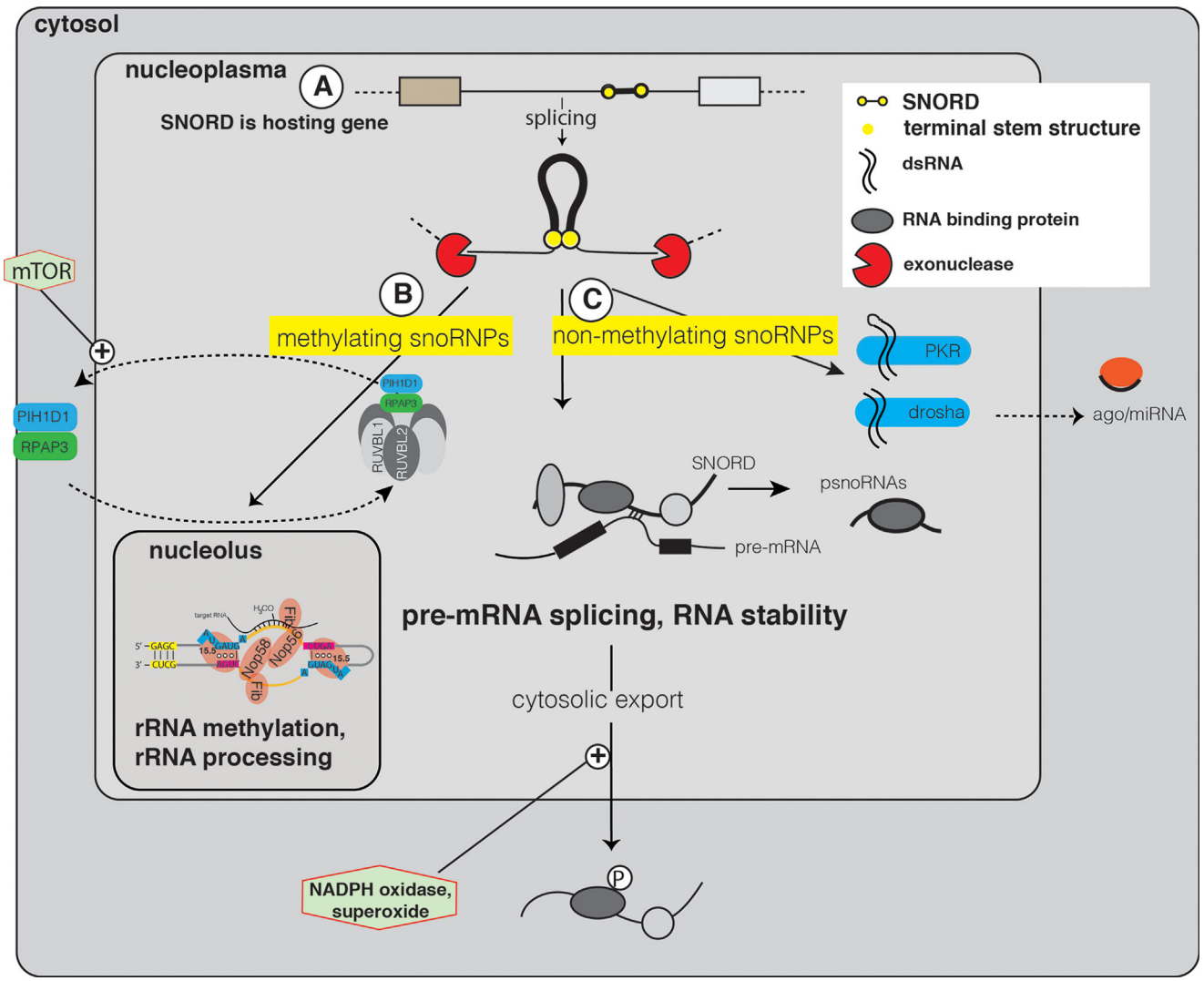

Figure 4. SNORDs generate methylating and non-methylating complexes. It is likely that there are differences in processing between individual SNORDs and this scheme summarizes general features. **A:** Generation of snoRNAs: almost all human SNORDs are intronic and are released from their hosting genes through pre-mRNA splicing, debranching of the lariat and exonucleic trimming. The location of a SNORD flanked by terminal stems in a hosting intron is shown; the boxes depict exons. The released RNA forms different ribonucleic protein (RNP) complexes: methylating snoRNPs and non-methylating snoRNPs. **B:** Methylating SNORDs are generated using the R2TP system (RUVBL1/2) [20], which is regulated by mTOR pathways. Methylating SNORDs perform 2′-O-methylation of rRNA in the nucleolus. In addition, SNORD3@, containing the methylase fibrillarin (fib) acts in rRNA cleavage. The structure of a methylating SNORNP, consisting of a terminal stem, two kink-turn motifs formed by C and D boxes are shown. The SNORD binds to the target rRNA via its antisense boxes and performs 2′-O-methylation at a specific rRNA ribose, 5 nt downstream of the D- or D′-box **C:** Non-methylating SNORDs are generated through the association of SNORDs with diverse proteins, either dsRNA binding proteins (blue, PKR: protein kinase R, drosha) or hnRNPs and other RNA binding proteins (gray circles, ovals). SNORDs activate PKR and can be processed by drosha similar to miRNAs, leading to their incorporation in to argonaute proteins (ago). SNORDs stabilized by hnRNPs bind directly to pre-mRNA via RNA-RNA interaction and change pre-mRNA alternative splicing [74] and mRNA expression [80], likely through changing the stability of the mRNA. Some of the SNORDs in non-methylating RNPs are further processed into psnoRNAs [70], possibly protected by hnRNPs from further degradation. The association of non-methylating SNORDs with shuttling proteins, such as HuR could explain their detection in the cytosol, which is promoted by NADPH superoxidase function [57].

23 "full-length, edited receptors, 5HT2C_FL_ed." The non-edited 5HT2C_FL_INI is constituitvely active and shows the strongest response to serotonin, whereas the edited isoforms show a weaker response to serotonin binding and their constitutive activity decreases with increased editing, with 5HT2C_FL_VGV having no constitutive activity [109, 111, 112]. Mouse models indicate that loss of the full length non-edited 5HT2C isoform causes hyperphagia [113], most likely because of the weak serotonin response of the edited receptors.

Central to this system is a stable secondary structure containing the alterative exon Vb that dictates the processing of the RNA [95, 96]. The default processing is skipping of Vb: exon Vb can be activated by disrupting this structure, either by the editing of guanosine to inosines, leading to full-length edited receptors, or by binding of SNORD115, which likely opens the dsRNA structure, leading to the non-edited full-length receptor (Fig. 3A).

The truncated RNA1-encoded protein is localized in the intracellular membrane systems, likely the endoplasmic reticulum and Golgi and sequesters the full-length RNA2-encoded receptor [110]. Without RNA1, the full-length receptor is localized at the plasma membrane and throughout the cell [114]. Due to the sequestration, RNA1-encoded receptors reduce the presence of the full-length RNA2 receptor at the surface (Fig. 3E). Thus, the RNA1/RNA2 isoform-ratio, which is controlled by SNORD115, regulates 5HT2C receptor activity by determining the surface concentration of the active receptor [110]. Because the SNORD115-5HT2C target interaction has been worked out (Fig. 2C), efforts to find a substitution for SNORD115 concentrated on this system.

SNORD115 can be substituted with an oligonucleotide

Using an oligo-walk [115], an oligonucleotide, oligo#5 that strongly promotes exon Vb inclusion in cell culture [103], similar to SNORD115 was identified. Oligo#5 was then tested in vivo, by injecting it into the third ventricle of wild-type C57/Bl6 mice. This treatment with oligo#5 increased 5HT2C_FL_INI and decrease RNA1 in the hypothalamic arcuate nucleus, as expected, and importantly, robustly reduced food intake. Thus, SNORD115 can be functionally substituted using an oligonucleotide, which further shows that a methylase is not necessary for SNORD115 action. The data also indicate that the ratio between the two 5HT2C proteins: a full-length receptor containing exon Vb and a truncated receptor lacking it, is involved in the regulation of food intake. Since it is known that the full-length receptor heterodimerizes with other transmembrane receptors [116, 117], it is possible that this ratio, controlled by the non-methylating SNORD115 contributes to the formation of the complex PWS by affecting different receptor systems.

Conclusion and outlook: snoRNAs can have multiple roles by forming different RNPs – a general role for ncRNAs?

The reviewed evidence shows that SNORDs have broader functions and more diverse properties than previously assumed. SNORDs not only form the well-studied methylating complexes, but in addition associate with hnRNPs and dsRNA binding proteins to form non-methylating complexes. The compositional analysis of several non-methylating SNORD-protein complexes showed a high degree of diversity, indicating multiple functions (Fig. 4).

Similar to other processes in RNA biology, such pre-mRNA splicing, a balance between RNA elements adhering to consensus sequences, distance requirements and trans-acting protein factors likely dictates whether a SNORD forms a methylating or non-methylating ribonucleoprotein protein complex. A stronger divergence from the perfect consensus sequences likely favors non-methylating snoRNPs, which is reflected by the presence of psnoRNAs. For some SNORDs, the same SNORD expressing unit can form both methylating and non-methylating complexes, and this balance could be caused by modification of factors needed to form methylating snoRNA complexes.

Once a SNORD or its fragment becomes metabolically stable in a non-methylating SNORD complex it can acquire new functions by binding to other nucleic acids. As in the methylating snoRNP, the function of the RNA is twofold: it acts as a scaffold for the proteins and it binds to other nucleic acids via base-pairing. Studies of SNORD27 showed that its pre-mRNA targets are not evolutionary conserved, suggesting that SNORDs are acquiring new functions during evolution. Non-methylating SNORDs could also explain why some SNORDs accumulate in the cytosol under stress [56, 57]. For example, HuR/ELVAL1 was found bound to all non-methylating SNORD complexes analyzed so far. HuR/ELAVL1 accumulates in the cytosol under oxidative stress condition [118] and thus could help transport associated non-methylating SNORDs out of the nucleus.

Due to the high expression of SNORDs – there are roughly as many SNORD3 transcripts as mRNA transcripts in a cell – SNORDs likely represent an important but underappreciated class of mRNA regulators.

Acknowledgments

We thank Drs. Emanuele Buratti and Edouard Bertrand for excellent discussions. This work was supported by NIH (R21HD080035 and R21NS098186) and the Foundation for Prader-Willi Research.

The authors have declared no conflicts of interest.

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