SURVEY AND SUMMARY

The structure and function of small nucleolar ribonucleoproteins

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ABSTRACT

Eukaryotes and archaea use two sets of specialized ribonucleoproteins (RNPs) to carry out sequence-specific methylation and pseudouridylation of RNA, the two most abundant types of modifications of cellular RNAs. In eukaryotes, these protein–RNA complexes localize to the nucleolus and are called *s*mall *n*ucleolar RNPs (snoRNPs), while in archaea they are known as *s*mall RNPs (sRNP). The C/D class of sno(s)RNPs carries out ribose-2′-*O*-methylation, while the H/ACA class is responsible for pseudouridylation of their RNA targets. Here, we review the recent advances in the structure, assembly and function of the conserved C/D and H/ACA sno(s)RNPs. Structures of each of the core archaeal sRNP proteins have been determined and their assembly pathways delineated. Furthermore, the recent structure of an H/ACA complex has revealed the organization of a complete sRNP. Combined with current biochemical data, these structures offer insight into the highly homologous eukaryotic snoRNPs.

BACKGROUND

Pseudouridine (ψ) and 2′-*O*-methylribose are the two most abundant post-transcriptional modifications of cellular RNAs, and are present in the ribosomal RNAs (rRNAs) of all species. Modification sites cluster within functionally important regions of the ribosome and are often located away from protein-binding sites (1). It is believed that these modifications facilitate the folding and stability of rRNA (2,3). In eukaryotes, these modifications are carried out by small nucleolar ribonucleoprotein (snoRNPs) within the nucleolus, a sub-nuclear compartment where ribosome biogenesis takes place. The class C/D snoRNPs are responsible for site-specific 2′-*O*-methylation of ribose (4–6) while the H/ACA snoRNPs catalyze isomerization of uridine to ψ (7,8).

The snoRNPs have functions beyond ribosomal RNA modification [see reviews by (9,10)]. A sub-set of snoRNPs from both classes are required for pre-rRNA cleavage events. These are the only snoRNPs required for yeast viability *in vivo* (11–13). In addition, the related *s*mall *Ca*jal body RNPs (scaRNPs) are responsible for chemically modifying spliceosomal *s*mall *n*uclear RNAs (snRNAs) and are retained within Cajal bodies (nuclear bodies in yeast), the likely site of RNP maturation, through a unique scaRNA sequence element, the CAB box (14–16). The scaRNAs may contain chimeric C/D and H/ACA RNA domains responsible for recruitment of both classes of snoRNP proteins (17). Human telomerase RNA (TR) is a scaRNA containing a chimeric 5′-reverse transcriptase domain involved in enzymatic activity and a 3′-H/ACA domain that recruits each of the core H/ACA snoRNP proteins (18). Although this domain is dispensable for *in vitro* activity of telomerase, it is required *in vivo* for the processing, stability and nuclear localization of TR (18–20).

The C/D and H/ACA snoRNPs are universally present in eukaryotes and have also been discovered in archaea, where they are referred to as *s*mall RNPs (sRNPs) (21,22). Archaeal sRNPs have both rRNA and tRNA modification activity (23). The archaeal RNA components of sRNPs (sRNAs) are typically smaller than their eukaryotic counterparts, possibly representing minimal structural units for both classes. Archaea have functional homologs to each of the core snoRNP proteins which possess clear sequence homology to their eukaryotic counterparts (21,24,25). Thus, the functional requirements of the core snoRNP proteins and the molecular mechanisms of ψ formation and 2′-*O*-methylation are ancient and have been conserved throughout evolution.

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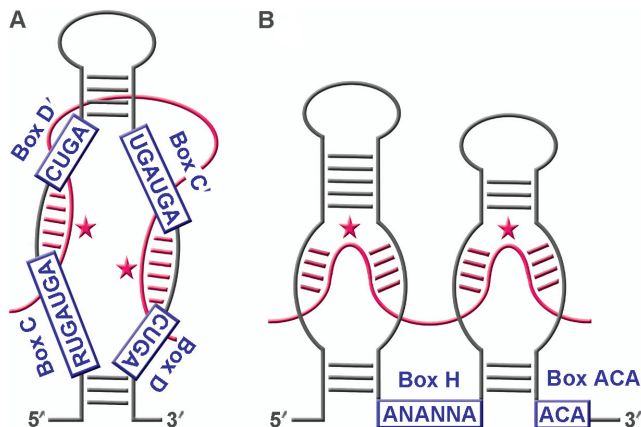
Figure 1. The guide snoRNAs and their target RNAs. The class (**A**) C/D and (**B**) H/ACA snoRNAs (grey) contain conserved and class-specific sequence motifs 'boxes' (blue) and unique guide regions that define their respective target RNA site(s) (magenta). The sites targeted for nucleotide modification are marked with a star.

The guide snoRNAs

Each C/D or H/ACA snoRNP is defined by its unique RNA component (the snoRNA) that can be identified by conserved sequence motifs, the C/D or H/ACA boxes. Regions of unique sequence within each snoRNA (the guide sequences) are responsible for targeting the mature and fully assembled snoRNPs to a specific substrate. The class-specific sequence elements are largely conserved from archaea to vertebrates and are critical for the assembly of functional sno(s)RNPs (26–29).

The class C/D snoRNAs are defined by two consensus motifs (Figure 1A). The 'box C' sequence RUGAUGA (R is purine) occurs near the mature 5′-end of the RNA, while the 'box D' sequence CUGA is located near its 3′ end. These two motifs are brought together by base pairing between the 5′ and 3′ termini of the RNA. Most C/D snoRNAs contain a second set of conserved sequences, the C′ and D′ boxes, located in the central region of the RNA. Both the C/D and C′/D′ boxes are generally well conserved in archaea and contain the hallmark K-turn sequence motif (30). In eukaryotes, the C′/D′ box is less conserved and often lack identifiable K-turn motifs. The site of modification is specified by the formation of base pairs between a sequence of 10–20 nucleotides that is found upstream of the D and/or D′ box motifs of the snoRNA and complementary sequences flanking the site of 2′-*O*-ribose methylation in the substrate RNA. The methylation reaction (Figure 2C) takes place exactly 5 nucleotides upstream of the conserved CUGA motif (4,31).

The H/ACA snoRNAs typically adopt a common secondary structure consisting of two hairpins (Figure 1B). The two stem-loops are connected by a hinge region that contains the 'box H' sequence ANANNA (N is any nucleotide) and the second hairpin is followed by a single-stranded segment with the 'box ACA' tri-nucleotide sequence located exactly three nucleotides upstream of the mature 3′-end of the snoRNA (Figure 1B). One or both of the stem-loops contain internal loops with 9- to 13-nucleotides on each strand that form the pseudouridylation
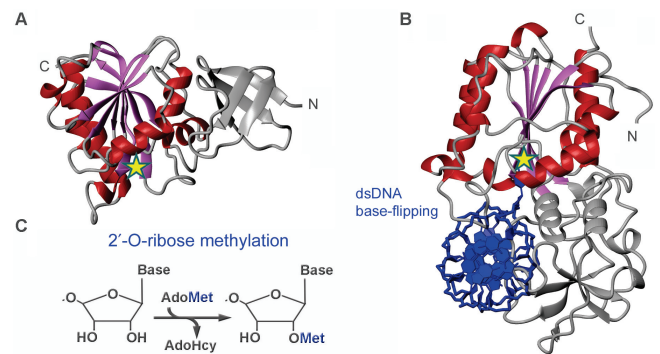

Figure 2. The conserved C/D methyltransferase fibrillarin. Crystal structure of (**A**) the archaeal fibrillarin (1FBN) and (**B**) of the double-stranded DNA methyltransferase HhaI (1MHT). The catalytic domains are colored according to their secondary structural elements (red: α-helices; violet: β-strand; grey: variable regions). *S*-adenosyl-methionine (AdoMet) binds at a conserved site (yellow star) of these related enzymes. Structural details of fibrillarin interaction with the sno(s)RNA-target RNA complex are unknown. HhaI flips out the nucleobase to be modified in dsDNA (blue). (**C**) Fibrillarin catalyzes the ribose-2′-*O*-ribose methylation of its RNA substrates by converting AdoMet to *S*-adenosylhomocycteine (AdoHcy).
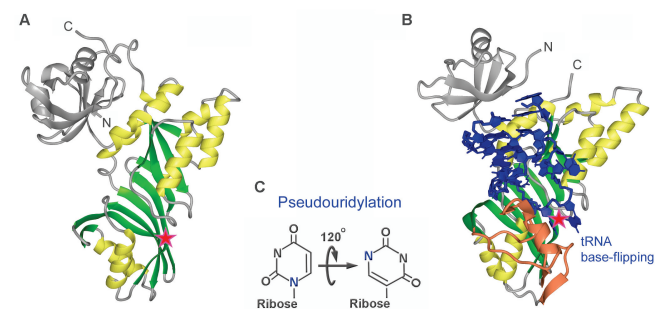

Figure 3. The conserved H/ACA Ψ synthase Cbf5. Crystal structures of (**A**) the archaeal H/ACA Ψ synthase Cbf5 (2APO) and (**B**) the *E. coli* tRNA Ψ synthase TruB (1K8W). The catalytic domains are colored according to their conserved secondary structural elements (yellow: α-helices; green: β-strand; grey: PUA domain and variable regions). The site of uridine isomerization (red star) is surrounded by highly conserved residues in Cbf5 and TruB. The TruB induces a base flipping of its tRNA substrate (blue). Structural differences between Cbf5 and TruB reflect differences in RNA substrate specificity; for example, a characteristic TruB peptide sequence (coral) is absent from Cbf5 homologs. (**C**) The isomerization of uridine to Ψ requires an overall 120° rotation of the uracil base.

pockets. These nucleotides are responsible for the site specificity of pseudouridylation (Figure 3C). The pseudouridylation site is situated 14–16 nucleotides upstream of either the H or ACA box motifs (7,8,27,32). Archaeal H/ACA sRNAs contain one to three stem-loops with less strictly conserved ANA box elements at both sites. Furthermore, the apical stem of these sRNAs may contain K-turn motifs (33) that are not detected in eukaryotic H/ACA snoRNAs.

The snoRNP core proteins

The C/D and H/ACA snoRNAs associate with their enzymatic components, known respectively as Nop1 and Cbf5 in yeast, that are responsible for the modifications carried out by each particle. Defects in snoRNP activity observed in yeast when these enzymes were mutated

supported this attribution (34,35). Nop1 is known as fibrillarin in all other species and Cbf5 is referred to as dyskerin or Nap57 in mammals. We refer to these enzymes as fibrillarin and Cbf5 for simplicity. Although Cbf5 and fibrillarin are responsible for catalysis, all mature H/ACA and C/D sno(s)RNAs further assemble with a conserved set of proteins that are common to each class. These class-specific core snoRNP proteins are required for the enzymatic activity, stability and nucleolar localization of the snoRNP (9,28,36,37).

In addition to the ribose-2′-O-methylase fibrillarin, C/D snoRNAs associate with the K-turn binding 15.5-kDa protein (Snu13 in yeast) and the two related proteins Nop56 and Nop58 in the mature snoRNP. 15.5 kDa/Snu13 is also a component of the spliceosomal U4 snRNP (38), where it recognizes a conserved K-turn motif within the snRNA (39). In archaea, the 15.5 kDa/Snu13 component is represented by the ribosomal L7Ae protein and the single archaeal Nop5 (Nop56/58) protein replaces Nop56 and Nop58 (40,41). In eukaryotes, each of the core proteins is required for C/D snoRNP activity and nuclear localization (42–44), but Nop56 is dispensable for snoRNA stability *in vivo* (45–47).

In addition to the Ψ synthase Cbf5, the H/ACA snoRNAs associate with the core proteins Gar1, Nhp2 and Nop10 in the mature snoRNP. Nhp2 shares sequence homology with the C/D protein 15.5 kDa/Snu13, but surprisingly lacks specificity for K-turn RNAs (48,49). In archaea, the K-turn-binding protein L7Ae functionally replaces Nhp2 (33). Thus, the C/D protein 15.5 kDa/Snu13 and the H/ACA protein Nhp2 are both functionally represented in archaea by a single ribosomal protein, L7Ae, suggesting a common evolutionary origin. All four H/ACA core proteins are essential for viability and activity of the snoRNP in yeast and, with the exception of Gar1, are also required for H/ACA snoRNA stability *in vivo* (50–56).

Bacteria lack homologs of the class C/D and H/ACA snoRNPs. Pseudouridylation and 2′-O-methylation of all cellular RNAs in these organisms is carried out by paralogous 'stand-alone' single-polypeptide enzymes that can recognize unique substrates without using a guide RNA or accessory proteins (reviewed in (57) and (58)). Related stand-alone enzymes are also responsible for the modification of tRNAs in eukaryotes. The core homology between bacterial stand-alone enzymes and the eukaryotic and archaeal sno(s)RNA-associated enzymes suggest they likely evolved from common ancestral proteins (30,59). Interestingly, ψ and 2′-O-methylribose are much less common in bacterial rRNA than in eukaryotic rRNA: only ~14 sites in *Escherichia coli* versus ~100 in *Saccharomyces cerevisiae* and ~200 in human. It appears that by separating catalytic function from target selection, evolution has made it possible to greatly expand the number of sites that can be modified specifically.

THE CONSERVED sno(s)RNP Enzymes

Fibrillarin and Cbf5 were originally identified as the enzymatic components of the C/D and H/ACA snoRNPs respectively, based on their sequence homology

with known stand-alone methylases and Ψ synthases. The enzymatic roles of these proteins have been demonstrated *in vitro* with purified archaeal components (60–62), and crystallographic analyses of the archaeal sRNP enzymes confirmed their structural homology with related stand-alone nucleotide modifying enzymes (63,64). These studies offer considerable insight into the conserved catalytic mechanisms utilized by the sno(s)RNPs.

The C/D snoRNP methyltransferase fibrillarin

Structural evidence for the role of fibrillarin as the C/D snoRNP methyltransferase first came from the crystal structure of that protein from the archaeon *Methanococcus jannaschii* (mjFib) (63), which revealed strong structural homology to enzymes known to catalyze methyl transfer from the cofactor *S*-adenosylmethionine (AdoMet) to a variety of substrates (57,65). Fibrillarin has since been characterized structurally in two other archaeal species: *Archaeoglobus fulgidus* (66) and *Pyrococcus furiosus* (67) (afFib and pfFib, respectively); in the former case, the protein was co-crystallized with the C/D sRNP core protein afNop5 and AdoMet bound to the catalytic site of the enzyme. In addition, the structure of the human fibrillarin core domain (hsFib) bound to AdoMet has recently been reported (PDB ID 2IPX, H. Wu *et al.*, unpublished results). Eukaryotic fibrillarin proteins contain an N-terminal *g*lycine-and-*a*rginine-*r*ich (GAR) domain of low sequence complexity that is not present in archaea and was removed from hsFib construct used for crystallographic studies. The archaeal proteins and hsFib are all structurally very similar and are represented by the mjFib structure in Figure 2A.

The crystal structure of mjFib revealed an amino-terminal domain of novel fold and an α/β carboxy-terminal domain structurally similar to previously characterized domains found in other AdoMet-dependent methyltransferases (e.g. HhaI (68) Figure 2B). Members of this enzyme family catalyze methyl transfer from AdoMet to a variety of different nucleophilic substrates, including nucleic acids, proteins and small-molecule metabolites. They are all typified by a catalytic domain consisting of a central seven-stranded β-sheet surrounded by three α-helices on each side (reviewed in (57)). Despite their structural similarity, methyltransferases display little overall sequence homology across different functional classes. However, they can all be identified by conserved regions (Motifs I–IV) that are responsible for AdoMet binding and support the catalytic reaction (57). Fibrillarin homologs possess a unique N-terminal domain that adopts a crescent-shaped five-stranded β-sheet motif in mjFib structure (Figure 2A). This N-terminal domain displays structural variability amongst the characterized archaeal homologs consistent with variability in their primary sequences. Additional conformational variations between fibrillarin structures can be found at the AdoMet-binding sites and are likely induced by interactions with the cofactor and with Nop5 (66).

The catalytic mechanism of fibrillarin remains to be determined; however, most AdoMet-dependent methyltransferase reactions occur via an S_N2-type

substitution of the activated methyl group in AdoMet by the nucleophilic acceptor (in this case, the ribose 2′-OH of the RNA substrate) (Figure 2C). The residues involved in AdoMet recognition are conserved in both archaeal and eukaryotic fibrillarin and occupy similar positions in each of the characterized fibrillarin structures, supporting the idea that the catalytic mechanism of this enzyme is both ancient and conserved. An absolutely conserved aspartate (D133 of afFib) is important for *in vitro* activity of the archaeal sRNP and has been proposed to act as the general base responsible for deprotonation of the 2′-OH nucleophile and/or stabilizing the AdoMet interaction (69).

The structural details of the interaction between fibrillarin and the guide or substrate RNA are currently unknown. DNA methylases, such as HhaI, utilize a large peripheral domain for substrate recognition (Figure 2B). Fibrillarin homologs lack similar domains and instead require core sno(s)RNP protein interactions for their recruitment to the C/D sno(s)RNA. *In vitro*, archaeal fibrillarin is incapable of direct recognition of sRNAs (60). However, since fibrillarin is the catalytic component of the C/D sno(s)RNP, it is predicted to form critical contacts with the guide and target RNA(s) in the assembled RNP. This is supported by UV cross-linking studies of eukaryotic snoRNPs (70,71).

Recognition of the RNA duplex formed between target and guide RNAs by fibrillarin may nonetheless resemble that of double-stranded DNA methyltransferases. A common structural feature of DNA methyltransferases, first observed in the crystal structure of HhaI bound to dsDNA (Figure 2B) (68), is a flipped out conformation of the base to be modified (57). This 'base-flipping' mechanism allows the enzyme access to the nucleobase for catalysis from within a normally base paired DNA structure. It is also plausible that the guide–substrate RNA complex may be positioned by interactions with the core proteins that induce an orientation of the target hydroxyl group for catalysis without the need for base flipping.

The H/ACA snoRNP Ψ synthase Cbf5

Crystallographic structures of the complex between archaeal Cbf5 and Nop10 confirmed the predicted homology between Cbf5 and the *E. coli* tRNA Ψ synthase TruB (64,72), a related protein that catalyzes the isomerization of uridine to Ψ at position 55 within the T-loops of all elongator tRNAs (73). Both Cbf5 and TruB are two-domain proteins: they contain a catalytic core domain that is common to all Ψ synthases (58) and a carboxy-terminal *p*seudo*u*ridine synthase and *a*rchaeosine transglycosylase (PUA) domain (Figure 3A and B). The catalytic core domain forms a six-stranded β-sheet with several α-helices located on the catalytic face contributing to the formation of the active site cleft. The C-terminal PUA domains adopt similar mixed α/β folds in these two proteins that pack against the catalytic core.

The active sites of TruB and Cbf5 are structurally similar; suggesting that the mechanism for converting uridine to Ψ is common to all these enzymes (Figure 3C)

(reviewed in (58)). Three residues (an aspartate, a basic residue and a tyrosine or phenylalanine) are conserved in all Ψ synthases, and therefore likely play conserved roles in catalysis. Mutation of the conserved aspartate eliminates catalytic activity of both TruB (74) and Cbf5 (35). The TruB structure supports the proposed function of this residue in being responsible for direct attack of the target uracil base (73,75). However, it is possible that the catalytic aspartate may target the anomeric position of the sugar like in some DNA glycosylases (76). TruB uses a base-flipping mechanism to reach the uracil base for catalysis (73) (Figure 3B). As with fibrillarin, it remains to be demonstrated that the snoRNA-associated Cbf5 enzyme utilizes a similar mechanism.

Comparison of Cbf5 with TruB reveals structural differences reflecting a divergent RNA-recognition mechanisms (64,72,77). Recognition of the target tRNA by TruB involves characteristic peripheral peptide segments within the catalytic domain that are missing from Cbf5 (Figure 3B). In addition, the entire catalytic face and PUA domain of Cbf5 possess a highly basic electrostatic surface potential, indicative of a substantial role in RNA binding. These observations are consistent with the requirement for Cbf5 to recognize a larger RNA substrate formed by the complex between the guide sno(s)RNA and the target RNA, compared to the tRNA acceptor loop recognized by TruB.

The PUA domain is considerably larger in Cbf5 than in TruB due to N- and C-terminal extensions in the former protein. The PUA domain is a common RNA-binding motif found in several nucleotide modifying enzymes (78). Structural comparisons to the unrelated *arc*haeosine *t*RNA *g*uanine *t*ransglycosylase (ArcTGT) (79) PUA domain and mutational analysis suggested that this domain in Cbf5 and its homologs recognizes the conserved 3′-ACA tri-nucleotide sequence (ANA in archaea) of H/ACA sno(s)RNAs (64,77). This predicted functional role of the Cbf5 PUA domain has now been demonstrated structurally (80). In humans, the dyskerin PUA domain is a common site of mutation in patients carrying the familial genetic disorder *dysk*eratosis *c*ongentia (DKC) (81). Furthermore, some patients inheriting this genetic disorder carry mutations within their telomerase RNA which contains an H/ACA snoRNA domain (18,82). DKC phenotypes have been attributed to both telomerase dysfunction and aberrant ribosome biogenesis, both of which may be attributed to defects in dyskerin–snoRNA interactions (83–85).

Recombinant Cbf5 proteins from archaea (and recently from yeast (86)) are able to directly and independently bind sno(s)RNAs (61,62). However, the studies with archaeal proteins show that this interaction is insufficient for guiding Ψ formation of a sRNA target sequence. Interestingly, the archaeal Ψ synthases Cbf5 and Pus10 (a novel archaeal enzyme unrelated to TruB), have recently been reported to catalyze Ψ formation at position 55 of archaeal tRNA *in vitro* (87). However, this 'stand-alone' activity of Cbf5 was greatly enhanced in the presence of the other core proteins (87). These observations support the idea that the snoRNP enzymes were derived from stand-alone ancestors and further suggest

that the core H/ACA proteins provide Cbf5 with functions that go beyond guide/substrate RNA recognition; for example, by providing interactions that structurally organize the active site for catalysis.

sno(s)RNP Assembly and Structure

The structural homology of fibrillarin and Cbf5 with their stand-alone counterparts suggests that their respective catalytic mechanisms are conserved. However, *in vitro*, neither archaeal fibrillarin nor Cbf5 can catalyze nucleotide modification of an sRNA target in the absence of the other core RNP proteins, even when the guide RNA is present (60–62).

In eukaryotes, the *in vivo* biogenesis of snoRNPs is a highly complex and dynamic process (88–90). The recruitment of the core snoRNP proteins to their respective snoRNAs is initiated co-transcriptionally (91–95) and is required for snoRNA maturation, stability and nuclear localization (28). Although unique functions have evolved amongst eukaryotic components, their core interactions are functionally represented in their archaeal counterparts (21). Thus, the structural characterization of the sRNP assembly intermediates in archaea have provided direct insight into the function of each core sno(s)RNP protein. These studies, combined with extensive biochemical data, have provided architectural models of the class C/D sno(s)RNPs. The recent crystal structure of the core H/ACA proteins bound to a guide sRNA (80) has provided the first atomic-level details of the interactions governing H/ACA sRNA (and snoRNA) recruitment and further insight into the mechanism of substrate RNA recognition by this class of snoRNP.

The C/D sno(s)RNP initiation complex

In vitro reconstitution of archaeal C/D sRNPs show that L7Ae is required for nucleating RNP assembly at the C/D and C′/D′ motifs of the sRNA (40,60). This role in RNP nucleation is functionally conserved in the eukaryotic 15.5-kDa protein that is similarly required for the core assembly of both C/D snoRNPs as well as U4 snRNP *in vivo* (44,96). These primary RNA-binding proteins directly recognize K-turn motifs, a common protein-binding site (97), found within the conserved domains of the RNA. Its secondary structure consists of a stem–bulge–stem motif where stem I (typically ending in two G:C base pairs) is connected with stem II (starting with two G:A base pairs) through an asymmetric internal bulge (~3 nucleotides with the sequence NRU, where N is any nucleotide and R is a purine) (Figure 4A).

The K-turn is recognized by proteins sharing a conserved RNA-binding motif that has been structurally characterized in several protein–RNA complexes, including L7Ae (98,99) (Figure 4B) and the eukaryotic C/D homolog 15.5-kDa protein (39). In all these complexes, stems I and II of the K-turn are brought close to each other by a sharp bend in the phosphate backbone (~60°) spanning the unpaired region and are stabilized by inter-helical A-minor interactions. The first two unpaired nucleotides are not conserved and participate in stacking
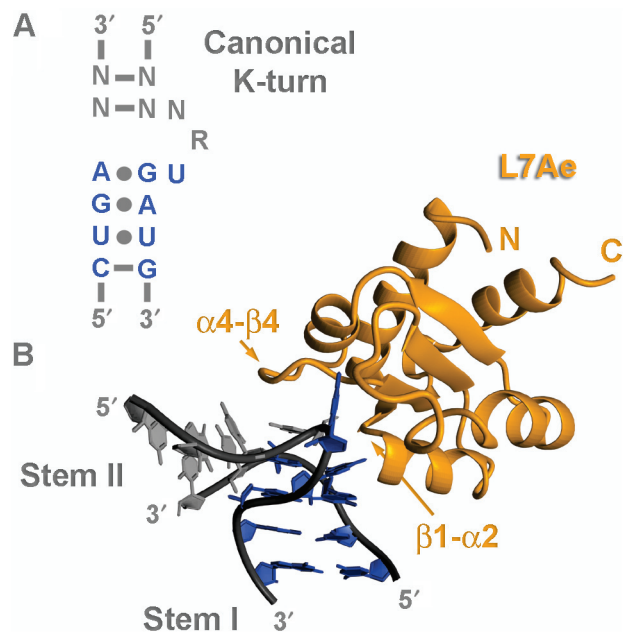

Figure 4. K-turn recognition by L7Ae. (**A**) Sequence of a canonical K-turn motif with conserved C/D nucleotides in blue. (**B**) Crystal structure of the archaea L7Ae protein (gold) bound to the canonical K-turn motif of C/D sRNAs (1RLG). RNA elements are colored as in (A). The K-turn adopts a characteristic ~60° bend in the phosphate backbone and L7Ae makes several interactions with conserved structural features of this motif.

interactions with stems I and II, while the conserved U adjacent to the tandem G:A pair is displayed outward facing the protein (Figure 4B). The sharp bend is facilitated by the interactions within stem II of the strictly conserved G:A sheared base pair. These base pairs are required for L7Ae and 15.5 kDa/Snu13 binding *in vitro* and for snoRNP function *in vivo* (40,44,100–104).

The K-turn motif is recognized by L7A and all its homologs through their conserved αβα sandwich motif, characterized by a four-stranded β-sheet core surrounded on both sides by a total of six α-helices. Residues located on α2, β1 and on the loops connecting β1-α2 and α4-β4 make several interactions with the conserved structural features of the kinked RNA motif (Figure 4B). The invariable U nucleotide is extruded from the apex of the RNA, buried in a hydrophobic pocket of the protein and stabilized by base-specific hydrogen bonding interactions. Together, these interactions provide shape complementarity for the K-turn fold and specificity for the strictly conserved U in the C/D sno(s)RNAs. Notably, sequence analysis of the homologous H/ACA snoRNP protein, Nhp2, suggests variation of key residues located at the predicted RNA-recognition site that may be responsible for the lack of K-turn recognition by this protein (99).

The eukaryotic 15.5-kDa/Snu13 proteins are strictly specific for canonical K-turns (Figure 4A), but L7Ae is able to recognize K-turns found with stem I replaced by internal loop nucleotides (referred to as K-loops (105)) observed in both archaea C/D and H/ACA

sRNAs (33,99,106). The specificity of 15.5 kDa/Snu13 allows binding to occur at only the more conserved C/D box motif of the snoRNA, but not at the C′/D′ box, because these second sites often lack identifiable K-turns (101). Structural comparisons of L7Ae with 15.5 kDa/Snu13 have attributed these differences to key residues within the α4-β4 loop (Figure 4B) as well as to dynamic characteristics that are predicted to fine-tune the RNA specificity displayed by eukaryotic homologs (99,106,107).

Solution-state studies using *fluorescence energy transfer* (FRET) (108) and *circular dichroism* (CD) (109) suggest that the tightly kinked RNA conformations observed in the crystal structures are only sampled transiently by the free RNAs. The fully kinked structure becomes stable only when the RNA is bound to a protein, or in the presence of large amounts of divalent cations such as magnesium. In contrast, L7Ae and 15.5 kDa/Snu13 are virtually identical in their RNA-bound (38,98,99) and RNA-free conformational states (107,109). These observations suggest both L7Ae and 15.5 kDa/Snu13 provide a fixed scaffold capable of selecting the appropriate RNA conformation in solution. Therefore, the C/D sno(s) RNP initiation complex formed by L7Ae or 15.5 kDa/Snu13 is likely important for stabilizing the sno(s)RNA in a conformation that favors the recruitment of the other core proteins.

The fibrillarin-Nop5 core protein complex

Formation of the L7Ae-sRNA initiation complex enables the recruitment of Nop5 to the assembling archaeal RNP, which in turn facilitates the association of fibrillarin to form the catalytically active sRNP *in vitro* (60). However, Nop5 can also directly associate with fibrillarin in the absence of RNA (66) and further reconstitution analysis suggests that this heterodimeric complex is more efficient than the isolated protein at assembling catalytically active particles (102). A functional association between Nop5 and fibrillarin is supported by the crystal structure of the Nop5-fibrillarin complex from *A. fulgidus* (Figure 5), which reveals an extensive heterodimeric interface (2637Å^2) between these proteins (66).

In the heterodimeric complex, Nop5 adopts a unique fold that includes an irregular N-terminal α/β domain that is involved in numerous interactions with fibrillarin. This irregular domain is connected to the highly conserved C-terminal α-helical core domain by an extended coiled–coiled motif. Surprisingly, the Nop5 coiled–coiled domain was found to self-associate, resulting in a quaternary complex containing two copies of the Nop5-fibrillarin heterodimer (Figure 5). The N-terminal domain of Nop5 is not well conserved in sequence amongst archaeal species, but seems to recognize fibrillarin largely through shape complementarity mediated by numerous main-chain interactions.

The C-terminal α-helical domain of Nop5 is likely to adopt the same fold in other archaeal and eukaryotic Nop56/Nop58 proteins, based on high sequence homology within this region (66). This domain contributes to fibrillarin recognition and interacts with residues directly involved in AdoMet recognition. A conserved aromatic
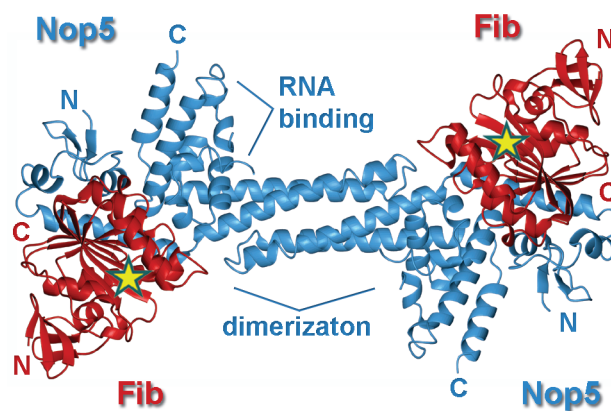

Figure 5. Crystal structure of the archaeal fibrillarin-Nop5 tetrameric complex (1NT2). Nop5 (blue) interacts with fibrillarin (Fib; red) through its N- and C-terminal domains. The C-terminal domain of Nop5 binds to the guide RNA and is positioned near the catalytic site (yellow star) of fibrillarin. The N- and C-terminal domains of Nop5 are connected by a coiled–coiled motif that facilitates dimerization of the Fib-Nop5 complex.

residue at the AdoMet site adopts a conformation that would preclude cofactor binding in free fibrillarin, but becomes oriented for AdoMet recognition when fibrillarin is co-crystallized with Nop5 but without cofactor (69). Furthermore, mutation of an arginine in Nop5 that forms a cation–π interaction with this fibrillarin aromatic residue severely disrupts catalysis (69). Together, these results strongly suggest that the communication between Nop5 and fibrillarin is important for efficient catalytic activity by mediating AdoMet interactions at the catalytic site.

The conserved C-terminal α-helical domain of Nop5 is required for recruiting this protein to the C/D sno(s)RNA. A basic patch, suggestive of RNA binding, is found on the surface of the afNop5 C-terminal domain and is located near the AdoMet-binding site of afFib (66). A single mutation of a highly conserved residue (Arg224 in afNop5) within this domain is sufficient to disrupt the interaction (66). sRNA nucleotide protection analysis indicates that Nop5 makes direct contacts with the conserved C/D and C′/D′ domains of the sRNA (110). Taken together, these results clearly show a critical role for this highly conserved domain of Nop5 in the recognition of the guide sRNA, and suggest that this interaction may be required to mediate the association of fibrillarin with the sRNP. Since this putative RNA-binding domain of Nop5 is located near the catalytic site of fibrillarin in the complex (Figure 5), it may also play a role in orienting the guide–target RNA duplex for catalysis (66).

The eukaryotic Nop5 homologs, Nop56 and Nop58, have retained the general functional properties of Nop5. For example, yeast Nop58 and Nop1 (yeast fibrillarin) are independently recruited to the snoRNA, suggesting a direct interaction with the RNA. However, the association of Nop56 requires the presence of Nop1, suggesting an interaction with the enzyme mediates its recruitment to the snoRNP (47). Thus, although both Nop56 and
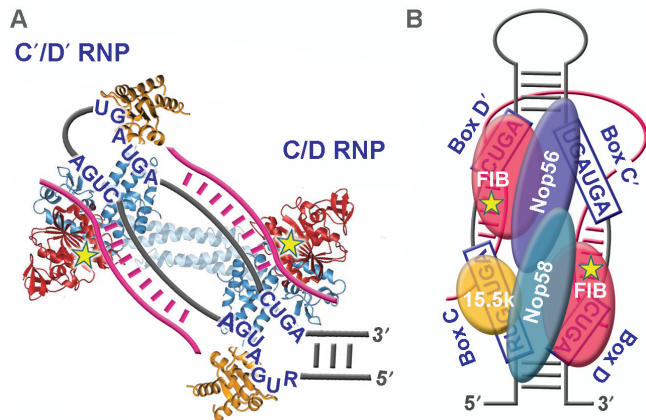
Figure 6. Class C/D sno(s)RNP architecture. (**A**) The archaeal C/D sRNP assembles with a copy of each core C/D proteins (colored as in Figures 4 and 5) at both the C/D and C′/D′ RNA motifs (blue) of the sRNA (grey), and guides methylation of a RNA nucleotide (pink) at each site. The observed quaternary structure of the fibrillarin-Nop5 complex would position two fibrillarin catalytic sites (yellow star) $\sim$80 Å apart. In contradiction to this observation, the distance between catalytic C/D and C′/D′ sites of the sRNAs is highly conserved and only $\sim$25–35 Å, suggesting the coiled–coiled interactions (transparent coloring) of Nop5 may be reorganized and/or disrupted in the fully assembled sRNP. (**B**) The C/D snoRNP is proposed to assemble into a pseudo-symmetric architecture. In contrast to the archaeal L7Ae protein, the Snu13/15.5 kDa protein appears to bind solely at the C/D site of the snoRNA. The Nop5 paralogs, Nop56 and Nop58, recognize the C and C′ Box elements, respectively, while a copy of fibrillarin (Fib) interacts with each D and D′ Box, consistent with its role in the catalytic center (yellow star).

Nop58 share core sequence homology to the archaeal Nop5 protein, these eukaryotic proteins have developed unique snoRNA-binding requirements and specific interactions with the enzyme required for snoRNP biogenesis.

The fully assembled C/D sno(s)RNPs

There is strong biochemical support for a symmetric architecture of archaeal C/D sRNPs, where a copy of each core protein is assembled at both the C/D and C′/D′ domain of the sRNA (60,100,102). This symmetric C/D sRNP architecture has recently been shown to be critical for achieving site-specific catalysis *in vitro* (111). Furthermore, sequential and cooperative activities between the two *trans*-acting modification sites have been demonstrated in an archaeal system (112). Cooperativity between the two catalytic sites is further supported by mutations made to disrupt K-turn formation at either the C/D or C′/D′ sites which are shown to adversely affect the methylation activity guided by the opposite D or D′ box (100,113). Such inter-domain communication in sRNPs (and snoRNPs) may ensure that, *in vivo*, both target sites are accurately modified.

The symmetric architecture of the full archaeal C/D sRNP containing each core protein assembled at the C/D and C′/D′ domains of the sRNA is illustrated in Figure 6A. This model is suggested by the quaternary structure of the *A. fulgidus* fibrillarin-Nop5 core protein

complex and supported by mutational analysis of this system that showed the requirement of coiled–coiled Nop5 interaction for symmetric assembly and efficient catalysis of the sRNP (66,100). This architectural model provides an elegant mechanism for organizing the assembly of the bipartite C/D sRNP. In this model, the fibrillarin-Nop5 tetramer recruits a copy of Nop5 at or near the C/D and C′/D′ motifs once L7Ae has bound the sRNA. The Nop5 interaction with fibrillarin would then correctly position a copy of the enzyme at both catalytic sites of the sRNP (Figure 6A). Once assembled, the coiled–coiled Nop5 interaction could further serve to mediate cooperativity between the C/D and C′/D′ sites of the sRNP.

Other archaea species, as well as the Nop56/Nop58 eukaryotic homologs, contain a large insertion ($\sim$60 amino acids) between the two helices of non-conserved sequence but conserved number of residues. However, the coiled–coiled domain of Nop5 proteins is largely conserved in sequence even amongst Nop56 and Nop58 proteins. At least in archaea, the architectural importance of this coiled-coiled domain has been supported biochemically (100,114). However, the conformational context of the coiled–coiled interaction in the assembled particle remains unclear. In archaea, the C/D and C′/D′ sRNA box motifs are positioned 12 nucleotides ($\sim$25–35 Å) apart (115), while in the afFib-afNop5 crystal structure, the catalytic pockets of the two fibrillarin molecules are separated by $\sim$80 Å (66). This difference suggests that the coiled–coiled interactions observed in the crystal structure may be reorganized (or possibly even disrupted) upon sRNP assembly (114). Interestingly, studies of eukaryotic snoRNPs indicate that a critical restructuring event occurs prior to nucleolar localization, and this has been proposed to activate the particle (116). Further structural analyses of completely assembled C/D sno(s)RNPs will solve this uncertainty and enhance our understanding of the core C/D sRNP interactions.

Eukaryotic C/D snoRNPs are predicted to adopt a pseudo-symmetric architecture similar to archaeal sRNPs (Figure 6B). UV cross-linking studies of C/D snoRNPs reconstituted in *Xenopus oocyte* nuclei place the related but distinct proteins Nop58 at the C box and Nop56 at the C′ box of the snoRNA, suggesting that these two proteins bind specific sites in the snoRNA (70). In this study, fibrillarin cross-linked to both D and D′ boxes, consistent with one copy of the enzyme being associated with each guide domain of the sRNA (Figure 6B). 15.5 kDa/Snu13 protein initiates snoRNP assembly and appears to bind solely at the more conserved C/D site of the snoRNA (101). However, it remains possible that 15.5 kDa/Snu13 is recruited to the C′/D′ domain through protein–protein interactions. Supporting this suggestion, recent mutational analyses of 15.5 kDa indicate that it contributes to the snoRNP architecture by making interactions with both Nop58 and Nop56 (96). Although a Nop56-Nop58 interaction remains to be demonstrated in eukaryotes, the observation that snoRNAs containing only the C/D motif associate with all four core proteins suggest such an interaction may indeed be important (41,71).
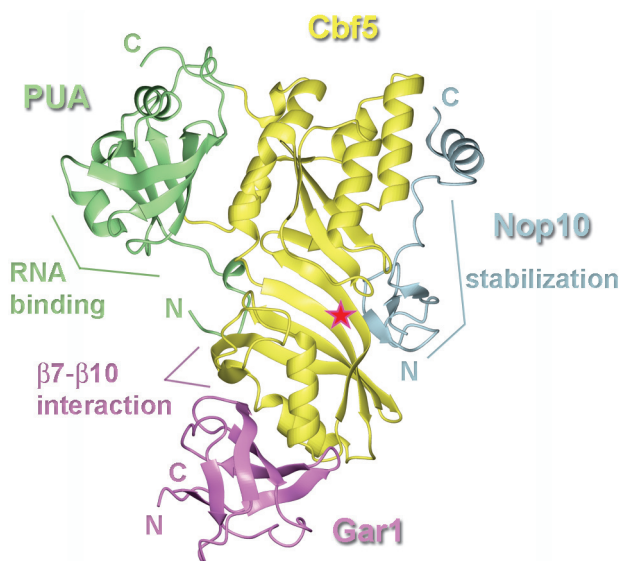
Figure 7. Crystal structure of the archaeal Cbf5-Gar1-Nop10 hetero-trimer (2EY4). The catalytic domain of Cbf5 (yellow) is surrounded by the Cbf5 PUA domain (green), Nop10 (blue) and Gar1 (purple) proteins. The Cbf5 PUA domain contributes to the extended RNA-binding surface of this protein. Nop10 packs against the back-side of the catalytic site (red star) and contributes to the organization and stabilization of the active site itself. Gar1 contacts Cbf5 through conserved hydrophobic interactions that may modulate substrate RNA loading and release.

The H/ACA core protein complex

Reconstitution analyses of H/ACA sRNPs (61,62) revealed that both archaea Cbf5 and L7Ae associate with the guide RNA directly and independently of each other. The two other core proteins, Nop10 and Gar1, instead require Cbf5 for recruitment to the guide RNA. These two core proteins associate independently of each other with Cbf5 in the absence of RNA, forming a core heterotrimer. Nop10 and Cbf5 are required *in vitro* to achieve basal levels of enzymatic activity in the presence of the guide and target RNAs (61). However, all four core proteins are required for full activity.

In the crystal structure of the Cbf5-Gar1-Nop10 heterotrimer (77) (Figure 7), the catalytic core domain of Cbf5 is decorated by its own PUA domain and by Nop10 and Gar1, each surrounding the catalytic site of Cbf5. Nop10 and Gar1 are separated by a distance of ~20 Å in the crystal structure, consistent with their independent association with the enzyme in both archaea and eukaryotes (49,61,62). Both the Nop10 and Gar1 interactions involve highly conserved residues within the catalytic domain of Cbf5, identifying key protein–protein interactions that may be required to modulate the function of the enzyme.

The structures of the Cbf5-Nop10 complexes from *M. jannaschii* and *P. abyssi* have also been described (64,72). In each complex, Nop10 is located directly behind the enzyme's catalytic site. NMR studies show that this small but highly conserved protein is conformationally dynamic on its own, yet retains structure in the N-terminal

β-ribbon zinc-binding motif and a short transient C-terminal helical tail connected by a ~20-Å-long flexible linker (64). Eukaryotic proteins are both structurally and dynamically similar to the archaea proteins, but have replaced the N-terminal zinc-binding motif with a simple β-turn (64,117). When bound to Cbf5, this flexible protein becomes highly ordered through numerous interactions with Cbf5 (~46% of the accessible surface of archaeal Nop10 is buried) but nonetheless retains the same secondary structure observed in the isolated protein (64).

The flexible linker is the most highly conserved region of Nop10 and makes several interactions with Cbf5 along a conserved stretch in Ψ synthases known as Motif I (118) that is important for the stability of these enzymes (119). The interaction with Nop10 may stabilize this motif in Cbf5 and explain its requirement for achieving basal activity of the sRNP. The N-terminal β-ribbon motif and C-terminal α-helix of Nop10 are separated by the extended linker domain and contribute to a basic trough that extends from the catalytic site of Cbf5. The conservation and physical properties of these domains suggested that Nop10 participates in additional inter-molecular interactions within the assembled RNP, and were predicted to contribute to the recognition of the guide sno(s)RNA and/or target RNA complex (64,72).

The structure of the archaeal H/ACA heterotrimeric Cbf5-Gar1-Nop10 complex offers insight into the functional role of Gar1 (77). Archaea Gar1 proteins lack the terminal GAR domains found in eukaryotic homologs but share the conserved functional core. In the complex, as well as on its own (K. Godin *et al.*, unpublished results), Gar1 adopts a six-stranded β-barrel motif and closely resembles RNA-binding domains found in bacterial translation initiation and elongation factors. The core domain of Gar1 was proposed to be responsible for binding guide RNA in yeast (120), and indeed the mammalian Gar1 protein cross-links to RNA near the catalytic site of the assembled snoRNP (49). However, the archaea Gar1 structure does not support a direct role in RNA binding. Although Gar1 is structurally homologous to RNA-binding proteins, the region of this protein that corresponds to the RNA-binding sites of IF2 and EF-G is involved instead in protein–protein contacts with Cbf5. Furthermore, Gar1 is ~20 Å away from the catalytic site. Thus, the cross-linking reported in mammals may be due to the eukaryotic-specific GAR domains or perhaps a temporary association with the substrate RNA during its loading or release (77,121,122).

Despite the lack of structural evidence for a direct role of Gar1 in RNA recognition from the archaea protein-only complex, its interaction with Cbf5 indicates a possible functional role for this protein in modulating RNA recognition. Cbf5 homologs lack a characteristic peptide segment found in TruB that is involved in tRNA T-loop recognition (73). The absence of this segment opens up the RNA-binding site of Cbf5 to include the β7/β10 hairpin of the enzyme. Gar1 interacts directly with this hairpin and may therefore influence the Cbf5–RNA interaction (77,80). Such a functional role would rationalize the observed requirement of Gar1 for full enzymatic activity of the archaeal sRNP (61,62).
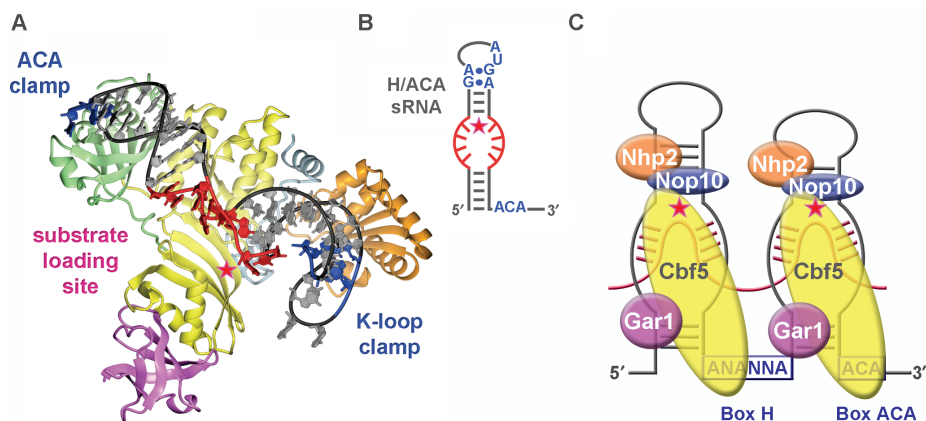
Figure 8. Class H/ACA sno(s)RNP architecture. (**A**) The crystal structure of the archaeal H/ACA core proteins with a guide sRNA (2HVY). (**B**) The H/ACA sRNA secondary structure and color scheme (base-paired stems and apical loop: grey; conserved ACA Box and K-loop nucleotides: blue; pseudouridylation pocket: red). The sRNA is recognized by Cbf5, L7Ae and Nop10 (colored as in Figures 4 and 7). The ACA Box and proximal stem are bound by the Cbf5 PUA domain, while the apical stem and K-loop are bound by Nop10 and L7Ae, respectively. These interactions act as conserved molecular clamps that precisely position the sRNA pseudouridylation pocket at the catalytic site of Cbf5 (red star). (**C**) The H/ACA snoRNP is proposed to adopt a bipartite structure with a copy of the core H/ACA proteins assembled at each hairpin motif of the snoRNA. The core H/ACA interactions are expected to be similar between eukaryotic and archaeal sRNPs, but some interactions with the snoRNA are expected to be unique to eukaryotes. For example, the Nhp2-snoRNA interaction differs from the L7Ae-sRNA K-turn (or K-loop) interaction.

The class H/ACA snoRNP

The recent crystal structure of the core H/ACA proteins assembled with a guide RNA (80) confirmed many of the proposed functional roles of the core proteins within the RNP and provided details of the interactions guiding sRNA recognition and sRNP architecture. In the archaea H/ACA complex (Figure 8A), the guide RNA is recognized by a composite surface formed by Cbf5, Nop10 and L7Ae, with the proximal stem and ACA tail motif positioned at the PUA domain of Cbf5 and the apical stem-loop recognized by Nop10 and L7Ae, respectively. The pseudouridylation pocket loosely associates across the catalytic face of Cbf5, consistent with the lack of sequence conservation among snoRNAs within this region. L7Ae binds at the predicted K-loop site found at the apical stem-loop domain of the sRNA and additionally interacts with Nop10 through inter-helical packing. The interaction with Nop10 covers a small surface area ($660 \, \text{Å}^2$), but involves largely conserved residues from both proteins. The highly conserved linker region of Nop10 that makes intimate contacts with Motif I in Cbf5 also makes contacts with the apical stem of the sRNA.

The PUA domain interacts specifically with the ACA box (ANA amongst archaeal species). Recognition of the ACA box by the PUA domain of Cbf5 was predicted based on structural comparisons with the ArcTGT PUA domain (64,72,77); however, the H/ACA complex reveals that their respective modes of recognition differ (80). The two strictly conserved adenines of the ACA box make extensive aromatic and hydrogen bonding interactions with the protein, while the less conserved cytosine in archaea adopts an exposed conformation. In addition, the PUA domain makes several interactions with the minor groove of the proximal sRNA stem.

During catalysis, the target site of the H/ACA sno(s)RNP is defined by a three-way helical junction formed by the apical stem of the guide sno(s)RNA and the duplex formed between the 5′- and 3′-guide sequences of the pseudouridylation pocket and the regions flanking the catalytic site, that is positioned ~14 nucleotides upstream of the ACA tail motif in archaea (~14–16 nucleotides in eukaryotes). Thus, the Nop10-L7Ae interaction with the apical stem-loop and the PUA recognition of ACA box serve as conserved molecular clamps to position the pseudouridylation pocket across the catalytic face of Cbf5. The conformational plasticity observed in the internal loop of the guide sRNA likely facilitates structural reorganization upon binding the substrate RNA that is needed to orient the resulting three-way helical junction precisely at the catalytic site of Cbf5 (80).

In the complete H/ACA structure, Gar1 makes no direct contacts with the guide RNA, but its proposed role in modulating interactions with the substrate RNA and the β7-β10 motif of Cbf5 is further supported. Although residues within this motif of Cbf5 remain partially disordered, additional interactions are observed with Gar1 compared to what is observed in the protein-only heterotrimer (80). When Li and Ye built a model to include the target RNA (after the TruB-tRNA complex), it was suggested that the additional interactions with Gar1 observed in their crystal structure would orient the β7-β10 motif to preclude an interaction with the substrate RNA (80). In contrast, when the β7-β10 loop adopts the conformation observed in the protein-only heterotrimer (77) (Figure 7), it is ideally positioned for recognizing the substrate RNA. Thus, this interaction with Gar1 may very well be critical for modulating target RNA loading and release.

H/ACA sRNAs (and snoRNAs) containing multiple stem-loop domains typically harbor pseudouridylation

guide sequences within each domain. The ability of a single archaeal H/ACA RNA hairpin domain to fully assemble into a catalytically active RNP suggests that H/ACA sRNAs with multiple stem-loops would contain a full complement of proteins at each domain. The resulting modular architecture of these sno(s)RNPs was predicted from low-resolution EM images of eukaryotic particles (51). In these multi-domain H/ACA sno(s)RNPs, inter-domain communication may be important for function. Archaeal and human H/ACA sno(s)RNAs containing two hairpin motifs are able to bind two copies of archaeal Cbf5, yet mutating only the terminal ACA box of a human snoRNA completely eliminates binding of this protein at both RNA-binding sites (62). Thus, association of Cbf5 (and therefore of Nop10 and Gar1) with the two RNA domains appears to be coupled; however, the details of the molecular interactions responsible for this coupling remain unknown.

Eukaryotic H/ACA snoRNPs are expected to adopt bipartite architectures that involve core RNP interactions similar to those in archaea (49) (Figure 8C). However, as with the C/D snoRNPs, H/ACA snoRNP proteins have evolved unique specificities and/or additional inter-particle interactions which are required for their biogenesis in these higher species. Recent nucleotide mapping of the conserved N-terminal domain of yeast Cbf5-snoRNA interaction identifies the expected RNA protection based on the archaea structure and identifies additional nucleotides 5′- to the stem-loops that appear to interact with the eukaryotic protein (86), perhaps through the N-terminal extension to the PUA domain found in eukaryotes. *In vitro* studies demonstrate a protein-only core H/ACA complex in yeast and mammalian systems that are similar to the archaeal core protein complex, yet involves all four core H/ACA proteins (49,123). In mammals, formation of the Nap57-Nop10 heterodimer is a prerequisite for recruitment of Nhp2 to the snoRNP, suggesting a Nop10-mediated association of Nhp2. This complex would be analogous to the Nop10-L7Ae interaction observed in the structure of the archaeal H/ACA sRNP (80), and likely further stabilized through eukaryotic-specific interactions.

Using mammalian proteins, specific snoRNA binding required the heterotrimeric complex formed by Nap57, Nhp2 and Nop10 (49). The archaea homologs to these proteins are the same three components that contact the guide RNA in the crystal structure (80). Notably however, H/ACA snoRNAs lack identifiable K-turns. It is possible that Nhp2 interacts with a similar kinked-RNA structure, but by evolving a stable association with the H/ACA enzyme complex, the genetic pressure to maintain a consensus K-turn site in H/ACA snoRNAs has been lost.

As in archaea, Gar1 interacts with the enzyme (Nap57) independently of the other core proteins, consistent with a late association of this protein during snoRNP biogenesis (49). Interestingly, the H/ACA assembly factor Naf1 contains significant sequence homology with Gar1 and associates with Cbf5/Nap57 as well (124–126).

Naf1 co-localizes at snoRNA transcription sites with the H/ACA enzyme and the other two core proteins Nhp2 and Nop10, but is excluded from Cajal bodies and nucleoli, thus it is not found in the mature snoRNP (90,92,93). Gar1 and Naf1 bind to the H/ACA enzyme *in vitro* and compete with each other for binding, suggesting a common recognition site (127). This competitive interaction may serve to regulate RNP activation by serving two distinct functional roles, where Naf1 could act to stabilize the initial snoRNP and perhaps inhibits premature enzymatic activity; then only when the snoRNP is fully matured, Gar1 is recruited to stimulate catalysis.

PERSPECTIVES

The structural and biochemical information on the H/ACA and C/D snoRNPs has been expanded greatly only in the last few years and has offered valuable insight into the catalytic mechanisms, the functional roles played by the conserved core proteins in modulating the activity of the enzyme and the assembly of the sno(s)RNP. However, a complete understanding of these functions will require further structural characterization of completely assembled sno(s)RNPs bound to their substrate RNAs. Furthermore, eukaryotic snoRNPs have evolved unique functional requirements distinct from archaea and require specific assembly pathways and trafficking factors for their biogenesis and *in vivo* functions. The structural characterization of eukaryotic snoRNPs and of their assembly factors are still needed to understand the functional differences and the mechanisms of snoRNP assembly and biogenesis.

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