Structurally Conserved Nop56/58 N-terminal Domain Facilitates Archaeal Box C/D Ribonucleoprotein-guided Methyltransferase Activity*  

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Background: Box C/D RNPs direct site-specific 2′-O-methylation of rRNA. 
Results: The Nop56/58 and fibrillarin core proteins establish a very stable dimer with Nop56/58 contributing to methyltransferase activity. 
Conclusion: The Nop56/58 core protein plays a role not only in RNP assembly, but also methyltransferase activity. 
Significance: Our observations reveal a novel role for the Nop56/58 core protein in box C/D RNP function.

Box C/D RNA-protein complexes (RNPs) guide the 2′-O-methylation of nucleotides in both archaeal and eukaryotic ribosomal RNAs. The archaeal box C/D and C′/D′ RNP subcomplexes are each assembled with three sRNP core proteins. The archaeal Nop56/58 core protein mediates crucial protein-protein interactions required for both sRNP assembly and the methyltransferase reaction by bridging the L7Ae and fibrillarin core proteins. The interaction of Methanocaldococcus jannaschii (Mj) Nop56/58 with the methyltransferase fibrillarin has been investigated using site-directed mutagenesis of specific amino acids in the N-terminal domain of Nop56/58 that interacts with fibrillarin. Extensive mutagenesis revealed an unusually strong Nop56/58-fibrillarin interaction. Only deletion of the NTD itself prevented dimerization with fibrillarin. The extreme stability of the Nop56/58-fibrillarin heterodimer was confirmed in both chemical and thermal denaturation analyses. However, mutations that did not affect Nop56/58 binding to fibrillarin or sRNP assembly nevertheless disrupted sRNP-guided nucleotide modification, revealing a role for Nop56/58 in methyltransferase activity. This conclusion was supported by the cross-linking of Nop56/58 to the target RNA substrate. The Mj Nop56/58 NTD was further characterized by solving its three-dimensional crystal structure to a resolution of 1.7 Å. Despite low primary sequence conservation among the archaeal Nop56/58 homologs, the overall structure of the archaeal NTD domain is very well conserved. In conclusion, the archaeal Nop56/58 NTD exhibits a conserved domain structure whose exceptionally stable interaction with fibrillarin plays a role in both RNP assembly and methyltransferase activity.

Eukaryotic small nucleolar RNAs (snoRNAs) guide the site-specific modification of ribonucleotides in target RNAs (1–4). The box C/D and H/ACA RNAs comprise the two major snoRNA families that guide 2′-O-methylation and pseudouridylation, respectively (1). Their primary target RNA for modification is ribosomal RNA but other target RNAs include the spliceosomal snRNAs and select mRNAs (5–7). SnoRNAs base pair with their target RNA to determine the specific nucleotide for modification with snoRNA-bound proteins catalyzing the modification reactions (4, 8). Homologous box C/D and H/ACA small RNAs (sRNAs) and RNA-protein complexes (sRNPs) guide the same nucleotide modification reactions in Archaea (8).

The archaeal box C/D sRNPs provide a model system for structural and functional studies because of their minimal size and their ability to be reconstituted in vitro as catalytically active complexes (9, 10). Archaeal box C/D sRNAs are 50–70 nucleotides in size and possess well conserved box C, C′, D, and D′ sequences (3, 4). Boxes C and D located at the 5′ and 3′ termini, respectively, base pair to form the box C/D motif folded into a kink-turn (K-turn) (11, 12). Internal boxes C′ and D′ base pair to form the C′/D′ motif, which exhibits a variant of the K-turn known as the K-loop (10, 13). In Archaea, these two RNA motifs are bound by the box C/D core proteins to assemble the terminal box C/D and internal C′/D′ RNPs. Target RNAs base pair with box C/D and C′/D′ RNP-associated guide sequences to direct the 2′-O-methylation of specific nucleotides in the target RNA (2, 9). Importantly, the inter-RNP spacing of these two subcomplexes is highly conserved at 12 nucleotides and alteration of this distance disrupts sRNP-guided nucleotide methylation (14).

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4 The abbreviations used are: snoRNA, small nucleolar RNA; RNP, ribonucleoprotein; Mj, M. jannaschii; DSC, differential scanning calorimetry; AdoMet, S-adenosylmethionine; Δ5aa, 5-amino acid deletion; NTD, N-terminal domain; CTD, C-terminal Nop domain; CC, coiled-coil domain; Af, A. fulgidus; Ss, S. sulfataricus; Pf, P. furiosus; Ni-NTA, nickel-nitrilotriacetic acid.

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The terminal box C/D and internal C’/D’ RNPs are each assembled with three core proteins (10, 15, 16). L7Ae initiates box C/D and C’/D’ RNP assembly by binding the K-turn and K-loop motifs. These L7Ae-sRNA complexes then serve as a binding scaffold to recruit Nop56/58, which uses its C-terminal domain (CTD or Nop domain) to bind the L7Ae-sRNA complex (10, 16, 17). The methyltransferase fibrillarin is then recruited by interaction with the Nop56/58 N-terminal domain (NTD) (18, 19). The Nop56/58 central coiled-coil (CC) domain promotes self-dimerization of this core protein (19), which provides intra-RNP protein–protein contact in the monoparticle as well as inter-RNP interaction of the dimeric complex. Thus Nop56/58 serves as a bridge between L7Ae and fibrillarin in both box C/D and C’/D’ RNP assembly as well as linking these two subcomplexes in monomeric and dimeric sRNPs (15, 16, 20).

Core protein–protein interactions play critical roles in both box C/D sRNP assembly and the methyltransferase reaction. Crystal structures of each of the three core proteins as well as various protein–protein and RNA-protein complexes have revealed the basic structure of this RNP enzyme (16, 17, 21). Although it is clear that fibrillarin is the methyltransferase enzyme that binds the S-adenosylmethionine and the target RNA substrates, it is not yet understood how the L7Ae and Nop56/58 core proteins contribute to or even facilitate catalytic activity. The interaction of Nop56/58 with fibrillarin has now been investigated using site-directed mutagenesis of the Nop56/58 NTD. Nop56/58-fibrillarin dimerization is remarkably stable and not easily disrupted. Interestingly, however, two NTD mutations that did not affect RNP assembly nevertheless greatly reduced or abolished methyltransferase activity, indicating the importance of Nop56/58 for the 2’-O-methylation reaction. Supporting this functional contribution, Nop56/58 can be cross-linked to the target RNA. Determination of the Methanothermococcus jannaschii (Mj) NTD crystal structure revealed that this domain is structurally well conserved among archaeal Nop56/58 homologs despite low sequence homology. Thus, the structurally conserved Nop56/58 NTD interacts with fibrillarin to play a role in not only box C/D sRNP assembly but also sRNP-guided nucleotide methylation.

**EXPERIMENTAL PROCEDURES**

**RNA Synthesis and Radiolabeling**—s8 box C/D sRNA was transcribed and gel purified as previously described (10). Target RNA substrates were purchased from Integrated DNA Technologies (IDT). The 4-thiouridine-substituted D target RNA (5’-GA(s4U)CAUGCUAACAGGUAG-3’) was purchased from Dharmacon. RNAs were 5’-terminal radiolabeled with 32P as described previously (18).

**Protein Expression and Purification**—The M. jannaschii (Mj) L7Ae, fibrillarin, and Nop56/58 genes were cloned as previously described (10). L7Ae and fibrillarin were expressed with N-terminal His6 tags and the Nop56/58 wild-type and mutant proteins were either N-terminal His6 tagged or untagged. Protein genes were cloned into either pET21a or pET28a vectors (Novagen) and mutations were introduced using the Stratagene QuickChange site-directed mutagenesis kit. Deletions or truncations were generated by PCR amplification from existing plasmids followed by subcloning into new pET28a vectors. The Nop56/58 coiled-coil deletion (ACC) was generated as previously described (20). All mutations, deletions, or truncations were verified by sequencing. Fibrillarin and L7Ae protein expression was induced in Rosetta DE3 cells (Novagen) using 1 mM isopropyl-β-D-galactopyranoside and grown for 3 h at 37 °C. For production of Nop56/58 and its mutant proteins, expression was induced in Rosetta DE3 cells with 0.2 mM isopropyl-β-D-galactopyranoside and grown for 24 h at 15 °C. Recombinant proteins were purified by Ni-NTA affinity chromatography or cation-exchange chromatography as previously described (10, 18). Prepared proteins were dialyzed against Buffer D (20 mM HEPES, pH 7.2, 3 mM MgCl2, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol) and stored at −80 °C. N-terminal His6 tags were removed by digestion with bovine thrombin before or during dialysis when desired (10).

**Protein and RNP Affinity Co-purification and Nucleotide 2’-O-Methylation Activity Assays**—Nop56/58-fibrillarin interaction was assessed via affinity purification or pulldown experiments examining dimer formation similar to that previously described (10). Histidine-tagged fibrillarin was incubated at a 1:1 molar ratio with wild-type or mutant Nop56/58 protein in 1× HBSSM buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl2) at 70 °C for 5 min, followed by cooling to room temperature and a brief centrifugation to remove precipitated protein. Soluble protein was incubated for 15 min at room temperature with Ni-NTA resin equilibrated in Buffer D containing 50 mM imidazole. Ni-NTA resin was then washed (3 times) with Buffer D supplemented with 0.02% SDS and 0.1% Triton X-100. Bound protein was eluted in Buffer D containing 250 mM imidazole and then precipitated with 4 volumes of acetone. Dried protein pellets were resuspended in SDS sample buffer and resolved on 12% SDS–polyacrylamide gels.

sRNP assembly and assessment of in vitro 2’-O-methylation activity were accomplished as previously described (10). sRNP assembly for affinity purification of the complex was carried out using histidine-tagged wild-type L7Ae mixed with equimolar concentrations of untagged fibrillarin and untagged wild-type and mutant Nop56/58 proteins and incubated with a half-molar equivalent of 5’-radiolabeled s8 RNA. sRNP complexes were assembled at 70 °C for 10 min in 1× HBSSM-300 (HBSSM with a final NaCl concentration of 300 mM). Assembled complexes were purified by Ni-NTA chromatography as described above but in HBSSM-300 buffer. Samples were resolved by SDS–polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining for protein and phosphorimaging for radiolabeled RNA. For in vitro 2’-O-methylation assays, time points at 0 and 60 min were evaluated in triplicate and the mean ± S.E. determined.

**Differential Scanning Calorimetry (DSC)**—DSC was performed using a MicroCal VP-DSC capillary cell microcalorimeter. Proteins were first dialyzed against 1× DSC buffer (10 mM cacodylate, pH 7.3, 250 mM NaCl, 250 mM KCl). Proteins at 25 or 50 μM in degassed 1× DSC buffer were scanned under pressure from 35 to 125 °C at a scan rate of 90 °C/h. Temperatures corresponding to the maximum observed heat capacity (Cp) were designated the Tm (22). Average Tm values and S.D. were determined by statistical analysis of replicate scans.
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Photocross-linking of 4-Thiouridine-substituted Target RNAs—Box C/D sRNP complexes were assembled by combining 200 pmol each of L7Ae, fibrillarin, and Nop56/58 proteins with 100 pmol of rRNA in buffer D in a final volume of 100 μl and heated at 70 °C for 10 min. D and D’ target RNAs were added at a final concentration of 1 μM and 20,000 cpn when radioactive RNA was included. Samples were cooled and stored at room temperature in the dark until irradiation. RNA was cross-linked to protein by irradiating samples with UVA light as previously described (23). The 100-μl samples in the microcentrifuge tubes were submerged in a water-filled well jacketed with a circulating solution of 20 °C Co(NO₃)₂ to regulate temperature and filter out wavelengths below 320 nm. The light intensity reaching the microcentrifuge tubes was ~200 milliwatts/cm². After irradiation, samples were precipitated with 4 volumes of acetone and resolved on 14% SDS-polyacrylamide gels. Resolved proteins were visualized by Coo massie Blue staining and radiolabeled RNA was visualized using a phosphorimager.

**Nop56/58 NTD Purification, Crystallization, Data Collection, and Processing**—The Mj Nop56/58 NTD coding sequence was PCR-amplified from the full-length Mj Nop56/58 gene and subcloned into a pET21a vector (Novagen). Recombinant NTD protein was expressed in Rosetta (DE3) cells by induction with 1 mm isopropyl 1-thio-β-d-galactopyranoside at 37 °C for 3 h. Cells were harvested and lysed by sonication in Buffer A (10 mm HEPES, pH 7.4, 250 mm NaCl, 10% glycerol). The sonicate was subsequently heated at 60 °C for 10 min to denature and precipitate *Escherichia coli* proteins. Selenomethionine-substituted protein was produced by inhibiting the methionine biosynthesis pathway (24). After removal of insoluble debris by centrifugation, the supernatant was concentrated and NTD protein was purified by FPLC over a Superdex 200 gel filtration column (GE Healthcare) equilibrated in 20 mM HEPES (pH 7.2) and 250 mm NaCl. Peak fractions were pooled and protein was concentrated to 2 mmo, then flash frozen in liquid nitrogen and stored at −80 °C.

Purified Mj Nop56/58 protein was initially screened for crystallization conditions by the hanging drop vapor diffusion method using a Hampton Research crystallization screen. Crystals were obtained in 0.2 M zinc acetate dehydrate, 0.1 M sodium cacodylate trihydrate (pH 6.5), 18% polyethylene glycol (PEG) 8,000. Both native as well as selenomethionine-substituted protein crystals were soaked in mother liquor containing 10% glycerol and flash frozen in liquid nitrogen. The datasets were collected at the SER-CAT beamline 22ID (Advanced Photon Light Source, Chicago, IL). Data were indexed and scaled using HKL2000 (25). Data reduction statistics and unit cell parameters are summarized in Table 1.

The structure of the Mj Nop56/58 NTD protein was solved by single wavelength anomalous dispersion. The selenomethionine-substituted protein crystals diffracted to 2.0 Å, whereas the native NTD crystal diffracted to a resolution of 1.7 Å. The program autosol in PHENIX was used to obtain an initial model (26). Autosol found five sites of which two were selenium and three were zinc. The model obtained from autosol was then used for molecular replacement with the higher resolution of the native crystal dataset. The initial map and model were obtained using PHASER (CCP4) (27) and manually fitted using COOT (28) followed by further refinement. Refinement was carried out in PHENIX (26) with 5% of the data excluded for the *R*_free calculation. Cross-validated, σa-weighted composite omit maps, or σa-weighted 2Fo − Fc and Fo − Fc maps were calculated in PHENIX and used for fitting and model building. TLS refinement in addition to individual ADP resulted in *R*_work and *R*_free values to 20 and 23%, respectively. The final model was analyzed using PROCHECK (29) and MOLPROBITY (30). Root mean square deviation calculations were made by using LSQMAN (31). All figures of crystal structures were constructed in PyMOL.

**RESULTS**

The Nop56/58-Fibrillarin Complex Is a Remarkably Stable Heterodimer—Box C/D RNA-protein complexes guide the 2’-O-methylation of specific nucleotides in target RNAs. Terminal box C/D and internal C’/D’ RNPs each guide methyltransferase reactions using three core proteins (Fig. 1A). We have investigated the importance of Nop56/58-fibrillarin dimerization for sRNP assembly and methyltransferase activity by mutating specific interface amino acids to alter the Nop56/58-fibrillarin interaction. The Nop56/58 core protein is com-
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prised of three structurally and functionally distinct domains (Fig. 1B) (17, 19). The C-terminal Nop domain (CTD) recognizes and binds the L7Ae core protein, which is the first core protein to bind the sRNA and initiate RNP assembly. The CC domain mediates self-dimerization of Nop56/58. This interaction couples the terminal box C/D and internal C’/D’ RNPs in a monomeric sRNP (20) and is responsible for the formation of a dimeric sRNP complex (15). The NTD binds fibrillarin and is responsible for heterodimer formation. In sRNP assembly, fibrillarin is the last core protein added to the complex and is the methyltransferase enzyme that catalyzes the nucleotide modification reaction.

A number of site-specific mutations or deletions were incorporated into the NTD of M. jannaschii (Mj) Nop56/58. Although disruption of heterodimerization with fibrillarin was the goal, mutants that would retain fibrillarin binding but compromise box C/D sRNP activity were also sought. Computational alanine scanning of the archael Nop56/58-fibrillarin crystal structure from Archaeoglobus fulgidus (Af) suggested specific amino acids for mutation that might weaken or disrupt Mj Nop56/58-fibrillarin heterodimer formation (Fig. 2A) (19, 32). Initial alignment of the two archael Nop56/58 primary sequences indicated that the NTD is not highly conserved. Nevertheless, superimposition of available crystal structures of the Nop56/58-fibrillarin heterodimer from A. fulgidus (Af), Pyrococcus furiosus (Pf), and Sulfolobus sulfataricus (Ss) revealed the overall conservation of the protein folded structure and the region of the NTD interface interaction with fibrillarin (Fig. 2B).

Select mutations clustered around a hydrophobic region in the Af/NTD crystal structure (corresponding to substitution of Tyr9 with a glycine, called Y9G, and a deletion of amino acids Ser69, Thr70, and Glu71, called ΔSTE, in the Mj Nop56/58 protein) that forms the interface with fibrillarin. Additional mutations were made in the Mj Nop56/58 α-helix corresponding to Af helix 3 (α-3), which also contacts fibrillarin. Mutations in helix α-3 included single substitutions (R100D and K112D), double substitutions (M103A/H104A and V114A/I115A), a quadruple substitution (M103A/H104A/V114A/I115A, called 4mut), as well as a deletion of the 5 amino acids Ser107–Thr111 (Δ5aa). Large domain deletions or truncations were also made in Mj Nop56/58 as controls. These were deletion of the Nop56/58 NTD domain (ΔNTD), the coiled-coil region (ΔCC), or the highly charged and poorly conserved C-terminal tail (ΔK-tail) (33, 34). Control constructs also included the individual NTD and CTD domains alone.

Interaction of the Nop56/58 mutants with fibrillarin was assessed using co-precipitation or “pulldown” experiments (Fig. 2C). Recombinant N-terminal His-tagged fibrillarin was incubated with the nontagged recombinant Nop56/58 mutants at 70 °C to encourage dimerization of these thermostable proteins and then slowly cooled before mixing with charged Ni-NTA resin. After washing, bound protein was eluted with imidazole and resolved by SDS-polyacrylamide gel electrophoresis. Unexpectedly, all site-specific mutations in the Mj Nop56/58 NTD were still able to dimerize with fibrillarin. Nop56/58 mutants where whole domains were deleted yielded the expected results. Only the Nop56/58 mutant lacking an NTD was unable to bind fibrillarin. (Control experiments lacking tagged fibrillarin ensured that the Nop56/58 proteins were not binding nonspecifically to the resin, see supplemental Fig. S1.) These initial results indicated that Nop56/58-fibrillarin dimerization is not easily disrupted by simple amino acid changes or small deletions in the Nop56/58 interface region.

Site-directed mutagenesis was consistent with a strong interaction between Nop56/58 and fibrillarin that was resistant to numerous amino acid replacements and deletions. Previous examination of the Af Nop56/58-fibrillarin crystal structure had suggested strong dimer stability based upon the largely nonpolar Nop56/58 NTD interface and high surface complementarity exhibited by these two proteins (19). To further investigate Nop56/58-fibrillarin stability, the heterodimer was subjected to various chemical reagents known to weaken or disrupt protein-protein interactions (Fig. 3A). Again, pulldown experiments using His-tagged fibrillarin assessed heterodimer stability. Dimers bound to the affinity column were washed with buffer containing high concentrations of salt (5 M NaCl), nonionic detergent (5% Triton X-100), or chaotropic agents (8 M urea or 6 M guanidine hydrochloride) before fibrillarin elution with imidazole. Only the strong chaotropic agent guanidine hydrochloride was able to disrupt dimerization.

The melting temperature of fibrillarin and select Nop56/58 proteins, either alone or when bound to fibrillarin, was determined by DSC. DSC can melt samples under pressure, allowing scans at temperatures up to 130 °C. Changes in heat capacity (C_p) are recorded and the peak change in C_p indicates the T_m, or temperature at which half of the protein population is unfolded or melted (22). Both fibrillarin and Nop56/58 proteins exhibited high unfolding temperatures with T_m values ranging from 85 to 98 °C. The Nop56/58 NTD itself was stable with a T_m of ~91 °C. Remarkably, dimerization of fibrillarin with the Nop56/58 NTD resulted in a protein complex with an elevated T_m of ~113 °C (Fig. 3B). All tested Nop56/58-fibrillarin dimers that contained an intact NTD yielded a similarly elevated T_m (Fig. 3C). In the case of the ΔK-tail and K112D Nop56/58 mutants, two other peaks in C_p beside that of the dimer were observed. These peaks appear to be free Nop56/58 or fibrillarin proteins based on the temperature at their C_p maxima. However, the major peak always corresponded to the highest T_m value (supplemental Fig. S2). These observations revealed that the Nop56/58-fibrillarin dimer exhibits an unusually stable protein-protein interaction.

Mutation of the Nop56/58 NTD Interface Does Not Disrupt sRNP Assembly But Can Impair Nucleotide Methylation—The methyltransferase fibrillarin is the last protein added in RNP assembly and establishes a catalytically competent nucleotide methylation complex (9, 10, 19). This recruitment acts through direct fibrillarin interaction with the NTD of Nop56/58. Nop56/58 is, in turn, bound to the L7Ae-sRNA complex through its C-terminal Nop domain (17, 19). The ability of Mj Nop56/58 NTD mutants to fully assemble complete box C/D sRNPs was assessed using pulldown experiments (Figs. 4, A and B). Radiolabeled Mj sR8 box C/D sRNA and Mj core proteins Nop56/58, fibrillarin, and His-tagged L7Ae were used to assemble the sRNP complex. Analysis of the various Nop56/58 domain deletion mutants demonstrated, as anticipated from
the previous co-precipitation experiments shown in Fig. 2, that assembly of a complete sRNP complex requires the Nop56/58 NTD (Fig. 4A). The Nop56/58 NTD alone (NTD) was not able to bind the assembling complex as it lacked the CTD domain critical for Nop56/58 interaction with L7Ae. The Nop56/58 CTD (CTD) did bind L7Ae although assembly of the sRNP was incomplete as this construct lacked the NTD for fibrillarin binding. Similarly, deletion of the NTD from Nop56/58 (ΔNTD) disrupted sRNP assembly as fibrillarin was again unable to bind the assembling complex. Deletion of either the ΔCC or the highly charged ΔK-tail had no effect and allowed complete sRNP assembly. In agreement with Nop56/58-fibrillarin pulldown experiments in Fig. 2, the Nop56/58 NTD site-specific amino acid substitution and deletion mutants assembled complete sRNP complexes (Fig. 4B). Only the Δ5aa, which likely shortens the -4 helix that directly contacts fibrillarin, showed a deficiency in sRNP assembly and reduced fibrillarin binding. Thus, extensive mutagenesis of the Nop56/58 NTD did not prevent fibrillarin binding and therefore box C/D sRNP assembly.
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Subsequently, the ability of each of these sRNP complexes assembled with a mutated Nop56/58 core protein was investigated for sRNP-guided nucleotide 2′-O-methylation by measuring the incorporation of [3H]CH₃ donated from S-adenosylmethionine (AdoMet) into target RNA substrates complementary to both the D and D′ guide regions (9, 10). RNP assembly with individual NTD or CTD domains failed to guide nucleotide methylation, similar to complexes assembled with Nop56/58 missing either the NTD or coiled-coil domain. Previous work has shown that the coiled-coil domain is essential for sRNP-guided nucleotide methylation (20). Deletion of the C-terminal Nop56/58 lysine-rich tail (ΔK-tail) had no effect on either assembly or methyltransferase activity. Most amino acid and deletion mutants on the Nop56/58 interface had no effect upon methyltransferase activity, consistent with the assembly of a functional sRNP complex. However, two interface mutations did disrupt catalytic activity of the assembled complex. The single substitution K112D and the Δ5aa deletion displayed significantly reduced and abolished activity, respectively. Although reduced methylation activity might be expected for the Δ5aa deletion due to reduced fibrillarin binding and sRNP assembly, the K112D single substitution showed no defects in fibrillarin binding, heterodimer stability, or sRNP assembly.

Nop56/58 Cross-links with Target RNA Substrate—The possible interaction of the Nop56/58 core protein with the target RNA substrate was explored in UV cross-linking experiments where the target RNA possessed a site-specific 4-thiouridine (s4U) to enhance cross-linking efficiency and specificity. The s4U substitution was positioned just 5′ to the region of sRNA-target RNA complementarity and therefore not involved in sRNA:target RNA base pairing (Fig. 5A) (35). This target RNA was 5′-radiolabeled and complementary to the D guide region of the sRNA. Assembled RNP complexes were incubated in the presence of radiolabeled s4U D target RNA and after UVA irradiation (>320 nm) (23) the sRNP complexes were disrupted with SDS and the proteins and RNA were resolved by SDS-polyacrylamide gel electrophoresis. The sRNP core proteins were visualized by Coomassie staining and the radiolabeled target RNA was visualized by phosphorimaging.

Superimposition of the stained protein and RNA phosphorimage revealed the formation of a specific radiolabeled product that migrated slightly slower than Nop56/58 indicating that this core protein was cross-linked to the s4U D target RNA (Fig. 5B). Increasing irradiation time resulted in correspondingly greater amounts of the cross-linked protein-RNA product. This cross-linked complex was not observed with simple Coomassie staining presumably due to the low sensitivity of Coomassie staining and the low efficiency of the UV cross-linking reaction. Control assembly reactions not irradiated or that were irradiated with a radiolabeled but unsubstituted D target RNA did not yield this radiolabeled protein. Competition with unlabeled or unsubstituted D target RNA reduced the amount of the cross-linked protein. A radiolabeled band appeared below fibrillarin whenever the s8 box C/D sRNA and the radiolabeled D target RNA, either s4U substituted or unsubstituted, were both present in the reaction. This band migrates at the size expected for an RNA duplex composed of base-paired s88 and target RNA. Addition of D target RNA lacking an s4U substitu-
tion produced a similar band but not the cross-linked protein migrating above Nop56/58. Unreacted, radiolabeled RNA migrated near the bottom of the gel below the L7Ae core protein.

**Archaeal Nop56/58 N-Terminal Domain Exhibits a Conserved Folded Structure Despite Low Sequence Similarity Among Homologs**—Alignment of the archaeal Nop56/58 and the eukaryotic Nop56 and Nop58 core proteins revealed that the N-terminal domain exhibits the lowest degree of conservation, whereas the coiled-coil and Nop domains are highly conserved (Fig. 2 and supplemental Fig. S3). Interestingly, existing crystal structures of the archael Nop56/58 NTD from *S. sulfataricus* (*Ss*) and *P. furiosus* (*Pf*) reveal high structural similarity, even though the *Af* NTD exhibits fewer structural elements when compared with the *Ss* NTD (Fig. 2A). To determine whether low sequence but high structural conservation is a general feature of archaeal Nop56/58 NTDs, as well as to help interpret the mutagenesis experiments at a molecular level, the crystal structure of the *Mj* NTD was solved to 1.7 Å (Fig. 6 and Table 1). Initial attempts to solve the structure using the known structures of the archaeal Nop56/58 proteins (PDB codes 2NNW, 3ID5, and 1NT2)
failed due to the low sequence homology among them. However, using a selenomethionine-substituted Mj Nop56/58 protein successfully yielded a crystal whose structure was solved by single wavelength anomalous dispersion analysis. The Mj Nop56/58 N-terminal domain is composed of four \( \beta \)-sheets surrounded by four \( \alpha \)-helices (Fig. 6, A and B). Two short \( \beta \)-strands are present between the \( \beta \)-2 and \( \beta \)-3 strands and at the N-terminal end of the \( \alpha \)-3 helix.

Structural conservation analysis comparing the Mj and Ss Nop56/58 NTDs using Dali (36) yielded a high Z-score of 10 even though these two domains exhibited only 18% sequence identity. Similarly, comparison of the Mj NTD with the Pf and Af Nop NTD structures exhibiting 19 and 8% sequence identity yielded Z-scores of 8 and 3, respectively. Superimposed Ss and Mj NTD structures revealed similarly folded protein domains (Fig. 6, C and D). The C-\( \alpha \) root mean square deviation values of Mj Nop56/58 NTD with that of Ss and Pf NTD structures was 1.9 \( \text{Å} \), whereas the Af Nop NTD structure had a root mean square deviation value of 9.9 \( \text{Å} \). Superimposing the Mj Nop56/58 NTD with other known archaeal Nop56/58 NTD structures revealed a well conserved three-dimensional fold overall (Fig. 7A). Of the four structures reported thus far, the Af Nop56/58 NTD is the most divergent. The Af NTD has only 2 \( \beta \)-strands and 3 \( \alpha \)-helices and the shortest N-terminal domain with 76 amino acid residues. In comparison, the Mj NTD is 121 residues and has 4 \( \beta \)-strands, 4 \( \alpha \)-helices, and a \( 3_{10} \) helix and the Pf and Ss NTDs are 128 and 133 residues, respectively, with 5 \( \beta \)-strands and 5 \( \alpha \)-helices each (Fig. 7B). Multiple sequence alignments combined with overlay of known Nop56/58 NTD-fibrillarin structures revealed that the Nop56/58 \( \alpha \)-4 helix (\( \alpha \)-3 helix in the Af structure), which mediates fibrillarin dimerization, is well conserved across Archaea (Figs. 2B, 6, C and D, and 7). It is predicted that the structure of the Mj fibrillarin-Nop56/58 heterodimer, as well as analogous heterodimers in other archaeal organisms, should conform to this conserved interaction. These results indicate a considerable amount of similarity in the folding of the N-terminal domain despite their very low primary sequence conservation.

**DISCUSSION**

Nop56/58 is one of three essential core proteins used to assemble the archaeal box C/D RNP nucleotide methylation complex. Nop56/58 plays a bridging role in RNP assembly using its C-terminal domain to bind the L7Ae-sRNA complex and...
then its NTD to recruit the methyltransferase fibrillarin. Examination of NTD interaction with fibrillarin has revealed an exceptionally stable dimer. Mutagenesis of key amino acids on the NTD interface with fibrillarin surprisingly did not block dimer formation. However, select mutations did affect the methyltransferase activity of the fibrillarin. The cross-linking of Nop56/58 to the target RNA substrate further argued that this core protein is important for engaging the target RNA substrate for 2′-O-methylation. Determination of the *M. jannaschii* NTD crystal structure revealed that despite low sequence homology of this domain among archaeal homologs, all exhibit a well conserved folded structure. Thus, Nop56/58 mediates critical protein-protein interactions that are exceptionally stable, well conserved in the box C/D RNP scaffold, and important for both RNP assembly and methyltransferase function.

Mutagenesis of the Nop56/58 interface with fibrillarin identified two alterations that were detrimental for methyltransferase activity, whereas having little or no effect upon sRNP assembly. Both the K112D substitution and the Δ5aa are located near the center of the α-4 helix (α-3 in Af) of the Mj Nop56/58 NTD. The α-4 helix is ~14 Å from the fibrillarin-bound AdoMet molecule (Fig. 6D). Thus, the α-4 helix is too distant from the fibrillarin catalytic center to participate directly in the methyltransferase reaction.

The crystal structure of the Mj Nop56/58 NTD does predict that the Δ5aa deletion mutant (STELT) would shorten helix 4 considerably, whereas the nearby K112D mutant would not be expected to significantly affect α-4 helix structure. Neither biochemical nor crystallographic analyses have provided clear
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FIGURE 7. **The archaeal Nop56/58 NTD is a structurally conserved domain.** A, the archaeal Nop56/58 NTD possesses a conserved domain structure. Superimposed Nop56/58 NTD crystal structures of *M. jannaschii* (MJ), *S. solfataricus* (Ss), *P. furiosus* (Pf), and *A. fulgidus* (Af). B, aligned NTD sequences and secondary structure elements of archaean Nop56/58 proteins whose crystal structures have been solved. Conserved (shaded dark blue) and similar (shaded light blue) amino acids are indicated. Secondary structural elements observed in the crystallized Nop56/58 NTDs are illustrated below.

insight into how the mutated Lys\(^{112}\) or STELT amino acids might facilitate the methyltransfer reaction. Lys\(^{112}\) is a charged and highly conserved residue in both archaeal and eukaryotic Nop56/58 homologs (supplemental Fig. S3). In contrast, the nearby deleted STELT residues of the S5aa mutant are not particularly well conserved in sequence. Notably, however, both mutations either remove a positively charged amino acid (K112D) or alter the spatial positioning of a charged residue (the Lys/Arg residue C-terminal to the deleted STELT residues of S5aa) in α-4 helix. Perhaps this helix with its positively charged amino acids facilitates methyltransferase activity by interacting with the base-paired, guide-target RNA duplex, especially given its close proximity as revealed in a recent crystal structure of a fully assembled monomeric box C/D sRNP (16). The cross-linking of Nop56/58 to the target RNA substrate in the fully assembled sRNP complex is certainly consistent with this possibility.

Alternatively, the Nop56/58 NTD could mediate important but poorly understood sRNP dynamics and structural changes that take place prior to or during the methyltransfer reaction. Crystal structures have revealed that a linker between the Nop56/58 NTD and CC domain gives the NTD a high degree of flexibility (16, 17, 33). This flexibility may facilitate substrate binding and release by forming “open” and “closed” states. In the open state, fibrillarin and the Nop56/58 NTD would be distal to the guide RNA, thus allowing the substrate RNAs access to the guide sequences. In the closed state, fibrillarin would swing into close proximity to site specifically catalyze addition of a methyl group to the base-paired substrate RNA. Thus, the α-4 helix of Nop56/58 and the Nop56/58 interface with fibrillarin could play important roles in facilitating the dynamic nature of this sRNP. The K112D and S5aa deletion mutants could conceivably alter the α-4 helix structure to affect the dynamic nature of this domain and thus methyltransferase function.

The Nop56/58-fibrillarin heterodimer is a remarkably strong protein-protein interaction. This interaction was not disrupted by significant mutagenesis of the Nop56/58-fibrillarin interface, exhibits an exceptionally high melting temperature of ~113 °C, and was stable to all but the harshest of chemical treatments. Nop56/58 and fibrillarin are highly conserved proteins of both archaeal and eukaryotic organisms. Notably, however, there are two Nop56/58 homologs in eukaryotes that presumably arose from gene duplication (9, 10, 37, 38). Both Nop56 and Nop58 interact with fibrillarin in the eukaryotic box C/D snoRNP and both exhibit ~60% similarity with the *M. jannaschii* Nop56/58 protein (supplemental Fig. S3) (34). Sequence alignment of the archaeal and eukaryotic homologs reveals well conserved coiled-coil and CTD domains, both of which participate in important protein-protein interactions within the assembled RNP complex. Interestingly, the NTD is less conserved for the archaeal and eukaryotic homologs. Despite this weaker sequence conservation, crystal structures reveal a highly conserved domain structure. This is not surprising given the importance of this domain for interaction with fibrillarin and the ultimate assembly of a catalytically active box C/D RNP complex. Although crystal structures of eukaryotic Nop56 or Nop58 proteins are not yet available, it is highly probable that the N-terminal domains of both will exhibit conserved structures with respect to their archaeal homologs. These folded structures will clearly be critical for protein-protein interaction with fibrillarin and the bridging role that the Nop56 and Nop58 proteins most certainly play in eukaryotic snoRNP assembly. Our work here suggests that these interactions are not only important for assembly but also for efficient methyltransferase function as well. A better understanding of the structural dynamics of Nop56/58 within the box C/D RNP during multiple rounds of catalysis and the mechanistic details of how the NTD influences nucleotide methylation activity awaits further investigation.

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