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Structure of H/ACA RNP protein Nhp2p reveals *cis/trans* isomerization of a conserved proline at the RNA and Nop10 binding interface

Bon-Kyung Koo^{1,2}, Chin-Ju Park^{1,2,§}, Cesar F. Fernandez¹, Nicholas Chim^{1,†}, Yi Ding^{1,‡}, Guillaume Chanfreau¹, and Juli Feigon^{1,*}

¹Department of Chemistry and Biochemistry, and the Molecular Biology Institute, PO Box 951569, University of California, Los Angeles, CA 90095-1569, USA

Abstract

H/ACA small nucleolar and Cajal body ribonucleoproteins (RNPs) function in site-specific pseudouridylation of eukaryotic rRNA and snRNA, rRNA processing, and vertebrate telomerase biogenesis. Nhp2, one of four essential protein components of eukaryotic H/ACA RNPs, forms a core trimer with the pseudouridylylase Cbf5 and Nop10 that specifically binds to H/ACA RNAs. Crystal structures of archaeal H/ACA RNPs have revealed how the protein components interact with each other and with the H/ACA RNA. However, in place of Nhp2p, archaeal H/ACA RNPs contain L7Ae, which binds specifically to an RNA K-loop motif absent in eukaryotic H/ACA RNPs, while Nhp2 binds a broader range of RNA structures. We report solution NMR studies of *S. cerevisiae* Nhp2 (Nhp2p), which reveal that Nhp2p exhibits two major conformations in solution due to *cis/trans* isomerization of the evolutionarily conserved Pro83. The equivalent proline is in the *cis* conformation in all reported structures of L7Ae and other homologous proteins. Nhp2p has the expected α - β - α fold, but the solution structures of the major conformation of Nhp2p with *trans* Pro83 and of Nhp2p-S82W with *cis* Pro83 reveal that Pro83 *cis/trans* isomerization affects the positions of numerous residues at the Nop10- and RNA-binding interface. An S82W substitution, which stabilizes the *cis* conformation, also stabilizes the association of Nhp2p with H/ACA snoRNPs *in vivo*. We propose that Pro83 plays a key role in the assembly of the eukaryotic H/ACA RNP, with the *cis* conformation locking in a stable Cbf5-Nop10-Nhp2 ternary complex and positioning the protein backbone to interact with the H/ACA RNA.

Keywords

Nhp2; L7Ae; NMR; snoRNP; scaRNP

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*Corresponding Author: Juli Feigon, Tel:1-310-206-6922, Fax:1-310-825-0982, feigon@mbi.ucla.edu.

²These authors contributed equally to this work

[§]Current address: Gwangju Institute of Science and Technology, 261 Cheomdan-gwagiro, Buk-gu, Gwangju 500-712 Republic of Korea

[†]Current address: Department of Molecular Biology and Biochemistry, 2212 Natural Sciences 1, University of California, Irvine, CA 92697

[‡]Current address: Galaxy Biotech, LLC, 1230 Bordeaux Dr., Sunnyvale, CA 94089.

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INTRODUCTION

Most H/ACA small nucleolar and small Cajal body ribonucleoproteins (snoRNPs and scaRNPs) function in the pseudouridylation of ribosomal and spliceosomal small nuclear RNAs, respectively, in eukarya and archaea.¹ Each H/ACA RNP consists of an H/ACA RNA and four evolutionary conserved proteins, Nhp2 (L7Ae in archaea),² Cbf5 (called Dyskerin³ or Nap57⁴ in mammals), Nop10, and Gar1. In most eukaryotes, each H/ACA RNA has two hairpins followed respectively by single stranded regions containing a conserved H-Box (ANANNA) and ACA-box (ACA).^{5,6} In archaea, the number of hairpins varies from 1 to 3, but is most often 1.^{7–10} Substrate RNAs bind to internal loops in the H/ACA hairpins, which are specific for each substrate, through base pairing^{11,12} with the target U and the adjacent 3' nucleotide unpaired at the top of the internal loop.^{13,14} Cbf5 functions as the pseudouridylase catalytic subunit, and the other three proteins are essential for optimal activity, stability, maturation, and nucleolar localization of the H/ACA RNPs.^{15–17} L7Ae does not interact with other H/ACA RNP proteins in the absence of the H/ACA RNA,^{18,19} while Nhp2p interacts directly with Cbf5 and Nop10 to form a ternary complex prior to binding H/ACA RNA.²⁰ The 3' half of human telomerase RNA (hTR) is an H/ACA scaRNA that is bound by the H/ACA proteins.^{21–24} Mutations in the human H/ACA RNP proteins Dyskerin, Nop10, and Nhp2 have been found in the genes of some patients with the bone marrow failure syndrome dyskeratosis congenita (DC).^{3,25–27}

Reconstitution *in vitro* and crystal structures of archaeal H/ACA protein complexes with and without RNA have provided a general structural framework of the molecular interactions in H/ACA RNPs.^{18,28–31} Cbf5 interacts with Nop10, a small protein that is largely unstructured in isolation,^{13,32} which forms an interface between L7Ae and Cbf5 to form an RNP complex that can be assembled independently of Gar1.²⁹ Gar1 binds to Cbf5 without contacts to the other proteins. The crystal structure of H/ACA RNPs from the archaeon *P. furiosus* (PDB ID: 2HVV), which contains all four proteins with a guide RNA, revealed that interactions between the H/ACA guide RNA and Cbf5, Nop10, and L7Ae occur at the two ends of the guide RNA.³³ L7Ae interacts with the upper stem-loop of the H/ACA RNA. In archaea, the upper stem-loop contains an RNA motif called a K-turn or K-loop,³⁴ with stacked A-G and G-A base pairs and a flipped out U, which interact specifically with L7Ae. At the opposite end, the 3' ACA tail interacts with the PUA domain of Cbf5. Comparison with the crystal structure of substrate RNA-bound H/ACA RNPs without L7Ae (PDB ID: 2RFK) and fluorescence spectroscopy revealed that L7Ae plays a key role in re-orienting the substrate RNA bound to the H/ACA guide pocket for catalysis.³⁵

To date, no structures of known eukaryotic H/ACA RNP proteins except for Nop10³⁶ have been reported. Eukaryotic H/ACA RNAs do not contain the conserved K-loop found in archaeal H/ACA RNPs, and Nhp2 interacts with irregular stem-loop structures without any apparent specificity for the K-loop motif.³⁷ Unlike L7Ae, in the absence of the other proteins Nhp2 binds non-specifically to RNA stem-loops *in vitro*, and the binding specificity for H/ACA RNAs appears to be conferred by the Cbf5-Nop10-Nhp2 core trimer, which forms prior to assembly with the RNA.^{20,37,38} Nevertheless, Nhp2 has a high level of homology with K-loop or K-turn binding proteins such as L7Ae, Snu13p, and 15.5kD protein, the human homologue of Snu13p.^{39,40} This raises an interesting paradox on the relationship between structure and function of Nhp2, since it bears sequence similarities with these proteins but appears to recognize a broader range of RNA structures.

Here we have investigated the structure of *S. cerevisiae* Nhp2 (Nhp2p) by solution NMR spectroscopy. Nhp2p adopts the α - β - α fold found in L7Ae and other homologous proteins. Surprisingly, however, the conserved Pro83 exhibits *cis/trans* isomerization. We report the structures of the major conformations of Nhp2p-WT (Pro83 *trans*) and Nhp2p-S82W (Pro83

cis), which show that Pro83 *cis/trans* isomerization results in conformational changes on the putative Nop10 and H/ACA RNA binding surface. The S82W mutation that stabilizes the Pro83 *cis* conformation also stabilizes the interaction of Nhp2p with H/ACA snoRNPs *in vivo*. In archaeal H/ACA RNPs, the homologous proline to Pro83 is part of a conserved “proline spine” that traverses the RNP from L7Ae through Nop10 to the active site of Cbf5,⁴¹ and interacts with both Nop10 and the flipped out U in the H/ACA RNA K-loop. We propose that Pro83 likely plays a similar role in Nhp2p, positioning the backbone of residue 82 to interact with the RNA, in spite of the absence of the K-loop motif. The *cis/trans* isomerization leading to flexibility at the RNA- and protein-binding interface may assure pre-assembly of the core Cbf5-Nop10-Nhp2p trimer prior to H/ACA RNA binding.

RESULTS

Nhp2p-WT has multiple conformations in solution due to proline *cis/trans* isomerization

Nhp2p contains an additional N-terminal extension (~14–23 residues) compared to other homologous 15.5kD family proteins. The full length Nhp2p behaved as a dimer in gel filtration chromatography and showed poor quality ¹H-¹⁵N HSQC spectra (data not shown). A version of Nhp2p containing a deletion of the first 23 residues (Nhp2p-24–156) was monomeric at concentrations used for NMR studies (~0.8mM). We hereby refer to this N-terminally truncated version of Nhp2p as Nhp2p-WT. An ¹H-¹⁵N HSQC spectrum of Nhp2p-WT is shown in Figure 1A. Although the cross peaks are for the most part well dispersed, indicative of a folded protein, many residues gave rise to two cross peaks. The backbone assignment of Nhp2p-WT revealed that ~36 of the amide signals are duplicated, indicating the presence of (at least) two conformations in slow exchange. We hypothesized that the two sets of peaks might arise from propagation of structural changes due to proline *cis/trans* isomerization. To test this hypothesis and identify the source of the structural heterogeneity, we generated six single proline-to-alanine substitutions in Nhp2p separately (P83A, P91A, P100A, P105A, P119A, and P127A, which includes all but the 3 prolines in the N-terminal region). Of these, P83, P91, and P100 are completely conserved in the Nhp2 orthologs 15.5kD, snu13p and L7Ae (Figure 2), as well as Nhp2 from other organisms. The ¹H-¹⁵N HSQC spectra of the Nhp2p proteins containing mutations of the less conserved prolines (Nhp2p-P105A, Nhp2p-P119A and Nhp2p-P127A), all had doubled peaks similar to Nhp2p-WT (Figure S1), indicating that two (or more) conformations were present in these mutants as well. The ¹H-¹⁵N HSQC spectra of Nhp2p-P91A and Nhp2p-P100A (Figure S1) all showed poor chemical shift dispersion and line broadening, indicating that the alanine substitutions in these proteins disrupted proper protein folding. Only Nhp2p-P83A gave a well-dispersed ¹H-¹⁵N HSQC with about one-third the number of doubled peaks (Figure 1B). Subsequent sequential assignment indicated that the remaining doubled cross peaks corresponded to residues near Pro33 and Pro105. These results indicate that the main source of the conformational heterogeneity in Nhp2p-WT is the presence of *cis/trans* isomerization of Pro83. This gives rise to two major conformations which are present at a ratio of about 60:40, based on integration of the ¹H-¹⁵N HSQC cross peak volumes (Figure 1C). The two non-conserved prolines, Pro33 and Pro105, which also show *cis/trans* isomerization have a more local effect on the structure.

Comparison of Nhp2p-WT and Nhp2p-P83A

Due to the presence of nine prolines in Nhp2p-WT (24–156) and the increased spectral overlap caused by the duplicated peaks, it was highly challenging to obtain the backbone assignment of Nhp2p-WT. Therefore, we initially tried to determine the structure of Nhp2p-P83A, assuming it would represent the conformation of Nhp2p-WT with Pro83 in the *trans* orientation. However, although the Pro83 to Ala mutation resulted in a unique conformation for most residues of the protein, it also induced the loss of NH resonances near the mutated

residue from Ala84 to Ile90. Based on sequence alignment with homologous proteins and preliminary structures of Nhp2p-P83A, these residues correspond to the N-terminal half of helix α_3 , which was consequently not well defined. In contrast to the case for Nhp2p-P83A, amide resonances for Asp85 to Ser88 were present in spectra of Nhp2p-WT. Since further analysis indicated that substitution of Pro83 with Ala partially destabilized helix α_3 , we proceeded to assign the resonances from the major conformation of Nhp2p-WT for structure determination. Resonance assignments were greatly aided by comparison of chemical shifts and assignments of Nhp2p-P83A. The other missing amides in the spectra of Nhp2p-P83A were also absent in the spectrum of Nhp2p-WT. Ala84 was assigned from the spectra of Nhp2p-S82W as discussed below. Backbone assignments were ~90% completed, and nearly complete side-chain assignments were obtained for those residues.

The major conformation of Pro83 is *trans* in Nhp2p-WT and *cis* in Nhp2p-S82W

Based on comparison with the spectra from Nhp2p-P83A, it appeared that the major conformation of Nhp2p-WT had Pro83 in the *trans* conformation. A BLAST⁴² search of the PDB database and a DALI⁴³ server search predicts that Nhp2p is structurally most similar to *H. sapiens* 15.5 kD (human homolog of Snul3p), followed by *S. cerevisiae* Snul3p, the putative *C. parvum* Nhp2, and *P. furiosus* L7Ae. Sequence homology for these proteins with Nhp2p is 63%, 58%, 74%, and 68%, respectively (Figure 2A). The residues that are equivalent to Nhp2p Pro83 have a *cis* conformation in the solution structure of 15.5kD (2JNB)⁴⁴ and the crystal structures of Snul3p (2ALE),⁴⁵ L7Ae (1PXW, 1XBI),^{46,47} L7Ae in complex with K-loop RNA (1SDS),⁴⁸ 15.5kD in complex with RNA (1E7K),³⁹ and L7Ae in various H/ACA RNP complexes with RNA (2HVY, 3HJW, 3HAX).^{28,29,33} We therefore wanted to confirm that the major conformation of Nhp2p-WT had a *trans* Pro83 and to determine the structure of Nhp2p with Pro83 in the *cis* conformation. Since the identity of the residue preceding a Pro can influence the conformation of the peptide bond,^{49–52} we investigated the effect of the S82E and S82W amino acid substitutions. These substitutions were chosen because homologous proteins have an acidic residue (Glu or Asp) at position 82, and Trp has been shown to increase the ratio of *cis:trans* proline conformers in α -hemoglobin stabilizing protein.⁵⁰ ¹H-¹⁵N HSQC spectra of Nhp2p-S82E showed more doubled peaks than Nhp2p-WT. Thus, the Glu substitution does not induce Nhp2p to form a single conformation with *cis* Pro83 as seen in structures of homologous proteins. The ¹H-¹⁵N HSQC spectra of Nhp2p-S82W showed a well-dispersed peak pattern with the same number of doubled peaks as Nhp2p-WT. However, the ratio of the cross peak volumes for almost all the doubled peaks changed from 60:40 to 40:60 (Figure 1C,D). This indicates that the minor conformation in Nhp2p-WT is the major conformation in Nhp2p-S82W.

The spectra of Nhp2p-S82W also allowed us to assign Ala84, whose amide was missing in Nhp2p-WT, and therefore confirmed that the major conformation of Pro83 is *trans* in Nhp2p-WT and *cis* in Nhp2p-S82W. The chemical shift difference $\delta(^{13}\text{C}\beta) - \delta(^{13}\text{C}\gamma)$ for proline is diagnostic for the conformation of the preceding peptide bond, with average values of 4.5 ± 1.2 and 9.6 ± 1.3 ppm observed for *trans* and *cis* isomers, respectively.^{50,53,54} The chemical shift difference $\delta(^{13}\text{C}\beta) - \delta(^{13}\text{C}\gamma)$ for the major conformation of Pro83 in Nhp2-WT was 5.34 ppm, indicating a *trans* Ser82-Pro83 conformation, and 8.11 ppm for Nhp2-S82W, indicating a *cis* peptidyl conformation. Further evidence for the proline conformations was obtained from ¹³C-edited NOESY spectra, which show a strong $\alpha\text{H}_{n-1} - \alpha\text{H}_n(\text{Pro})$ NOE cross peak for the *cis* conformation and a weak or no visible NOE cross peak for the *trans* conformation.

We were unable to unambiguously determine the peptide bond conformation for Pro119, since we could not assign the Pro119 ¹³C β and ¹³C γ chemical shifts or the H α resonance of Arg118. The chemical shift analyses of ¹³C β and ¹³C γ show that the conformation of all

other prolines is *trans* except for Pro83, Pro33, and Pro105, which show a mixture of *cis* and *trans* as discussed above.

Solution structures of Nhp2p with the *cis* and *trans* conformation of Pro83

The structure calculations for Nhp2p-WT and Nhp2p-S82W (residues 36–156) were performed using 915 and 1001 interproton distance restraints, respectively, and 177 and 178 dihedral angle restraints, respectively (Table 1). The N-terminal unstructured part of the construct (Met24 to Ala35, 12 a.a.) was not included in the structure calculations due to lack of restraints. For the residues with two sets of peaks, indicative of two conformations, we used restraints associated with the larger populated state only, which corresponded to the *trans* conformation of Pro83 in Nhp2p-WT and the *cis* conformation of Pro83 in Nhp2p-S82W. Due to the spectral overlap from the doubling of many resonances, the backbone and side chain assignments were only about 90% complete for both proteins. Superposition of the 20 lowest energy structures for Nhp2p-WT and Nhp2p-S82W for residues 36–156 are shown in Figure 2B,C. While most of the secondary structure of Nhp2p-WT and Nhp2p-S82W is well defined (Table 1) except for the beginning of $\alpha 3$, the $\beta 4$ - $\alpha 5$ loop (residues 126–138) and $\alpha 4$ - $\beta 4$ loop (residues 112–118) are less well determined.

The protein structure for both Nhp2p-WT (*trans* Pro83) and Nhp2p-S82W (*cis* Pro83) is composed of four β -strands and five α -helices organized in a $\alpha\beta\alpha\beta\alpha\beta\alpha$ order (Figure 2). It is a three-layered α - β - α sandwich, as seen in the homologous proteins, with one layer comprising α -helices 4, 1, and 5, the middle layer comprising a four-stranded β -sheet with 1($\uparrow$), 4($\downarrow$), 2($\uparrow$), and 3($\uparrow$) order, and the other layer comprising α -helices 2 and 3, which are parallel with the β -strands. Based on the sequence alignment with homologous proteins (Figure 2A) Nhp2p has a relatively longer amino acid sequences between $\beta 4$ and $\alpha 5$ than the homologous proteins, and in the structure this region contains a short helical element (N130 to K133) in the middle. However, the location of $\alpha 5$ is very similar to that of the homologous proteins (see Figure 5).

In the structure of Nhp2p-WT, the Ser82-Pro83 αH distance is 4.89 Å, while in Nhp2p-S82W the equivalent αH distance is 2.31 Å. For comparison, the Glu61-Pro62 αH distance in 15.5kD is 2.5 Å and 1.02 Å in the free and RNA bound structures, respectively. Analysis of the 1H - ^{15}N HSQC spectra of Nhp2p-WT and Nhp2p-P83A shows that Pro83 *cis/trans* isomerization affects the chemical shifts of many residues on helix $\alpha 2$, $\alpha 3$, $\alpha 4$, and the β -sheet (Figure 3A). Remarkably, the residues that have doubled peaks due to *cis/trans* isomerization of Pro83 are localized to the face of the protein that interacts with Nop10 and H/ACA RNA in the homologous L7Ae complexes^{28,29} (Figure 3A,B). There is a strong correspondence between the doubled peaks on helix $\alpha 3$ and helix $\alpha 2$ and the residues that would be expected to interact with Nop10 and RNA, respectively. Pro83 is in the $\beta 2$ - $\alpha 3$ turn and caps the N-terminal end of helix $\alpha 3$. Superposition of Nhp2p-WT and Nhp2p-S82W shows that changing the prolyl peptide bond from *trans* to *cis* has a significant effect on the position of helix $\alpha 3$, which moves closer to helix $\alpha 2$ (Figure 3C,D). The distance between $\alpha 2$ and $\alpha 3$ is ~9.5 Å in Nhp2p-WT (*trans* Pro83) and ~8.0 Å for Nhp2p-S82W (*cis* Pro83). These two helices are separated by ~7.0 Å in L7Ae in the H/ACA RNP.³³ Helix $\alpha 3$ of Nhp2p-WT is also angled up ~10° relative to the position of $\alpha 3$ of Nhp2p-S82W. The positions of the *trans* and *cis* proline side chains are substantially different, with *cis* Pro83 much closer to helix $\alpha 2$, and the backbones of the preceding residue 82 point in opposite directions (Figure 3D). Of the two other prolines that also show *cis/trans* isomerisation, Pro105 is in the loop between $\beta 3$ and $\alpha 4$, and is on the opposite side of the protein from Pro83 (Figure 2D,E), and the *cis/trans* isomerization has only small local effects on the structure. Pro33 is in the disordered N-terminus, which is not shown in the final structures.

Although the NMR (and CD) spectra indicate that Nhp2p-WT, Nhp2p-P83A, and Nhp2p-S82W all form folded structures, CD spectra (Figure S2) showed that Nhp2p-P83A is much more stable and Nhp2p-S82W is somewhat less stable than Nhp2p-WT. These results indicate that even though the absence of the Pro83 in Nhp2p-P83A partially destabilizes helix α_3 , overall the *trans* conformation at position 83 results in a more stable protein fold.

The S82W substitution stabilizes the association of Nhp2p with H/ACA snoRNPs, while the Nhp2p-P83A mutant shows reduced interaction with H/ACA snoRNPs

We hypothesized that adoption of the *cis* conformation is important for Nhp2p function and H/ACA RNP formation *in vivo*, since L7Ae as well as other homologous proteins all have a *cis* proline at the same position. To test this hypothesis, we constructed plasmids expressing full-length FLAG-tagged versions of Nhp2p, either wild-type or with the S82W or P83A mutations. These mutations were chosen because Nhp2p-S82W has a higher proportion of *cis* Pro83 (60%) than Nhp2p-WT, while position 83 in Nhp2p-P83A is locked in the *trans* conformation. The plasmids were transformed into a wild-type strain and the genomic copy of *NHP2* was deleted, such that the only sources of cellular Nhp2p were the plasmid borne versions.

The strains expressing full-length FLAG-tagged Nhp2p, Nhp2p-P83A, and Nhp2p-S82W all showed comparable growth rates and levels of Nhp2p expression (Figure S3), showing that these FLAG-tagged versions of Nhp2p are functional, and that the mutations do not grossly affect Nhp2p function or expression *in vivo*. However, significant differences were observed when we investigated the stability of the interaction of Nhp2p mutants with H/ACA snoRNPs *in vivo*. As Nhp2p is a stable core component of yeast H/ACA snoRNPs, we assessed the association of the S82W and P83A mutants with H/ACA snoRNPs by monitoring their interaction with H/ACA snoRNAs in co-immunoprecipitation experiments. We used anti-FLAG antibodies to pull-down FLAG-tagged Nhp2p wild-type, -S82W, or -P83A and assessed the level of Nhp2-associated snoRNAs by Northern blot (Figure 4A). Immunoprecipitation experiments were performed at either physiological salt concentration (150 mM NaCl), or at higher, more stringent salt concentration 500 mM NaCl. As H/ACA snoRNP complexes are stable, we found that Nhp2p is capable of interaction with H/ACA snoRNAs at 500 mM NaCl (Figure 4A), consistent with previous reports.^{17,55} No immunoprecipitation of the U1 snRNA was observed in any of these conditions (Figure 4A), confirming the specificity of these co-immunoprecipitations studies. Only small differences in the level of snoRNA pulled down with Nhp2p, Nhp2p-P83A, and Nhp2p-S82W were observed in low salt conditions (Figure 4A). However under more stringent high salt conditions, Nhp2p-S82W showed a higher level of association than the wild-type version with snoRNAs, especially for snR37 and snR42 and to a lesser extent snR32. In contrast, at this salt concentration Nhp2p-P83A exhibited an almost complete loss of interaction with all snoRNAs. The stronger association of S82W with some specific snoRNAs should be considered in light of the observation that Nhp2p-S82W is somewhat less stable than Nhp2p-WT (see above). These results correlate well with the level of *cis* peptide bond conformation between residues 82 and 83 in the different Nhp2p versions.

To confirm that the RNA pull-downs accurately reflect the association of Nhp2p with the intact H/ACA snoRNPs and not just with the H/ACA snoRNAs, we assessed the interaction of these various versions of Nhp2p with Cbf5p. We performed a second set of co-precipitation experiments using wild-type and mutant Nhp2p constructs expressed in a strain containing a TAP-tagged version of Cbf5p. After pull down with Calmodulin-Sepharose beads to precipitate the TAP-tagged Cbf5p, we monitored the association of wild-type and mutant versions of Nhp2p by western blot using anti-FLAG antibodies. While the amount of Cbf5p precipitated was similar for all constructs (Figure 4B), we found that the S82W version of Nhp2p associated more robustly with Cbf5p than the wild-type Nhp2p, especially

at more stringent salt conditions (Figure 4B). This result shows that despite the variability of association with individual snoRNAs observed in Figure 4A, Nhp2-S82W is more strongly associated with the bulk of H/ACA snoRNPs than the wild-type version. In contrast the P83A mutant showed a much-reduced association with Cbf5p in these conditions (Figure 4B, which is consistent with the results observed in the RNA co-immunoprecipitation experiments. In conclusion, we found that increasing the conformation of Pro83 to *cis* using the S82W mutation stabilizes the association of Nhp2p with most H/ACA RNPs, while the P83A mutation strongly decreases its association with the RNPs. We cannot rule out that the deletion of the conserved Pro83 in Nhp2p-P83A, rather than the obligate *trans* conformation induced by the alanine substitution, is responsible for the decreased association with H/ACA RNPs. Nevertheless, the results with Nhp2p-S82W strongly support the hypothesis that the *cis* conformation of Pro83 of Nhp2p strengthens the association of Nhp2p with the other components of the H/ACA RNPs *in vivo*.

DISCUSSION

Comparison of the structure of Nhp2p to functional orthologues

Box C/D and H/ACA RNPs function in site-specific 2'O methylation and pseudouridylation of ribosomal and snRNAs.^{31,56,57} The archaeal L7Ae, *S. cerevisiae* Snu13p, and *H. sapiens* 15.5 kD proteins bind specifically to K-turn motifs present in box C/D RNAs.^{38,39,48,58–60} L7Ae also has specificity for the related K-loop motif found in archaeal H/ACA RNAs.^{18,30,34,61} In eukaryotic H/ACA RNPs, which do not contain a K-loop motif, L7Ae is replaced by Nhp2. In contrast to L7Ae, which binds specifically to K-loop or K-turn RNA in the absence of other box C/D or box H/ACA proteins, Nhp2p binds non-specifically to irregular stem-loops *in vitro*.³⁷ *In vivo*, Nhp2p assembles with Nop10p and Cbf5 (the pseudouridylase), and the ternary complex confers specificity for the H/ACA RNA.²⁰

While the structures of archaeal H/ACA RNPs, proteins and RNA complexes have provided detailed insight into their architecture and functional roles,⁴¹ there is little structural information on eukaryotic H/ACA proteins. Comparison of the structures of Nhp2p and of the homologous proteins 15.5 kD, Snu13p, L7Ae, and the putative *C. parvum* Nhp2 in the free state (Figure 5A) shows that, on the RNA and protein binding surface, all these proteins have a similar helix $\alpha 2$ length but there are differences in the length and structure of helix $\alpha 3$. The N-terminal end of helix $\alpha 3$ has a kink or bend in Nhp2p-S82W, Snu13p, *C. parvum* putative Nhp2, and L7Ae and the helix is shorter in 15.5 kD. The putative Nhp2 from *C. parvum*, identified by structural genomics,⁶² has a structure and sequence more similar to L7Ae than Nhp2p. Nhp2p has a higher pI (9.73) than the homologous proteins (Snu13p, L7Ae, *C. parvum* putative Nhp2p, and 15.5kD have pIs of 6.57, 5.35, 5.85, and 8.36, respectively) and the binding surface is more positively charged (Figure 5B). Comparison of the electrostatic potential surface of Nhp2p reveals that the protein-binding region (primarily helix $\alpha 3$) is less negatively charged and the RNA binding region (primarily helix $\alpha 2$) is more positively charged than the homologous proteins. The more positively charged RNA binding region on Nhp2p may facilitate stronger electrostatic interactions to compensate for lower specificity of interaction with a broader range of RNA substrates that do not have a K-loop or K-turn.

Our structure also provides insights into the effects of two human Nhp2 mutations associated with dyskeratosis congenita, V126M and Y139H, which have been shown to affect RNP assembly.⁶³ Analysis of the structure shows that the equivalent residues, V122 and F146 in Nhp2p, are at the beginning of $\beta 4$ and within $\alpha 5$, respectively. F146 is in the hydrophobic core, and the human mutation to histidine would be expected to destabilize the protein. V122 is in the RNA binding region and is the first residue in $\beta 4$ of Nhp2p, so a mutation to methionine could destabilize both the hydrophobic core and RNA binding.

Conformational flexibility at the H/ACA RNA and Nop10 binding interface

The structural studies of Nhp2p presented here revealed that three prolines, Pro33, Pro105, and the universally conserved Pro83, are undergoing *cis/trans* isomerization, leading to conformational flexibility. Pro33 is in the flexible N-terminus of the protein, such that *cis/trans* isomerization of this proline has little effect on the folded structure. Pro105 is at the beginning of the β 3- α 4 loop. It is localized on the opposite surface of the protein from the Nop10- and RNA-binding surface (Figure 2), and also has only a local effect on the structure. In contrast, the *cis/trans* isomerization of the conserved Pro83 results in significant chemical shift differences for residues on the protein and RNA binding interface, and a large change in the positions of the proline and adjacent loop residues and of helix α 3 relative to helix α 2 (Figure 3 and 6), as discussed further below.

Among Nhp2p orthologues studied to date, only Nhp2p has been found to show *cis/trans* isomerization of the universally conserved Pro83. Of these proteins, only the structure of 15.5kD protein has been solved in solution by NMR. Published ^1H - ^{15}N HSQC spectra of 15.5kD⁴⁴ and Snu13⁶⁴ clearly show no indication of *cis/trans* isomerization of the residue equivalent to Pro83. Nevertheless, the NMR study of 15.5kD protein also showed that helix α 3, which interacts with the 61kD protein, is conformationally dynamic, and the flexibility of α 3 was suggested to be an important attribute of the protein interaction interface.⁴⁴ As discussed above, helix α 3 is less well defined and much more variable in length and structure than helix α 2 (Figure 5) among the homologous proteins, perhaps indicating variability in the intrinsic flexibility of the free proteins, with L7Ae being the most rigid and Nhp2p being the most flexible. Many eukaryotic proteins adopt multiple conformations in the absence of binding partners.^{65,66} In the case of Nhp2p, interconversion between the major conformations due to Pro83 *cis/trans* isomerization may drive the formation of the ternary Cbf5-Nop10-Nhp2 complex prior to assembly with the H/ACA RNA. This in turn assures that Nhp2p binds specifically to H/ACA RNAs rather than non-specifically to any RNA stem-loop.

The *cis* conformation of Pro83 plays a key role at the H/ACA RNA and Nop10 binding interface

Figure 6 illustrates the positions of the side chains on the putative RNA and Nop10 binding surface, which are localized primarily on the helix α 2 and helix α 3 surface, of Nhp2p-WT (*trans*) and Nhp2p-S82W (*cis*) compared with L7Ae in the archaeal H/ACA RNP (PDB ID: 2HVY). In Nhp2p-WT with *trans* Pro83, helix α 3 is positioned ~9.5 Å from helix α 2, while in Nhp2p-S82W, with *cis* Pro83, helix α 3 is positioned ~8.0 Å from helix α 2. This shifts the side chains of the putative Nop10 binding residues H89, L93 (α 3), and V64 (α 2) closer together to form a more compact binding surface, as observed for the equivalent residues H66, L70, and T41 in L7Ae. The repositioning of helix α 3 relative to helix α 2 also puts the RNA binding residues, which are on helix α 2 and the α 4- β 4 loop, closer to the Nop10 binding surface.

L7Ae interacts with the archaeal RNAs at the K-loop motif, with additional contacts to some phosphates in the stem below the A-G base pairs. The K-loop motif contains tandem G-A, A-G base pairs at the top of a stem-loop with a conserved G/A-N-G/U sequence on the 3' side of the loop.³⁴ These stem-loops are related by sequence and structure to the K-turn, which consists of two RNA stems separated by a short asymmetric loop with a characteristic sharp bend (kink) between the two stems, one with tandem A-G base pairs, the other with Watson-Crick pairs.⁶⁷ The residues that have side-chain or backbone hydrogen bond or electrostatic interactions with the RNA K-loop and stem are R34, K35, N38, E39, K42, R46, D59, A96, A98 in L7Ae,³³ which correspond to K57, R58, K61, E62, K65, K69, S82, S121, and V123 in Nhp2p. The lysine and arginine residues are conserved, and the other residues

hydrogen bond via their backbone atoms. Significantly, as discussed in the following, the *cis* conformation of Pro83 changes the position of residue 82 so that its backbone amide and carbonyl point toward instead of away from the putative RNA binding surface on helix $\alpha 2$ (Figure 3D).

In the archaeal *P. furiosus* H/ACA RNP, a series of prolines in Cbf5, Nop10, and L7Ae stack on each other to form a proline spine (Figure 6D),⁴¹ which connects the flipped out nucleotides at the active site to the functionally important L7Ae. The universally conserved Pro83 corresponds to L7Ae Pro60, the last proline in the proline spine. Pro60 interacts with Pro33 of Nop10 and stacks over the flipped out U of the K-loop. In addition, the *cis* conformation of Pro60 positions the peptide backbone NH and C=O of Asp59 to hydrogen bond to the C2 carbonyl and N3 imino of the flipped out U (Figure 6D). The amino acid at position 59 (Ser82 in Nhp2p) is not conserved, but since the hydrogen bonds with the flipped out uracil are from the peptide backbone, the identity of the side chain at this position is not important. In fact, in the archaeal *M. jannaschii* L7Ae the equivalent residue is a lysine and makes identical interactions with the RNA base. The same contacts are seen in the complexes of the homologous proteins 15.5 kD and Snu13 with K-turns in box C/D snoRNAs.

The Nhp2p-S82W substitution stabilized the binding to H/ACA RNPs, consistent with our proposal that the stabilization is due to the *cis* conformation of Pro83, while Nhp2p-P83A reduces binding to the RNP. Remarkably, the *cis* Pro83 and Ser82 are positioned to interact with Nop10 and the RNA in the same way as for L7Ae. However, eukaryotic H/ACA RNPs do not have a K-turn or K-loop motif. Since the universally conserved proline (at position 83 in Nhp2p) and the preceding residue appear to have a conserved structure and interaction with a flipped out U among a diverse set of homologous proteins, we examined the secondary structure of H/ACA RNAs in *S. cerevisiae* for which substrates have been identified (<http://yeastgenome.org> and <http://people.biochem.umass.edu/fournierlab/snornadb/main.php>).⁶⁸ Approximately 73% (27 of 37) have a U, ten or eleven base pairs from the top of the pseudouridylation pocket at or near a loop, that could potentially bulge out to hydrogen bond with the Ser82 backbone. In the K-loop found in archaeal H/ACA RNAs, the universally conserved bulge U is ten base pairs from the top of the pseudouridylation pocket, counting the two G•A base pairs. We hypothesize that Pro83 in Nhp2p serves the same function as Pro60 in L7Ae, adopting the *cis* conformation in the complex with Nop10-Cbf5, which correctly positions Pro83 next to Pro33 of Nop10 and the backbone of the Ser82 to hydrogen bond with a bulged out U on the upper stem-loop of the H/ACA RNA. We conclude that Nhp2p Pro83 may play a key role in the assembly of the eukaryotic H/ACA RNP, with the *cis* conformation locking in a stable Cbf5-Nop10-Nhp2 ternary complex and positioning the protein backbone to interact with the H/ACA RNA, thus stabilizing the overall structure of H/ACA RNPs.

MATERIALS AND METHODS

Protein Purification for Structural Studies

The gene encoding *S. cerevisiae* Nhp2p-WT(Nhp2p₂₄₋₁₅₆) was cloned into the pET24a vector as a C-terminal histidine-tagged fusion (Novagen), and the construct was used to transform *E. coli* strain BL21(DE3) gold cells (Stratagene). Uniformly ¹⁵N- and ¹⁵N/¹³C labeled proteins were obtained by growing the transformed *E. coli* cells in M9-minimal media containing ¹⁵NH₄Cl (Cambridge Isotopes Inc.) and unlabeled/¹³C₆-labeled-D-glucose (Cambridge Isotope Inc.) as the sole nitrogen and carbon sources, respectively. Selectively ¹⁵N-Ile-labeled Nhp2p-S82W was prepared by incorporating 98% [¹⁵N]Ile in growth medium containing a mixture of the other unlabeled amino acids. A final concentration of 0.2 % (v/v) polyethylenimine applied to the crude lysate largely removed

nucleic acid without precipitating the proteins. The proteins were purified by FPLC column chromatography with a Ni-NTA affinity column (GE Healthcare) followed by Resource S ion exchange and Superdex-75 (GE Healthcare) gel filtration. The purity and homogeneity of all samples were confirmed by SDS-PAGE.

Site-directed mutagenesis for six single P→A mutants (P83A, P91A, P100A, P105A, P119A and P127A), S→W mutant (S82W) and S→E mutant (S82E) was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. NMR samples for the Nhp2p-P83A and Nhp2p-S82W were prepared with the same purification procedure of the wild-type protein (Nhp2p-WT).

NMR spectroscopy and solution structure calculations

All spectra for assignments and structure determination were obtained on Bruker DRX 600 and 800 MHz spectrometers at 293 K (Nhp2p-P83A) and 298 K (Nhp2p-WT and Nhp2p-S82W). Backbone assignments of Nhp2p-WT and Nhp2p-S82W were obtained following standard procedures⁶⁹ from CBCANH, HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO using uniformly ¹⁵N, ¹³C-labeled proteins in a 90% H₂O/10% D₂O solution containing 20 mM HEPES-KOH, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT. The same series of experiments were done for Nhp2p-P83A in a 90% H₂O/10% D₂O solution containing 100 mM sodium phosphate, 100 mM NaCl (pH 6). Side-chain assignments were obtained using HBHA(CO)NH⁷⁰ and HCCH-TOCSY⁷¹ for each protein. Many residues have two sets of cross-peaks caused by *cis/trans* isomerization of Pro83 and to a lesser extent Pro33 and Pro105. Sidechain assignments were obtained only for the most populated conformer in each protein, which corresponds to Pro83 *trans* for Nhp2p-WT and Nhp2p-P83A and Pro83 *cis* for Nhp2p-S82W. The proline side chain carbon resonances were assigned from CC(CO)NH and HNCACB experiments, and the chemical shift difference $\delta(^{13}\text{C}_\beta) - \delta(^{13}\text{C}_\gamma)$ for proline was used to determine the proline peptide bond conformation (average values of 4.5 ± 1.2 and 9.6 ± 1.3 ppm for *trans* and *cis* isomers, respectively^{50,53,54}). Side chain assignments for the 7 isoleucines were obtained or confirmed by analysis of ¹H-¹⁵N HSQC spectrum of ¹⁵N-Ile- labeled Nhp2p-S82W.

The NOE cross-peaks for distance restraints were obtained from 3D ¹³C-edited NOESY-HSQC ($\tau_m=150$ msec) and 3D ¹⁵N-edited NOESY-HSQC ($\tau_m=120$ and 150 msec).⁷² Only cross peaks from the most populated conformer were used in the structure calculation. NOE-derived interproton distance restraints were classified into three ranges: 1.8–2.8, 1.8–3.4 and 1.8–5.0 Å, corresponding to strong, medium, and weak NOE cross-peak intensities, respectively. An additional 0.5 Å was added to the upper bound for NOEs involving methyl groups. The dihedral angle restraints were extracted from TALOS chemical shift analysis.⁷³ The hydrogen bond restraints were determined based on the slowly exchanging amide protons identified by recording ¹H-¹⁵N HSQC after a ¹⁵N-labeled protein sample had been exchanged into a D₂O buffer. All data were processed with Topspin (Bruker) and NMRPipe,⁷⁴ and analyzed with the program Sparky (<http://www.cgl.ucsf.edu/home/sparky>).

Structure calculations were performed with CYANA 2.1⁷⁵ and validated using Pro-Check NMR.⁷⁶ Unambiguous side-chain NOE cross-peak assignments were obtained manually and used in an initial round of structure calculations to identify the secondary-structure topology. These initial structures were used to filter possible assignments for previously ambiguous peaks. Seven cycles of CYANA generated further side-chain assignments, each cycle having 10,000 steps of torsion-angle dynamics with a simulated annealing protocol. A set of 100 structures was calculated, and the 20 structures with the lowest target function values were chosen for analysis. Structures were visualized using the programs PyMOL (<http://www.pymol.org>) and MOLMOL.⁷⁷

Yeast expression plasmids and immunoprecipitation assays

NHP2 full-length constructs were PCR-amplified for insertion into the centromeric plasmids pUG34 and pUG36⁷⁸ by the addition of *Xba*I and *Eco*RI sites at the 5' and 3' end, respectively, of the oligonucleotide primers. The sequence for a FLAG epitope tag flanked by glycine residues (GGCGATTACAAGGATGACGACGATAAGGGT) was inserted at the C-terminus of the *NHP2* constructs by inclusion of the complementary sequence in the oligonucleotide 3' primer. Cloning of the *NHP2* constructs into pUG34 and pUG36 with *Xba*I and *Eco*RI resulted in removal of *eGFP* but maintained the MET25-P. Constructs were confirmed by sequencing.

Yeast media and manipulations were as described.⁷⁹ *NHP2* plasmid constructs were transformed in BY4741 and Cbf5TAP (Open Biosystems). *NHP2* was disrupted using the KanMX6 marker from pFA6a-kanMx6⁸⁰ amplified with a forward primer containing 49 nt of *NHP2* sequence upstream of the 5' end and a reverse primer containing 56 nt of *NHP2* sequence downstream of the 3' end. The amplified DNA was transformed into strains containing *NHP2* full-length FLAG tagged wild-type and mutant constructs, plated on YPD overnight and replica-plated on YPD-G418 (200 µg/ml). Deletion of *NHP2* was confirmed by PCR. We found that the low levels of expression of Nhp2p from the MET25 promoter when strains were grown in methionine-containing medium (basal levels of transcription from the MET25 promoter) were sufficient to complement the deletion of the *NHP2* gene. Therefore, to avoid any issues linked to overexpression of Nhp2p, strains were grown in the presence of methionine to keep the expression of Nhp2p to minimal levels.

Immunoprecipitations, western and northern blots

Immunoprecipitations of Nhp2pFLAG were carried out by growing strains in appropriate selective media at 30°C to OD₆₀₀ between 0.4 and 0.6, washed once with H₂O, and resuspended in NET-2 (40 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% IGEPAL) and protease inhibitor cocktail (complete EDTA-free, Roche). Cell disruption was done by addition of glass beads and vortexing. Samples were sedimented at 2500 r.p.m. in a micro-centrifuge followed by supernatant sedimentation at 16,000 g for 20 min in a micro-centrifuge. Lysates were added to anti-FLAG M2 agarose beads (Sigma) with NET-2 at a final concentration of 150 mM or 500 mM NaCl for immunoprecipitations and incubated at 4°C for 1 hr. Beads were washed in NET-2 with either 150 mM or 500 mM NaCl final concentration. RNAs were extracted by phenol, ethanol precipitated and resuspended in H₂O. RNAs were fractionated in 5% polyacrylamide/8 M urea gels. Northern blots and snoRNA probes were as described.⁸¹

Immunoprecipitations of Cbf5pTAP strains were carried out as above but cells were resuspended in IPP150 without EDTA (10 mM β-Me, 10 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM MgAcetate, 1 mM Imidazole, and 0.1% IGEPAL) and pull-downs were carried out with Calmodulin Sepharose 4B (GE Healthcare). After washing, beads were split in half for RNA and protein sample extraction. Protein samples were heated at 100°C for 10 min in SDS-sample buffer. For western blots, anti-FLAG monoclonal antibodies (Sigma) at 1:5000 with goat anti-mouse HRP linked IgG at 1:10000 and Peroxidase anti-peroxidase at 1:1000 were used and revealed by Super Signal West Femto (Thermo Scientific) ECL system.

Accession numbers

Coordinates and restraints for the 20 lowest energy structures of Nhp2p-WT (residues 36–156) and Nhp2p-S82W (residues 35–156) have been deposited in the Protein Data Bank with accession numbers 2LBX and 2LBW, respectively. NMR assignments have been deposited in the Biological Magnetic Resonance Bank (accession numbers 17579 and 17578).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ^1H - ^{15}N HSQC of Nhp2p show many doubled cross peaks due to proline *cis/trans* isomerization

(A) ^1H - ^{15}N HSQC spectrum of Nhp2p-WT. Some of the doubled peaks are circled.

(B) ^1H - ^{15}N HSQC spectrum of Nhp2p-P83A. Doubled peaks circled in (A) appear as single resonances. (C–D) Regions of the ^1H - ^{15}N HSQC spectra of (C) Nhp2p-WT and (D) Nhp2p-S82W, illustrating the difference in relative intensities of some of the cross peak pairs. The peaks associated with the *trans* and *cis* conformation of Pro83 are labeled in blue and red, respectively.

Figure 2. Sequence alignment and structure of Nhp2p-WT and Nhp2p-S82W

(A) Primary sequence alignment of Nhp2p with homologous proteins *H. sapiens* 15.5kD, *S. cerevisiae* Snu13p, *C. parvum* Nhp2p (putative) and *P. furiosus* L7Ae. Sequence alignment was performed using T-Coffee⁸² and ClustalW⁸³ and the figure was rendered using ESPrpt.⁸⁴ The residues in red are highly conserved among proteins. The position of Pro83, which shows *cis/trans* isomerization in Nhp2p, is boxed in blue. The well-conserved proline residues are marked with black filled star. Doubled peaks in the Nhp2p-WT ¹H-¹³C HSQC are indicated by black ovals below the sequences. Secondary structure elements from the determined structure of Nhp2p are indicated above the alignment with filled cylinders (helices) and filled arrows (β -strand). Colors correspond to those on the structures shown in (B–E). (B–C) Superimposition of the backbone (N, Ca, CO) atoms for the 20 lowest-energy structures (residues 37–156) of (B) Nhp2p-WT and (C) Nhp2p-S82W. (D–E) Ribbon diagram of the lowest energy structure of (C) Nhp2p-WT and (D) Nhp2p-S82W, respectively. All prolines are shown in light blue.

Figure 3. Effect of proline isomerization on the structure of Nhp2p

(A) Ribbon representation of Nhp2p-WT with the positions of the residues whose amides that show doubled peaks in Nhp2p-WT but not in Nhp2p-P83A highlighted in purple and residues whose amides which are doubled in both Nhp2p-WT and Nhp2p-P83A highlighted in light violet. (B) Ribbon representation of Nhp2p-WT with the positions of residues equivalent to those in L7Ae which bind to Nop10 and H/ACA RNA highlighted in yellow and cyan, respectively. (C) Superimposition of structures of Nhp2p-WT (gray) with Nhp2p-S82W (blue). Pro83 and the peptide backbone of Ser82 or Trp82 are highlighted in green for Nhp2p-WT and magenta for Nhp2p-S82W. (D) Close-up view of the region of Nhp2p-WT and Nhp2p-S82W around P83A (region boxed in (A), 90° rotated). In the *cis* conformation, Pro83 and the backbone amide and carbonyl of residue 82 point toward the helix α_2 , while in the *trans* conformation they point away.

Figure 4. Nhp2-P83A shows decreased association with H/ACA snoRNA and proteins, while Nhp2p-S82W stabilizes the association with H/ACA snoRNPs

(A) Northern blot analysis of RNAs co-immunoprecipitated with the various versions of Nhp2p. Extracts from a wild-type strain containing the pUG34 vector (lanes 1, 5, 9) and *nhp2Δ* strains containing the pNHP2FLAG WT (lanes 2, 6, 10), pNHP2FLAG S82W (lanes 3, 7, 11) or pNHP2FLAG P83A (lanes 4, 8, 12) plasmid were subjected to immunoprecipitation with anti-FLAG agarose beads. RNAs were extracted with phenol, precipitated, fractionated on a 5% polyacrylamide/8 M urea gel and subjected to Northern analysis to detect the H/ACA snoRNAs snR10, snR32, snR37 and snR42 and the U1 snRNA. Quantifications of the levels of associations of the different mutants with individual snoRNAs is provided in Table S1. The snR37 northern blot was obtained from a separate

experiment than for the other snoRNAs, therefore the U1 snRNA control is shown twice. (B) Western blot analysis of the association of the various versions of Nhp2p with Cbf5p. Extracts from a wild-type strain containing the pUG36 vector (lanes 1, 7) or Nhp2FLAG WT in pUG36 (lanes 2, 8) and a *CBF5TAP* strain containing pUG36 (lanes 3, 9) or Nhp2FLAG WT (lanes 4, 10), Nhp2FLAG S82W (lanes 5, 11) or Nhp2FLAG P83A (lanes 6, 12) plasmids in pUG36 were subjected to affinity precipitation using Calmodulin sepharose beads. Proteins were extracted from the beads by heating at 100°C in SDS sample buffer were fractionated on either a (a) 12% or (b) 6% SDS-polyacrylamide gel and probed by western blot analysis with (a) anti-FLAG antibodies to detect Nhp2pFLAG or (b) PAP to detect Cbf5pTAP.

Figure 5. Comparison of Nhp2p with homologous proteins

(A) Ribbon diagram and (B) electrostatic surface potential surface of Nhp2p-S82W (Pro83 *cis* conformation) and orthologues, solution structure of *Homo sapiens* 15.5kD (PDB ID 2JNB),⁴⁴ and crystal structures of *S. cerevisiae* Snu13p (PDB ID 2ALE),⁴⁵ *M. jannaschii* L7Ae (PDB ID 1XBI),⁴⁶ and *C. parvum* putative Nhp2 (PDB ID 2AIF).⁶² All of the proteins shown are structures solved for the free protein alone. Electrostatic potential surface calculated by APBS. Blue means a positive charge and red a negative charge.

Figure 6. Comparison of the Nop10 and RNA binding interface of Nhp2p-WT, Nhp2p-S82W, and L7Ae

(A–C) Ribbon diagrams of (A) Nhp2p-WT (gray), (B) Nhp2p-S82W (blue), and (C) L7Ae (light green) from the archaeal *P. furiosus* H/ACA RNP with Cbf5, Nop10, H/ACA RNA, and substrate RNA (PDB ID 2HVY).²⁹ The residues in L7Ae that interact with Nop10 and RNA, and the homologous residues in Nhp2p, are highlighted in yellow and aqua, respectively. The RNA binding residues in L7Ae are R34, K35, T37, E39, K42, R46, D59, (N38, I93, V95, A96, A98 have van der Waals contacts),³³ which correspond to K57, R58, K61, E62, K65, K69, S82, (V60, T116, T120, S121, V123) in Nhp2p. The Nop10p binding residues in L7Ae are T41 in $\alpha 2$, A65, H66, P69, L70 and E73 in $\alpha 3$, which correspond to V64, P83, S88, H89, V92, L93 and D96 in Nhp2p. (D) Zoomed in view of the *P. furiosus* H/ACA RNP showing the residues comprising the proline spine. Pro60 (equivalent residue to Nhp2p Pro83) stacks over the flipped out U in the K-loop, and the backbone of Asp59 has two hydrogen bonds with the U base (red). The H/ACA RNA is orange, substrate RNA is magenta, L7Ae is light green, Nop10 is cyan, Cbf5 is tan.

Table 1

Structural statistics for Nhp2p-WT and Nhp2p-S82W

	Nhp2p-WT (Residues 36–156)	Nhp2p-S82W (Residues 36–156)
<i>NOE restraints</i>	915	1001
Short-range ($ i-j \leq 1$)	567	599
Medium-range ($1 < i-j < 5$)	140	161
Long-range ($ i-j \geq 5$)	208	241
Hydrogen bonds distance restraints ^a	114	116
<i>Dihedral Angle Restraints</i>	177	178
Phi	89	89
Psi	88	89
<i>Violations</i>		
Distances $> 0.5 \text{ \AA}$	None	None
Dihedral angles $> 5^\circ$	None	3
<i>Average target function ($\text{\AA}^2$)</i>	3.64 ± 0.31	10.94 ± 0.54
<i>Ramachandran analysis (%)</i>		
Most favored regions	83.7	85.9
Additional allowed regions	16.0	12.3
Generously allowed regions	0.2	1.8
Disallowed regions	0.0	0.0
<i>RMS deviations from the mean ($\text{\AA}$)</i>		
Backbone atoms (N,C $^\alpha$,C',O)	0.74 ± 0.16	0.75 ± 0.14
Heavy atoms	1.30 ± 0.17	1.39 ± 0.15

^aTwo restraints per one hydrogen bond