Human Fibrillarin Forms a Sub-complex with Splicing Factor 2-associated p32, Protein Arginine Methyltransferases, and Tubulins α3 and β1 That Is Independent of Its Association with Preribosomal Ribonucleoprotein Complexes

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Fibrillarin (FIB, Nop1p in yeast) is an RNA methyltransferase found not only in the fibrillar region of the nucleolus but also in Cajal bodies. FIB is essential for efficient processing of preribosomal RNA during ribosome biogenesis, although its precise function in this process and its role in Cajal bodies remain uncertain. Here, we demonstrate that the human FIB N-terminal glycine- and arginine-rich domain (residues 1–77) and its spacer region 1 (78–132) interact with splicing factor 2-associated p32 (SF2A-p32) and that the FIB methyltransferase-like domain (133–321) interacts with protein-arginine methyltransferase 5 (PRMT5, Janus kinase-binding protein 1). We also show that these proteins associate with several additional proteins, including PRMT1, tubulin α3, and tubulin β1 to form a sub-complex that is principally independent of the association of FIB with preribosomal ribonucleoprotein complexes that co-immunoprecipitate with the sub-complex in human cells expressing FLAG-tagged FIB. Based on the physical association of FIB with SF2A-p32 and PRMTs, as well as the other reported results, we propose that FIB may coordinate both RNA and protein methylation during the processes of ribosome biogenesis in the nucleus and RNA editing such as small nuclear (nucleolar) ribonucleoprotein biogenesis in Cajal bodies.

Fibrillarin (FIB) is the most abundant protein in the fibrillar regions of the nucleolus where ribosomal RNA transcription and early preribosomal RNA (pre-rRNA) processing take place (1, 2). FIB is also found in Cajal bodies, subnuclear organelles that contain distinct components involved in RNA transcription and editing such as mRNA splicing and small nuclear (nucleolar) ribonucleoprotein (sn/o)RNP) biogenesis (3, 4). FIB is a component of a ribonucleoprotein (RNP) complex that contains U3, U8, and U13 small nuclear RNAs that exhibit consensus sequence elements denoted box C (5'-UGAUAGA-3') and box D (5'-CUGA-3') (5). The FIB RNP associates with Nop56p, Nop5p/58p, and a 15.5-kDa protein (a counterpart of yeast Snu13p) to form box C/D sn/oRNP complexes that function in site-specific 2'-O-methylation of pre-rRNA (6–9). FIB is the methyltransferase that catalyzes this 2'-O-methylation (10).

FIB, or Nop1p in the yeast Saccharomyces cerevisiae, is highly conserved in eukaryotes with respect to sequence, structure, and function (11–17). Deletion of the Nop1 gene in yeast results in inhibition of 2'-O-ribose methylation and pre-rRNA processing at sites A0 to A2, indicating that Nop1p is directly involved in both pre-rRNA methylation and processing and ultimately in ribosome assembly (18). Although human FIB is the functional homolog of yeast Nop1p, it only partially complements a yeast nop1-defective mutant (15). Human FIB is a nucleolar autoantigen for the non-hereditary immune disease scleroderma (14). FIB co-localizes with the survival motor neuron (SMN) gene product in both nucleoli and Cajal bodies/gems of primary neurons (19, 20). SMN is linked to one of the most common inheritable causes of childhood mortality, spinal muscular atrophy (19). In fact, a direct interaction between FIB and SMN has been demonstrated, although no functional basis for this interaction has been established, including any involvement of FIB in the pathogenesis of spinal muscular atrophy. Another protein, the nuclear DEAD box protein p68, an RNA-dependent ATPase and RNA helicase, co-localizes with FIB specifically in nascent nucleoli during telophase (21). As with SMN, no physiological role of its interaction with FIB has been established.

Human FIB (~36 kDa) comprises 321 amino acids and three structural domains (14) and is 66% identical to yeast Nop1p. The N-terminal 80 residues comprise a glycine- and arginine-rich (GAR) domain (14) that is also present in Nop1p and Xenopus FIB (Fig. 1) but not in Tetrahymena FIB (17) or Methanococcus jannaschii FIB (22). The GAR domain is methylated at arginine residues, although the arginine methyltransferase responsible for in vivo methylation has not been identified (23, 24). The GAR domain is responsible for the interaction

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of FIB with both SMN protein and the DEAD box RNA helicase p68 (19, 21). A centrally located 90-residue sequence resembles an RNA-binding domain (RBD) present in various snRNPs. This RBD together with the C-terminal α-helix domain and the intervening spacer (spacer region 2) constitutes a methyltransferase-like domain that contains an S-adenosyl methionine-binding motif (14, 22). Replacement of two residues in the Nop1p S-adenosyl methionine-binding motif results in temperature sensitivity and a drastic reduction in nascent rRNA transcript methylation under restrictive conditions (18). Thus, the methyltransferase-like domain is responsible for FIB methyltransferase activity. The C-terminal α-helix domain is composed of ∼30 residues, and although this domain probably targets FIB to Cajal bodies, spacer region 2 appears to target FIB to the fibrillar regions. However, the targeting of FIB in both instances occurs only in the presence of the RBD (3). Although it is well established that FIB plays a role in ribosome biogenesis within the nucleolus, its role in Cajal bodies is not understood.

Our recent studies have used proteomic methodology to characterize a series of preribosomal ribonucleoprotein (pre-rRNP) complexes formed in mammalian cells. We have thus far isolated and analyzed the pre-rRNP complexes associated with human nucleolin (25), parvinulin (26), and Nop56p (27). These studies demonstrated the applicability of proteomic analysis to human nucleolin (25), parvinulin (26), and Nop56p (27). These studies demonstrated the applicability of proteomic methodology to characterize a series of preribosomal ribonucleoprotein (pre-rRNP) complexes formed in mammalian cells. We have thus far isolated and analyzed the pre-rRNP complexes associated with human nucleolin (25), parvinulin (26), and Nop56p (27).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human kidney cell line 293EBNA, Opti-MEM, and LipofectAMINE were purchased from Invitrogen (Grand Island, NY). Dulbecco’s modified Eagle’s medium, anti-FLAG M2 affinity gel, FLAG peptide, IGEPAL CA630, RNase A, and α-cyano-4-hydroxycinnamic acid were from Sigma-Aldrich Chemical (Steinheim, Germany). Alkaline phosphatase-conjugated anti-mouse IgG was from Amersham Biosciences (Uppsala, Sweden). Alexa Fluor 488-conjugated rabbit anti-mouse IgG antibody was from Molecular Probes, Inc. (Eugene, OR). Trypsin (sequence grade) was from Promega (Madison, WI) and Achromobacter lyticus protease I (Lys-C) was from WAKO Pure Chemicals (Osaka, Japan). ZipTipC18 was from Millipore (Billerica, MA). LA Taq DNA polymerase was from Takara (Shiga, Japan). Protease inhibitor mixture Complete Mini was from Roche Diagnostics (Mannheim, Germany). Collagen I-coated Biocoat 8-well culture slides were from BD Biosciences (Franklin Lakes, NJ). All other reagents were from WAKO Pure Chemicals.

**Vectors for Epitope-tagged FIB and FIB Truncation Mutants and Expression in 293EBNA Cells**—The FIB expression plasmid was constructed using a PCR-amplified DNA fragment comprising the FIB open reading frame with the FLAG tag at its N terminus (Fig. 1). This fragment was introduced into the NheI and the BamHI sites of the mammalian expression vector, pcDNA3.1 (+) (Invitrogen). The PCR primer set was 5'-ATATATCTAGAGCCACCATGGACTACAAGGACG-ACGACGAGAAAGCCAGGGAGGATTCAGTCCCCGT-3' and 5'-TATTAGGATCCTGACGATCTTTTGCACCCTGGG-3', and human placenta cDNA (Origen Technologies, Inc., Rockville, MD) was used as the template. The DNA fragment encoding the FLAG tag along with the nucleolar localization signal (NLS) of HIV Rex (RRRRPQRSRQRK) (28) and the SV40 nuclear localization signal (NS) (PKKKRKV) (29) was synthesized by PCR using the oligonucleotide sets 5'-TATATGCTAGCCGCTGCAATGATGAGCCTAGGGGACGCCGAGTTG-3' and 5'-TCTTTTCTTTTGGGATCGGCGGCCGTCAGCGGGG-3', and 5'-GATCCAAAAAGAAAAAGAGGCTAGGATCCAAAAAGAAAAGGAGAA-3' and 5'-ATAGGATACTACACTTTTCTTCTTCTTTTTTT-3'. The amplified fragment was subcloned between the NheI and the BamHI sites of pcDNA3.1 (+), and the resulting plasmid was designated pcDNA3.1-NLS. All the expression plasmids of the FIB deletion mutants were constructed by introducing PCR-amplified fragments between the BamHI and XhoI sites downstream of the FLAG tag in pcDNA3.1-NLS. Primer sets used for the amplification of FIB deletion mutants were as follows: 5'-ATATATGCTAGCCGCTGCAATGATGAGCCTAGGGGACGCCGAGTTG-3' and 5'-TATTAGGATCCTGACGATCTTTTGCACCCTGGG-3', and 5'-TATTAGGATCCTGACGATCTTTTGCACCCTGGG-3', and 5'-ATATATGCTAGCCGCTGCAATGATGAGCCTAGGGGACGCCGAGTTG-3'. The amplified fragment was subcloned between the NheI and the BamHI sites of pcDNA3.1 (+), and the resulting plasmid was designated pcDNA3.1-NLS. All the expression plasmids of the FIB deletion mutants were constructed by introducing PCR-amplified fragments between the BamHI and XhoI sites downstream of the FLAG tag in pcDNA3.1-NLS. Primer sets used for the amplification of FIB deletion mutants were as follows: 5'-ATATATGCTAGCCGCTGCAATGATGAGCCTAGGGGACGCCGAGTTG-3' and 5'-TATTAGGATCCTGACGATCTTTTGCACCCTGGG-3', and 5'-ATATATGCTAGCCGCTGCAATGATGAGCCTAGGGGACGCCGAGTTG-3'.
Human Fibrillarin-associated Protein Complexes

Isolation of FIB- and Its Truncated Mutant-associated Complexes—At 48 h post-transfection, 293EBNA cells were harvested and washed with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% IGEPAL CA630) containing a protease inhibitor mixture on ice for 30 min. The soluble fraction was obtained by centrifugation at 15,000 rpm for 30 min at 4 °C and was incubated with 20 μl of anti-FLAG M2-agarose beads for 4 h at 4 °C for immunoprecipitation of FIB-associated complexes or overnight at 4 °C for deletion mutant-associated complexes. After washing the agarose beads five times with lysis buffer and once with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, the complex bound to the agarose beads were eluted with 20 μl of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 500 μg/ml FLAG peptide. The eluted complexes were analyzed by SDS-PAGE.

Ribonuclease Treatment of the FIB- and Truncation Mutant-associated Complexes—The immunoprecipitated FIB and truncation mutant-associated complexes described above were incubated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 1 μg/ml RNase A for 10 min at 37 °C, washed twice with lysis buffer, and then once with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and eluted with buffer containing the FLAG peptide as described above.

Immunocytochemistry—293EBNA cells were grown on Collagen I-coated 8-well culture slides and transfected with expression plasmids using LipofectAMINE. Prior to fixation, cells were washed with PBS followed by incubation with 3.7% formaldehyde in PBS. After several washes with PBS-T (PBS containing 0.05% (w/v) Tween 20), the cells were incubated with PBS containing 0.1% (w/v) Triton X-100 for 5 min at room temperature. The cells were then blocked by 3% (w/v) nonfat dried milk in PBS and were incubated with anti-FLAG for 1 h at room temperature. The cells were rinsed in PBS-T and then incubated with Alexa Fluor 488-conjugated anti-mouse IgG for 1 h at room temperature, followed by three washes with PBS-T. The resulting cells were examined with a confocal laser-scanning microscope TCS (Leica Microsystems AG, Wetzlar, Germany).

Protein Identification by the Peptide Mass Fingerprinting Method—Protein-containing SDS-PAGE gel fragments were subjected to in-gel digestion with trypsin as previously described (25). The resulting peptides were recovered and analyzed for peptide mass fingerprints using a PE Biosystems MALDI-TOF MS (Voyager DE-STR) as described previously (25). Peptide masses were searched with 50 ppm mass accuracy using the data base fitting program MS-Fit (available at prospector.ucsf.edu), and protein identification was performed according to the criteria described previously (25).

Protein Identification by LC-MS/MS Analysis—The FIB- and truncation mutant-associated complexes were digested with Lys-C, and the resulting peptides were analyzed using a nanoscale LC-MS/MS system as described (30). The peptide mixture was applied to a Mightysil-RP-18 (300 μm × 75 mm, 5 μm, Kanto Chemical, Osaka, Japan) frit-less column (45 mm × 0.150 mm i.d.) and separated using a 0–40% gradient of acetonitrile containing 0.1% formic acid over 80 min at a flow rate of 50 or 20 nl/min. Eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-Tof 2, Micromass Wythenshawe, UK). MS/MS spectra were acquired by data-dependent collision-induced dissociation, and MS/MS data were analyzed using the Mascot software (Matrix Science, London, UK) for peptide assignment. The criteria were in accordance with the manufacturer’s definitions. If necessary, match acceptance of automated batch processes was confirmed by manual inspection of each set of raw MS/MS spectra in which the major product ions were matched with theoretically predicted product ions from the data base-matched peptides. Proteins in the sample were identified from anti-FLAG antibody with FLAG-peptide were analyzed by the same LC-MS/MS method as used for the fibrillarin-associated complexes and then subtracted from the proteins identified in the total fibrillarin-associated complexes. Thus, those proteins identified in the mock eluate were not included in the fibrillarin-associated proteins unless the quantitative increase was confirmed.

Ultracentrifugation of FIB-associated RNP Complexes—The anti-FLAG immunoprecipitate obtained from FLAG-tagged FIB gene-transfected 293EBNA cells after elution with the FLAG peptide was analyzed on a 12–50% sucrose gradient in 50 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl2. The gradients were centrifuged in an SW65 rotor at 50,000 rpm at 15°C for 3 h at 4°C. A total of 18 fractions of 300 μl each were collected. The migration of the 40 S/60 S/80 S ribosomal complexes was determined by comparison to the ultraviolet absorption profile of cytosolic ribosomes fractionated by ultracentrifugation under identical experimental conditions.

RESULTS

Isolation of FLAG-tagged FIB-associated Protein Complexes—Because endogenous FIB is found primarily in the nucleus of mammalian cells, the subcellular localization of the FLAG-tagged protein in transfected 293EBNA cells was confirmed by immunofluorescence microscopy using an antibody to FLAG (Supplementary Fig. 1). Although weak staining was observed in the cytoplasm and nucleoplasm of the FLAG-FIB-transfected cells, the nucleus exhibited intense staining thus confirming the correct localization of FLAG-tagged FIB.

Complexes associated with FIB were isolated from FLAG-FIB-transfected cells via immunoprecipitation using the FLAG antibody. A typical silver-stained SDS-PAGE gel of FLAG-FIB-associated complexes immunoprecipitated with anti-FLAG after expression of FLAG-tagged full-length FIB. Lanes 1 and 2, FIB-associated complexes (FIB full) with (+) or without (−) RNase treatment; lanes 3 and 4, control immunoprecipitate with or without RNase. Molecular weight markers are indicated to the right. The protein bands identified by MALDI-TOF analysis after in-gel digestion of protein bands with trypase are indicated on both sides of the gel. Boxed proteins denote FIB-associated proteins following RNase treatment. Proteins in gray indicate those identified in the control. PRMT1 and 5, protein arginine methyltransferases 1 and 5, respectively; SF2A-p32, splicing factor-2-associated protein p32; FIBb48, FIB-binding protein 48 kDa; HKSP, kinesin-like spindle protein; PP2C, protein phosphatase 2C.

RNA Integrity in FIB-associated Complexes—RNA integrity is requisite for protein association in pre-rRNP complexes associated with human nucleolin, parvulin, and Nop56p, all of which may be involved in ribosome biogenesis as reported in our previous studies (25–27). Therefore, the requirement for RNA integrity was also analyzed for FLAG-FIB-associated complexes. RNase treatment prompted the dissociation of the
| Protein | Accession No. (GI) | MW (Da) | Function in mammals | Yeast Homolog | Percent Identity to yeast homolog | Function in Yeast |
|---------|------------------|---------|---------------------|---------------|---------------------------------|------------------|
| Fibrillarin | 11425985 | 33763.4 | A component of a snRNP particle thought to participate in the first step of preribosomal RNA processing. | NOP1 | YDL014W | 66 | 3SR primary transcript processing; rRNA modification. |
| Nucleolar protein 5A (56 kDa with KKEED repeat) | 12832025 | 66008.7 | Similar to S. cerevisiae SIK1p, a nucleolar KKEED repeat protein involved in pre-rRNA processing. | SIK1(NOP56) | YLR197W | 50 | 3SR primary transcript processing; rRNA modification. |
| Nucleolar protein NOP58/NOP58 | 7766254 | 59540.5 | Putative snoRNA binding protein | NOP58 | YOR310C | 47 | 3SR primary transcript processing; rRNA modification. |
| NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae) | 4826860 | 14164.6 | Binds to the 5' stem-loop of U4 snRNA. Nucleolar protein that associates with the checkpoint protein RAD17, highly similar to S. cerevisiae Snu13p. | SNU13 | YEL069W | 71 | Pre-mRNA splicing factor; part of small (ribosomal) subunit (SSU) processosome (contains U3 snRNA). |
| Splicing factor, arginine/serine-rich 4 | 5032089 | 56759.3 | mRNA splicing; Splicing factor 4; member of the SR protein family; has an RNA recognition motif (RRM). | SRF40 | YKR092C | 32 | Nucleocytoplasmic transport; Nop140 homolog, a nonribosomal protein of the nucleolus and coiled bodies; nucleolar protein. |
| Hypothetical protein FFL10774 | 12697963 | 116025.6 |  | KRE33 | YNL132W | 55 | 40S subunit assembly and export to cytoplasm; killer toxin resistance. |
| Hypothetical protein FFL10534 | 8922496 | 75069.7 | Unknown | TIR1 | YDL060W | 32 |  |
| Hypothetical protein AD034 | 13899340 | 64594.2 | Unknown | RID1/RPR1 | YOR119C | 38 | 20S pre-rRNA processing |
| DEAD12H (Asp-Glu-Ala-AspKHS) box polypeptide 5 (RNA helicase, 5 kDa) | 4758138 | 69104.7 | RNA helicase p68 | DBP2 | YNR112W | 56 | RNA helicase |
| DEAD12H box polypeptide 21 | 2135315 | 89195.5 | RNA helicase Gu | DBP3 | YGL078C | 32 | RNA helicase; 3SR primary transcript processing; large ribosomal subunit assembly and maintenance. |
| Poly(A) binding protein, cytoplasmic 1 | 3183544 | 70825.9 | Poly(A) binding | PAB1 | YER165W | 54 | Poly(A) binding |
| Poly(A) binding protein, cytoplasmic 4 (inducible form) | 4504715 | 70738.1 | RNA-binding protein, binds to the mRNA poly(A) tail; may play a role in translation and mRNA stability. | PAB1 | YER165W | 53 | Poly(A) binding |
| Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A) | 14114163 | 90423.0 | Binds RNA and scaffold attached region DNA; contains an RGG box domain; component of hnRNP complexes; may play a role in mRNA structure or processing. | NOP3 | YDR432W | 37 | Required for efficient 27S pre-rRNA processing; Nop3p shuttles between the nucleolus and the cytoplasm. |
| Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor) | 5902076 | 27727.8 | Splicing factor 10; essential for constitutive pre-mRNA splicing; member of the SR family; alternative splicing factor. | NOP3 | YDR432W | 26 | Required for efficient 27S pre-rRNA processing; Nop3p shuttles between the nucleolus and the cytoplasm. |
| Splicing factor, arginine/serine-rich 6 | 13656376 | 39563.4 | Splicing factor | NOP3 | YDR432W | 25 | Required for efficient 27S pre-rRNA processing; Nop3p shuttles between the nucleolus and the cytoplasm. |
| Pescadillo homolog 1, containing BRCT domain (zebrafish) | 7657455 | 67960.0 | Plays a crucial role in cell proliferation and may be necessary for oncogenic transformation and tumor progression. | NCCP | YGR103W | 39 | rRNA processing |
| DKGFP546M182 protein | 3686141 | 58096.6 |  | CIC1 | YHR052W | 26 | 26S proteasome; ribosome biogenesis. |
| Nucleolar GTPase | 12652715 | 83629.5 | Nucleolar GTPase | NGO2 | YNR053C | 55 | Nuclear/Nucleolar GTP-binding protein 2 |
| Putative nucleotide binding protein, estradiol-induced | 7657048 | 63528.4 | Nucleotide binding | NUG1 | YER006W | 30 | Nuclear GTPase |
| Nucleolin | 4885511 | 76298.2 | Ribosome biogenesis | NSR1 | YGR115C | 32 | rRNA processing; small ribosomal subunit assembly. |
| RNA-binding region (RNP1, RRM) containing 2 | 4757926 | 58905.5 | Nuclear protein that may be a splicing factor; contains motifs characteristic of splicing factors. | NSR1 | YGR115C | 23 |  |
| Ras-GTPase activating protein SH3 domain-binding protein 2 | 14916573 | 54077.8 | Probable scaffold protein that may be involved in mRNP transport. | NSR1 | YGR115C | 25 |  |
majority of the protein components of FLAG-FIB-associated complexes. However, at least four major protein-staining bands as well as several minor bands remained associated with FLAG-FIB after RNase treatment (Fig. 2). The four major protein bands appeared to exhibit approximate equivalent stoichiometry with respect to staining intensity and were representative of the more abundant proteins present in the non-ribose complexes. However, at least four major protein bands and several minor bands that remained associated with FLAG-FIB after RNase treatment (data not shown). These results suggested that at least two distinct groups of proteins are present in FIB-associated complexes, one whose association is dependent on RNA integrity (the FIB-associated RNPs) and another that associates directly with FIB (independent of RNA integrity). However, it was uncertain as to whether these two groups of proteins were associated with each other or present independently.

Identification of Protein Components in FIB-associated RNPs—Given the involvement of FIB in ribosome biogenesis, we expected isolated FIB-associated RNP complexes to contain trans-acting factors involved in this process, as well as ribosomal proteins. We therefore identified the protein components of the FIB-associated RNPs. We previously described a highly sensitive "direct nano-flow LC-MS/MS" system to identify proteins in limited amounts of multiprotein complexes (30). Immunoprecipitated FIB-associated complexes were digested with Lys-C and analyzed directly using the nano-LC-MS/MS system. In addition to the criteria for match acceptance described under "Experimental Procedures," more stringent criteria were adopted to conclusively identify proteins. Namely, at least two different peptides had to be identified in a single nano-LC-MS/MS analysis, and/or at least one peptide had to be identified at least twice (with highly significant data base matching scores) among four separate analyses. A total of 1426 peptides were identified via sequence data base searches using the collision-induced dissociation spectra obtained from four nano-LC-MS/MS runs of a Lys-C digest of the FIB-associated complexes. These peptide data identified 170 proteins (excluding the bait protein and the proteins present in mock) that met our identification criteria. Although we do not exclude that some of the proteins identified may be nonspecifically associated proteins, we believe most of the protein components identified in the fibrillarin-associated complexes are specifically associated with fibrillarin. Of the 170 proteins, 73 were ribosomal proteins (43 from the large subunit and 30 from the small subunit; Supplementary Table SI) and 97 were non-ribosomal proteins (Supplementary Tables SII and SIII). Of the non-ribosomal proteins, 24 were assigned as probable trans-acting factors involved in ribosome biogenesis based on their homology to yeast proteins known or expected to be involved in ribosome biogenesis (Table I and Supplementary Table SIII).

In addition to the putative trans-acting factors that were assigned, the FIB-associated complexes contained 68 more non-ribosomal proteins, including a number of RNA-binding proteins, splicing factors, DNA-topoisomerase, Myb-binding protein, components of DNA-dependent protein kinase, components of the signal recognition particle, nuclear matrix proteins as well as many hypothetical/unknown proteins (Supplementary Table SIII). Of these, at least 44 proteins reportedly localize to the nucleolus or the nucleus (31, 32) (Supplementary Table SIII).

Identification of Proteins Associated with FIB without RNA Integrity—The proteins that remained associated with FIB after the RNase treatment were identified by MALDI-TOF analysis after in-gel digestion of excised SDS-PAGE gel bands with trypsin. The identified proteins were SKB1 homolog (protein arginine-methyltransferase 5, PRMT5) (GI:5174683; 18 peptides matched; 29.6% peptide coverage; mean error = -3.41 ppm), tubulin a3 (GI:5174733; 9 peptides matched; 27.2% peptide coverage; mean error = -0.42 ppm), tubulin b1 (GI:135448; 13 peptides matched; 33.3% peptide coverage; mean error = 0.70 ppm), PRMT1 (GI:2499803; 16 peptides matched; 55.6% peptide coverage; mean error = -4.21 ppm), and splicing factor SF2-associated protein p32 (SF2A-p32) (GI:4502491; 8 peptides matched; 50.7% peptide coverage; mean error = -5.08 ppm) (Fig. 2). These proteins were also identified by nano-LC-MS/MS analysis of the isolated FIB-associated RNPs without...
protein complexes associated with each of the truncated proteins expressed in the 293EBNA cells were purified by immunoprecipitation with anti-FLAG-conjugated beads. Silver staining of an SDS-PAGE gel showed that a number of proteins were present in each of the three immunoprecipitates, and many of the bands were common among the three isolated protein complexes as well as in the full-length FIB-associated complex. Still, some protein bands were present uniquely in each of the complexes (Fig. 3A). When the immunoprecipitates were treated with RNase A, only SF2A-p32 remained associated with in the FIB I- and FIB II-associated complexes, whereas only PRMT5 remained associated with the FIB III-associated complexes (Fig. 3A). This result showed that SF2A-p32 interacts directly with the FIB GAR domain and spacer region 1 and that PRMT5 interacts with the methyltransferase-like domain (Fig. 1). Although PRMT5 was also detected in a mock immunoprecipitate prepared from control cells, its level was clearly elevated upon expression of FIB III. Despite the binding of SF2A-32p and PRMT5 to the corresponding truncated proteins, the binding of PRMT1 and other proteins associated with full-length FIB was not detected.

Two additional FIB truncation mutant expression vectors were constructed. FIB IV contained sequences encoded by FIB I and II, whereas FIB V contained FIB II and III sequences (Fig. 1). Both FIB IV and FIB V were expressed in 293EBNA cells, although FIB IV suffered some degradation that was most likely due to proteolytic cleavage from the C terminus (Supplementary Fig. S2). Immunoisolation using anti-FIB showed that these two truncated mutants were also associated with a number of proteins, most of which became dissociated upon RNase A treatment as was the case for the other truncation mutants (Fig. 3B). As expected from the FIB I and FIB II results, FIB IV associated with only SF2A-p32. On the other hand, in addition to SF2A-p32, FIB V also associated with tubulin α3 and β1 as well as several other proteins, including one with the molecular mass of 48 kDa (FIB-48; Fig. 3B). PRMT5 and PP2C were detected more strongly in the control (Fig. 3B) than in previous experiments (compare with Fig. 3A) due to the use of different lots of anti-FLAG M2 affinity gel (purchased from Sigma-Aldrich Chemical, Steinheim, Germany). However, densitometry analysis indicated that PRMT5 was clearly increased in FIB V-associated protein complexes (and full-length FIB-associated complexes) compared with the control; i.e., the staining intensities of PRMT5 after RNase treatment were 19,149 (arbitrary values) for full-length FIB, 12,776 for FIB IV, 20,046 for FIB V, and 7,634 for the control (Fig. 3B). These results support the assertion that the methyltransferase-like domain corresponding to the FIB III mutant is responsible for the binding of FIB to PRMT5. These results also indicate that the presence of both the spacer region 1 and the methyltransferase-like domain is required for the binding of FIB to tubulin α3 and β1 as well as FIB-48. With respect to the association of FIB with PRMT1, none of the five FIB truncation mutants bound this protein indicating that its binding is dependent on the domain structure of full-length FIB and/or that the binding of the other proteins (e.g., SF2A-p32 and PRMT5) is a prerequisite to PRMT1 binding. Together, these results suggest that FIB along with SF2A-p32, PRMT5, tubulins α3 and β1, FIB-48, and PRMT1 constitute a subcomplex that functions as an integrated module.

Fractionation of FIB-associated RNP Complexes by Ultracentrifugation—The above results did not address whether the subcomplex that resulted from RNase A treatment constituted a protein complex that was independent of FIB-associated RNPs. Therefore, the immunoisolated FIB-associated complexes were fractionated by ultracentrifugation through a su-
The FIB GAR domain (corresponding to truncation mutant FIB I) and the spacer region 1 (FIB II), whereas PRMT5 binds the methyltransferase-like domain (FIB III) (Fig. 3). Given that FIB V, but not FIB III, associated with tubulin α3, tubulin β1, and FIBb-48kD protein (Fig. 3), both the methyltransferase-like domain and the spacer region 1 are necessary for the association of these proteins with FIB. However, the methyltransferase-like domain and the spacer region 1 were still not sufficient to recruit PRMT1. These results indicate that, in addition to these regions, the FIB GAR domain is required for the association with PRMT1. However, the data do not address whether PRMT1 binding is dependent on the recruitment of SF2A-p32, PRMT5, and the other proteins to FIB. Because these FIB-associated proteins fractionated as a much smaller complex relative to the FIB-associated pre-rRNPs that likely represent a mixture of 40 S and 60S/80S preribosomal particles (Fig. 4), these results indicate that FIB and these associated proteins constitute a sub-complex that is independent of the larger FIB-associated pre-rRNPs.

The association of FIB with two protein arginine methyltransferases is very intriguing, because FIB itself is an RNA methyltransferase. Thus, the association provides for the possible coordinated regulation of RNA and protein methylation events such as those in which both the RNA and protein molecules are involved. Two types of arginine methyltransferases were identified as FIB-associated proteins. First, PRMT5 (JBP1) is a type II methyltransferase that catalyzes the formation of N\(^{\text{G}}\),N\(^{\text{G}}\)-dimethylarginine (35). Thus, given that FIB is involved in ribosome biogenesis and is itself an RNA methyltransferase, our results provide biochemical evidence that these three different types of methyltransferases physically associate and therefore may function in concert. Given that, during ribosome biogenesis, not only are pre-rRNAs methylated but several nucleolar and ribosomal proteins as well (37–39), three types of methylation reactions may be required to coordinate certain stages of ribosome biogenesis and/or related processes.

FIB is known to interact with SMN protein (19), and we found that FIB interacts with PRMT5 (JBP1). Because SMN...
in FIB. Another intriguing finding of this study is that FIB also interacts with SF2A-p32, which regulates RNA splicing by inhibiting the binding of splicing factor ASF/SF2 to pre-mRNA and the phosphorylation of ASF/SF2 (41). Because it may compete with ASF/SF2 for SF2A-p32 binding, mRNA splicing may also be regulated by FIB.

In addition to SF2A-p32 and PRMTs, tubulins α3 and β1 were also identified in the FIB-associated sub-complex. These microtubule subunits are heterodimers composed of one polypeptide each of α- and β-tubulin. Therefore, their presence in the sub-complex may provide a link to components of the microtubule cytoskeleton that consists of a highly organized network of microtubule polymers bound to accessory proteins, including microtubule-associated proteins, molecular motors, and microtubule-organizing proteins. Although no other components of microtubule cytoskeleton were found, the tubulins may be involved in transporting the FIB-associated sub-complex among subnuclear domains, including the nucleolus, mRNA, nucleolus, and Cajal bodies.

Despite the preferable subcellular localization of FIB in Cajal bodies, its role in Cajal bodies is completely unknown. However, our results provide some clues on the possible roles of FIB in sn or snoRNP biogenesis and pre-mRNA processing in Cajal bodies. In addition, our finding that FIB associates with protein-arginine methyltransferases is also consistent with the recent report that symmetrical dimethylation of arginine is required to localize SMN to Cajal bodies as well as for its involvement in pre-mRNA splicing (42). FIB may function as a transporter of protein-arginine methyltransferases to Cajal bodies and/or as a scaffold to perform coordinated methylation of both RNA and protein substrates during RNA editing in Cajal bodies as well as in ribosomogenesis in the nucleolus.

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