The pre-mRNA-splicing factor SF3a66 functions as a microtubule-binding and -bundling protein

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SF3a (splicing factor 3a) complex is an essential component of U2 snRNPs (small nuclear ribonucleoprotein particles), which are involved in pre-mRNA splicing. This complex consists of three subunits: SF3a60, SF3a66 and SF3a120. Here, we report a possible non-canonical function of a well-characterized RNA-splicing factor, SF3a66. Ectopic expression experiments using each SF3a subunit in N1E 115 neuroblastoma cells reveals that SF3a66 alone can induce neurite extension, suggesting that SF3a66 functions in the regulation of cell morphology. A screen for proteins that bind to SF3a66 clarifies that SF3a66 binds to β -tubulin, and also to microtubules, with high affinity, indicating

that SF3a66 is a novel MAP (microtubule-associated protein). Electron microscopy experiments show that SF3a66 can bundle microtubules, and that bundling of microtubules is due to cross-bridging of microtubules by high-molecular-mass complexes of oligomerized SF3a66. These results indicate that SF3a66 is likely to be a novel MAP, and can function as a microtubule-bundling protein independently of RNA splicing.

Key words: microtubule-associated protein (MAP), neurite extension, RNA splicing, SF3a complex.

INTRODUCTION

Genes in eukaryotes are often interrupted by intervening sequences (introns) that must be removed during gene expression. Pre-mRNA splicing is a multi-step pathway by which introns are removed precisely, and functional exons are joined together [1,2]. The snRNPs (small nuclear ribonucleoprotein particles) are essential for the formation of an active spliceosome, which mediates intron excision. The snRNPs play major roles in the accuracy and specificity of intron removal by recognizing conserved sequence elements in the pre-mRNA. The snRNP consists of snRNAs and a number of common and unique proteins. Seven common proteins (Sm proteins) bind to the Sm-binding sites in U1, U2, U4 and U5 snRNAs. The Lsm proteins bind to U6 snRNA. All snRNPs also contain unique proteins, some of which have essential roles in RNA splicing [3–6].

SF3a (splicing factor 3a) complex is one of the unique proteins in the U2 snRNP [7,8]. SF3a complex is incorporated into immature 15 S U2 snRNP in the presence of SF3b to generate the mature active 17 S U2 snRNP [7], which is essential for spliceosome assembly. SF3a complex consists of three subunits: SF3a60, SF3a66 and SF3a120 [9], and these proteins have all been identified as spliceosome-associated proteins [10–12]. In *Saccharomyces cerevisiae*, genes corresponding to all subunits have been identified, and genetic analysis has revealed that Prp9p, Prp11p and Prp21p (the yeast homologues of SF3a60, SF3a66 and SF3a120 respectively) are essential for RNA splicing [13–18]. SF3a60 and SF3a66 interact indirectly via SF3a120, generating the SF3a heterotrimer [16,18,19]. Similarly, Prp9p, Prp11p and Prp21p form a heterotrimer, where Prp9p and Prp11p interact via Prp21 [20–22]. These findings indicate that both the structural features of the SF3a subunits and the manner in which they interact

are conserved from yeasts to mammals, emphasizing the crucial role of SF3a subunits in pre-mRNA splicing.

To date, there have been no reports of functions of the SF3a complex in addition to RNA splicing. During a screen for proteins that bind to IRSp53 (insulin receptor tyrosine kinase substrate p53), an adaptor protein that links Rho family small GTPases to rearrangements of the actin cytoskeleton [23–26], we fortuitously identified SF3a subunits as binding proteins of IRSp53. These results suggested that the SF3a complex has functions in addition to its role in the formation of spliceosome and splicing. It was recently reported that the Sm proteins, the common proteins of snRNPs [5,6], also regulate localization of germ granules, called P granules, to germ cell precursors during early development of *Caenorhabditis elegans* independently of RNA splicing [27]. P granules are large cytoplasmic particles that contain some component proteins and a low level of RNA [28]. P granules are segregated to germ cell precursor cells during embryogenesis by asymmetric cell division, and are associated with the nuclear envelope after the 16-cell stage [29]. Barbee et al. [27] reported that some Sm proteins localize to P granules and that disruption of SmE or a combination of Sm proteins (SmB + D3 or SmF + E + G or SmD1 or SmD2), but not other splicing factors, causes defects in localization of P granules to germ cell precursors, and later in nuclear association. Although the molecular mechanism underlying this function is not well known, these findings raise the possibility that general mRNA-splicing factors, such as components of snRNPs, which are traditionally thought to function only in the control of RNA splicing, could have functions besides RNA splicing activity, supporting the idea that the SF3a complex also has a novel function independent of pre-mRNA splicing.

In the present study, we found that one of the SF3a subunits, SF3a66, binds to microtubules and can bundle microtubules

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GST, glutathione S-transferase; His₆-, hexahistidine-tagged; IRSp53, insulin receptor tyrosine kinase substrate p53; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MAP, microtubule-associated protein; SF3a, splicing factor 3a; snRNP, small nuclear ribonucleoprotein particle; sulpho-EGS, ethylene glycol bis(sulphosuccinimidyl succinate).

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in vitro, suggesting a non-canonical function of RNA-splicing factors in mammals.

EXPERIMENTAL

Protein expression and antibody production

Hexahistidine-tagged (His₆-)SF3a60, His₆-SF3a66 and His₆-SF3a120, and GST (glutathione S-transferase)-tagged SF3a60 and GST-SF3a66, were expressed with a baculovirus system. Rabbit polyclonal antibodies against SF3a66 were prepared by immunization with the His₆-SF3a66.

Pull-down assay and identification of binding proteins

N1E 115 cells were ³⁵S-labelled and lysed with ice-cold lysis buffer [40 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/0.2 mM PMSF]. The lysate was incubated with GST fusion proteins immobilized on glutathione beads (Amersham Biosciences) at 4 °C. The beads were washed three times with lysis buffer, and the binding proteins were eluted with SDS sample buffer and subjected to SDS/PAGE. Bands were visualized by autoradiography.

To identify the proteins that bind to SF3a66 in N1E 115 cells, N1E 115 cell lysate was prepared in large batches, and incubated with GST-SF3a66N2 at 4 °C. The binding proteins were then subjected to SDS/PAGE. The band of interest was excised from the gel and digested with trypsin. The digested peptide fragments were analysed by LC-MS/MS (liquid chromatography-tandem mass spectrometry) analysis (Q-ToF2; Micromass, Altrincham, Cheshire, U.K.).

Transfection and immunocytochemistry

N1E 115 cells were maintained in 10 % (v/v) FBS (fetal bovine serum)/DMEM (Dulbecco's modified Eagle's medium). For transfection, cells were seeded on to coverslips in 3.5 cm dishes 1 day before transfection. N1E 115 cells were transfected with each construct with LIPOFECTAMINE™ 2000 (Invitrogen), according to the manufacturer's protocol. All constructs except SF3a120 were subcloned into pEF-BOS vectors. SF3a120 was subcloned into pME vector. At 24 h after transfection, cells were fixed with 3.7 % formaldehyde for 15 min and permeabilized with 0.2 % Triton X-100 in PBS for 5 min. Cells were then incubated with anti-FLAG polyclonal antibody (Sigma). The secondary antibody used for detection was Alexa-488-conjugated rabbit IgG (Molecular Probes). F-actin was stained with rhodamine phalloidin (Molecular Probes).

Purification of tubulin and microtubule-binding assay

Purified tubulin was prepared from pig brains with two cycles of temperature-dependent polymerization/depolymerization. Briefly, fresh pig brains were homogenized in Buffer A [100 mM Pipes/KOH (pH 6.8)/2 mM EGTA/1 mM MgCl₂] containing 0.5 mM GTP and ultracentrifuged at 4 °C for 1 h. Soluble tubulin was incubated in Buffer B [100 mM Pipes/KOH, (pH 6.8)/2 mM EGTA/1 mM MgCl₂/8 M glycerol] containing 0.5 mM GTP at 37 °C for 1 h, followed by ultracentrifugation at 37 °C for 1 h. The resulting polymerized tubulin was depolymerized by gentle homogenization in Buffer A containing 0.5 mM GTP on ice, and then ultracentrifuged at 4 °C for 45 min. Soluble tubulin was repolymerized as described above. The polymerized tubulin was depolymerized on ice with Buffer C [20 mM Pipes/KOH (pH 6.8)/0.4 mM EGTA/0.2 mM MgCl₂] containing 0.5 mM

GTP. The purified depolymerized tubulin was purified further on a phosphocellulose column (P11; Whatman) to deplete MAPs (microtubule-associated proteins).

Microtubules were formed in Buffer A containing 1 mM GTP, followed by stabilization with 40 µM Taxol (Sigma). Purified His₆-SF3a66 was added to a constant concentration of Taxol-stabilized microtubules (2 µM) and incubated at room temperature for 1 h. Microtubules and bound SF3a66 were separated from the solution by ultracentrifugation (30 000 g for 10 min at 25 °C). Samples were subjected to SDS/PAGE and analysed by Coomassie Blue staining.

Microtubule bundling assay

Purified tubulin was labelled with 5-(and-6)-carboxytetramethyl rhodamine succinimidyl ester (Molecular Probes), as described previously [30]. Labelled and unlabelled tubulins were mixed (combined concentration 13 µM) and polymerized at 37 °C in polymerization buffer [80 mM Pipes/KOH (pH 6.8)/1 mM MgCl₂/0.3 mM EGTA/1 mM GTP/10 % (v/v) DMSO] for at least 30 min. The polymerized microtubules were stabilized with 40 µM Taxol at room temperature. These microtubules (final concentration 8 µM) were then incubated with His₆-SF3a66 (final concentration 1.8 µM) or His₆-SF3a66 dialysis buffer [100 mM Pipes/KOH (pH 6.8)/2 mM EGTA/1 mM MgCl₂/150 mM NaCl] at 37 °C for 30 min. One volume of the incubated mixture was diluted in 10 volumes of dilution buffer [100 mM Pipes/KOH (pH 6.8)/2 mM EGTA/1 mM MgCl₂/40 µM Taxol] and deposited on a polylysine-coated (100 µg/ml) coverslip. The morphology of microtubules was observed with a confocal microscope (Radiance 2000; Bio-Rad).

Electron microscopy

For negative staining, Taxol-stabilized microtubules and His₆-SF3a66 were incubated for 30 min for 37 °C. Diluted sample solutions were mounted on Formvar-coated copper grids, fixed with 1 % (v/v) glutaraldehyde in Buffer A and then stained with 1 % (w/v) uranyl acetate. Observation was performed with a JEOL JEM-2000EX electron microscope.

Chemical cross-linking and gel-filtration analysis

Purified His₆-SF3a66 was re-dialysed in PBS and subjected to ultracentrifugation (200 000 g at 4 °C for 10 min) to remove debris. His₆-SF3a66 (4.5 µM) was incubated with a cross-linking reagent, sulpho-EGS [ethylene glycol bis(sulphosuccinimidylsuccinate); Pierce] in PBS at room temperature for 20 min. The reaction mixtures were stopped by adding an excess amount of Tris/HCl, pH 7.5 (final concentration 100 mM), before subjecting to SDS/PAGE followed by Western blot analysis with anti-SF3a66 antibody. For gel-filtration analysis, His₆-SF3a66 was separated on a Superdex 200 HR column (Pharmacia) equipped with an HPLC apparatus (PU-2080; JASCO), followed by Western blot analysis with anti-SF3a66 antibody.

Immunoprecipitation analysis

COS7 cells were maintained in 10 % FBS/DMEM and seeded in 10 cm dishes on the day before transfection. Cells were transfected with LIPOFECTAMINE (Invitrogen) with pEF-BOS-FLAG-tagged SF3a66 and pEF-BOS-Myc-tagged SF3a66, according to the manufacturer's protocol. One day after transfection, cells were harvested and lysed with lysis buffer [40 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/0.2 mM PMSF].

The lysate was incubated with Protein G beads (Pierce) for 1 h to remove the non-specific binding proteins. Beads were separated by centrifugation from the supernatant, and the pre-cleared lysate was incubated with anti-FLAG antibody (M2; Sigma) for 2 h at 4 °C. Protein G beads were added to the lysate and incubated for 2 h. The immunoprecipitates were washed with lysis buffer three times and subjected to Western blot analysis with anti-Myc antibody (9E10; Santa Cruz) and anti-FLAG antibody.

RESULTS

SF3a66 induces neurite extension by itself

To investigate a novel function of the SF3a complex, we examined the effect of ectopic expression of three SF3a subunits in N1E 115 cells. FLAG-tagged SF3a60, SF3a66 and SF3a120 were overexpressed in N1E 115 cells, and the morphologies of cells expressing these proteins were visualized by phalloidin staining. Untransfected control cells showed a round shape. In contrast, cells overexpressing SF3a66, but not SF3a60 or SF3a120, were flattened, and some had extended neurites (Figure 1A). These results suggested that SF3a66 might be involved in regulation of cell morphology by itself. Thus we investigated SF3a66 in greater detail. We next examined which region of SF3a66 is important for neurite extension. The N-terminal region of SF3a66 contains a zinc-finger motif that is necessary for association with 15 S U2 snRNP [31], and a proline-rich motif is present in the C-terminus [16]. The deletion mutants shown in Figure 1(B) were overexpressed in N1E 115 cells, and the cell morphology was observed. N1E 115 cells expressing the N-terminal region of SF3a66 (shown as 'N' in the Figure) and the latter half of the N-terminal region of SF3a66 (shown as 'N2' in the Figure) had extended neurites, as found for cells expressing wild-type SF3a66. In contrast, overexpression of the C-terminal region of SF3a66 ('C') or the former half of the N-terminal region of SF3a66 ('N1') did not induce neurite formation, although some N1 transfectants were flattened. These results indicated that the latter half of the N-terminal region of SF3a66 (N2) is sufficient for neurite formation in N1E 115 cells (Figure 1C). We next investigated proteins that interact with SF3a66 through its N2 region, which might be responsible for neurite formation in N1E 115 cells.

Identification of β -tubulin as an SF3a66-associated protein which may be responsible for neurite extension

To screen proteins that bind to SF3a66, we used GST fusion proteins corresponding to the deletion mutants of SF3a66 shown in Figure 1(B) as probes for pull-down assays. 35 S-labelled N1E 115 lysate was incubated with these probes (Figure 2A, left panel), and the binding proteins to these probes were separated by SDS/PAGE and visualized by autoradiography. As shown in Figure 2(A), when we used GST-SF3a66 as a probe, we detected a major band with a molecular mass of approx. 50 kDa (shown by the arrow, right panel). This band was also detected when GST-SF3a66N and GST-SF3a66N2 were used as probes. All of the probes that yielded this 50 kDa band contained the N2 region, which could induce neurite extension when expressed in N1E 115 cells.

These results strongly suggested that this 50 kDa binding protein plays an important role in neurite extension in N1E 115 cells. To identify this protein, N1E 115 cell lysate was prepared on a large scale and subjected to pull-down assay, and the corresponding band was excised from the gel and digested with trypsin. The digested peptide fragments were subjected to LC-MS/MS

analysis, and we identified this protein as β -tubulin (results not shown).

SF3a66 interacts with SF3a120 directly and with SF3a60 indirectly, thus generating the SF3a heterotrimer. Because it was reported that the N-terminal region of SF3a66 (which overlaps with the N2 region) binds to SF3a120 to form the SF3a complex [31], we next examined the effects of SF3a120 and SF3a60 on the association of SF3a66 with β -tubulin by pull-down assay. GST-SF3a66 was incubated with 35 S-labelled N1E 115 cell lysate in the presence or absence of His₆-SF3a120 and His₆-SF3a60, and protein binding was analysed (Figure 2B, right panel). We detected the 50 kDa band, which was identified as β -tubulin (shown by the arrow in Figure 2B, right panel), in the absence of SF3a120 or SF3a120 plus SF3a60, indicating that only free SF3a66 could bind to β -tubulin. In addition, when we used GST-SF3a60 instead of SF3a66 as a probe, we did not detect this band (Figure 2B). These results strongly suggested that the binding ability of SF3a66 to β -tubulin is independent of the RNA-splicing function, and that SF3a66 plays an important role in neurite extension in N1E 115 cells though association with β -tubulin.

SF3a66 is a novel MAP

α/β -Tubulin heterodimers polymerize to form microtubules. These microtubules are a major cytoskeletal component of growing neurites. The observation that SF3a66 could induce neurite formation in N1E 115 cells, and the identification of β -tubulin as a binding protein for SF3a66, suggested that SF3a66 binds to microtubules, leading us to investigate the ability of SF3a66 to bind to microtubules by co-sedimentation assays. Tubulin was extracted from pig brains by two cycles of polymerization and depolymerization, followed by purification on a phosphocellulose column to remove MAPs. Purified tubulin (Figure 3A, left) was polymerized and stabilized with Taxol to form Taxol-stabilized microtubules. The Taxol-stabilized microtubules were incubated with purified His₆-SF3a66, as shown in Figure 3(A) (right panel), and the mixture was separated by ultracentrifugation into supernatant and pellet, which contained the microtubules. Addition of microtubules to His₆-SF3a66 resulted in incorporation of SF3a66 into the pellet (Figure 3B). In contrast, His₆-SF3a66 incubated under the same conditions, but without microtubules, was present exclusively in the supernatant, indicating that His₆-SF3a66 binds to microtubules. Scatchard plot analysis with a constant amount of microtubules and increasing amounts of His₆-SF3a66 revealed that the binding affinity (K_d) is approx. 0.4–0.6 μ M (results not shown). Because the K_d values of tau and MAP2, two major MAPs, were reported to be approx. 1.0 μ M and 5–25 μ M respectively [32,33], these results suggest that SF3a66 binds to microtubules with a higher affinity than that of other MAPs.

SF3a66 bundles microtubules *in vitro*

Many MAPs have been reported to have effects on microtubule dynamics, including promoting polymerization, stabilizing microtubules and bundling microtubules. Because SF3a66 is a novel MAP, we next examined whether SF3a66 had any effect on microtubules. Rhodamine-labelled tubulin was polymerized and then stabilized with Taxol. The Taxol-stabilized, rhodamine-labelled microtubules were incubated with or without purified His₆-SF3a66, and the morphology of the microtubules was observed under a microscope. As shown in Figure 3(C), many microtubules gathered in the presence of His₆-SF3a66, whereas this was observed only rarely in the absence of His₆-SF3a66.

Figure 1 SF3a66 induces neurite extension in N1E 115 cells

(A) N1E 115 cells were transfected with FLAG-tagged SF3a60, SF3a66, or SF3a120. At 24 h after transfection, cells were fixed and stained with anti-FLAG antibody, and the morphologies of the transfectants were observed with a confocal microscope. Cell morphologies were classified as three types: round, flat and neurite extension. Cells with neurites that were longer than the cell bodies were classified as having neurite extension. The results shown in the right panel are the means of six independent experiments. In each experiment, at least 60 transfectants were counted. Error bars indicate the S.D. $^{**}P < 0.01$ (Student's *t* test, compared with non-transfected cells). (B) Schematic representation of FLAG-tagged SF3a66 deletion mutants. a.a., amino acids; WT, wild-type; N, N1, N2 and C refer to different regions of SF3a66. (C) N1E 115 cells were transfected with FLAG-tagged wild-type or the various deletion mutants of SF3a66 shown in (B). Cell morphology of each transfectant was observed, and the proportion of cells with each cell morphology was classified as in (A). Results are the means of four independent experiments in which at least 100 transfectants were counted. Error bars are shown as S.D. $^{*}P < 0.03$; $^{**}P < 0.01$ (Student's *t* test compared with non-transfected cells).

To analyse the morphology of gathered microtubules in greater detail, we employed electron microscopy. Microtubules were incubated with or without His₆-SF3a66, and samples were stained with uranyl acetate. Electron microscopic observation of the negatively stained samples revealed that microtubules were bundled in the presence of His₆-SF3a66 (Figure 4A, middle

panel). Furthermore, 'cloud-like' objects (shown by the arrows in Figure 4A) were observed along the bundled microtubules. Higher magnification revealed structures that resembled ends of threads between the bundled microtubules and appeared to cross-bridge them (shown by the arrows in Figure 4B, middle panel). These structures were also observed with His₆-SF3a66 alone (arrows

Figure 2 Identification of β -tubulin as an SF3a66-binding protein

(A) ³⁵S-labelled N1E 115 cell lysate was subjected to pull-down assays with various GST fusion deletion mutants of SF3a66 (see the legend to Figure 1B for further details), which are shown in the Coomassie staining. The arrow in the right-hand panel indicates the bands thought to be β -tubulin. (B) ³⁵S-labelled N1E 115 cell lysate and GST-SF3a66 were incubated with or without His₆-SF3a60 and His₆-SF3a120. GST-SF3a60 was also incubated with ³⁵S-labelled N1E 115 cell lysate with or without His₆-SF3a66 and His₆-SF3a120. The band that corresponds to β -tubulin (indicated by the arrow) was visible only when N1E 115 cell lysate was incubated with GST-SF3a66 alone. Coomassie Blue staining as a quality and loading control is shown on the left.

in Figure 4B, right panel). These results suggested that SF3a66 could form homo-oligomers to generate a high-molecular-mass complex that might be visible at higher magnifications.

SF3a66 oligomerizes *in vivo* and *in vitro*

To determine whether SF3a66 can form an oligomer and exist as a high-molecular-mass complex, we examined the molecular mass of purified His₆-SF3a66. Purified His₆-SF3a66 was ultra-centrifuged, and the supernatant was loaded on to a Superdex column for separation by molecular mass. His₆-SF3a66 was eluted in higher-molecular-mass fractions in addition to the monomer fractions, indicating that purified recombinant SF3a66 can also exist as an oligomer (Figure 5A). Next, we treated purified His₆-SF3a66 with a chemical cross-linking reagent, sulpho-EGS, to cross-link His₆-SF3a66. His₆-SF3a66 treated either with or

Figure 3 SF3a66 is a microtubule binding and bundling protein

(A) Tubulin was extracted from pig brains and purified on a phosphocellulose column. His₆-SF3a66 was expressed in a baculovirus system and purified with Ni²⁺-agarose beads. The purities of these proteins for *in vitro* assay was confirmed by Coomassie blue staining. (B) His₆-SF3a66 (1 μ M) was incubated with or without 2 μ M Taxol-stabilized microtubules (MT; the concentration represents that of α/β -tubulin heterodimer used for microtubule formation) for 1 h. The mixture was ultracentrifuged and separated into supernatant (S) and pellet fraction (P). (C) Rhodamine-labelled tubulin was polymerized and stabilized with Taxol. Labelled microtubules were mixed with His₆-SF3a66 or dialysis buffer (for the negative control) and incubated for 30 min at 37 °C. The reaction mixtures were diluted and deposited on coverslips and observed with a confocal microscope. 'Pre-treat' indicates sample containing labelled microtubules without His₆-SF3a66 or dialysis buffer. Scale bars indicate 20 μ m in the upper panels, and 4 μ m in the lower panels.

without sulpho-EGS was separated by SDS/PAGE and subjected to Western blot analysis with anti-SF3a66 antibody. As shown in Figure 5(B), although a large proportion of SF3a66 existed as a monomer, cross-linked SF3a66 was present primarily as a trimer, with a molecular mass of approx. 200 kDa. The results of the chemical cross-linking assay appear to conflict with the results of the gel-filtration analysis, which showed that SF3a66 formed an oligomer with a molecular mass of more than 660 kDa. However, it is possible that SF3a66 initially oligomerizes as a trimer, which may be susceptible to cross-linking, and these trimers then assemble loosely to form a high-molecular-mass complex. In this case, each SF3a66 trimer in a high-molecular-mass complex might not be cross-linked.

Figure 4 Electron microscopy of SF3a66-induced bundled microtubules

Negatively stained electron microscopy samples of Taxol-stabilized microtubules incubated with or without SF3a66. His₆-SF3a66 alone was also observed. The scale bar represents 500 nm in (A) and 125 nm in (B). Arrows in (A) indicate the 'cloud-like' structures, and arrows in (B) indicate structures that look like the ends of threads and may be composed of SF3a66. MT, microtubules.

Lastly, we examined whether SF3a66 oligomerizes *in vivo*. Myc-tagged SF3a66 was transfected into COS7 cells with or without FLAG-tagged SF3a66, and the cell lysate was immunoprecipitated with anti-FLAG antibody, followed by Western blot analysis. FLAG-tagged SF3a66 and Myc-tagged SF3a66 were co-immunoprecipitated, indicating that SF3a66 forms oligomers *in vivo* (Figure 5C).

DISCUSSION

In the present study, we have shown that SF3a66, an essential component of U2 snRNP, binds to β -tubulin and microtubules. The estimated affinity of SF3a66 for microtubules is higher than that of conventional MAPs such as tau and MAP2 [32,33], suggesting that SF3a66 is a novel MAP.

Although conventional MAPs, such as MAP2, MAP4, and tau, have three to four repeated tubulin-binding motifs that consist of 18 weakly conserved core amino acids [34,35], SF3a66 does not contain such tubulin-binding motifs, suggesting that an unidentified sequence in the N2 region, which binds to β -tubulin, is responsible for tubulin binding. This suggests that SF3a66 is a MAP that is uniquely different from other MAPs in terms of conservation of the tubulin-binding domain. Tau and MAP2 have been reported to cross-bridge and bundle microtubules *in vitro* and

in vivo [36–41]. In the present study, *in vitro* analyses revealed that SF3a66 also has microtubule-bundling activity. Electron microscopic analysis suggested that SF3a66 oligomerizes to form a high-molecular-mass complex between microtubules, resulting in cross-bridging of microtubules *in vitro*. In contrast, tau forms straight, short and 'arm-like' projections, which line up on the surface of microtubules in a periodic manner and cross-bridge them [36]. This indicates that there are some qualitative differences between SF3a66 and tau in the mechanism of microtubule bundling, suggesting that SF3a66 has a role distinct from that of tau in microtubule bundling *in vivo*. In contrast with tau expression, which does not induce neurite extension in N1E 115 cells [42], ectopic expression of SF3a66 in N1E 115 cells induced neurite extension. This result suggests that the function of SF3a66 differs from that of tau with respect to the regulation of microtubule arrangement in N1E 115 cells.

SF3a66 is thought to localize and function in the nucleus, which is problematic in terms of its function as a MAP, which would require localization in the cytoplasm. The well-characterized physiological function of SF3a66 is RNA splicing. A yeast mutant, with a mutation in the gene encoding Prp11p (the homologue of SF3a66), accumulates abnormally spliced pre-mRNAs [13]. Reconstitution experiments with recombinant SF3a66 protein revealed that SF3a66 is essential for the formation of spliceosomes [31]. SF3a66 was also reported to be present in

Figure 5 SF3a66 oligomerizes and is present as a high-molecular-mass complex

(A) Gel-filtration analysis of His₆-SF3a66. His₆-SF3a66 was loaded on to a Superdex 200 HR column and separated by HPLC. The eluted fractions were collected and subjected to Western blot analysis with anti-SF3a66 antibody to determine which fraction contained SF3a66. Thyroglobulin (660 kDa), aldolase (158 kDa), and ovalbumin (43 kDa) were used as molecular-mass markers, and their positions are shown by the upwards-pointing arrows. (B) Chemical cross-linking of His₆-SF3a66. His₆-SF3a66 was incubated with a chemical cross-linking reagent, sulpho-EGS, and the products were analysed by Western blotting with anti-SF3a66 antibody. (C) COS7 cells were transfected with Myc-tagged SF3a66 plus FLAG-tagged SF3a66 or Myc-tagged SF3a66 plus empty vector. Cell lysate was harvested and immunoprecipitated with anti-FLAG antibody followed by Western blot analysis.

the nucleus (nuclear speckles), and to co-localize with splicing factors [43]. These findings all show that SF3a66 functions as an RNA-splicing factor in the nucleus. Here, considering our finding that SF3a66 can function as a MAP, we will discuss three possible ways by which SF3a66 could interact with microtubules or tubulin topologically, and the physiological functions of SF3a66 in each case. Because microtubules are located in the cytoplasm, the first possibility is that SF3a66 also localizes in the cytoplasm in addition to the nucleus, and regulates microtubule dynamics *in vivo*. To investigate this hypothesis, we have attempted to determine the endogenous localization of SF3a66 in several cell lines, including N1E 115 cells, by generating several antibodies specific for SF3a66. Studies with one of these antibodies revealed signals at the tips of neurites in differentiated N1E 115 cells (results not shown); however, we have not confirmed these results with other anti-SF3a66 antibodies. Schmidt-Zachmann et al. [44] presented data for the localization of SF3a66 in cells. As far as we can judge, a slight signal is detected in the cytoplasm of cells stained with anti-SF3a66 antibody compared with cells

stained with anti-Sm antibody although, as Schmidt-Zachmann et al. [44] described in their paper, SF3a66 is predominantly localized in the nucleus, and they do not mention the slight signal. Considering these findings, it is possible that a small amount of SF3a66 localizes in the cytoplasm and functions as a MAP to bundle microtubules.

The second possibility is that SF3a66 functions as a MAP during cell division. Because nuclear proteins are released into the cytoplasm when the nuclear membrane breaks down during mitosis, SF3a66 could move into the cytoplasm and associate with microtubules during mitosis. PRC1, a microtubule-binding and -bundling protein, is localized in the nucleus during interphase, but associates with the spindles during early mitosis and metaphase and the spindle mid-zone during late mitosis [45,46]. It is possible that SF3a66 acts in a manner similar to PRC1, and associates with the mitotic spindle during mitosis and regulates cell cycle progression.

Tubulin was previously thought to exist only in the cytoplasm, but recent studies have shown that β II-tubulin, which is one isotype of β -tubulin, is present in the nuclei of several cell types, including cancer cells [47–49]. Interestingly, nuclear β II-tubulin is reported to exist only as α/β heterodimers and not in microtubule networks [48]. Though the function of β II-tubulin in the nucleus is unclear, it is thought that β II-tubulin plays a role in maintaining nuclear shape as part of the nuclear matrix [48]. Nuclear β II-tubulin may also be involved in control of cell proliferation [47]. Thus the third possibility is that SF3a66 in the nucleus may bind to nuclear β II-tubulin and influence its function. Because β II-tubulin is thought to be involved in maintenance of the nuclear shape, SF3a66 may also influence nuclear morphology through an interaction with β II-tubulin. It is also possible that SF3a66 functions as an anchoring protein that traps β II-tubulin in the nucleus.

In conclusion, we have found that the RNA-splicing factor SF3a66 also acts as a microtubule-binding and -bundling protein. This is the first demonstration in mammals that a component of snRNP, which has been thought to function only as an RNA-splicing factor, can function independently of RNA splicing.

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