Neurofibromatosis 2 tumour suppressor schwannomin interacts with βII-spectrin

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NF2 **is the most commonly mutated gene in benign tumours of the human nervous system. The NF2 protein, called schwannomin or merlin, is absent in virtually all schwannomas, and many meningiomas and ependymomas[1–3]. Using the yeast two-hybrid system, we identified βII-spectrin (also known as fodrin) as a schwannomin-binding protein. Interaction occurred between the carboxy-terminal domain of schwannomin isoform 2 and the ankyrin-binding region of βII-spectrin. Isoform 1 of schwannomin, in contrast, interacted weakly with βII-spectrin, presumably because of its strong self-interaction. Thus, alternative splicing of *NF2* may regulate βII-spectrin binding. Schwannomin co-immunoprecipitated with βII-spectrin at physiological concentrations. The two proteins interacted *in vitro* and co-localized in several target tissues and in STS26T cells. Three naturally occurring *NF2* missense mutations showed reduced, but not absent, βII-spectrin binding, suggesting an explanation for the milder phenotypes seen in patients with missense mutations. STS26T cells treated with *NF2* antisense oligonucleotides showed alterations of the actin cytoskeleton. Schwannomin itself lacks the actin binding sites found in ezrin, radixin and moesin, suggesting that signalling to the actin cytoskeleton occurs *via* actin-binding sites on βII-spectrin. Thus, schwannomin is a tumour suppressor directly involved in actin-cytoskeleton organization, which suggests that alterations in the cytoskeleton are an early event in the pathogenesis of some tumour types.**

Schwannomin is structurally similar to the ezrin-radixin-moesin (ERM) family of membrane-organizing proteins that link plasma membrane and cytoskeleton[4,5]. Apart from its role in cell morphogenesis and adhesion[6], there is little knowledge of schwannomin function. Schwannomin interacts with at least five other proteins of unknown identity[7]. Two schwannomin isoforms are generated by alternative splicing of exon 16 and encode proteins that differ in their C-terminus. Isoform 2 is five amino acids shorter than isoform 1 and contains eleven strongly hydrophilic amino acids at its C-terminus[8]. Evolutionary conservation of the isoforms suggests functional significance, but so far, no direct functional differences have been identified.

We used the yeast two-hybrid system[9] to identify proteins that interact with isoform 2. One of the positive clones encoded βII-spectrin (residues 1716 to 1998; ref. 10). Upon re-transformation of individual plasmids, full-length isoform 2 showed strong βII-spectrin binding, and only those parts of the protein that included the C-terminal domain bound βII-spectrin (Fig. 1*a*). Full-length isoform 1 bound βII-spectrin much less efficiently (less than 1% affinity compared with isoform 2; Fig. 1*a*). The

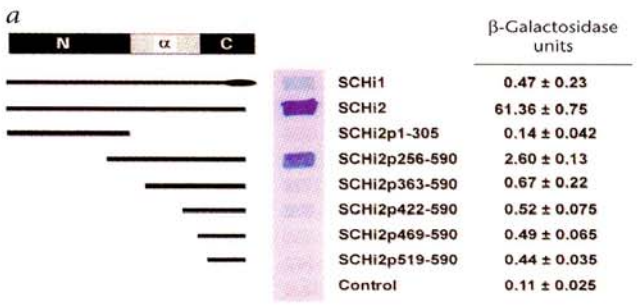

Fig. 1 Yeast two-hybrid tests of interaction. Blue colour indicates the presence of β-galactosidase and a positive test of interaction between encoded proteins. ***a***, Interaction between βII-spectrin encoded by pGAD10Sp and the indicated schwannomin proteins encoded by pGBT9. ***b***, Tests for schwannomin self-interaction. Full-length schwannomin isoform 1 (SCHi1) encoded by pGBT9 interacted strongly with the same protein encoded by pGAD10. No interaction was seen between full-length schwannomin isoform 2 (SCHi2) proteins encoded by pGBT9 and pGAD10, nor between a variety of controls. (AD, GAL4 activation domain; BD, GAL4 binding domain). ***c***, Interaction between parts of βII-spectrin and full-length schwannomin. Semi-quantitative liquid assays for β-galactosidase activity demonstrated that βII-spectrin repeats 15 and 16 are involved in the interaction with schwannomin. The control used pGAD10 without insert. β-galactosidase units[9] = $1000 \times [OD_{420}/(OD_{600} \times \text{time (in min)} \times \text{culture volume (in ml)})]$.

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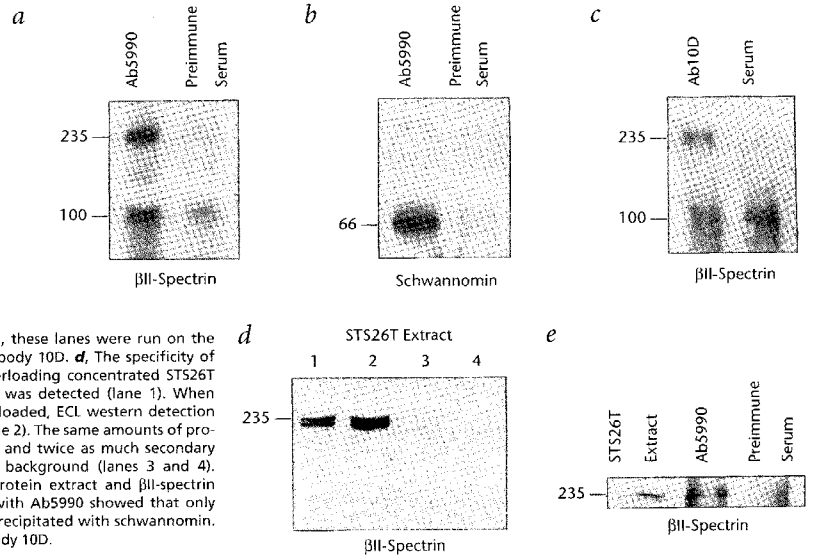

Fig. 2 Co-immunoprecipitation of βII-spectrin and schwannomin. ***a***, A 235-kD band (the size of full-length βII-spectrin) was immunoprecipitated from STS26T cell extracts with schwannomin Ab5990 but not with rabbit preimmune serum. A non-specific band of 100 kD (indicating equal loading of lanes) was also seen. Detection was conducted with antibody 10D. ***b***, When the same immunoprecipitates were detected with Ab5990 but not preimmune serum, a single band of 66 kD (the size of schwannomin), was detected. ***c***, A 235-kD band was immunoprecipitated from STS26T cell extracts with anti-βII-spectrin antibody but not with rabbit serum. To confirm the size of the βII-spectrin band, these lanes were run on the same gel as in *a* and detected with antibody 10D. ***d***, The specificity of antibody 10D was demonstrated by overloading concentrated STS26T extract. A doublet of βII-spectrin bands was detected (lane 1). When three times the amount of protein was loaded, ECL western detection revealed little additional background (lane 2). The same amounts of protein detected without primary antibody and twice as much secondary antibody (1:3000 dilution) revealed no background (lanes 3 and 4). ***e***, Side-by-side comparison of STS26T protein extract and βII-spectrin immunoprecipitated from STS26T cells with Ab5990 showed that only the larger βII-spectrin band co-immunoprecipitated with schwannomin. The detection was conducted with antibody 10D.

shortest C-terminal part of schwannomin which still interacted stronger than controls encoded residues 519–590 (Fig. 1*a*). Binding increased with increasing length, up to the longest C-terminal part tested which encoded residues 256–590. Thus, although regions toward the N-terminal domain do not bind βII-spectrin directly, they appear to stabilize the interaction.

The reduced βII-spectrin binding by schwannomin isoform 1 is likely the result of strong self-binding of isoform 1 (Fig. 1*b*). The addition of a hydrophilic C-terminal domain in isoform 2 prevents such an interaction and renders it available for binding to βII-spectrin. Recently, interaction between N- and C-terminal schwannomin domains has been independently observed[11]. Like ezrin, schwannomin may exist as an elongated dimer and a folded monomer[12].

βII-spectrin belongs to a family of proteins that bind actin, ankyrin and several other cytoskeletal proteins[13,14]. Erythrocyte (βI-) spectrin interacts with protein 4.1 which is related to schwannomin. Despite high homology with βII-spectrin (77.0% similarity and 60.6% identity), erythrocyte spectrin does not interact with schwannomin in the yeast two-hybrid system (D.R.S. *et al.*, unpublished data). Similar to schwannomin, only isoforms of protein 4.1 that contain 21 alternatively-spliced residues can bind erythrocyte spectrin[15].

The cDNA clone identified in the initial screen (encoding βII-spectrin residues 1716–1998) represented close to the minimal part of the coding sequence necessary for binding. Full-length schwannomin isoform 2 interacted with a slightly smaller part of βII-spectrin (residues 1774–1992), but not with residues 1774–1893 or 1885–1992 (Fig. 1*c*). This demonstrates that βII-spectrin repeats 15 and 16 are involved in the interaction with schwannomin.

To demonstrate that the full-length proteins interact *in vivo* at physiological concentrations, we co-immunoprecipitated schwannomin and βII-spectrin from STS26T Schwann-like cells. A band of 235 kD, close to the predicted molecular weight of βII-spectrin, was immunoprecipitated with schwannomin antibody, but not with preimmune serum (Fig. 2*a*). When we analysed the same immunoprecipitates with anti-schwannomin (Ab5990) or

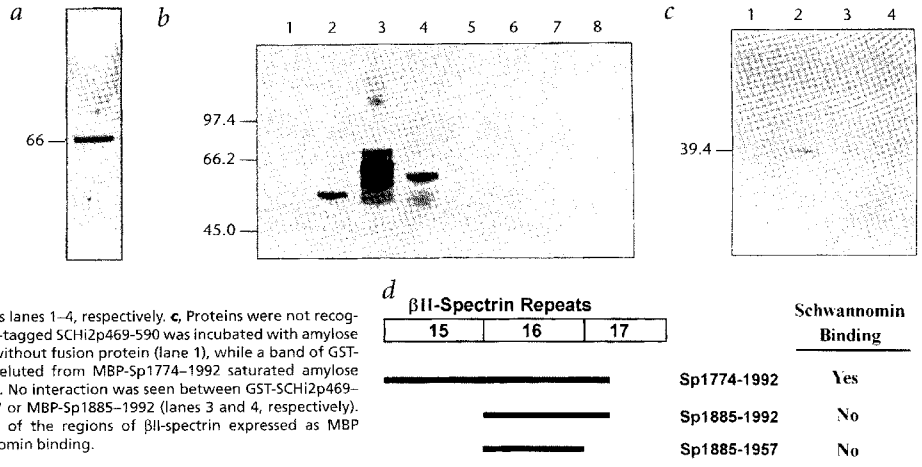

Fig. 3 Interaction *in vitro*. ***a***, Ab5990 specifically detected a single band of 66 kD, the size of schwannomin. ***b***, βII-spectrin antibody did not recognize MBP (lane 1), but recognized the MBP-spectrin fusions MBP-Sp1885–1957 (50.7 kD), MBP-Sp1774–1992 (66.8 kD), and MBP-Sp1885–1992 (54.6 kD; lanes 2–4, respectively). Partial degradation is seen in lanes 3 and 4. As a control, only secondary antibody was used in lanes 5–8, loaded as lanes 1–4, respectively. ***c***, Proteins were not recognized by Ab5990 when GST-tagged SCHi2p469-590 was incubated with amylose resin saturated with MBP without fusion protein (lane 1), while a band of GST-SCHi2p469–590 (39.4 kD) eluted from MBP-Sp1774–1992 saturated amylose resin was detected (lane 2). No interaction was seen between GST-SCHi2p469–590 and MBP-Sp1885–1957 or MBP-Sp1885–1992 (lanes 3 and 4, respectively). ***d***, Graphic representation of the regions of βII-spectrin expressed as MBP fusions tested for schwannomin binding.

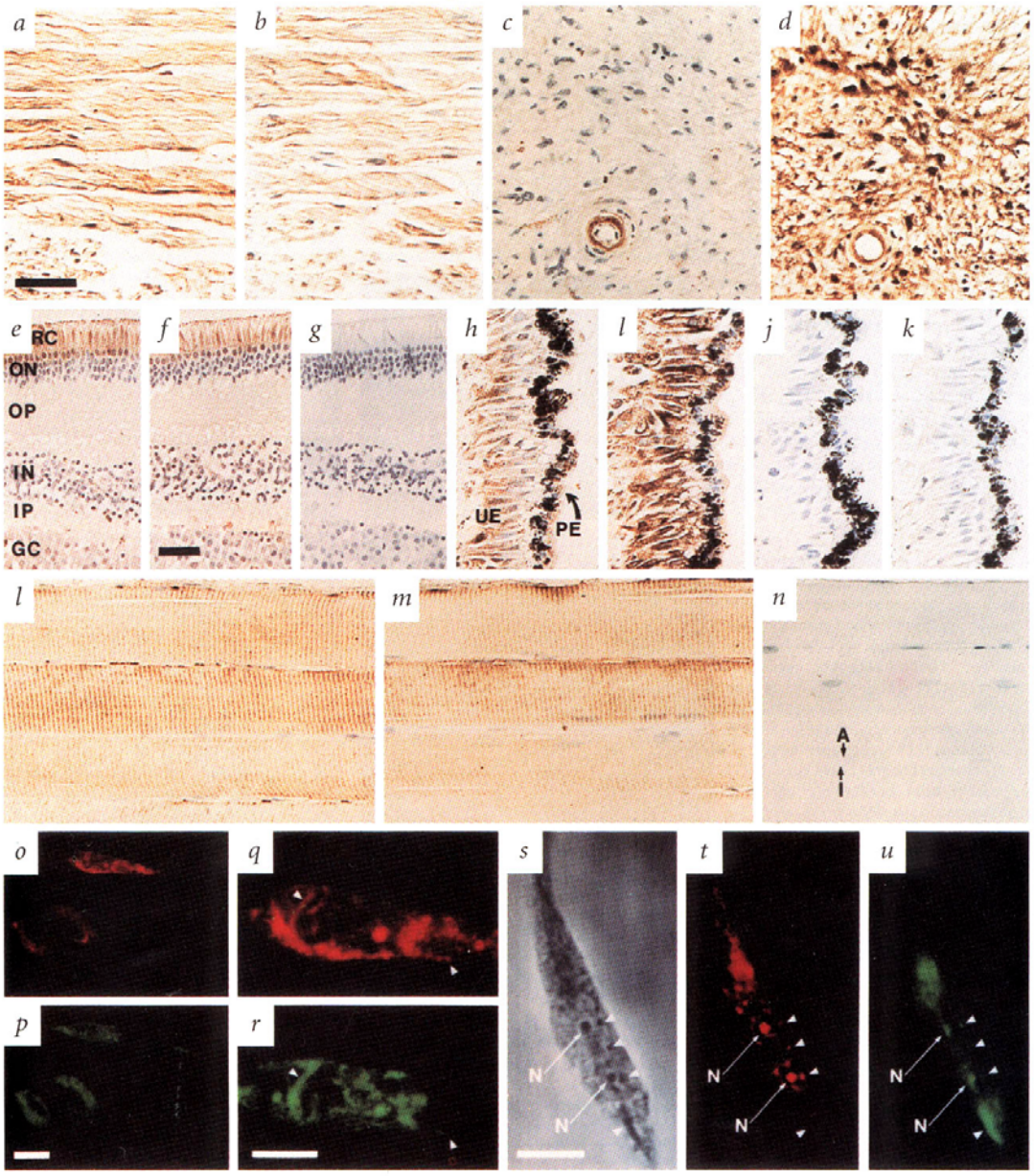

Fig. 4 Tissue distribution of spectrin and schwannomin in tissues and cell lines. Tissue distribution (*a–n*). Adjacent sections of normal human eighth cranial nerve stained with anti-schwannomin (Ab5990) (***a***) and anti-spectrin (***b***) antibodies. Adjacent sections of a vestibular schwannoma revealed no staining with Ab5990 (***c***), but staining was observed when using anti-spectrin antibody (***d***). A blood vessel penetrating the tumour stained with both antibodies. Retina stained very light in the ganglion cell (GC), inner plexiform (IP), inner nuclear (IN), outer plexiform (OP), and outer nuclear (ON) layers, and strongly in rods and cones (RC) with both Ab5990 (***e***) and anti-spectrin (***f***). No retinal staining was observed when using Ab5990 preabsorbed with peptide antigen (***g***). Strong staining for both schwannomin (***h***) and spectrin (***i***) was observed in unpigmented epithelium (UE), all pigment epithelia (PE), and corneal epithelium (not shown). No staining was observed with Ab5990 preabsorbed with peptide antigen (***j***) or rabbit serum (***k***). Adjacent sections of striated muscle revealed staining with Ab5990 (***l***) and anti-spectrin antibody (***m***), while a control using Ab5990 preabsorbed with peptide antigen revealed no staining (***n***). Striated muscle treated with rabbit serum also revealed no staining (not shown). Staining of both proteins occurred in I bands; the A and I bands are seen in (*n*). Bar = 100 μm (*a–d*, *h–n*), 100 μm (*e–g*). Double-staining of spectrin and schwannomin in Schwann-like STS26T cells (*o-u*). Immunofluorescence staining of STS26T cells revealed co-localization of schwannomin (***o*** and ***q***) and spectrin (***p*** and ***r***) in several regions (arrowheads). Phase-contrast imaging of a pre-cytokinesis cell with interphase nuclei (***s***). Confocal microscopy showed co-localization of schwannomin (***t***) and spectrin (***u***) in several cytoplasmic regions, nucleolus (N) and other nuclear regions (arrowheads). No staining was observed when Ab5990 was preabsorbed with peptide or with pre-immune rabbit serum (not shown). Bar = 10 μm (*o–p*), 5 μm (*q–r*), 10 μm (*s–u*).

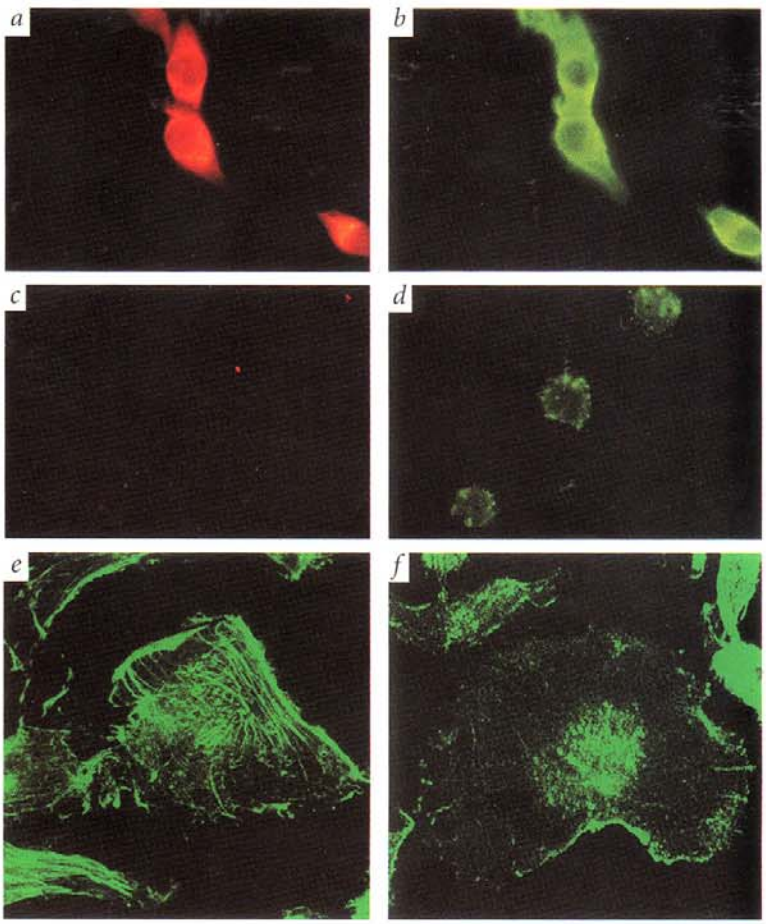

Fig. 5 Changes in the actin cytoskeleton upon antisense treatment of STS26T cells. *NF2* sense-oligonucleotide–treated cells had normal morphology and continued to express schwannomin when stained with schwannomin Ab5990 (***a***). Schwannomin largely co-localized with β-actin when the same cells were stained with anti-β–actin antibody (***b***). In contrast, *NF2* antisense-oligonucleotide–treated cells became spherical, had reduced adhesion and lost schwannomin expression (***c***). Staining with an actin antibody showed that the structure of the actin cytoskeleton was severely affected (***d***). Confocal microscopy showed that *NF2* sense-oligonucleotide–treated cells stained with FITC-labelled phalloidin had a complex structure of F-actin stress fibers (***e***), that were lost in cells treated with *NF2* antisense oligonucleotides (***f***). Bar = 20 μm (*a–d*), 20 μm (*e,f*).

anti-spectrin (10D) antibodies, we detected bands of the expected sizes (Fig. 2*b,c*). In addition to a non-specific band of approximately 100 kD, the βII-spectrin antibody recognized a doublet of proteins of approximately 235 kD in STS26T cells (Fig. 2*d*). The larger protein was the same size as that co-immunoprecipitated with schwannomin (Fig. 2*e*). This may indicate the presence of two spectrin isoforms, only one of which interacts with schwannomin. Alternatively, the lower βII-spectrin band may be a product of degradation.

Table 1 • Reduced binding of mutant schwannomins with βII-spectrin*

GAL4 binding-domain construct	GAL4 activation-domain construct	
	pGAD10Sp	pACT-SBP2
pGBT9 (control)	0.15±0.20	0.16±0.08
pGBT9NF2i2	79.29±0.63	99.79±2.77
pGBT9NF2i2Leu360Pro	25.86±0.28	95.99±1.08
pGBT9NF2i2Leu535Pro	19.77±0.20	89.79±1.41
pGBT9NF2i2Gln538Pro	1.08±0.17	116.03±0.76

*pGBT9 constructs encoding schwannomin isoform 2 (NF2i2), or schwannomin isoform 2 mutants Leu360Pro, Leu535Pro or Gln538Pro were co-transformed with pGAD10Sp encoding βII-spectrin or pACT-SBP2. Positive and uniform levels of β-galactosidase activity for all schwannomin mutants tested against SBP2 demonstrated that the decreased values seen for schwannomin mutants were not the result of loss of expression.

Both the schwannomin and βII-spectrin antibodies are highly specific. The schwannomin antibody has been extensively characterized, and recognizes a 66-kD band in STS26T cell extracts (Fig. 3*a*; refs 1,6). The βII-spectrin antibody recognizes bacterially-expressed recombinant proteins of the expected size (Fig 3*b*). *In vitro*, βII-spectrin (residues 1774–1992) bound to the C-terminal domain of schwannomin isoform 2 (residues 519–590; Fig. 3). There was no binding upon interaction with maltose-binding protein (MBP) or with shorter βII-spectrin proteins (residues 1885–1992 or 1885–1957; Fig. 3). These data confirmed that interaction required portions of βII-spectrin repeats 15 and 16, which encompass the ankyrin-binding region of spectrin[16]. Thus, the interaction of schwannomin and βII-spectrin was direct and did not require other domains of schwannomin, spectrin or other proteins.

Although most *NF2* germline and somatic mutations result in truncated proteins, several missense mutations in the C-terminal half of the protein have been described which may be associated with milder phenotypes. We generated full-length constructs encoding naturally-occurring missense mutations Leu360Pro (ref. 17), Leu535Pro (ref. 18) or Gln538Pro (ref. 19) by PCR-mediated site-directed mutagenesis[20]. These mutations reduced binding between 67% and 99% (Table 1). Missense mutations altering the schwannomin N-terminal domain did not affect βII-spectrin binding (data not shown). The decreased binding was not due to

lack of expression or stability of mutant proteins, as shown by intact interaction of mutant proteins with an N-terminal binding protein (SBP2; Table 1). Reduced (but not absent) binding of schwannomin proteins containing missense mutations provides a molecular explanation for the observation of milder NF2 phenotypes associated with non-truncating *NF2* mutations[17].

To determine whether βII-spectrin and schwannomin were co-expressed in tissues affected by the NF2 phenotype, we analysed several human tissues by immunocytochemistry. As previously shown[1,6], we detected schwannomin expression in normal Schwann cells, but not in schwannomas containing *NF2* mutations, consistent with the role of schwannomin as a tumour suppressor (Fig. 4*a*,*c*). Spectrin was detected in both normal Schwann cells of the eighth nerve and in vestibular schwannomas (Fig. 4*b*,*d*).

In addition to giving rise to tumours, *NF2* germline mutations also cause cataracts and retinal hamartomas[21]. Schwannomin is expressed in the lens[22], and spectrin degradation is typically seen in rat lenses induced to form cataracts[23]. Both proteins were also detected in rods and cones of the retina, but little expression was seen in neurons of other retinal layers (Fig. 4*e*–*g*). The unpigmented epithelium of the ciliary body and all pigment epithelia strongly stained for both spectrin and schwannomin (Fig. 4*h*–*k*). In the retinal pigment epithelium (RPE), one function of the βII-spectrin-ankyrin complex is the positioning of $(Na^+ + K^+)$ATPase in the apical membrane[24]. Signals received by cadherins also affect $(Na^+ + K^+)$ATPase distribution and stimulate assembly of the spectrin skeleton in RPE (ref. 25). Co-localization of spectrin and schwannomin in the RPE suggests that both may have a role in the development of NF2 retinal hamartomas[6]. Another hamartoma type, congenital hypertrophy of the RPE, is common in patients with *APC* gene mutation[26], and may point to common final pathways for APC and schwannomin.

Muscle cells have a spatially dispersed cytoskeleton, and βII-spectrin is known to be localized to Z lines within the striated skeletal muscle I-bands[27]. The *NF2* gene is abundantly expressed in muscle tissue[8,28]. Schwannomin was detected only in I-bands and clearly co-localized with spectrin (Fig. 4 *l*–*n*).

We also detected overlapping staining patterns for spectrin and schwannomin in the cytoplasm of STS26T cells by confocal fluorescent microscopy (Fig. 4*o*–*r*). In addition, undergoing division STS26T cells strongly expressed both schwannomin and spectrin in the nucleolus (Fig. 4*s*–*u*). Another human tumour suppressor, the RB protein, down-regulates ribosomal RNA production in the nucleolus by binding the RNA-polymerase-I transcription factor UBF (ref. 29). In addition, we have identified a previously unknown protein (SBP2) with homologies to nuclear transport proteins that binds to the N-terminal domain of schwannomin and may facilitate nuclear transport (D.S. & S.P., manuscript in prep.)

Based on homology with other ERM proteins[30,31], schwannomin has been predicted to interact with the cytoskeleton. In several cell types, schwannomin co-localized with F-actin[31–33], but a direct interaction could not be demonstrated. This was not surprising as schwannomin lacks the actin binding domains present in other ERM proteins[30]. The identification of βII-spectrin as a direct interactor of schwannomin now explains these seemingly contradictory observations as binding of βII-spectrin to F-actin is well established[34]. Solubility studies of wild-type and mutant schwannomins revealed that mutant schwannomins have increased solubility, presumably due to loss of cytoskeleton binding[33]. Among the studied mutants was schwannomin Leu360Pro, which exhibits reduced binding to βII-spectrin, and schwannomin proteins truncated at the C-terminal domain (which contains the Leu535Pro and Gln538Pro substitutions that reduce binding to βII-spectrin drastically).

Interactions between schwannomin and the cytoskeleton was previously suggested by antisense studies. Treatment of STS26T cells with *NF2* antisense oligonucleotides caused schwannomin depletion with a resultant early change in cellular morphology[6]. When antisense-treated cells were stained with FITC-phalloidin or β-actin antibodies, a reorganization of the actin cytoskeleton and loss of stress fibers was detected that was not seen with sense oligonucleotides (Fig. 5).

Schwannomin is a human tumour suppresser directly affecting cytoskeletal distribution. This supports the notion that changes in cytoskeletal organization may represent a primary event in tumourigenesis rather than a consequence of changes in other signal transduction cascades. The binding of βII-spectrin to both actin and schwannomin may make it a point of convergence for signals mediated by schwannomin as well as other spectrin-binding proteins such as ankyrin. The ankyrin- and schwannomin-binding sites on βII-spectrin partially overlap, suggesting possible competition for spectrin binding. Future studies will need to define additional proteins that interact with schwannomin, signals that are mediated by the spectrin skeleton, and the functional differences between schwannomin bound to the cytoskeleton and free schwannomin.

Methods

Identification of proteins interacting with schwannomin. The *NF2* isoform-2 cDNA was generated by RT-PCR of the end of the *NF2* cDNA with a forward primer that annealed upstream of the unique *Sfi*I site and a reverse primer that annealed at the isoform-2 alternatively spliced exon 16, modified to introduce a *Bam*HI site for cloning. The fragment was ligated in the respective position excised from the *NF2* isoform-1 cDNA. A yeast two-hybrid screen[9] of a human adult brain cDNA library cloned in GAL4–activation-domain vector pGAD10 was accomplished using pGBT9NF2i2, encoding schwannomin isoform 2 fused to the GAL4-binding domain (vectors and library from Clontech). Upon screening 6.6×10^6 colonies, a plasmid encoding βII-spectrin, pGAD10Sp, was purified and retransformed with pGBT9NF2i2, pGBT9NF2i1, encoding schwannomin isoform 1, or pGBT9NF2i2 partial deletions. Yeast strain Y190 double-transformants were grown on SC media without leucine, tryptophane, or histidine, and with 3-amino-1,2,4-triazole (25 Mm) and 2% glucose[9]. β-galactosidase production was assayed by incubating freeze-fractured colonies on nitrocellulose in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 0.03 mM β-mercaptoethanol, 2.5 μM X-gal) at 37 °C for 15 min to 8 h.

Antibodies. Antibody 5990 was previously described[6]. The polyclonal rabbit affinity-purified βII-spectrin antibody (antibody 10D) was raised against a GST fusion to βII-spectrin residues 1676–2204, and was a gift of Jon Morrow[16]. Other antibodies were purchased from Sigma.

Immunoprecipitation. STS26T cells were grown in DMEM with 10% fetal bovine serum, and penicillin/streptomycin. Cultures with minimal cell-cell contact were harvested and homogenized in buffer A (20 mM Tris pH 8.0, 150 mM NaCl, and 0.25 or 0.5% Triton X-100, 0.05% sodium azide) or B (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.05% deoxycholic acid, 1.0% Tween 20, 0.05% sodium azide), and 2 μg/ml aprotinin, 0.5 mg/ml Pefabloc SC, 1.0 μg/ml pepstatin, 1.0 μg/ml leupeptin. Cell lysates were made from formalin-fixed (4%) *Staphylococcus aureus* cells (Sigma) and incubated for 30 min at 4 °C to clear antibodies. The *S. aureus* cells were removed by centrifugation and this step was repeated. For each immunoprecipitation, cleared lysate protein (10 μg) was mixed with antiserum 5990 or preimmune serum (5 μl) in buffer A (1 ml), or antibody 10D or rabbit serum (1 μl) in buffer B (1ml), and rotated overnight at 4 °C. Thereafter, 50 μl of 50% slurry of protein A Sepharose (Sigma) was added and the mixtures were rotated for 2 h at 4 °C. Protein complexes binding the protein A Sepharose were collected by centrifugation for 5 s and washed in buffer A four times, after which SDS-PAGE gel sample buffer (50 μl) was added (50 mM Tris pH 6.8, 20% glycerol, 2% SDS, 50 mM β-mercaptoethanol, 0.05% bromophenol blue). Proteins were eluted by boiling for 10 min. One to 20 μl were used for SDS-PAGE and western blotting.

***In vitro* protein binding assays.** Segments of βII-spectrin were PCR-amplified and cloned in pMALC2 (New England BioLabs). MBP and MBP fusions with βII-spectrin were expressed in *Escherichia coli* DH5α and purified using amylose resin (New England BioLabs). Residues 469–590 of schwannomin isoform 2 (SCHi2p469–590) were expressed in DH5α as fusions to glutathione S-transferase (GST) using pGEX-5X-1 (Pharmacia) and were purified using Sepharose 4B-glutathione (Pharmacia). *In vitro* protein binding assays were conducted by incubating purified MBP or MBP-spectrin fusions with fresh amylose resin for 5 min at RT and 25 min at 4 °C in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 2 μg/ml aprotinin and 0.5 mg/ml Perfabloc SC). The bound proteins were washed three times with binding buffer (160 mM NaCl, 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2.5 mM KCl, 50 mM hepes, pH 7.4, 1% Triton X-100, 2 μg/ml aprotinin and 0.5 mg/ml Perfabloc SC). The saturated amylose resin was then incubated with GST-SCHi2p469–590 for 20 min at 4°C and washed three times with binding buffer. The quantities of MBP and GST proteins were normalized by conducting dot blots of serial dilutions detected with anti-MBP (New England BioLabs) and anti-GST (Pharmacia) antibodies. Resin-bound proteins were eluted, electrophoresed in 4–20% precast polyacrylamide gradient gels (Bio-Rad), and detected by western blot analysis with βII-spectrin antibody (antibody 10D) or anti-schwannomin (Ab5990) polyclonal antibodies by ECL (Amersham).

Immunohistochemical staining. Rabbit anti-spectrin antibody (1:2,000 dilution), Ab5990 (20 μg/ml), or rabbit preserum (1:2,000 dilution) were incubated with tissue sections overnight at 4 °C as previously described[3]. Primary antibodies were detected using the ABC *elite* Peroxidase Kit (Vector), enhanced by diaminobenzidine (DAB) enhancer, and visualized with DAB (Biomeda). Sections were counterstained using aqueous hematoxylin (Xymed). Absorption controls were conducted using Ab5990 preabsorbed with peptide antigen at 100 μM for 2 h at RT.

Immunofluorescence. STS26T cells were grown, harvested, and stained as previously described[6] with Ab5990 (20 μg/ml) and mouse monoclonal anti-spectrin (1:40 dilution; Sigma), mouse monoclonal anti-β–actin (1:200 dilution; Sigma), or rabbit preimmune serum. Following three washes with cold DPBS, cells were incubated with TRITC-conjugated affinity-purified goat-anti-rabbit IgG (Sigma) or FITC-conjugated affinity-purified goat anti-mouse IgG (Sigma), washed four times in cold DPBS, and mounted. Confocal fluorescence microscopy was performed using a Zeiss LSM 310 confocal microscope.

PCR-based site-directed mutagenesis. Three PCR reactions were performed in two steps to produce an *NF2* fragment with nucleotide-base changes to introduce single amino-acid substitution mutations as previously described[20]. Primers used to generate individual mutations and PCR conditions are available upon request. The mutated PCR products were ligated in place of the normal counterpart excised from pGBT9NF2i2.

Antisense treatment of STS26T cells. Phosphorothioated oligodeoxynucleotides (pODNs) were synthesized, purified and used to treat STS26T cells as described previously[6]. Briefly, cells were incubated with 50 μM pODN (antisense, CGGGAACGGTCTGGGCTG, or sense, CAGCCCAGACCGTTCCCG) and 0.8 units/ml streptolysin O (Sigma) for 5 min at RT. The reaction was stopped by adding 10 ml of DMEM with 10% fetal bovine serum and 1× antimycotic solution (Gibco-BRL). The cells were pelleted, resuspended in culture medium and grown in four-well slides.

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