The tumor suppressor merlin interacts with microtubules and modulates Schwann cell microtubule cytoskeleton

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The lack of neurofibromatosis 2 tumor suppressor protein merlin leads to the formation of nervous system tumors, specifically schwannomas and meningiomas. Merlin is considered to act as a tumor suppressor at the cell membrane, where it links transmembrane receptors to the actin cytoskeleton. Several tumor suppressors interact with another component of the cytoskeleton, the microtubules, in a regulated manner and control their dynamics. In this work, we identify merlin as a novel microtubule-organizing protein. We identify two tubulin-binding sites in merlin, one residing at the N-terminal FERM-domain and another at the C-terminal domain. Merlin’s intramolecular association and phosphorylation of serine 518 regulate the interaction between merlin and tubulin. Analysis of cultured glioma cells indicates colocalization between merlin and microtubules especially during cell division. In primary mouse Schwann cells only minor colocalization at the cell periphery of interphase cells is seen. However, these cells drastically change their microtubule organization upon loss of merlin indicating a functional association of the proteins. Both in vitro assays and in vivo studies in Schwann cells indicate that merlin promotes tubulin polymerization. The results show that merlin plays a key role in the regulation of the Schwann cell microtubule cytoskeleton and suggest a mechanism by which loss of merlin leads to cytoskeletal defects observed in human schwannomas.

INTRODUCTION

Inactivation of the neurofibromatosis 2 (NF2) tumor suppressor gene leads to the development of multiple benign tumors of the nervous system, particularly meningiomas and schwannomas (1). The NF2 gene encodes a 595-amino acid protein merlin (schwannomin), which is related to the ezrin–radixin–moesin (ERM) protein family. Merlin and ERM proteins are located primarily underneath the cell membrane where they anchor transmembrane proteins to the actin cytoskeleton (2,3). They form homo- and heterotypic interactions (4) which in turn regulate their binding to other proteins (5–7). Head-to-tail binding leads to a closed conformation of the ERM proteins (4); for merlin, the closed form is thought to act as a tumor suppressor, whereas the open protein is unable to regulate growth. Phosphorylation of a C-terminal serine (S518) by p21-activated kinase (PAK) or cAMP-dependent protein kinase A (PKA) weakens merlin’s self-association and is believed to inactivate the growth-suppressing activity of merlin (8,9). However, the functional regulation of merlin is still not completely understood.

Merlin is also involved in receptor recycling and endocytosis. It inhibits platelet-derived growth factor receptor degradation (10) and binds hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (11), which is known to regulate receptor tyrosine kinase trafficking to the degradation pathway (12). Recently, merlin was shown to regulate EGF receptor recycling and turnover in Drosophila (13). Thus, increasing evidence positions merlin at the membrane where it can bind membrane receptors and regulate their expression and localization.

A common feature of many tumor suppressors is their ability to interact with microtubules and regulate microtubule
stability (14,15). Microtubules, themselves, are also known to regulate cell growth. They, for instance, control cell division and regulate endocytosis and recycling of growth factor receptors (16). We set out to study merlin–tubulin interaction when we noticed that endogenous merlin colocalizes with tubulin in mitotic structures of U251 glioma cells (17). Although merlin has previously been suggested to bind tubulin in vitro (3,18), no further evidence has been provided to support the binding. Here, we have studied the association between merlin and tubulin, the regulation of the interaction, the effects of merlin on microtubule dynamics and the consequences of loss of merlin on microtubules in Schwann cells. Our results show that merlin plays an important role in regulating microtubule cytoskeleton of mouse primary Schwann cells.

RESULTS

Merlin associates with microtubules

We have previously shown that in synchronized U251 glioma cells merlin localizes to mitotic structures (17,19). This led us to analyze the distribution of endogenous merlin and tubulin at various cell cycle stages in U251 glioma cells (Fig. 1A–H). We noticed parallel accumulation of merlin and tubulin around the nucleus in interphase cells before mitosis (A). During mitosis, merlin colocalized with microtubules at the mitotic spindles, although some diffuse merlin staining was also noticed in the cytoplasm (B and C). During cytokinesis especially the midbody demonstrated high degree of colocalization between merlin and tubulin (D and E). The colocalization was lost at early G1, when merlin accumulated in the nucleus (F). The staining patterns remained separate during the entire G1 and S phase (G). At G2, a partial colocalization was seen (H). We also studied cultured primary mouse Schwann cells, whose purity was verified by a Schwann cell-specific marker p75 (Supplementary Material, Fig. S1). In interphase cells merlin had a predominantly submembranous localization, whereas tubulin was localized at the cytoplasm. Areas of potential colocalization were identified from confocal sections using image analysis software. The analysis revealed occasional sub-plasmalemmal regions and (I and J) and cell extensions (K and L) (colocalized points shown in white in Fig. 1J, L), in which codistribution was seen. In mitotic Schwann cells staining was mostly diffuse.

Merlin binds polymerized microtubules in vitro

We then wanted to identify the tubulin-binding regions in merlin. We mapped the interaction sites with a tubulin pull-down assay using various purified glutathione S-transferase

Figure 1. Distribution of merlin and microtubules in U251 glioma and primary mouse Schwann cells. (A–H) U251 glioma cells were synchronized and stained for merlin (green, A19) and tubulin (red, α-tubulin). The cells were fixed at different points of the cell cycle, the cell cycle phase was verified by FACS analysis and the localization of the two proteins was analyzed by confocal microscopy. Merlin and tubulin start to colocalize already in interphase cells when cells are approaching mitosis (A). During mitosis merlin localizes to mitotic spindles (B, C) and during cytokinesis at the midbody (D, E and insets). After mitosis the two proteins have separate staining patterns (F, G). At late G2, some colocalization can be seen again (H). (I–L) In the primary mouse Schwann cells merlin (green, A19) shows a predominantly submembranous distribution, whereas microtubules (red, β-tubulin) are distributed within the cells body. Analysis of confocal sections with Image J analysis software demonstrates occasional submembranous regions, where the two proteins colocalize (white areas in J and L). Scale bar 10 μm.
Figure 2. Merlin binds polymerized tubulin in vitro. (A) A schematic drawing on merlin’s domains. (B) Tubulin binding sites in merlin were mapped by a tubulin pull-down assay, in which recombinant GST-merlin was pulled down with polymerized microtubules by ultracentrifugation. Merlin N (amino acids 1–314), α-helical merlin (amino acids 314–492) and merlin C (amino acids 492–595) constructs were used in the assay. Total (t), supernatant (s) and pellet (p) fractions with or without tubulin were run into SDS-PAGE, blotted either with merlin (A19 or C18) or GST-antibody and analyzed. (C) The amount of merlin in the pellet fraction was quantified by western blots by Typhoon Imager and compared to the total amount of merlin. (D) The binding sites in merlin were further mapped with merlin constructs 1–100 and 492–547, GST alone, MAP2 and ezrin N (1–309) were used as controls in the pull-down assay. The tubulin blots can be found from Supplementary Material, Figure S2. (E) In vitro translated 35S methionine labeled merlin was produced and used for in vitro tubulin pull-down experiments. Merlin was pulled down with polymerized microtubules and the amount of merlin in the pellet was analyzed and compared with total amount of merlin (%). Merlin isoforms 1 and 2 and C-terminally deleted merlin constructs demonstrated reduced binding in comparison with isoform I. However, a further truncation of 10 residues returned the binding activity (Fig. 2E) showing the complexity of the binding interphase.

Intramolecular association and phosphorylation of merlin regulate its binding to microtubules

Merlin undergoes conformational regulation and can form intramolecular association with its N- and C-terminal domains (6). This intramolecular association can mask many of the binding sites in merlin. Therefore, we studied whether intramolecular binding affects merlin–tubulin binding. Purified C-terminal GST-merlin was incubated together with increasing amounts of N-terminal merlin in order to saturate the C-terminal binding site. When C-terminus was saturated with the N-terminus it could no longer bind tubulin (Fig. 3A). GST alone incubated with the C-terminus was used as a negative control.

The C-terminal S518 of merlin can be phosphorylated by PAKs and cAMP-dependent PKA (8,9,20). This phosphorylation is thought to affect merlin’s growth-regulating activity (21–23). We studied whether the phosphorylation plays a role in merlin–tubulin binding. We treated the C-terminus (492–595) of wild-type merlin or non-phosphorylatable merlin S518A mutant in vitro with PKA and performed the tubulin pull-down assay (Fig. 3B). Phosphorylation was verified by 32P ATP labeling (not shown). Significantly lower amount of merlin was pulled down after PKA treatment indicating that the phosphorylation of the serine 518 decreases the in vitro binding of merlin to tubulin.

Merlin enhances microtubule polymerization in vitro

Many tumor suppressor proteins have been reported to affect tubulin polymerization (14) and therefore we studied whether merlin has an effect on tubulin polymerization. Purified tubulin was polymerized in vitro and the polymerization was monitored at OD350. Microtubule-associated proteins (MAPs) and Taxol were used as positive controls and GST alone as a zero control. Nocodazole was used to control tubulin depolymerization (not shown). When recombinant merlin was added to the polymerization reaction, the rate of tubulin polymerization was increased (Fig. 4). Merlin N-, α- or C alone were not able to induce tubulin polymerization (not shown) and merlin 1–547, which has reduced affinity for tubulin, was also unable to induce tubulin polymerization.

Merlin affects the structure of Schwann cell microtubule cytoskeleton

As schwannomas, the hallmark of Neurofibromatosis 2, originate from Schwann cells, we studied merlin’s effect on microtubule cytoskeleton in this cell type. We used primary mouse Schwann cells lacking merlin (Nf2flx2/flx2), after addition of
Cre-recombinase leading to genotype Nf2^/^- from now on marked as Nf2^-/-, see also discussion) (24) and re-introduced wild-type merlin via adenovirus infection (Nf2 add-back). The microtubule cytoskeleton of the Nf2^-/- mouse Schwann cells differed markedly from cells re-expressing merlin via adeno-infection (Nf2 add-back). In the Nf2 add-back cells, microtubules were assembled as dense cables reaching from one end of the cell to the other and the cells displayed a normal spindle-like structure (Fig. 5A, D). Instead, the microtubules of the Nf2^-/- cells were disorganized, and the overall cell shape was more spread (Fig. 5B, D). Similar morphological differences have also been described in cultured human Schwann cells versus schwannoma cells (25).

The amount of polymerized versus unpolymerized (i.e. soluble) tubulin was analyzed from Schwann cells extracted with Triton X-100. More soluble \( \alpha \)- and \( \beta \)-tubulin were present in Nf2^-/- cells than in wild-type (Nf2^flox2/flox2, marked as Nf2^+/+) or add-back cells (MWT) (Fig. 5C). The amount of soluble tubulin was associated with the expression level of merlin; increasing merlin expression resulting in reduced soluble tubulin. The total amount of tubulin in Nf2^-/- and the add-back Schwann cells was evaluated after solubilization in 6 M urea buffer. When the urea-lysates were analyzed in SDS–PAGE the add-back (MWT) and the Nf2^+/+ cells appeared to contain at least equal amount of tubulin as the Nf2^-/- cells. From this data we concluded that the ratio of polymerized/unpolymerized tubulin differs between Nf2^-/- and add-back cells; i.e. in Nf2^-/- Schwann cells the proportion of free tubulin (\( \alpha/\beta \)) is higher than in the wild-type cells.

Figure 4. Merlin enhances tubulin polymerization in vitro. Purified tubulin from bovine brain was polymerized in vitro at +37°C and the polymerization was monitored at OD_{350}. Merlin GST-fusion proteins were incubated together with tubulin. Taxol and MAP2 were used as positive controls for tubulin polymerization, tubulin alone and tubulin together with GST were used as zero controls. When full-length merlin (WT) was added to the polymerization reaction, tubulin polymerized more rapidly. Merlin 1–547 was not able to induce tubulin polymerization. The graph shows an average of three experiments.
Tubulin polymerization dynamics differ in \(2/2\) and Nf2 add-back Schwann cells

The difference in the integrity of the tubulin cytoskeleton in \(2/2\) and add-back Schwann cells led us to test whether a difference in the polymerization rates of tubulin is also seen in vivo. We treated the Schwann cells with Nocodazole, which sequesters tubulin monomers and thereby depolymerizes microtubules. Incubation with Nocodazole reversed the normal morphology of Schwann cells and the cells obtained a fibroblast-like morphology (Fig. 6A, B; Supplementary video 1 and 2, for add-back and \(2/2\) cells, respectively). After 5 min of Nocodazole block release both cell types began to regain their normal shape by losing the flattened morphology. The add-back Schwann cells began to obtain their normal morphology (i.e. long extensions) 2 h after the block release, whereas the \(2/2\) cells had only unorganized microtubules at this time-point (Fig. 6C, D; 120 min). After 4 h of block release both cell types had their microtubules re-grown. In addition, the add-back cells had regained their spindle-like shape (Fig. 6C, D; 240 min). This implies that the normal Schwann cell morphology is dependent on intact microtubule cytoskeleton and that merlin enhances microtubule organization in Schwann cells.

In addition, we used fluorescence recovery after photobleaching (FRAP) technology to study, whether tubulin dynamics were altered in primary Schwann cells lacking merlin. Schwann cells were transfected with EGFP-tubulin and 24 h after transfection the cells were analyzed by FRAP. Cells expressing merlin had a 25% faster tubulin recovery rate than the Nf2 \(-/-\) cells (Fig. 6E) \((P < 0.001)\). The data suggest that merlin is able to enhance microtubule dynamics of Schwann cells in vivo.

**DISCUSSION**

In this study we describe a novel function for merlin in the regulation of microtubule organization and dynamics. We show that merlin directly binds tubulin and regulates microtubule dynamics *in vitro* and *in vivo*. Our results show that merlin contains two tubulin-binding sites, one in the FERM-domain and another in the C-terminal domain. The existence of two separate binding sites in merlin indicates that merlin could bind either laterally to the sides of the microtubules or cross-link individual microtubules to each other, this way bundling the microtubules to thicker entities. In addition, the entire protein is needed to induce tubulin polymerization, as shorter merlin fragments were not able to induce polymerization. Interestingly, the truncating patient mutation 1–547 showed reduced tubulin binding and did not induce polymerization, although it harbors the two identified tubulin binding sites. A further 10 residue deletion returned the binding activity indicating that mere loss of the binding site does not

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**Figure 5.** Microtubule cytoskeleton is altered in NF2 \(-/-\) Schwann cells. Primary mouse Schwann cells with merlin null background (NF2\(\Delta\)exon2, here marked as \(-/-\) (B) and the same cells with adeno-infected wild-type merlin (MWT) (A) were stained for merlin (green, A19) and \(\beta\)-tubulin (red) and imaged by fluorescence microscope and the images were deconvoluted. Microtubule organization is different in merlin \(-/-\) cells and in cells expressing merlin. Magnification \(\times 630\). (C) Triton X-100 lysates made from the \(-/-, +/-\) and add-back (MWT) cells. Protein amounts were analyzed by Bradford assay and equal protein amounts used. As an additional loading control, the membranes were stained with Ponceau red. The lysates were probed for merlin (A-19), \(\alpha\), \(\beta\)- and acetylated-tubulin by western blot. Total lysates of the \(-/-, +/-\) and merlin add-back cells (MWT) were solubilized in 6 M Urea buffer and probed for \(\beta\)-tubulin and merlin (A-19). (D) A high-magnification image of tubulin in MWT and \(-/-\) cells. Scale bar 10 \(\mu\)m.
Figure 6. Microtubule recovery is faster in merlin-expressing primary Schwann cells. (A, B) Primary mouse Schwann cells lacking merlin (+/−) (B) or with adeno-infected merlin add-back (MWT) (A) were treated with Nocodazole to depolymerize microtubules. The block was released and cells were either imaged with DIC filter for 4 h once every 30 s (Supplementary videos 1 and 2, 200x magnification) or still images were captured from the indicated time points. Alternatively add-back MWT (C) and −/− (D) cells were fixed at indicated time points after which cells were stained with β-tubulin and a Schwann cell marker p75 and imaged with fluorescence microscope (magnification 630x) after which the images were deconvoluted. Nuclei were stained with Hoechst (blue). (E) For FRAP experiments primary Schwann cells (+/− and add-back) were transfected with EGFP-tubulin and 24 h after transfection the cells were imaged for FRAP with confocal microscope. Pre-bleach images were obtained and the region of interest (equal size region of interest was used with all the cells) was bleached. Post-bleach images were acquired at 2 s intervals. From the data, background was subtracted and normalized similarly for all the samples. Eight cells of each construct (+/− and add-back/MWT) were imaged and the averaged data was used for data analysis. Cells that were expressing merlin had a faster recovery rate of tubulin than the −/− cells (P < 0.001).
explain the result. It is possible that the 1–547 mutation leads to an altered folding of the protein and thereby prevents the interaction with tubulin.

An intramolecular association and phosphorylation of S518 in merlin can regulate its binding to microtubules. The binding of several other proteins to microtubules is regulated by phosphorylation. For example a major phosphoprotein in the brain, MAP2, is phosphorylated by PKA and upon phosphorylation dissociates from microtubules (26). Both MAP2 and merlin function as PKA-anchoring proteins (AKAPs) (27,28) and therefore merlin might have a similar function as MAP2, targeting PKA to microtubules. It is of interest that both kinases, PKA and PAK, known to regulate merlin phosphorylation, localize to centrosomes at mitosis and affect microtubule formation, in analogy with merlin (8,9,20,29,30). Previous results have suggested that phosphorylation of merlin results in the conformational opening of the molecule allowing it to interact with other proteins (23). However, in light of our study (and our unpublished observations from other interaction studies) it seems that the regulation is more complex than previously thought. We hypothesize that merlin can be ‘open’ but non-phosphorylated at S518 since in our results only the open but S518 non-phosphorylated merlin binds tubulin.

To study the role of merlin on microtubule dynamics in vivo, we used cells from genetically engineered mouse, in which wild-type Nf2 gene has been replaced with a NF2 exons2/loxP2loxP2 variant. Addition of Cre-recombinase to the cell cultures leads to deletion of exon 2 (NF2exons2/Δexon2 genotype) and to complete loss of merlin (Fig. 5). Overexpression of a Δexon 2 mutant in cultured cells is known to disturb cell adhesion (31). However, in our model, replacement of the wild-type allele with the NF2Δexon2 leads to a greatly reduced merlin expression, when compared with the wild-type Nf2 gene as shown in mouse tissues (24). Indeed, Cre-recombinase treated MEF cells from the same mice have previously been used as a model of genetically engineered Nf2−/− cells (32).

Our data suggest that merlin plays an important role in regulating Schwann cell microtubule cytoskeleton. Loss of merlin in Schwann cells results in a change of the spindle-like morphology and alteration in microtubule organization. Also the ratio of polymerized versus unpolymerized tubulin is affected by merlin, i.e. Schwann cells that lack merlin have more un polymerized tubulin than cells expressing merlin. The Nf2 −/− cells contain more acetylated tubulin, which is regarded as a marker of older and more stable structures. This might imply that tubulin polymerization/turn-over is not as efficient in the Nf2 −/− cells as in merlin-expressing cells. The results from the Nocodazole-repolymerization experiment confirm these data. Our FRAP data further supports this finding as tubulin turnover appears to be higher in merlin-expressing Schwann cells. Unfortunately, in our experimental FRAP set-up, it was not possible to monitor the dynamics of individual microtubules due to the thickness of the cells and to the high amount of free tubulin dimers. Therefore, by FRAP we have only analyzed tubulin recovery as a tubulin pool and not as individual microtubule polymers. A difference might have been even greater if we were able to study individual microtubule polymers. It also seems that microtubules are a major contributor to the Schwann cell morphology, since Schwann cell morphology resembled that of fibroblasts after depolymerization of microtubules.

Microtubules regulate endocytic pathways (16,33). Recent papers have linked merlin to endocytic events and receptor recycling (10–13). It has been shown that cells use a specific sorting mechanism of fast and slow maturation of the endocytic vesicles. Certain receptors, such as EGFR, are recycled through a dynamic pool of early endosomes that are highly mobile on microtubules and that mature rapidly towards the late endosomes. This rapid movement and maturation is completely dependent on microtubules; if microtubules are disrupted, the dynamic vesicles will non-selectively join all early endosomes and are less-efficiently degraded (34). Two papers have localized merlin to early endosomes (11,13). Thus, merlin may function as a linker at the plasma membrane, helping receptor-containing endocytosed vesicles to attach to microtubules. If cells lack merlin, this receptor recycling might be slower, and the receptors would be cleared from the membrane less efficiently. We propose that merlin plays a dual role at the membrane of Schwann cells, partly by transferring early endocytosed vesicles to rapidly growing microtubules with the help of its interaction partner HRS and partly by increasing the microtubule polymerization rate, thus enhancing the vesicle maturation process.

In the past years several tumor suppressor proteins, including p53, APC, VHL and BRCA1 have been shown to bind and regulate microtubules (14). In line with merlin, also APC and VHL show only limited colocalization with microtubules, but yet affect their dynamics in vivo (35,36). Here, we show that also merlin binds microtubules, regulates their polymerization and has an important role in the establishment of normal Schwann cell morphology. Schwannoma cells from NF2 patients display altered morphology with long, multiple extensions (37). Based on our study this altered morphology may be associated with a disturbed microtubule cytoskeleton due to the lack of merlin, thus linking the interplay of merlin and tubulin to normal Schwann cell development and possibly to schwannoma formation.

MATERIALS AND METHODS

Cell lines and antibodies

U251 glioma cells were maintained in Dulbecco’s Minimum Essential Medium (MEM), supplemented with 10% fetal calf serum (FCS) (PromoCell, Heidelberg, Germany), 1% l-glutamine and 50 μg/ml gentamycin (Invitrogen). Cells were fixed in 3.5% paraformaldehyde, pH 7.5. Primary Schwann cells were isolated and cultured as previously described (38) from NF2 knock-out mice with both alleles of the NF2 exon 2 loxed, thus producing after Cre recombination the NF2ΔΔ genotype (24). The cells were used either at passage two or three. Anti-merlin polyclonal rabbit antibodies A-19 sc-331 (epitope 2-21), C-18 sc-332 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1398 NF2 (39), anti-schwannomin (40) and mouse mAb KF10 (39) were used. TO-PRO 3-iodide probe (Invitrogen, Molecular Probes) was used for DNA staining. Monoclonal anti-α-, β- and -acetylated-tubulin antibodies (Sigma–Aldrich) were used to detect tubulin. Anti-GST polyclonal...
goat antibody (GE Healthcare) was used to detect GST proteins. p75 antibody (41) was used as a Schwann cell marker. Alexa 488-, 568-, 594- and 633-conjugated goat anti-mouse and goat anti-rabbit antibodies (Invitrogen, Molecular Probes) were used as secondary antibodies in immunofluorescence and HRP-conjugated rabbit anti-mouse and swine anti-rabbit and anti-goat (Santa Cruz) secondary antibodies (DAKO A/S, Glostrup, Denmark) in western blot analysis.

**Plasmids and protein expression constructs**

The following merlin constructs: isoform I (amino acids 1–595), isoform II and isoform I variants S518A, S518D, 1–309, merlin a-helical domain (amino acids 314–477), C-terminus (amino acids 492–595) and C-terminus with S518A mutation as described previously (9), merlin 1–537 and merlin 1–547.

Adenovirus construct were made using Stratagene’s AdEasy system. Full-length merlin was cloned into the adenovector and the virus was produced according to manufacturer’s instructions in 293A cells. The infection titer was optimized to have 100% efficiency and verified by immunofluorescence staining. Schwann cells lacking merlin were infected with adenoviruses 3 days before the experiments were conducted. EGFP-tubulin construct was obtained from Invitrogen, and transfection to primary Schwann cells was done with Lipofectamine PLUS (Invitrogen).

**In vitro translation and quantification of labeled proteins**

Merlin pcDNA3 plasmids (Invitrogen) were used as a template for a T7-coupled rabbit reticulocyte transcription–translation system (Promega, Madison, WI, USA) in the presence of 35S-methionine. Five microlitres of 50 μl reaction were run in to SDS–PAGE, gel was dried and exposed to film to determine the size and amount of the labeled protein. For quantification the gel was also exposed to PhosphoImager low energy-plate protocol. The proteins were used immediately after purification.

**GST-fusion protein production**

GST-fusion proteins were expressed in *Escherichia coli* DH5α and purified following standard protocol. Fusion protein was eluted from the Glutathione Sepharose beads (GE Healthcare) by 5 mM reduced glutathione in 50 mM Tris–HCl pH 8.0, over night at +4°C. Before some tubulin pull-down assays different amounts of N- and C-terminus were incubated in 50 mM Tris–HCl, 150 mM NaCl (pH 8.0) for 30 min at room temperature. The eluted fusion proteins for the PKA assay were dialyzed against 20 mM Tris–HCl, 10 mM MgCl₂, pH 7.4 at +4°C o/n. Wild-type merlin was produced in SF9 insect cell line. Merlin with an N-terminal GST-tag was cloned into a baculovirus transfer vector pAcG2T (BD Biosciences) and then produced with the BaculoGOLD system (BD Biosciences).

**In vitro phosphorylation**

GST-proteins were dialyzed against the reaction buffer and run into SDS–PAGE, protein amounts estimated and equal amounts of each construct were used in the *in vitro* phosphorylation assay. Total volume of the reaction was 40 μl including PKA reaction buffer (20 mM Tris–HCl, 10 mM MgCl₂, pH 7.4), 200 mM ATP and purified human or bovine catalytic subunit of PKA (Sigma–Aldrich). Reaction was incubated 30 min at +30°C and was stopped by adding 20 mM PKA inhibitor H89 (Sigma–Aldrich).

**Tubulin pull-down assay**

Tubulin pull-down was performed with 35S-labeled *in vitro* translated protein, eluted GST-fusion protein or with *in vitro* PKA phosphorylated eluted GST-fusion protein. Equal protein amounts were used in all experiments. Purified bovine tubulin (Cytoskeleton) (70 μg) was added to tubulin polymerization buffer (80 mM PIPES, 0.5 mM MgCl₂, 1 mM ethylene glycol-bis, 1 mM GTP, pH 6.9, 10% glycerol, 10 mM Taxol) to a final volume of 200 μl and the microtubules were allowed to polymerize at +37°C for 30 min in the presence of different merlin constructs. After this 50 μl were removed and labeled as ‘total’ fraction and the remaining 150 μl were centrifuged at 11 503 g for 30 min at +30°C to collect the polymerized microtubules. Supernatant was removed and the pellet was resuspended in 150 μl of polymerization buffer. Twelve microlitres of each fraction were analyzed on SDS–PAGE.

**Western blot**

Primary Schwann cells were lysed in ice-cold ELB buffer (150 mM NaCl, 50 mM Hepes pH 7.4, 5 mM EDTA, 0.5% NP40 and complete protease inhibitor cocktail tablet, Roche) or in mild Triton-X buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1 mM PMSF) including protease inhibitors (Complete), cells were incubated on ice for 15 min and centrifuged at full speed in +4°C for 15 min. Alternatively, cells were scraped in ice-cold PBS, centrifuged briefly and the cell pellet was suspended into Urea-buffer (50 mM Tris, 6 mM Urea, pH 7.4). The cells were lysed for 15 min and briefly sonicated. The protein amounts were analyzed either by the Bradford assay or by Coomassie staining. Equal amounts of proteins were run into gel transferred into nitrocellulose membrane and blotted with antibodies. As an additional loading control, loading was verified after western blot by staining the filters with Ponceau red. A representative band of the whole filter of approximately 150 kD was chosen as the loading band for Figure 5C.
Immunofluorescence, laser scanning confocal microscopy, live cell imaging and fluorescence recovery after photobleaching

U251 and Schwann cells were fixed in 3.5% PFA for 10 min, washed in PBS and permeabilized for 5 min in 0.1% Triton X-100/PBS and blocked at 5% BSA in PBS. Cells were stained with merlin and tubulin antibodies (diluted 1:100 and 1:200, respectively) followed by secondary antibodies or TO-PRO 3-iodide or DAPI nuclear stain. Double stainings were performed sequentially. Coverslips were mounted in DABCO (Sigma) and Mowiol (Calbiochem) or Vectashield (Vector Laboratories, Burlingame, CA, USA). Cells were examined by confocal microscope: U251 cells with Leica SP2 equipped with Ar and Kr lasers (Leica Microsystems, Heerbrugg, Switzerland) and Schwann cells with Zeiss LSM 510 META (Fig. 1) using the sequential scanning mode. Schwann cell images (Figs 5 and 6D) were acquired by immuno-fluorescence microscope (Zeiss Axioskop equipped with AxioCam cooled CCD-camera, Carl Zeiss, Esslingen, Germany).

Live cell imaging was performed in pre-warmed microscope chambers at +37°C with 20 mM Hapes as a buffering agent in the medium. Cells were plated on LabTek borosilicate no. 1.5 imaging chambers (Nunc, Naperville, IL, USA) and imaged either with Olympus inverted IX81 microscope (supplemented with 5% CO₂ for the Nocodazole experiment) and CellR program or with confocal Zeiss LSM 510 META microscope. For videos, cells were imaged every 30 s for 4 h (exposure 122 ms). FRAP analysis was performed with the Zeiss confocal microscope, pre-bleach images were obtained after which lasers were turned to full power and region of interest was bleached 50 times, the same settings were used for all FRAP experiments including the region of interest. Data analysis was performed in Zeiss META and in Microsoft Excel.

Statistical and image analysis

All statistical analyses were performed in Excel with Student’s t-test using two-tailed distribution and image analysis was performed with Image J 1.36b. Images were processed with Adobe Photoshop, or with Image J 1.36b. Background was subtracted from DIC images with FFT bandpass filter and with Pseudo flat-field filter in Image J. Wide-field immunofluorescence images (Figs 5A, B and 6C, D) were deconvoluted with Huygens Deconvolution software (Scientific Volume Imaging), 30 iterations for each channel.

In vitro tubulin polymerization and other experiments

In vitro tubulin polymerization was performed on UV-permeable 96-well plates. Tubulin was purified from bovine brain as previously described (42) and recombinant merlin was produced in SF9 insect cells as described in the GST-fusion protein section. Nocodazole 20 μM, Taxol 20 μM (Sigma) and MAP’s (purified from bovine brain) were used as controls in the polymerization reaction. The polymerization buffer (80 mM PIPES, 0.5 mM MgCl₂, 1 mM Ethylene glycol-bis, 1 mM GTP, pH 6.9, 10% glycerol) was mixed with tubulin (25 μM) and merlin constructs (different amounts) were added on ice. At the beginning of the experiment the 96-well plate was transferred to pre-warmed 96-well plate reader that measured the OD at 350 nm every 5 min at +37°C for 60 min. The Nocodazole experiment was performed on primary Schwann cells with or without the re-expression of merlin via adenovirus-infection. Cells were incubated with 8 μM Nocodazole overnight after which the block was released and the cells were fixed at indicated time points. The cells were stained for tubulin and merlin.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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