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Point mutation in the *NF2* gene of HEI-193 human schwannoma cells results in the expression of a merlin isoform with attenuated growth suppressive activity

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Abstract

Neurofibromatosis type 2 (NF2) is a genetic disorder characterized by the formation of bilateral schwannomas of the eighth cranial nerve. Although the protein product of the *NF2* gene (merlin) is a classical tumor suppressor, the mechanism by which merlin suppresses cell proliferation is not fully understood. The availability of isolated tumor cells would facilitate a better understanding of the molecular function of merlin, but primary schwannoma cells obtained from patients grow slowly and do not yield adequate numbers for biochemical analysis. In this study, we have examined the *NF2* mutation in HEI-193 cells, an immortalized cell line derived from the schwannoma of an NF2 patient. Previous work showed that the *NF2* mutation in HEI-193 cells causes a splicing defect in the *NF2* transcript. We have confirmed this result and further identified the resultant protein product as an isoform of merlin previously designated as isoform 3. The level of isoform 3 protein in HEI-193 cells is comparable to the levels of merlin isoforms 1 and 2 in normal human Schwann cells and several other immortalized cell lines. In contrast to many mutant forms of merlin, isoform 3 is as resistant to proteasomal degradation as isoforms 1 and 2 and can interact with each of these isoforms *in vivo*. Cell proliferation assays showed that, in $NF2^{-/-}$ mouse embryonic fibroblasts, exogenously expressed merlin isoform 3 does exhibit growth suppressive activity although it is significantly lower than that of identically expressed merlin isoform 1. These results indicate that, although HEI-193 cells have undetectable levels of merlin isoforms 1 and 2, they are, in fact, not a merlin-null model because they express the moderately active growth-suppressive merlin isoform 3.

Keywords

merlin; schwannomin; NF2; ERM proteins

Introduction

Neurofibromatosis type 2 (NF2) is an inherited disorder that predisposes patients to the formation of bilateral schwannomas of the eighth cranial nerve and an increased propensity

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for the formation of meningioma and spinal ependymoma [1]. The gene responsible for NF2 was cloned in 1993 [2,3]. The *NF2* transcript can be alternatively spliced to form many *NF2* variants [4,5], the most abundant of which are isoforms 1 and 2, which comprise approximately 90% of the mature *NF2* transcript within cells ([6], see Fig. 1). Only isoform 1 has been shown to suppress cell growth in cell model systems [7].

The mechanism by which merlin regulates cell proliferation is not fully understood. Merlin can block Rac-mediated signaling [8], perhaps directly through inhibition of Pak activity [9]. Consistent with this notion, tumor-derived $NF2^{-/-}$ schwannoma cells display aberrant membrane ruffling that is characteristic of hyperactivated Rac [10]. This phenotype can be corrected by addition of dominant negative Rac [10] or wild type merlin [11], suggesting that loss of merlin in schwannoma cells may lead to cellular hypertrophy as a result of elevated Rac signaling.

Merlin also is known to play an important role in contact-dependent growth arrest [12,13]. Embryonic fibroblasts derived from *Nf2*-deficient mice continue to grow beyond confluency, and their inability to form stable adherens junctions appears to be at least partially responsible for this aberrant phenotype [13].

Although studies using primary schwannoma cells have yielded significant information, their use also has significant drawbacks. Typical tumor specimens yield extremely low numbers of cells, and the cells grow very slowly in culture. In addition, primary schwannoma cells are refractory to gene transfection, making mechanistic studies difficult. Thus, the inherent difficulties in obtaining, maintaining, and manipulating primary schwannoma cells led us to evaluate the immortalized schwannoma cell line HEI-193 as an alternative cell system with which to study merlin function.

The HEI-193 cell line was derived from an NF2 patient with spontaneous bilateral vestibular schwannomas as well as a history of meningioma [14]. The schwannomas were surgically removed and their constituent cells subsequently immortalized using the human papilloma virus E6-E7 genes [15]. Previous work detected a germ-line mutation in the *NF2* gene at position −1 of the intron 14 / exon 15 border. This mutation is predicted to destroy the donor sequence of exon 15 and result in exon skipping [16]. The presence of a shorter *NF2* transcript in HEI-193 cells was confirmed by RT-PCR [15]. However, the molecular alterations in the *NF2* transcript and the encoded merlin protein were not fully described.

In this paper we report that the merlin protein expressed in HEI-193 cells has amino acid sequence identical to that of a splice variant previously designated as isoform 3 [17]. This isoform was first described in a family with a history of a mild form of NF2 and was shown to arise because of an A→T mutation within the *NF2* gene at the +3 position of the donor site of intron 15 [17]. Interestingly, isoforms 1, 2 and 3 are simultaneously and equivalently expressed both at the RNA and protein levels in fibroblasts derived from this family, but in schwannoma cells only isoform 3 is expressed [17]. Based on the mild nature of the NF2 disease phenotype seen in this family, the authors of this study concluded that merlin isoform 3 retained mild tumor suppressive activity.

Here we present evidence that HEI-193 cells express merlin isoform 3 with no detectable isoform 1 or 2. The level of merlin isoform 3 in HEI-193 cells is comparable to levels of merlin found in normal human Schwann cells and several immortalized cell lines, and merlin isoform 3 appears to be as stable as isoforms 1 and 2. Although the presence of merlin isoform 3 has no apparent negative effect on the growth of HEI-193 cells, when exogenously expressed in $NF2^{-/-}$ mouse embryonic fibroblasts, isoform 3 suppresses growth, but much less effectively than similarly expressed merlin isoform 1. Merlin isoform 3 also interacts readily with both isoforms I and 2 *in vitro* and *in vivo*.

Materials and Methods

Reagents and Supplies

Unless otherwise indicated, all reagents were purchased from Invitrogen (Carlsbad, CA). Anti-merlin C18 and A19 antibodies and anti-Cyclin D were from Santa Cruz (Santa Cruz, CA). Anti-caveolin antibody was purchased from BD Biosciences (San Diego, CA) and anti-ezrin antibody, anti-FLAG (M2), and the proteasome inhibitor MG-132 were from Sigma (St-Louis, MO). FuGENE 6 transfection reagent and protease inhibitor cocktail were from Roche (Indianapolis, IN). HRP-conjugated secondary antibodies were purchased from Pierce (Rockford, IL) and chemiluminescent immunoblot detection was performed using the ECL kit from Amersham Biosciences (Piscataway, NJ). Plasmids were isolated using the Wizard® Plus DNA purification system and *In vitro* transcription/translation was performed using the TNT® system; both systems were purchased from Promega (Madison, WI).

Cell Culture

HEI-193, NIH3T3, MEF3^{flox2}, MEF3$^{\Delta 2}$, U251 and Cos-7 cells were maintained in DMEM supplemented with 10% fetal calf serum (U.S. Bio-Technologies Inc., Parkerford, PA) and 100 U/ml penicillin/streptomycin. Normal human Schwann cells were maintained as described by Bashour et al. [11]. Primary mouse embryonic fibroblasts (MEFs), harboring the conditional mutant, *Nf2$^{flox2/flox2}$* [30], were a kind gift of Dr. Marco Giovannini (INSERM, France). The immortalized mouse embryonic fibroblast cell line, MEF3^{flox2},was derived from primary *Nf2$^{flox2/flox2}$* MEFs. To generate the merlin-null MEF cell line designated MEF3$^{\Delta 2}$, MEF3^{flox2} cells were infected with an adenoviral vector encoding *Cre* recombinase. Transient transfection was carried out using FuGENE 6 or Lipofectamine 2000 according to manufacturer's directions.

Genomic DNA isolation and PCR

Genomic DNA was isolated from U251 and HEI-193 cells using the DNeasy™ tissue kit from Qiagen (Valencia, CA). Using Platinum® *Taq* DNA Polymerase High Fidelity and 0.5 μg of genomic DNA from either HEI-193 or U251 cells, a 525 bp fragment containing 153 bp of intron 14, exon 15 (163 bp) and 209 bp of intron 15 was PCR-amplified with the primers Mer_Ex15F (5′-AAGCTCCCATGGCCTGTG-3′) and Mer_Ex15R (5′-ACAAGCTCAACTTCCATCCAG-3′). The amplification program was as follows: a denaturation step at 94°C for 2 min followed by 30 cycles of amplification (94°C for 30 sec, 55°C for 15 sec, 68°C for 1 min) and a final elongation step at 68°C for 10 min. PCR products were cloned using the pCR®4-TOPO vector and TOPO TA cloning kit and sequenced at the Genomics Core at Cincinnati Children's Hospital Medical Center.

RNA isolation and RT-PCR

Confluent HEI-193 or U251 cells from a 10 cm plate were washed twice with ice cold PBS (Ca^{2+}/Mg^{2+} free). Total RNA was isolated using TRIzol® Reagent according to manufacturer's recommendations and resuspended in DEPC-treated water. RT-PCR using the SuperScript™ One-Step RT-PCR with Platinum® *Taq* was performed on 1 μg of total RNA. The full length coding region of merlin was amplified using the primersMerFexonI (5′-CATGGCCGGGGCCATCGCTTCC-3′) and MerR3′utr (5′-GCGGTCCCGGTAGCAGGAGAAGTG-3′). Exon 13 to exon 17 of merlin was amplified using the primers Mer_sense (5′-CGAAGAGCCAAGCAGAAGCTC-3′) and MerR3′utr. The glyceraldehyde-3-phosphate dehydrogenase (GPDH) cDNA was amplified using the primers hGPDH5′ (5′-ATGGGGAAGGTGAAGGTC-3′) and hGPDH3′ (5′-TTACTCCTTGGAGGCCATG-3′). Synthesis of cDNA was performed at 50°C for 30 min followed by a pre-denaturation step at 94°C for 2 min. PCR amplification consisted of 35 cycles

(94°C for 15 sec, 55°C for 30 sec, 68°C for 2 min) with a final elongation step at 68°C for 10 min. RT-PCR products were resolved on a 0.8% agarose gel followed by staining with ethidium bromide. PCR products were cloned into the pCR®4-TOPO vector using the TOPO TA cloning kit and sequenced.

Immunoblot Analysis and Inhibition of Ubiquitin-mediated Protein Degradation

Immunoblot detection was performed as described earlier [18]. Anti-merlin antibodies and Cyclin D1 were diluted 1:1,000, anti-ezrin 1:4,000, anti-caveolin 1:5,000 and secondary antibodies 1:10,000. All antibodies were diluted in blocking buffer. To inhibit ubiquitin-dependent protein degradation, the 26S proteasome inhibitor MG-132 was dissolved in DMSO and added to cells at the concentrations and time points indicated. DMSO alone was used as a vehicle control.

Dot Blot Analysis of Protein Levels

Serial dilutions of cell lysates and known amounts of purified recombinant merlin isoform 1 were spotted on nitrocellulose and subjected to immunoblot analysis using the anti-merlin antibody A19 (Santa Cruz sc-331). Intensities of all spots were quantified using ImageQuant (Molecular Dynamics) software. A standard curve was generated using spot intensities of purified, bacterially expressed merlin, and the amount of merlin present in cell lysates was estimated using the standard curve.

In Vitro Transcription/Translation

Plasmids (pcDNA3) encoding wild type merlin isoform 1 or 2 or FLAG-tagged isoform 3 were transcribed and translated *in vitro* according to manufacturer's directions using the TNT® Quick coupled transcription/translation system. For immunoprecipitation, 20 μl of TNT® mixture containing merlin isoform 1 or 2 was combined with 20 μl of a control TNT® mixture in which no DNA had been transcribed/translated or with 20 μl of a TNT® mixture containing FLAG-merlin 3. After incubation on ice for 30 min, 5 μl were removed and mixed with 5 μl of 2X SDS-PAGE sample buffer and boiled 5 min. To the remaining 35 μl of protein mixtures, 320 μl of 0.5% TX-100 in PBS were added along with 5 μl of anti-FLAG (M2) antibody and the solution placed on ice for 30 min. Protein G-agarose slurry was added as described below and the sample incubated overnight before washing agarose 3 times with 0.5% TX-100/PBS and preparation for SDS-PAGE.

Immunoprecipitation

To immunoprecipitate FLAG epitope-tagged merlin, cell lysates were prepared using IP buffer containing 50mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 0.05% sodium deoxycholate, 0.1% SDS, 60 mM n-octyl-β-D-glucopyranoside, 1% Triton X-100, and protease inhibitor cocktail. After 15 min lysis in 1 ml of IP buffer, insoluble material was pelleted for 15 min in a microcentrifuge. The soluble supernatant was removed and 80 μl was mixed with 20 μl of 5X SDS-PAGE sample buffer and boiled 5 min. Five μl of anti-FLAG antibody was added to the remainder of the lysate and the mix was placed on ice for 30 min. Next, 50 μl of Protein G agarose slurry was added and the tubes placed on a rotator at 4°C for 4 h. The Protein G agarose was pelleted and washed 3 times with IP buffer, then boiled for 5 min after resuspension in 200 μl of 2X SDS-PAGE sample buffer.

Cell growth assay

The effect of recombinant adenoviral expression of merlin isoforms on the growth of merlin mutant MEF3$^{\Delta 2}$ cells was measured in 96-well plates by MTT assay. Five 96-well plates were prepared with 2×10^3 cells per 100 μl per well, five wells per set, four sets per plate. Each plate had one set of mock infected cells and three sets of cells infected cells with recombinant

adenovirus expressing merlin isoform 1, merlin isoform 3 or control GFP. Infections were performed by adding to each well 10 μl of media containing 2×10^5 pfu virus for an m.o.i of 100:1. MTT assays were performed on one plate on day 0, day 1, day 2, days 3 and day 4. Twenty μl of a 5 mg/ml solution of MTT in PBS was added per well, incubated for 1 h, the media was then aspirated, the cells were solubilized in 100 μl of DMSO for 30 min and the absorbance at 570 nm was measured on a Bio-Tek EL-311 Microplate Reader (Bio-Tek Instruments, Winooski, VT).

Results

Confirmation of the NF2 mutation in HEI-193 cells

To evaluate HEI-193 cells as a model for studying merlin function, we carried out a thorough investigation of the *NF2* gene, transcript, and protein in HEI-193 cells. It has been reported previously that the *NF2* gene in HEI-193 cells possesses a point mutation at the border of intron 14 and exon 15 [15]. To confirm the presence of the previously described G→A mutation within the *NF2* gene (position 1571-1, Fig. 1 and Table 1), we amplified the intron 14/exon 15 region from HEI-193 genomic DNA by PCR and sequenced the product. The result confirmed the presence of the transition mutation (Table 1). This mutation replaces the G that is present in 100% of the −1 position of the intron/exon borders catalogued by Mount [16], preventing recognition of this sequence as an intron/exon border. As a control, we sequenced the identical region of the *NF2* gene from U251 cells, a glioma cell line known to express full-length merlin [19]. As expected, the *NF2* gene from U251 cells did not possess this mutation (Table 1).

The predicted effect of this mutation in HEI-193 cells is a splicing error leading to skipping of exon 15 during processing of the *NF2* pre-mRNA, resulting in a shortened *NF2* transcript [15]. Our RT-PCR amplification of the open reading frame of the *NF2* mRNA using HEI-193 and U251 total RNA as templates (Fig. 2) confirmed that such a transcript exists. As shown in Fig. 2, the major *NF2* transcript from U251 cells was 1.8 kb as predicted, whereas the full length *NF2* transcript from HEI-193 cells was slightly shorter, at 1.6 kb. The smaller transcripts in both cell lines reflect other alternative splicing, especially of exons 2 and 3, which is commonly observed in many cell types [4,6,20-22]

Full length, wild type merlin is a mixture of isoforms 1 and 2, themselves generated by alternative splicing of the 3′ end of the *NF2* transcript (Fig. 1). Previous data have suggested that these isoforms are expressed at roughly equivalent levels, with isoform 2 being slightly more prevalent [6]. These two forms can be distinguished from one another by RT-PCR amplification of the *NF2* transcript near the 3′ end of the open reading frame [23]. Using primers designed to amplify the *NF2* transcript from exon 13 to the 5′ end of exon 17 (Fig. 1), a 491 bp product was formed as the result of amplification of merlin isoform 1, whereas a 536 bp product was formed from isoform 2 transcript due to the presence of the 45 bp exon 16 (Fig. 1). As expected, both products are present in U251 cells (Fig.2), indicating the presence of merlin isoforms 1 and 2 in these cells. In contrast, when a similar reaction was performed using HEI-193 RNA, a single band of approximately 330 bp was present.

Taken together, our data suggest that the *NF2* mutation in HEI-193 results in a shortened *NF2* transcript due to a splicing error in the 3′ end of the *NF2* mRNA. Additionally, alternative splicing at the 3′-end of the *NF2* mRNA is abolished in HEI-193 cells as evidenced by the single RT-PCR product.

HEI-193 cells express a variant NF2 transcript termed isoform 3

To establish the identity of the major isoform of merlin expressed in HEI-193 cells, we sequenced the RT-PCR products. Most clones obtained from HEI-193 mRNA possessed wild

type 5′ ends, with some clones lacking exons 2 and/or 3 (not shown). However, as predicted, the 3′ end of *NF2* transcript from HEI-193 cells consisted of a sole isoform lacking both exons 15 and 16 (Fig. 1). Surprisingly, the predicted amino acid sequence of this form of merlin corresponded exactly with that of a merlin isoform previously reported in an NF2 patient, designated isoform 3 [17]. However, isoform 3b, a second transcript that lacks only exon 15, detected in the same NF2 patient, was not detected in HEI-193 cells.

Levels of merlin isoform-3 protein in HEI-193 cells are not regulated by protein instability

Previous work has shown that mutant forms of merlin are more susceptible than their wild-type counterpart to proteolytic degradation and this may account for the observation that mutant merlin is rarely detectable in cells [24]. When we compared merlin levels in HEI-193 cells, primary normal human Schwann cells and several immortalized cell lines including NIH3T3 and Cos-7, we found that HEI-193 cells express roughly equivalent level of merlin isoform 3 (Fig. 3a). To compare the stability of merlin isoform 3 with that of isoforms 1 or 2, we transfected Cos-7 cells with cDNA encoding merlin isoforms 1, 2, or 3. Cos-7 cells are readily amenable to transfection and have been used previously in studies testing the detergent solubility and subcellular localization of overexpressed merlin [19,25]. Fig. 3b shows that, when ectopically expressed in a heterologous cell line, merlin isoform 3 is as efficiently expressed as isoform 1 or 2, suggesting that isoform 3 is not inherently unstable. When HEI-193 cells were transiently transfected with each isoform of merlin (Fig. 3b), there appeared to be a low but detectable level of proteolytic degradation of all three isoforms, as suggested by the presence of an additional, lower M_w, immunoreactive band in the immunoblot. Therefore, while HEI-193 cells are capable of expressing high levels of merlin isoforms 1, 2 and 3, a low degree of proteolysis affecting all isoforms cannot be ruled out.

Although these results suggest that merlin isoform 3 is as stable as isoforms 1 and 2, previous data have shown mutant forms of merlin to be particularly susceptible to ubiquitin-mediated degradation [24]. To test whether endogenous merlin isoform 3 was sensitive to ubiquitin proteolysis, we treated HEI-193 cells with the proteasome inhibitor MG-132, which had been shown previously to increase the half life of merlin [24]. When MG-132 was added at 5 or 50 μM for 7 or 26 h, cyclin D1 protein levels increased as expected for a protein that is efficiently degraded by the 26S proteasome (Fig. 3c) indicating that the ubiquitin pathway is fully functional in HEI-193 cells. However, treatment with MG-132 had no effect on either merlin isoform 3 or ezrin levels in HEI-193 cells. These data supported the idea that the level of merlin isoform 3 in HEI-193 cells is not regulated by a ubiquitin-dependent proteolytic mechanism.

Merlin isoform 3 can interact with isoforms 1 and 2

We and others have shown previously that merlin isoforms 1 and 2 can directly bind to one another [19,23] and that interactions amongst merlin isoforms can modulate merlin:ezrin interactions *in vivo* [23]. Thus, it would be of interest to know whether merlin isoform 3 could directly interact with isoforms 1 and 2. To address this question, untagged forms of merlin 1 and 2 and a FLAG epitope-tagged version of isoform 3 were produced using a coupled *in vitro* transcription/translation system (TNT®). Untagged merlin 1 or 2 was then mixed with control or FLAG-merlin 3 reactions and the lysates subjected to immunoprecipitation using anti-FLAG antibodies. Subsequent immunoblot analysis using the polyclonal anti-merlin antibody A19 (Fig. 4a) revealed that isoforms 1 and 2 could only be immunoprecipitated in the presence of FLAG-merlin 3, indicating that merlin isoform 3 indeed interacted with wild type isoforms (1 and 2) of merlin.

To test if the different merlin isoforms could interact *in vivo*, Cos-7 cells were transfected with isoforms 1 or 2 alone or co-transfected with a FLAG epitope-tagged version of isoform 3. Merlin isoform 3 complexes were isolated using anti-FLAG antibodies and the presence of all

isoforms detected using the anti-merlin antibody A19. As shown in Fig. 4b, all isoforms expressed well and at similar levels and, as expected, no merlin isoform 1 or 2 protein was immunoprecipitated in the absence of FLAG-merlin 3. However, when FLAG-merlin 3 was co-transfected, isoforms 1 and 2 were readily detected in FLAG immunoprecipitates. These results indicate that merlin isoform 3 is capable of forming *in vivo* complexes with isoforms 1 and 2.

Merlin isoform 3 is growth suppressive but less effective than isoform 1

The alternative splicing at the 3′end of the wild type merlin gene leads to the replacement of the 16 amino acid C-terminus of isoform 1 by a different 11 amino acid C-terminus for isoform 2 (Fig. 1). This difference is apparently sufficient to prevent isoform 2 from being growth suppressive [7]. To determine whether merlin isoform 3 possesses growth suppressive activity, mutant merlin MEF3$^{\Delta 2}$ cells were infected with recombinant adenovirus expressing isoform 1, isoform 3 or a GFP expressing control and their effect on cell proliferation was measured by MTT assay. As shown in Fig. 5, cells infected with merlin isoform 1 grew at the same rate as controls under subconfluent conditions on days 1 and 2 but had a reduced growth rate coincident with confluence on days 3 and 4. This merlin-induced contact inhibition of growth was not observed in uninfected and control GFP infected cells. In contrast, cells infected with isoform 3 showed a somewhat reduced growth rate on days 3 and 4 but did not have the dramatic reduction in growth that was apparent in cells infected by merlin isoform 1. We conclude that merlin isoform 3 does not suppress cell growth as efficiently as isoform 1.

Discussion

The use of merlin-deficient cell and animal models has contributed significantly to our understanding of merlin function. The importance of merlin to growth regulation and development was convincingly demonstrated by the targeted disruption of the *NF2* gene, which resulted in embryonic lethality at day 6.5 in mice [26], whereas, *Nf2* heterozygous mice develop a variety of highly metastatic tumors [27]. Early studies using primary cultured merlin-deficient schwannoma cells isolated from human tumors revealed distinct mutant phenotypic traits at the cellular level, including cytoskeletal abnormalities [10,11] and growth characteristics [28]. Unfortunately, such cells are very slow-growing and refractory to DNA transfection, thus making biochemical and mechanistic studies difficult. The HEI-193 cell line, immortalized from the schwannoma cells of an NF2 patient tumor, does not suffer from these disadvantages, although until now, its characterization was incomplete. Our study addresses this shortcoming.

By sequencing the exon 15 region of the *NF2* locus from HEI-193 cells, we confirmed the previously reported G→A mutation at the −1 position of the intron 14/exon 15 border. Although this mutation was predicted to promote skipping of exon 15, it was not expected to abolish alternative splicing of exon 16 which would normally lead to isoforms 1 and 2 (Fig. 1). Thus, it was surprising to discover that HEI-193 cells express a single *NF2* mRNA consisting of exons 1-14 followed by exon 17. Due to novel splicing, the amino acid sequence of this splice variant is significantly shorter and differs completely from *either* isoform 1 or 2 near the C-terminus. Unexpectedly, the amino acid sequence is identical to a merlin isoform described previously in a family with mild NF2 and designated isoform 3 [17]. It is also interesting to note that, unlike the mutation found in HEI-193 cells, the mutation reported by Sainio et al. [17] is at the +3 position of the exon 15/intron 15 border (Table 1). At this time, how the two disparate mutations result in the same mature transcript known as isoform 3 remains unexplained.

As noted above, the merlin isoform 3 transcript has been previously described as arising in a family with a very mild form of NF2 [17]. The mild manifestation of the disease in the affected family led the authors of this study to conclude that isoform 3 retained some level of growth

suppressive activity. HEI-193 cells, which express isoform 3 exclusively, may provide the optimal experimental system to determine the growth suppressive potential of this isoform. Our cell proliferation experiments using Nf2-null MEFs infected with adenoviral vectors encoding either merlin isoform 1 or isoform 3, provide a direct comparison between the growth suppressive activity of the two merlin isoforms. They reveal that isoform 3 does not suppress cell growth nearly as strongly as isoform 1 does, but it does retain a level of activity significantly above baseline. These results provide experimental validation of the conclusion of the genetic study of Sainio et al. [17] and are also consistent with the growth characteristics of HEI-193 cells. They also suggest that, although HEI-193 cells lack merlin isoforms 1 and 2, they are, in reality, not a null model completely devoid of functional merlin protein.

We made two additional interesting observations in our characterization of merlin isoform 3. First, the levels of merlin mRNA and protein in HEI-193 cells are equivalent to the levels of merlin isoforms 1 and 2 in many other cell types, including normal human Schwann cells, monkey Cos-7 cells, and NIH3T3 mouse fibroblasts. Second, we found that merlin isoform 3 is as stable as isoforms 1 and 2 when overexpressed in HEI-193 cells as well as Cos-7 cells, suggesting that isoform 3 is not preferentially susceptible to degradation. In fact, isoform 3 protein does not appear to be susceptible to degradation via the ubiquitin-proteasome pathway, as previously reported for several merlin mutants [24]. This suggests that isoform 3 can co-exist with isoforms 1 and 2 in most cells, consistent with the notion that it can regulate the function of isoforms 1 and 2.

In addition, our characterization of merlin isoform 3 and our *in vivo* and *in vitro* binding studies revealed that merlin isoform 3 interacts with isoforms 1 and 2. This interaction may have interesting functional implications in light of published evidence that merlin isoforms 1 and 2 can interact with one another [19,23] and that this interaction can compete effectively with the interaction of isoform 1 with ezrin [23]. The idea that merlin isoforms can bind one another or ERM proteins and thereby modulate their activity is also inherent in a study of the hierarchical strengths of interaction among the N- and C-termini of merlin, ezrin and their binding partners [29]. Thus, it is possible that merlin isoform 3 may be co-expressed with isoform 1 in some cells, and the interaction of the two isoforms may act to attenuate the growth suppressive activity of isoform 1, resulting in a mild form of the disease.

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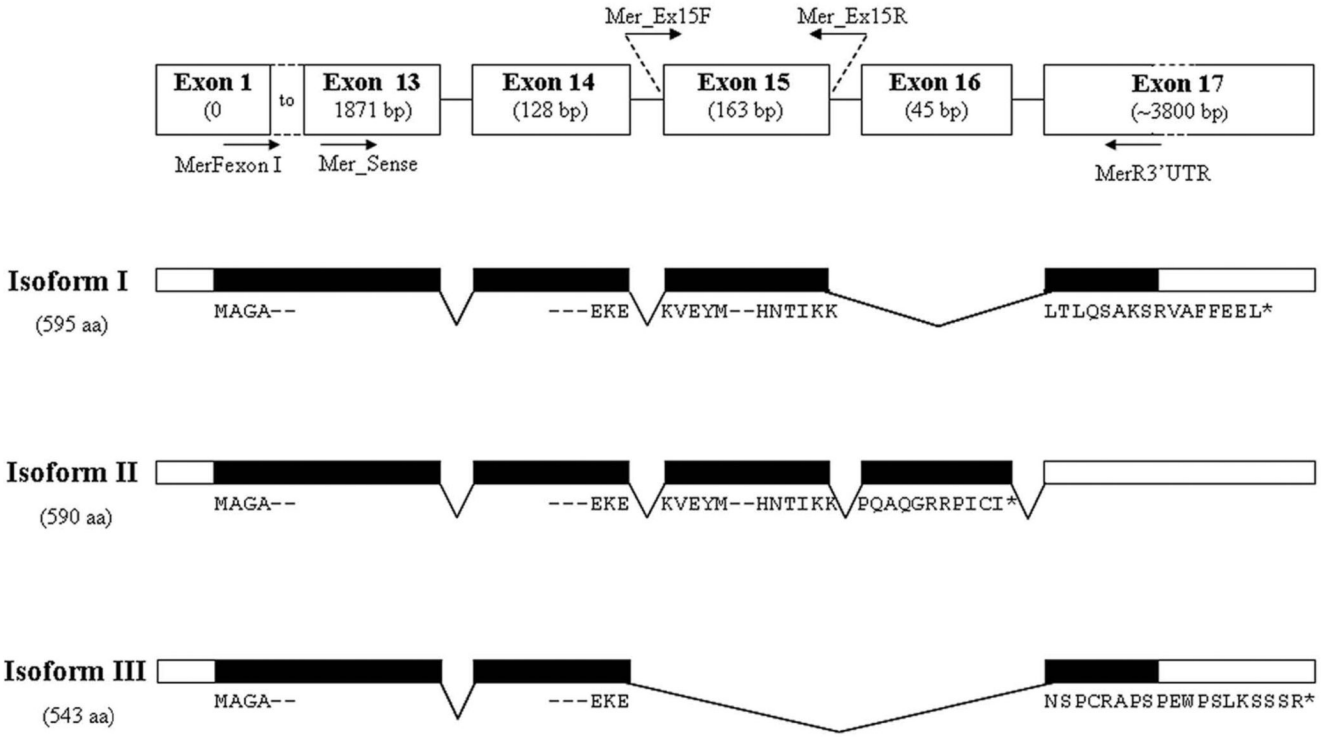

Figure 1. Schematic of *NF2* isoforms

NF2 isoforms 1, 2 and 3 are diagrammatically represented and aligned with the *NF2* gene to show the contribution of the different exons to the resultant mRNA structure and protein sequence. *NF2* isoform 2 differs from isoform 1 by the addition of exon 16, resulting in the substitution of the last 16 amino acids of isoform 1 with 11 different ones. Isoform 3 lacks both exon 15 and 16, resulting in a protein C-terminus different from both merlin 1 and 2. The arrows represent the relative positions of primers used for PCR and RT-PCR analysis. The open rectangles represent the non-translated region whereas the black rectangles represent the translated region of the mRNA. The asterisks denote positions of stop codons.

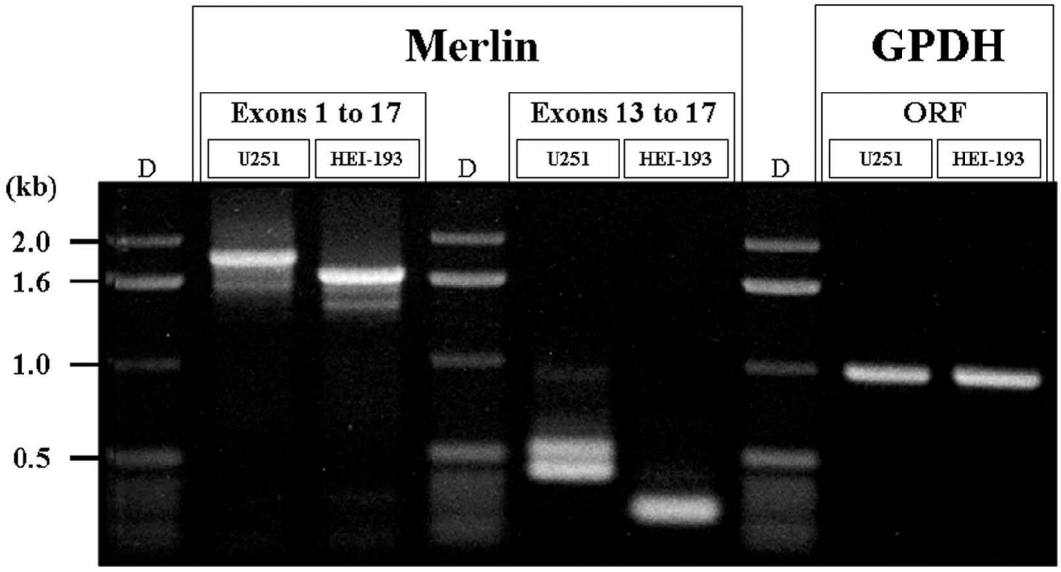

Figure 2. U251 cells express *NF2* isoforms 1 and 2 whereas HEI-193 cells express a shorter *NF2* transcript

RT-PCR was performed on 1 μg of total RNA isolated from U251 and HEI-193 cells. RT-PCR reactions included amplification of the *NF2* transcript from exons 1 through 17 (primers MerFexonI and MerR3′UTR, Fig. 1), exons 13 to 17 (primers Mer_Sense and MerR3′UTR, Fig 1) or glyceraldehyde-3-phosphate dehydrogenase (GPDH) as a control. HEI-193 cells express a shorter isoform of *NF2* compared to the isoforms expressed by U251 cells. Lanes labeled “D” are a DNA ladder used for size estimation of RT-PCR products.

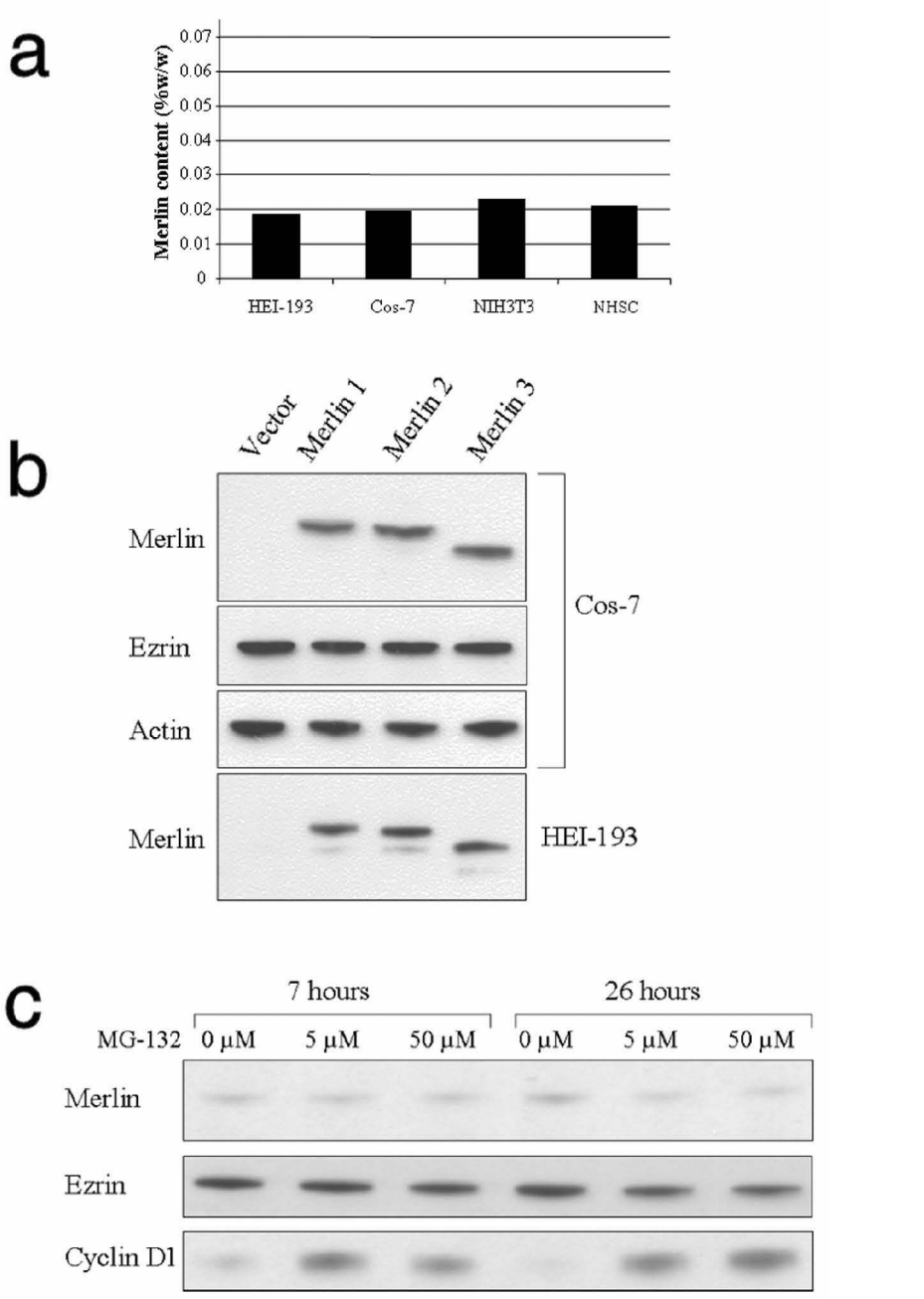

Figure 3. Levels of merlin isoform 3 protein are not regulated by protein instability

(a) Dot blot analysis, using the A19 antibody, shows the levels of merlin in HEI-193, Cos-7, NIH3T3 and primary normal human Schwann cells (NHSC) are comparable. Merlin is expressed as a percentage of total cellular protein. (b) Cos-7 or HEI-193 cells, at 2×10^5 cells, per well of a 6-well plate were transfected with 1 μg of pcDNA3 (vector) or 1 μg of pcDNA containing the cDNA encoding merlin isoforms 1, 2, or 3. Expression was analyzed 24 h after transfection using immunoblot analysis. Merlin isoform 3 can be overexpressed at similar levels as isoforms 1 and 2 and is not inherently unstable. (c) HEI-193 cells, at 5×10^5 per 60mm plate were DMSO treated (vehicle) or treated with the 26S proteasome inhibitor MG-132 for the concentrations and time points indicated. Protein levels were then assessed by immunoblot analysis. The lack

of effect of $_{MG-132}$ suggests that endogenous HEI-193 merlin isoform 3 is not degraded by the ubiquitin pathway.

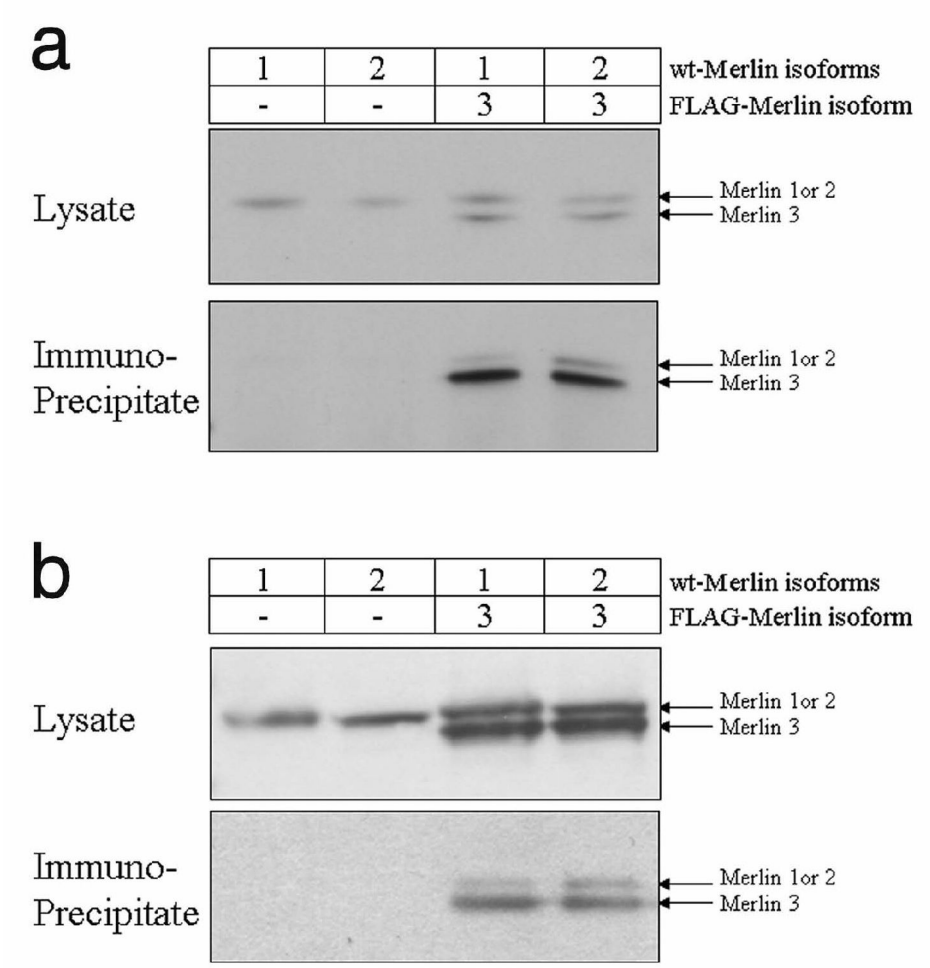

Figure 4. Merlin isoform 3 can interact with isoforms 1 and 2 *in vitro* and *in vivo*

(a) *In vitro* transcribed/translated merlin proteins were mixed, immunoprecipitated using anti-FLAG antibodies and subjected to western blot analysis. Merlin proteins were detected using anti-merlin antibody A19. Note that untagged isoforms 1 and 2 only precipitate in the presence of FLAG-isoform 3, indicating interaction of isoforms 1 and 2 with isoform 3. (b) 4×10^5 Cos-7 cells were plated on 60mm plates and transfected with 1 μg of DNA encoding merlin isoforms 1 or 2 and co-transfected with either 1 μg of empty vector or 1 μg of DNA encoding FLAG epitope-tagged isoform 3. Lysates were prepared and immunoprecipitated using anti-FLAG antibody and merlin proteins detected using the anti-merlin antibody A19 as in (a). In both (a) and (b) the upper panel (lysate) shows expression of all isoforms of the expected size and the lower panel (ImmunoPrecipitate) shows that isoforms 1 and 2 are found in complex with the flagged merlin isoform 3.

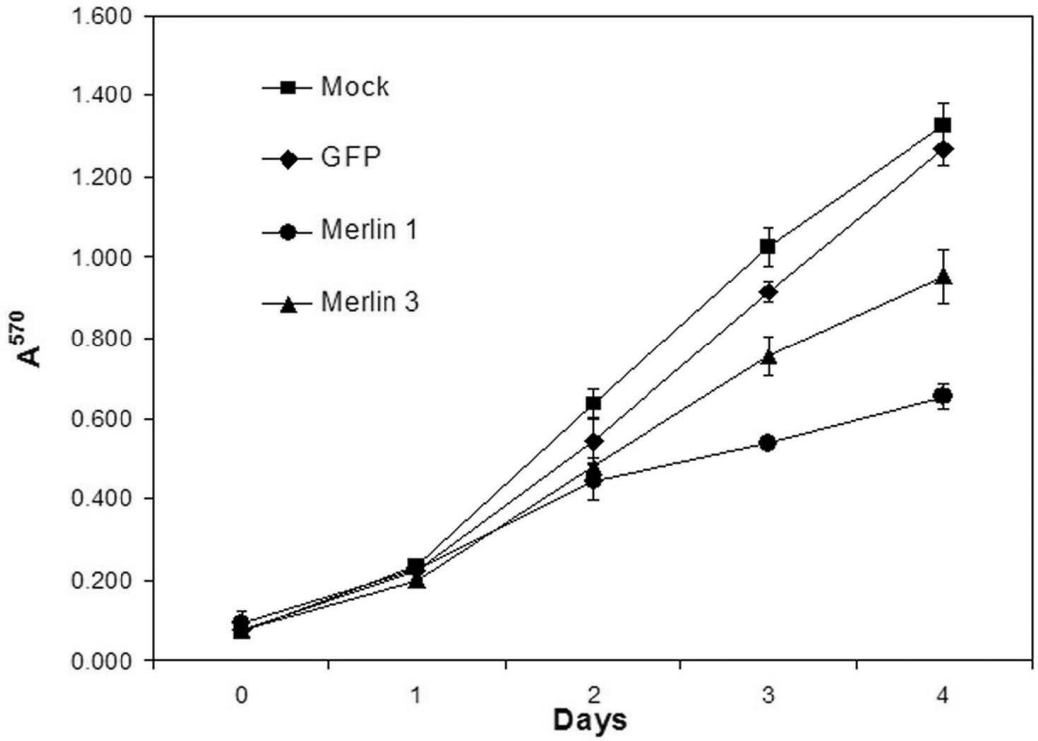

Figure 5. Merlin isoform 3 has a weakly suppressive effect on cell proliferation

MEF3$^{\Delta 2}$ cells, which lacked functional merlin protein, were infected with recombinant adenovirus expressing isoform 1, isoform 3 or a GFP-expressing control as described in the text. The effect of isoform 1 and isoform 3 expression on cell proliferation was measured by MTT assay for four days.

Table 1

Intron 14/Exon 15 border of the *NF2* gene in HEI-193 cells

Intron 14 / Exon 15 border of HEI-193 *NF2* gene.

Exon 15 of merlin was amplified using Genomic DNA isolated from HEI-193 and U251 cells using intronic primers flanking exon 15 (Mer_15F and Mer_15R, Fig. 1). HEI-193 cells possess a transition from G to A at the −1 position of the intron 14 / exon 15 border, leading to the destruction of the splice acceptor site of exon 15. The mutation in the HEI-193 cells is clearly different from the one reported by Sainio *et al*. [17] which is a transversion from A to T at the exon 15 +3 position. Nevertheless, the two mutant merlins have identical amino acid sequence.