NF2/Merlin Is a Novel Negative Regulator of mTOR Complex 1, and Activation of mTORC1 Is Associated with Meningioma and Schwannoma Growth[▽][†]

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Inactivating mutations of the neurofibromatosis 2 (NF2) gene, *NF2*, result predominantly in benign neurological tumors, schwannomas and meningiomas, in humans; however, mutations in murine *Nf2* lead to a broad spectrum of cancerous tumors. The tumor-suppressive function of the NF2 protein, merlin, a membrane-cytoskeleton linker, remains unclear. Here, we identify the mammalian target of rapamycin complex 1 (mTORC1) as a novel mediator of merlin's tumor suppressor activity. Merlin-deficient human meningioma cells and merlin knockdown arachnoidal cells, the nonneoplastic cell counterparts of meningiomas, exhibit rapamycin-sensitive constitutive mTORC1 activation and increased growth. NF2 patient tumors and *Nf2*-deficient mouse embryonic fibroblasts demonstrate elevated mTORC1 signaling. Conversely, the exogenous expression of wild-type merlin isoforms, but not a patient-derived L64P mutant, suppresses mTORC1 signaling. Merlin does not regulate mTORC1 via the established mechanism of phosphoinositide 3-kinase–Akt or mitogen-activated protein kinase/extracellular signal-regulated kinase-mediated TSC2 inactivation and may instead regulate TSC/mTOR signaling in a novel fashion. In conclusion, the deregulation of mTORC1 activation underlies the aberrant growth and proliferation of NF2-associated tumors and may restrain the growth of these lesions through negative feedback mechanisms, suggesting that rapamycin in combination with phosphoinositide 3-kinase inhibitors may be therapeutic for NF2.

Meningiomas are mesenchymal tumors that arise from the arachnoid layer covering the brain and spinal cord and account for approximately 30% of all primary intracranial neoplasms (30). Most sporadic meningiomas (60%) display somatic inactivation of the *NF2* gene. Germ line mutations of *NF2* are associated with neurofibromatosis 2 (NF2), a dominantly inherited disorder characterized by multiple nervous system tumors, including schwannomas and meningiomas (33). Although most meningiomas are benign (WHO grade I), they often cause significant morbidity due to compression of the adjacent brain or spinal cord. Benign meningiomas also have recurrence rates of up to 20% over 10 years. Ten percent of meningiomas are classified as atypical (WHO grade II) or anaplastic (WHO grade III) and display more aggressive clinical behavior, with rapid growth and increased recurrence rates (6, 21). The current standard of care is maximal surgical resection, with adjuvant radiation reserved for progressive tumors or those with aggressive features (e.g., WHO grade II or III). The treatment strategy for meningiomas that progress

despite surgery and radiation remains limited, and currently there is no effective chemotherapy.

The development of effective therapies has been hampered, in part, by our incomplete understanding of the signals influencing meningioma cell growth. Enhanced expression of certain peptide and steroid growth factors and receptors in meningioma tissue suggests that specific autocrine growth-stimulatory loops may be functionally important in meningioma cell proliferation (20, 38). The scarcity of established meningioma models that would allow for the assessment of growth-regulatory mechanisms has also hampered progress. Recently, we have developed reliable meningioma models that overcome the challenges of the low growth rates and senescence of primary benign meningioma cells (19).

Biallelic inactivation of the *NF2* gene is detected in the majority of sporadic meningiomas and nearly all schwannomas (11). The tumor suppressor gene *NF2* encodes merlin (also called schwannomin), a member of the ezrin-radixin-moesin (ERM) protein family that functions to link membrane proteins to the cortical actin cytoskeleton (31, 41). Like the ERM proteins, merlin has been implicated in the regulation of membrane organization and cytoskeleton-based cellular processes such as adhesion, migration, cell-cell contact, spreading, proliferation, and signal transduction (27). The loss of contact-dependent inhibition of proliferation is seen in several types of *NF2*-deficient cells (23, 29). Merlin controls cell proliferation in response to cell contact via CD44 (28) and functions together with the related tumor suppressor Expanded via the

Hippo/Mst pathway in both *Drosophila* and some types of mammalian cells (14, 49). Although merlin is implicated in a wide range of cellular activities, the precise mechanism by which merlin mediates growth-inhibitory functions in human arachnoidal and Schwann cells and the way in which its loss results in tumor formation in NF2 remain poorly understood.

We recently reported that primary human merlin-deficient meningioma cells exhibit a striking, enlarged-cell phenotype compared to nonneoplastic arachnoidal cell counterparts derived from the same patient (19). Interestingly, the tuberous sclerosis complex (TSC) tumor suppressor syndrome is characterized by widespread benign tumors that possess abnormally large cells (22). Mutations in the tumor suppressor genes *TSC1* and *TSC2* result in TSC syndrome, and the corresponding protein products, hamartin and tuberin (referred to as TSC1 and TSC2), function together as a complex that potently inhibits mammalian target of rapamycin complex 1 (mTORC1) (17). mTOR is an evolutionarily conserved Ser/Thr kinase that exists in one of two distinct functional complexes, TORC1 and TORC2. TORC1, which regulates autophagy, protein translation, and ribosome biogenesis, is potently and specifically inhibited by rapamycin (10, 46). TORC2, which is less sensitive to rapamycin, is important for cytoskeletal regulation and Akt/protein kinase B activation (16, 18, 36).

The TSC1-TSC2 complex inhibits mTORC1 by acting as a GTPase-activating protein for the small GTPase Rheb (Ras homolog enriched in brain). Inactivation of the TSC1-TSC2 complex results in the accumulation of GTP-bound Rheb, which activates mTORC1 (10). In addition to naturally occurring mutations in the *TSC1* and *TSC2* genes, growth factor stimulation of the phosphoinositide 3-kinase (PI3K)-Akt pathway, as well as Ras/mitogen-activated protein kinase (MAPK) pathways, leads to the phosphorylation and inactivation of the TSC1-TSC2 complex and consequent activation of mTORC1 (17). The activation of mTORC1 results in the phosphorylation of two well-characterized effectors, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and S6 kinase 1 (S6K1), leading to an increase in ribosomal biogenesis and the selective translation of specific mRNA populations. As a critical regulator of cell growth and proliferation, the mTORC1 pathway is dysregulated in several hamartoma syndromes, as well as in many cancers (10).

In this report, we identify the NF2 tumor suppressor protein, merlin, as a novel negative regulator of the mTORC1 pathway to control cell growth (cell size). We show that mTORC1 is constitutively activated in merlin-deficient human meningioma cells, leading to increased cell size. Furthermore, we suggest that the slow growth of merlin-deficient meningioma cells is due to a rapamycin-sensitive, mTORC1-S6K-dependent negative feedback loop that diminishes PI3K-Akt signaling in response to growth factor stimulation. The findings of these studies provide insight into the mechanism of merlin tumor suppressor activity and, moreover, indicate that rapamycin or rapamycin analogs in combination with PI3K inhibitors may provide promise as new therapeutics in the treatment of meningiomas and schwannomas.

MATERIALS AND METHODS

Antibodies and reagents. Unless otherwise stated, all antibodies were obtained from Cell Signaling Technology. Exceptions include cyclin D1 (BD Bioscience),

FLAG epitope (M2, Sigma), hemagglutinin (HA) epitope (Covance), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Chemicon International) antibodies. Merlin rabbit polyclonal antibody (C26) was described previously (19). Growth factors and inhibitors were from the following sources: insulin, wortmannin, and nocodazole, Sigma; insulin-like growth factor 1 (IGF1), Austral Biologicals; and rapamycin, LY294002, and UO126, Calbiochem. Kinase inhibitors were reconstituted in dimethyl sulfoxide (DMSO) or methanol per the manufacturers' recommendations.

Sample collection. Tissue samples were obtained from the Massachusetts General Hospital tumor repository and consisted of excess discarded tissues collected from patients undergoing clinically indicated surgery for tumor resection or specimens from autopsies. Tissues were harvested fresh for the establishment of cell lines as described previously (19) and/or were formalin fixed or flash-frozen on liquid nitrogen for histological and immunohistochemical analyses. Clinical information regarding defining sporadic and NF2-associated tumors was based on diagnoses from referring physicians, according to the published guidelines for the diagnosis of NF2 (12). The Institutional Review Board of the Massachusetts General Hospital/Partners HealthCare approved this study, and informed consent was obtained from all study subjects.

Immunohistochemistry. Four benign merlin-negative meningiomas and five vestibular schwannomas were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for immunocytochemistry analyses. Appropriate controls for antibodies included DU145 cells and normal nerve tissue (negative controls) and PTEN-deficient PC3 cells (positive controls). Paraffin-embedded tissue sections were immunostained using commercial rabbit polyclonal antibodies to phospho-S6, recognizing S235/236 and/or S240/244, under conditions described previously (15).

Cell culture. Cultures of primary meningioma and arachnoidal cells from fresh tissues were established as described previously (19). Meningioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with a 4.5-g/liter glucose solution containing 15% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin. Arachnoidal cells were maintained in a mixture of improved MEM with L-glutamine (Richter's modified medium [Cellgro; Mediatech, Inc.]), 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and insulin (Gibco Life Technologies, Inc.) at 4 mg/liter. Human embryonic kidney 293T (HEK293T) cells were maintained in DMEM containing 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. $Nf2^{-/-}$ and $Nf2^{+/+}$ mouse embryonic fibroblasts (MEFs) were kindly provided by A. I. McClatchey (Massachusetts General Hospital, Boston) and described previously (23). The littermate-derived pair of $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEF samples (both with the $p53^{-/-}$ genotype) were provided by D. J. Kwiatkowski (Brigham and Women's Hospital, Boston, MA) and described previously (47). The AC007-hTERT cell line was developed by retrovirally transducing primary arachnoidal cells with the human telomerase reverse transcriptase (hTERT) gene by using the retroviral plasmid pBABE-puro-hTERT (7).

For amino acid withdrawal studies, control and merlin knockdown arachnoidal cells were cultivated in full-growth medium prior to the shifting of cells into Dulbecco's PBS for the times indicated in Fig. 2E. For kinase inhibitor studies, merlin knockdown arachnoidal cells were deprived of serum (in 0.2% FBS) and treated with rapamycin, LY294002, wortmannin, or U0126 at the concentrations indicated in Fig. 4C to F for 30 min prior to lysis. For wortmannin control experiments, arachnoidal cells were deprived of serum (in 0.2% FBS), pretreated for 15 min with 100 nM wortmannin, and then stimulated for 15 min with 25 ng/ml IGF1. For the analysis of negative feedback inhibition of Akt, merlin-deficient meningioma and merlin knockdown arachnoidal cells in complete medium were treated with 10 nM rapamycin for 24 h prior to lysis. For the insulin stimulation assay, control and merlin knockdown arachnoidal cells were stimulated with 100 nM insulin.

Constructs, virus production, and siRNAs. The generation of FLAG-tagged wild-type (WT) merlin isoforms 1 and 2 and merlin mutants expressing S518 mutations (S518A and S518D) was described previously (44). FLAG-tagged L64P mutant merlin was generated from full-length isoform 1 by using the QuikChange site-directed mutagenesis kit (Stratagene) and sequenced to verify the incorporation of the change. FLAG-tagged wild-type TSC1 and TSC2 were provided by B. D. Manning. FLAG-tagged Rheb and HA-S6K were kindly provided by Joseph Avruch (Massachusetts General Hospital, Boston). The generation of a lentivirus construct expressing WT merlin isoform 1 in pCSCW2 and of merlin RNA interference (RNAi) constructs in pLKOpuro.1 was described previously (19, 37). Lentiviral particles were produced by the Neuroscience Vector Core at Massachusetts General Hospital. Endogenous TSC2 knockdown in HEK293T cells was carried out using SMARTpool small interfering RNAs (siRNAs) per the suggestion of the manufacturer (Dharmacon).

Transfections and infections. Mammalian transfections using HEK293T cells, including those with siRNAs, were performed using Lipofectamine 2000 as recommended by the manufacturer (Invitrogen). Transfected cells were maintained in full-growth medium for 48 h prior to lysis. For the insulin stimulation experiments, cells were serum starved (in 0% FBS) for the last 24-h period and then stimulated with 100 nM insulin for 15 min. The immunoprecipitation of HA-S6K was performed using anti-HA antibody (see Fig. 5D).

Isogenic pairs of control and merlin knockdown arachnoidal cell samples were generated by the infection of primary arachnoidal cells or AC007-hTERT cells with lentiviruses containing a nonspecific scrambled target sequence (scr) or a merlin-specific RNAi target sequence (m5, m6, or m8) (19). Cells were infected at a multiplicity of infection (MOI) of 10 and selected with puromycin (0.4 μg/ml) to obtain stable pools. Unless otherwise stated in the text, control scr and merlin (m5) RNAi arachnoidal cells were serum deprived (in 0.2% FBS) on day 7 postinfection and harvested the following day to obtain protein lysates.

Merlin-deficient meningioma cells and $Tsc2^{-/-}$ MEFs were infected with merlin isoform 1 at a MOI of 50 or 100 by lentivirus (pCSCW2)-based delivery and then harvested 3 days postinfection to obtain protein lysates.

Proliferation assays. To detect $Nf2^{+/+}$ and $Nf2^{-/-}$ MEF cells in S phase, 5-bromo-2′-deoxyuridine (BrdU) incorporation was quantified using the in situ cell proliferation kit FLUOS according to the specifications of the manufacturer (Roche, Mannheim, Germany). $Nf2$ MEF cells were plated onto poly-D-lysine-coated coverslips, treated with DMSO or rapamycin (20 nM) for 24 h, and incubated with 10 μM BrdU for the final 4 h. BrdU incorporation was detected using a fluorescein-conjugated anti-BrdU antibody. BrdU-positive and DAPI (4′,6-diamidino-2-phenylindole)-positive cells in five randomly chosen fields per condition were counted using a TCS SP5 confocal microscope (Leica, Wetzlar, Germany), and the percentages of BrdU-positive cells were calculated. All the experiments were performed in triplicate. Statistical analyses were carried out using Student's two-tailed t test.

Cell lysis and immunoblotting. Meningioma and arachnoidal cells and $Tsc2$ MEF cells were harvested to obtain cell lysates as described previously (19). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad), and subjected to immunoblot analyses using appropriate antibodies, horseradish peroxidase-conjugated secondary antibodies, and an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotechnology).

Cell size and cell cycle analysis. The sizes of primary merlin knockdown arachnoidal (AC030) cells relative to those of scr control cells with or without rapamycin treatment were determined by fluorescence-activated cell sorter (FACS) analyses with a FACSCalibur instrument (Becton Dickinson) using forward scatter height (FSC-H) values. Stable cell populations were treated with 5 nM rapamycin every other day for 7 days. Cells were trypsinized and resuspended in PBS containing 3% FBS and 5 mM EDTA. A total of 20,000 cells were collected, and the mean FSC-H values were obtained using CellQuest software. Cells were stained by propidium iodide for FACS analyses to determine the cell sizes in G_1- and G_2/M-phase populations.

Cultured meningioma and merlin knockdown arachnoidal cells treated with and without rapamycin were visualized by phase-contrast imaging using a Nikon Eclipse TE2000-U inverted microscope and photographed using a SPOT RT slider charge-coupled device camera and SPOT imaging software (Diagnostic Instruments Inc.).

RESULTS

Rapamycin inhibits the growth of merlin-deficient arachnoidal and meningioma cells in vitro. We recently reported that human primary merlin-deficient meningioma cells are enlarged during culturing compared to matched normal arachnoidal cells. Similarly, arachnoidal cells in which merlin expression is suppressed become dramatically larger than cells treated with control RNAi constructs (19). These findings suggest that pathways regulating increased cell size may be negatively regulated by merlin. Since the activation of the mTORC1 pathway has emerged as a conserved regulatory mechanism in the control of cell size (45), we examined whether the mTORC1-specific inhibitor rapamycin would counter the increased sizes of merlin-deficient NF2 target cell types. Treatment with rapamycin, at a concentration as low as 1 nM,

concurrent with the initiation of stable knockdown of merlin in arachnoidal cells blocked an increase in cell size compared to that of untreated merlin knockdown cells (Fig. 1A). An analysis of relative cell sizes by flow cytometry using the mean FSC-H as a parameter demonstrated that arachnoidal cells in which merlin was suppressed were significantly larger than control cells, as indicated by a rightward shift in the mean FSC-H (Fig. 1B, left). Rapamycin abolished the effect of merlin knockdown on cell size (Fig. 1B, right). Additionally, we observed that enlarged, actively dividing merlin knockdown arachnoidal cells treated with rapamycin for 4 days reverted to a strikingly smaller size (see Fig. S1 in the supplemental material). The reversal of the phenotype of cell enlargement, relative to the sizes of untreated cells, in cultures of merlin-deficient meningioma cells treated with rapamycin was also observed (Fig. 1C). These findings indicate that the inhibition of mTORC1 by rapamycin can not only prevent but also reverse increases in the sizes of merlin-deficient arachnoidal and meningioma cells.

Merlin-deficient cells exhibit constitutive mTORC1 signaling. We next examined whether the mTORC1 signaling pathway in merlin-deficient NF2 target cells was dysregulated. The levels of phosphorylation of ribosomal protein S6, an indicator of mTORC1 activity, in three primary merlin-deficient meningioma cell cultures were elevated compared to those in three normal primary arachnoidal cell lines under conditions of serum deprivation (Fig. 2A). To examine whether the increase in mTORC1 signaling was due to merlin loss, we suppressed merlin in arachnoidal cells by using three different targeting short hairpin RNAs (shRNAs; m5, m6, and m8). This suppression produced an increase in the phosphorylation of S6 at Ser235/236 and at Ser240/244 (sequential S6K substrate phosphorylation sites of S6) compared to that in control (scr) RNAi arachnoidal cells under conditions of serum deprivation. Consistent with increased mTORC1-dependent phosphorylation of S6, we observed increased phosphorylation at S2448 of mTOR and T389 of p70-S6K, a direct mTORC1 substrate, in merlin knockdown cells compared to that in control cells (Fig. 2B). Merlin suppression in these cells also resulted in decreased 4EBP1 mobility (an indicator of phosphorylation) compared to that in control cells, confirming that both arms regulating mTORC1-mediated protein synthesis are enhanced (Fig. 2C). Additionally, we detected increased expression of cyclin D1, a downstream target of mTORC1 (1), in merlin knockdown cells. These findings suggest that mTORC1 is activated in a growth factor-independent manner in merlin-deficient cells.

We tested whether merlin suppression resulted in increased cell size in different phases of the cell cycle. The suppression of merlin with two different shRNAs in arachnoidal cells caused an increase in cell size in G_1 and G_2/M phases of the cell cycle (Fig. 2D), indicating that cell size and aberrant mTORC1 signaling in merlin-deficient cells do not reflect cell cycle changes and represent bona fide effects on mTORC1. We also examined if merlin-deficient cells exhibited elevated mTORC1 signaling compared to that in control arachnoidal cells under low-serum conditions after S phase arrest, by treating cells with nocodazole for 24 h prior to replating the cells in 0.2% FBS–DMEM for an additional 24 h. Under these conditions, merlin-deficient cells exhibited the activation of mTORC1, eliminating the possibility of mitogenic input under low-serum
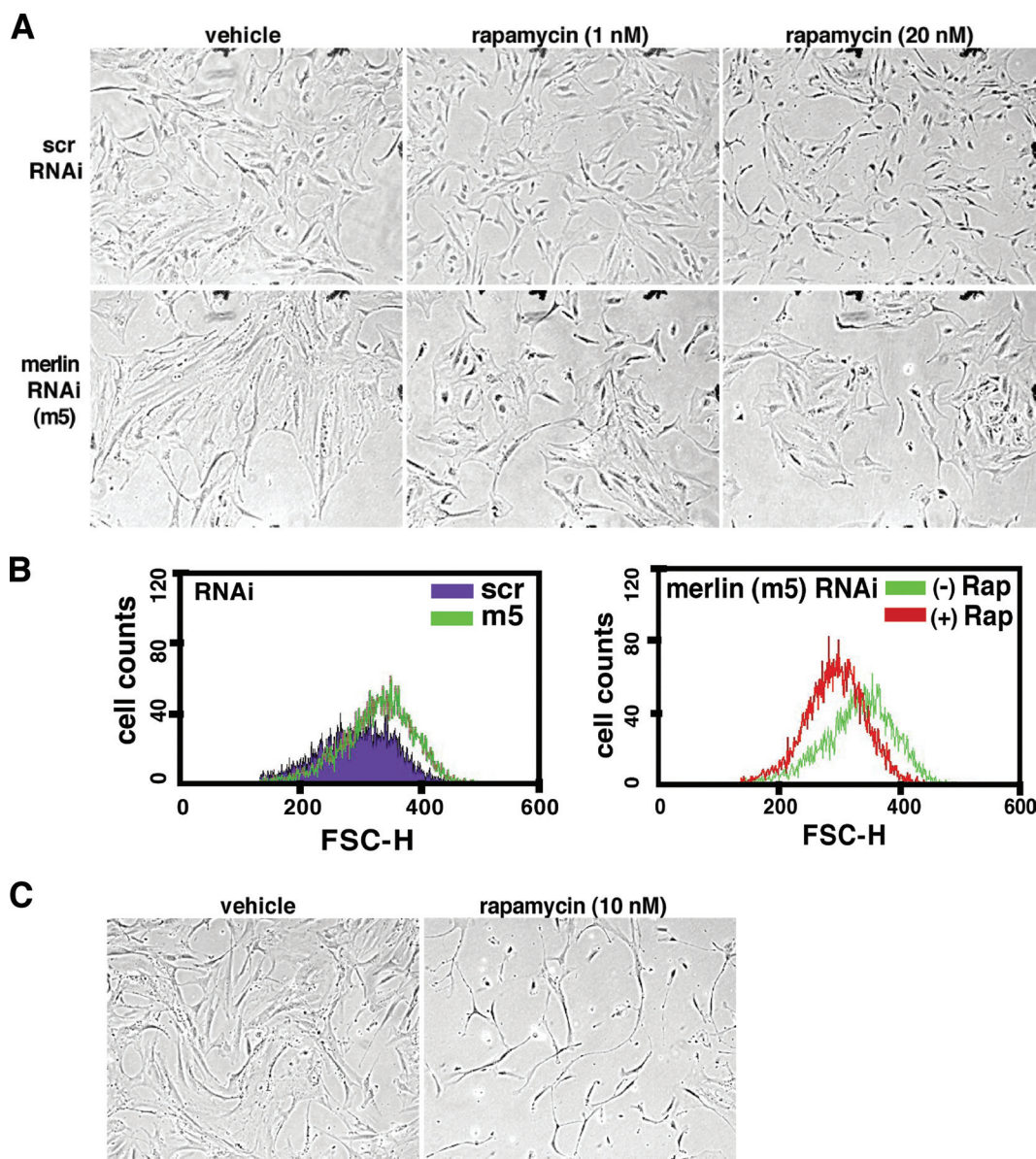
FIG. 1. Rapamycin inhibits the growth of primary merlin-deficient arachnoidal and meningioma cells. (A) Primary control (scr RNAi) and merlin knockdown (m5 RNAi) arachnoidal (AC030) cells were treated with or without rapamycin starting at day 2 postinfection with lentiviruses expressing shRNAs and every other day thereafter for 12 days. Phase-contrast images demonstrate an increase in the sizes of untreated (vehicle-treated) merlin RNAi arachnoidal cells compared to those of scr RNAi arachnoidal cells (original magnification, ×10). Rapamycin (1 and 20 nM) inhibits the increase in the sizes of merlin RNAi cells compared to those of vehicle-treated merlin RNAi arachnoidal cells. (B) Primary control and merlin knockdown arachnoidal (AC030) cells, treated with (+) or without (−) rapamycin (Rap; 10 nM) every other day for 10 days, were evaluated by FACS analyses to determine relative cell sizes. The x axes indicate relative cell sizes according to FSC-H values. (Left) Merlin m5 RNAi increases the sizes of arachnoidal cells, as determined by the rightward shift in the mean FSC-H histogram compared to that for control scr-treated arachnoidal cells. (Right) Rapamycin abolishes the effect of merlin m5 RNAi on cell size. (C) The enlargement of cells in primary merlin-deficient MN304 meningioma cultures treated with vehicle only is inhibited by treatment with rapamycin (10 nM) every other day for 7 days (original magnification, ×10).

conditions (see Fig. S2 in the supplemental material). We next examined whether S6 phosphorylation was appropriately terminated in the absence of nutrients in *NF2*-deficient cells. Amino acid deprivation blocked mTORC1 signaling in both the control and merlin knockdown arachnoidal cells (Fig. 2E), suggesting that merlin does not regulate mTORC1 through nutrient-sensitive pathways.

Merlin-deficient meningiomas and vestibular schwannomas exhibit aberrant mTORC1 signaling in vivo. To determine whether mTORC1 signaling is aberrantly regulated in NF2-associated tumors, we examined the status of S6 phosphorylation in four *NF2*-deficient benign meningiomas and five vestibular schwannomas by immunohistochemistry analyses. Phospho-S6 immunostaining of *NF2*-deficient meningiomas
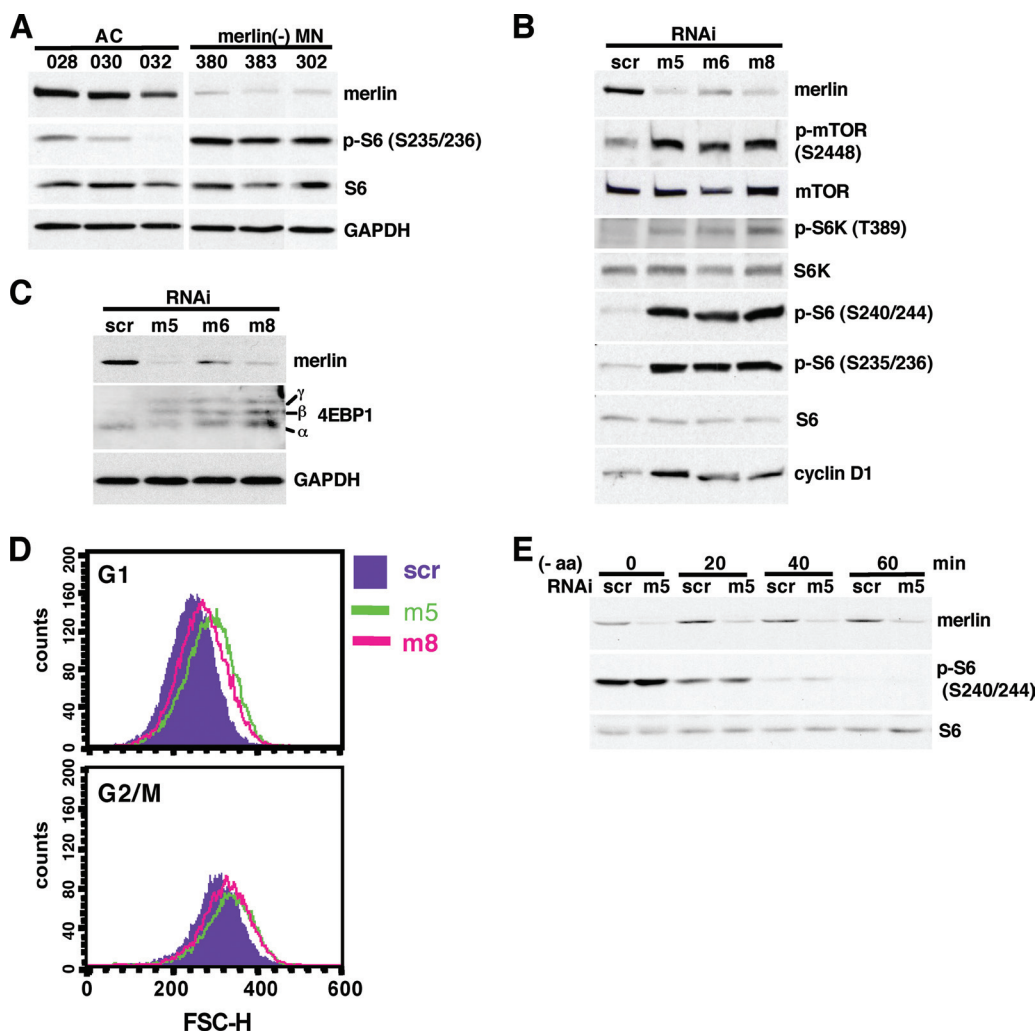
FIG. 2. The mTORC1 pathway is aberrantly activated in merlin-deficient NF2 target cell types in a growth factor-independent manner but is sensitive to nutrient deprivation. (A) Three primary arachnoidal (AC028, AC030, and AC032) and three primary merlin-deficient [merlin(−)] meningioma (MN380, MN383, and MN302) cell lines were deprived of serum (in 0.2% FBS) overnight prior to being harvested to obtain protein lysates. S6, a common marker of mTORC1 activation, exhibits elevated phosphorylation in merlin-deficient MN cells compared to that in normal arachnoidal cells, with no change in total S6 protein levels. GAPDH protein levels demonstrate equivalent sample loading. p-S6 (S235/236), S6 phosphorylated at S235/236. (B) Arachnoidal cells stably expressing three different lentivirus-mediated shRNAs (m5, m6, and m8) targeting merlin exhibit increased phosphorylation of mTOR (S2448) and mTORC1 effectors p70-S6K (at Thr389) and S6 (at S240/244 and S235/236), as well as cyclin D1, a downstream target of mTORC1, upon merlin knockdown compared to that in control (scr RNAi) cells under conditions of serum deprivation (in 0.2% FBS). Total levels of mTOR, S6, and S6K proteins were unchanged. p-mTOR (S2448), mTOR phosphorylated at S2448; p-S6K (T389), S6K phosphorylated at T389; p-S6 (S240/244) and p-S6 (S235/236), S6 phosphorylated at S240/244 and S235/236, respectively. (C) Merlin knockdown in arachnoidal cells (by m5, m6, and m8) results in increased accumulation of phosphorylated species of 4EBP1 compared to that in scr control RNAi cells under conditions of serum deprivation (in 0.2% FBS). 4EBP1 phosphorylation (detected by anti-4EBP1 antibody) is reflected by 4EBP1 electrophoretic mobility results. (D) Cell cycle evaluation by FACS analysis demonstrates increased sizes of merlin knockdown cells (expressing m5 and m8 shRNAs) compared to those of scr control cells in G_1 and G_2/M phases of the cell cycle. (E) To examine effects of merlin deficiency on nutrient deprivation, control (scr) and merlin RNAi (m5) knockdown arachnoidal cells were grown overnight with full serum (15% FBS) prior to being shifted into Dulbecco's PBS for 0, 20, 40, or 60 min. The phosphorylation of S6 (at S240/244) is appropriately terminated in merlin RNAi arachnoidal cells, similar to that in control scr cells. − aa, amino acid deprivation.

showed diffuse cytoplasmic positive staining, consistent with the activation of the mTORC1 pathway (Fig. 3). The vestibular schwannomas displayed a focal staining pattern with areas of strong phospho-S6 positivity. Normal nerve tissue employed as a negative control did not show phospho-S6 staining. Similar patterns of phospho-S6 immunostaining were obtained using antibodies recognizing phosphorylation at both Ser235/236 and Ser240/244. These data suggest that mTORC1 is activated in vivo in NF2-associated schwannomas and meningiomas.

mTORC1 activation upon merlin loss is PI3K-Akt and MAPK/extracellular signal-regulated kinase (ERK) independent. We next sought to understand the mechanism of constitutive, growth factor-independent activation of mTORC1 signaling in merlin-deficient cells. Since growth factor-initiated mTORC1 signaling is commonly mediated by upstream activation of PI3K-Akt, we examined whether the PI3K-Akt signaling cascade was dysregulated in NF2 target cells. We observed that Akt was not phosphorylated at Ser473 in either
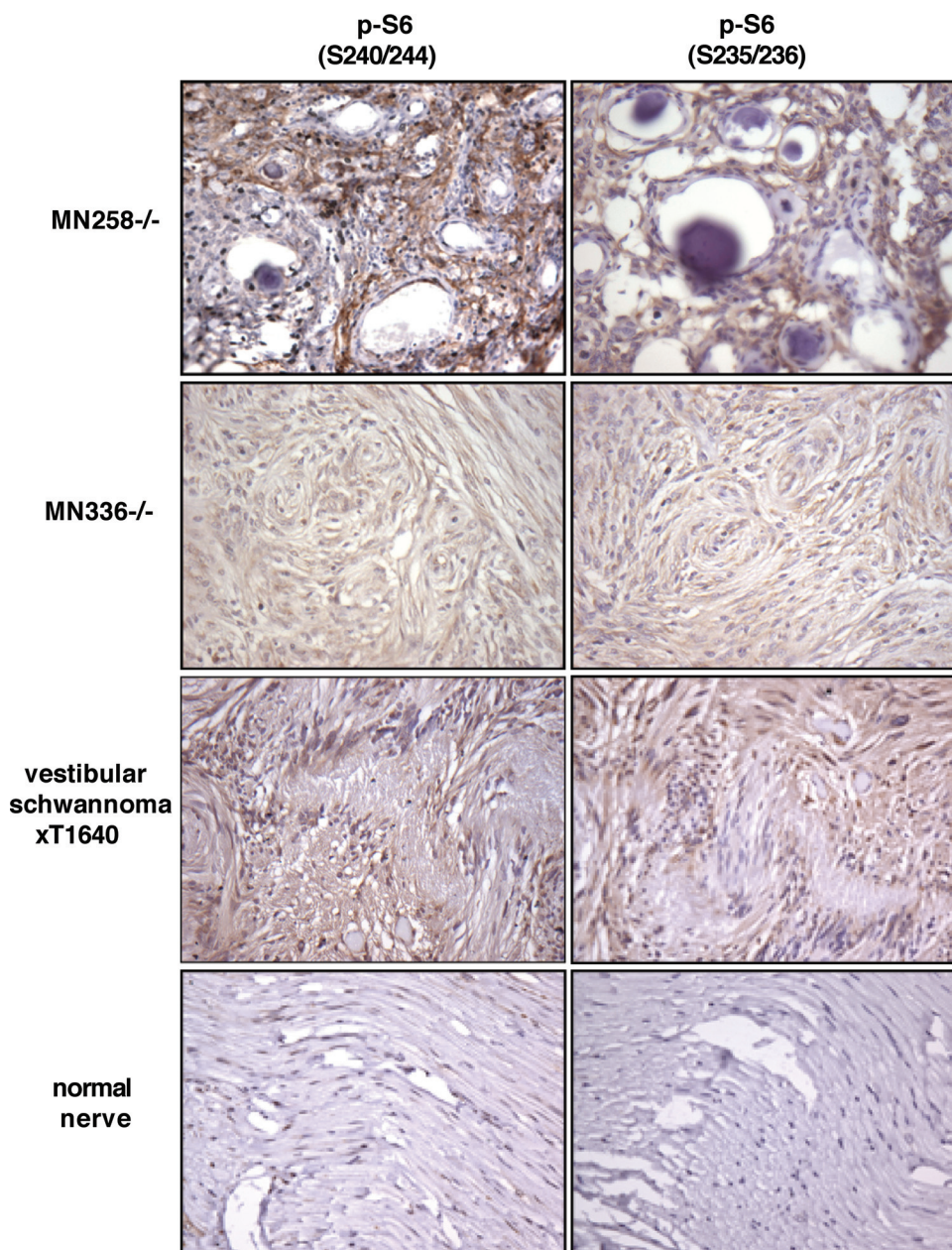
FIG. 3. Merlin-deficient meningiomas and vestibular schwannomas exhibit constitutive S6 phosphorylation. Patient-derived merlin-deficient meningioma (MN258$^{-/-}$ and MN336$^{-/-}$) and vestibular schwannoma (xT1640) tissues were stained immunohistochemically to detect S6 phosphorylation (at S240/244 and S235/236). Data are representative of results for four benign merlin-deficient meningiomas and five benign vestibular schwannomas. Sections of normal nerve tissue (negative control) did not exhibit S6 phosphorylation (original magnification, ×40).

merlin-deficient meningioma cells or merlin knockdown arachnoidal cells, similar to that in control arachnoidal cells (Fig. 4A and B). Consistent with these findings, PRAS40, a downstream effector of Akt (35, 42), was not phosphorylated (Fig. 4B). Tuberin, a direct substrate of Akt, also was not phosphorylated at T1462 in merlin knockdown arachnoidal cells (data not shown). In addition, we did not observe aberrant activation of various growth factor receptors upon merlin loss, consistent with the absence of Akt activation (see Fig. S3 in the supplemental material). Collectively, these data suggest that PI3K-Akt signaling is not dysregulated in merlin-deficient meningi-

oma cells and therefore does not contribute to aberrant mTORC1 signaling.

We then examined merlin-deficient cells for constitutive MAPK signaling since positive growth signals from the Ras/MAPK pathway can also stimulate mTORC1 activity. ERK-specific activation of mTORC1 involves multisite phosphorylation of tuberin by both ERK and its downstream target p90 ribosomal S6 kinase (25, 32). Interestingly, we detected constitutive ERK1/ERK2 phosphorylation in both merlin-deficient meningioma cells and merlin knockdown arachnoidal cells, compared to control arachnoidal cells, under conditions
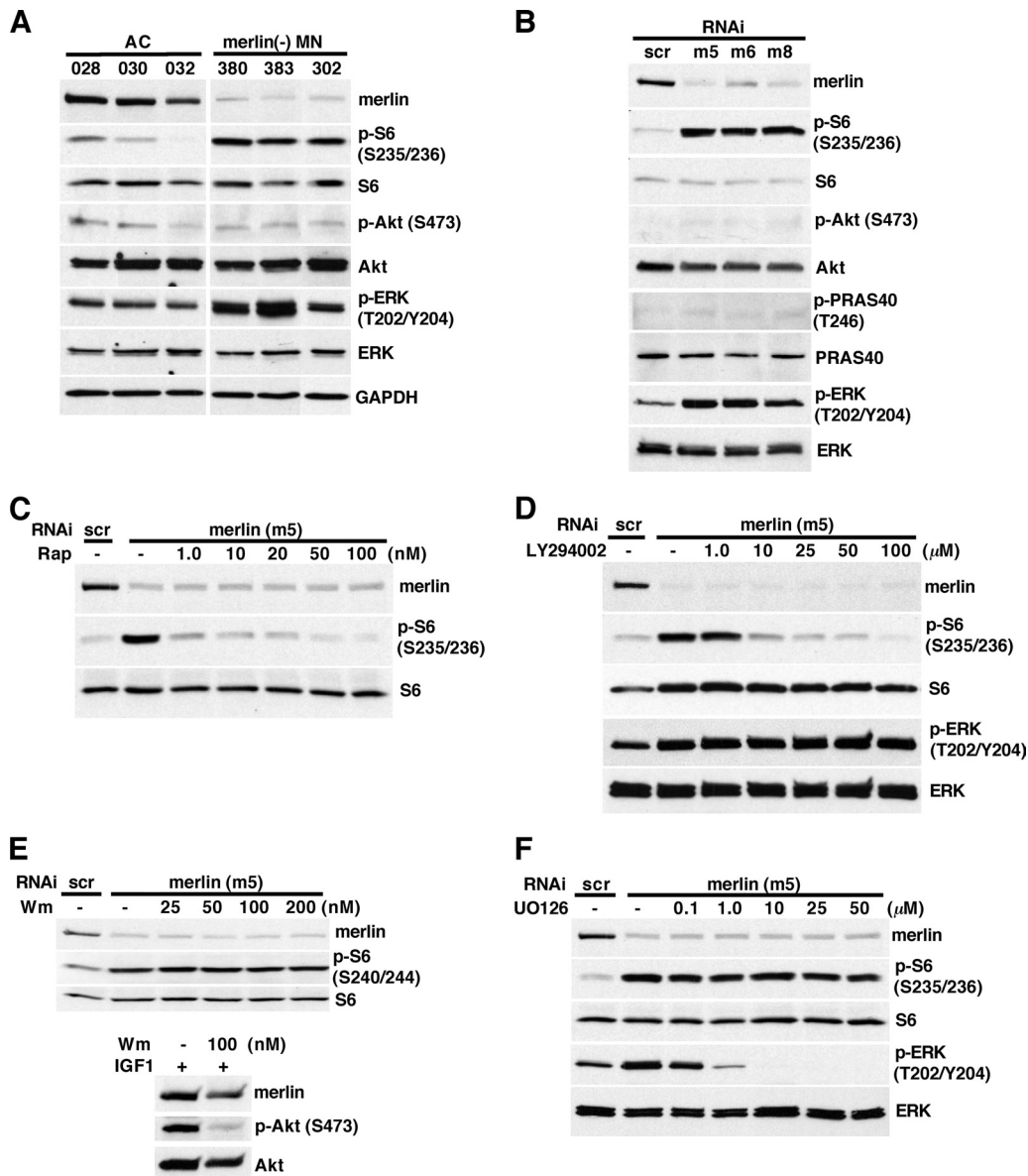
FIG. 4. Merlin-deficient mTORC1 activation is PI3K-Akt and MAPK/ERK independent. (A and B) Merlin-deficient meningioma [merlin(−) MN] (A) and merlin knockdown (m5, m6, or m8 RNAi) (B) arachnoidal cells exhibit elevated ERK1/ERK2 phosphorylation compared to that in control (scr RNAi) arachnoidal cells. In contrast, merlin-deficient target cells exhibit no change in the phosphorylation of Akt (A and B) or PRAS40 (B), a substrate of Akt and a negative regulator of mTORC1, compared to that in control arachnoidal cells. Cells were treated as described in the legend to Fig. 2A and B. (C to F) Control (scr RNAi) and merlin knockdown (m5 RNAi) arachnoidal cells were deprived of serum (in 0.2% FBS) overnight, and the merlin knockdown cells were then incubated with the indicated concentrations of rapamycin (Rap), LY294002, wortmannin (Wm), and UO126 for 30 min. Cell lysates were assayed for S6 phosphorylation by immunoblot analyses. −, absent. (C) The mTORC1-specific inhibitor rapamycin blocks constitutive S6 activation. (D) The PI3K inhibitor LY294002 blocks S6 activation but does not affect MAPK/ERK signaling. (E, top) Wortmannin does not block S6 activation, indicating that mTORC1 activation is independent of PI3K-Akt signaling. (Bottom) Wortmannin potency is demonstrated by the inhibition of IGF1-stimulated Akt phosphorylation in arachnoidal cells pretreated with wortmannin (100 nM), in contrast to that in untreated cells. (F) U0126, a MEK1/MEK2 inhibitor, blocks ERK1/ERK2 phosphorylation but has no effect on S6 phosphorylation.

of serum deprivation (Fig. 4A and B), suggesting that merlin may regulate mTORC1 activity through the Ras/MAPK signaling pathway.

To further assess the involvement of upstream signaling pathways in the aberrant activation of mTORC1 signaling in merlin-deficient cells, we examined S6 phosphorylation in the presence of pharmacological inhibitors that specifically target

these pathways. As expected, the inappropriate activation of S6 in merlin knockdown arachnoidal cells was inhibited by rapamycin, demonstrating dependence on mTORC1 (Fig. 4C). In addition, we found that the PI3K inhibitor LY294002 blocked S6 activation (Fig. 4D). Since mTOR, a PI3K-related enzyme, is directly inhibited by LY294002 to the same extent as PI3Ks (4, 43), we examined the response of S6 activation to wortman-

nin, which exhibits relatively high specificity for PI3Ks at concentrations of 20 to 50 nM. In the presence of 25 to 200 nM wortmannin, we detected no inhibition of S6 activation in merlin-deficient cells (Fig. 4E), confirming that PI3K-Akt signaling does not contribute to constitutive mTORC1 activation. Wortmannin potently inhibited IGF1-stimulated Akt activation in pretreated arachnoidal cells, demonstrating wortmannin's effectiveness (Fig. 4E, bottom). Additionally, we found that neither LY294002 nor wortmannin had any effect on ERK1/ERK2 phosphorylation, indicating that ERK activation was not dependent on PI3K signaling (Fig. 4D and data not shown). To determine whether the mechanism of constitutive mTORC1 activation in merlin-deficient cells is dependent on Ras/MAPK signaling, we treated cells with the MEK1/MEK2 inhibitor compound UO126. UO126 completely inhibited ERK1/ERK2 signaling in a dose-dependent manner without affecting S6 phosphorylation, indicating that merlin does not regulate mTORC1 through Ras/MAPK-dependent signaling pathways (Fig. 4F). Together, these results confirm that mTORC1 activation in merlin-deficient cells is not mediated by the activation of either PI3K-Akt or Ras/MAPK signaling pathways.

Exogenous merlin expression inhibits mTORC1 signaling. To determine whether merlin overexpression could inhibit growth factor-mediated mTORC1 activation, full-length merlin isoform 1 or isoform 2 was coexpressed with the HA-S6K reporter in insulin-stimulated HEK293T cells. Consistent with the findings of previous studies, Rheb overexpression potently activated mTORC1 signaling, as indicated by S6K (T389) phosphorylation, whereas TSC1 and TSC2 coexpression inhibited S6K phosphorylation (Fig. 5A). Both merlin isoforms strongly inhibited S6K activation (Fig. 5A). We next examined whether an NF2 patient-derived L64P missense mutant or either of two merlin S518 phosphomutants expressing S518A or S518D, thought to render merlin active or inactive, respectively, as a tumor suppressor protein (39, 40), is able to block mTORC1 activation. Under growth conditions with full serum, WT merlin blocked mTORC1 activation relative to that in cells expressing a control vector; however, the L64P NF2 mutant protein did not. Similar to WT NF2 protein, both S518 phosphomutants were capable of suppressing mTORC1 activity (Fig. 5B). In agreement with this finding, the reintroduction of WT NF2 protein into NF2-deficient meningioma cells by lentivirus-mediated delivery inhibited endogenous S6 phosphorylation under conditions of serum deprivation (Fig. 5C). Collectively, these findings show that merlin loss leads to the activation of mTORC1 and that the ectopic expression of either merlin isoform 1 or 2 inhibits mTORC1 signaling.

We next examined whether merlin could inhibit mTORC1 activation in TSC1-TSC2-deficient cells. As expected, TSC2 knockdown via siRNA expression resulted in increased S6K (T389) phosphorylation in HEK293 cells; however, NF2 protein expression in TSC2 knockdown cells was unable to block S6K activation under growth conditions with full serum (Fig. 5D). Similarly, NF2 protein overexpression did not inhibit constitutive S6K activity in TSC2 null MEFs (Fig. 5E), indicating that merlin does not reside downstream of the TSC1-TSC2 complex. To determine whether NF2 protein overexpression could block Rheb-mediated activation of mTORC1, we coexpressed Rheb and NF2 protein. As shown in Fig. 5F, merlin did

not inhibit elevated mTORC1 activity resulting from Rheb overexpression, confirming that merlin is not downstream of TSC-Rheb.

Merlin-deficient cells exhibit negative feedback regulation of PI3K-Akt signaling. Like TSC, NF2 is a tumor syndrome involving predominantly benign lesions. One line of thought is that the limited malignant potential of TSC-associated tumors is due to the attenuation of Akt signaling (17). We examined whether merlin-deficient cells were defective in growth factor-mediated activation of Akt. We observed that, in response to insulin stimulation, a time-dependent increase in Akt and S6 phosphorylation in control RNAi arachnoidal cells occurred but that Akt stimulation was impaired in merlin knockdown arachnoidal cells (Fig. 6A). Merlin knockdown cells demonstrated constitutive S6 activation, with no further activation in response to insulin stimulation. Prolonged exposure to the mTORC1-specific inhibitor rapamycin enhances PI3K-Akt activation in many cell lines by relieving phospho-S6K-mediated PI3K-Akt feedback inhibition (26). The exposure of merlin-deficient meningioma cells and merlin RNAi arachnoidal cells to rapamycin for 24 h resulted in the activation of Akt (Fig. 6B). Collectively, these data are consistent with the existence of a basal mTORC1-dependent negative feedback loop in merlin-deficient cells. Furthermore, rapamycin treatment resulted in decreased cyclin D1 expression in both merlin-negative meningioma cells and arachnoidal cells in which merlin was suppressed, indicating that the elevation of cyclin D1 in merlin-deficient cells is partially mTORC1 dependent (Fig. 6B, bottom).

$Nf2^{-/-}$ **MEFs exhibit constitutive mTORC1 activation and rapamycin-sensitive proliferation.** We also examined mTORC1 signaling in $Nf2^{-/-}$ MEFs and found that, similar to human cell types, $Nf2$-deficient MEFs exhibited constitutive activation of mTORC1 signaling, in contrast to WT $Nf2^{+/+}$ MEFs, as indicated by phospho-S6 levels and the phosphorylation status of 4EBP1 (Fig. 7A and B). Furthermore, the observed increase in the proliferation of $Nf2$-deficient MEFs compared with that of $Nf2^{+/+}$ MEFs was significantly reduced when the $Nf2$-deficient MEFs were treated with 20 nM rapamycin (Fig. 7C), suggesting that the activation of mTORC1 signaling partly contributes to the enhanced proliferation of these cells.

DISCUSSION

In this study, we provide evidence that the NF2 tumor suppressor merlin acts as a novel negative regulator of mTORC1. Merlin deficiency results in the constitutive activation of mTORC1 signaling in human arachnoid/meningioma cells in the absence of growth factors but has a negligible effect on nutrient-dependent mTORC1 activation. Contrary to the concept that unphosphorylated merlin isoform 1 (lacking phosphorylation at S518 and exhibiting a closed conformation) and not isoform 2 is responsible for the tumor suppressor function (39, 40), we found no functional distinction in the abilities of the merlin isoforms or the phospho-S518 merlin mutants (the S518A and S518D proteins) to suppress mTORC1 signaling. Our findings indicate that neither the isoform type nor the S518 phosphorylation status predicts tumor suppressor activity as defined by aberrant mTORC1 signaling. This result is consistent with the absence of NF2-causing mutations in the car-
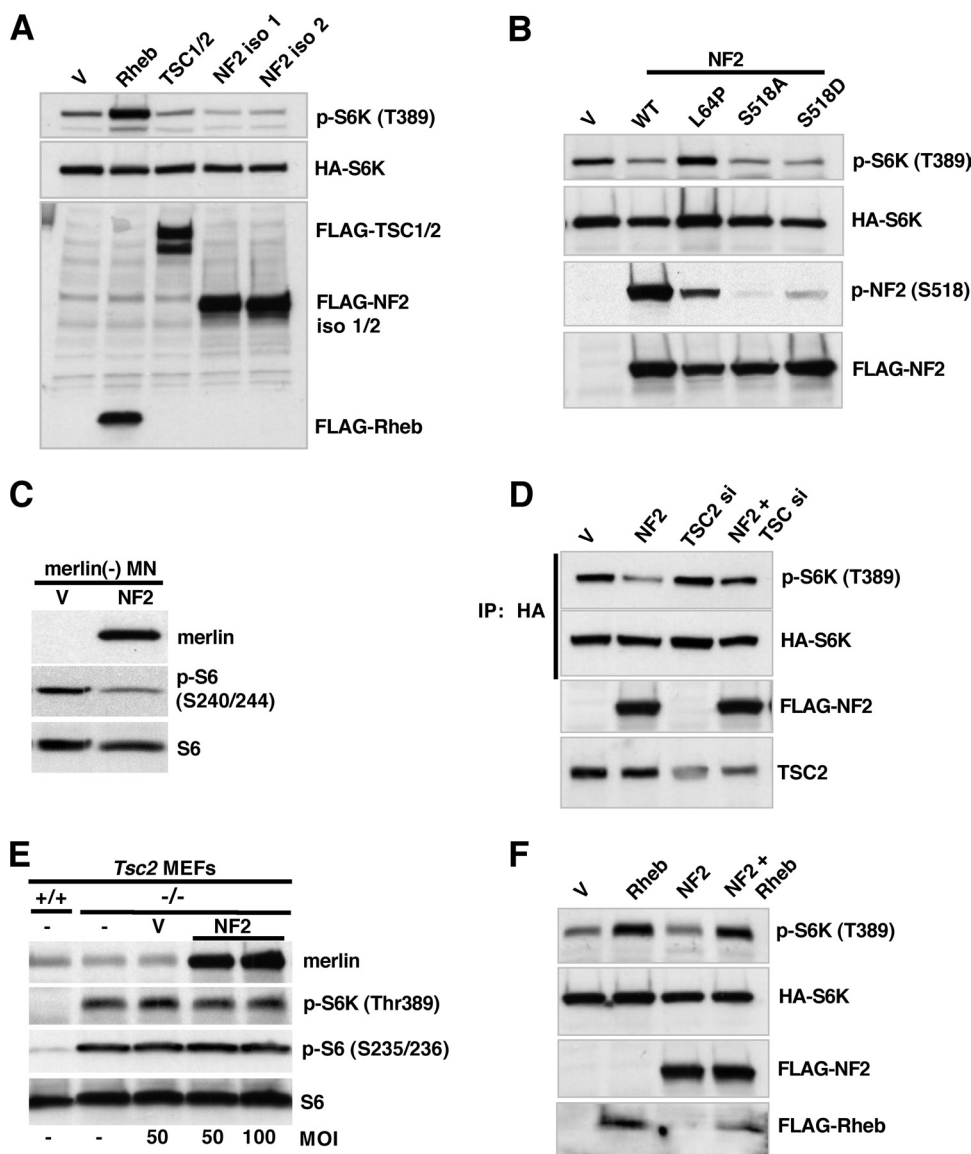
FIG. 5. Merlin expression inhibits mTORC1 signaling. (A) HEK293T cells were cotransfected with HA-S6K and vector only (V) or FLAG-tagged Rheb, TSC1-TSC2, or NF2 protein isoform 1 (NF2 iso 1) or isoform 2 (NF2 iso 2). After 24 h, cells were subjected to serum starvation (0% FBS) overnight and stimulated with insulin (150 nM) for 15 min. Both NF2 isoforms inhibit S6K phosphorylation compared to that in cells expressing the vector only. Rheb expression activates and TSC1-TSC2 expression inhibits the mTORC1 pathway. (B) HEK293T cells were cotransfected with HA-S6K and vector only or FLAG-tagged WT NF2 protein or mutant NF2 protein constructs expressing L64P, S518A, or S518D. Similar to WT NF2 protein, NF2 mutant proteins expressing S518A and S518D inhibit S6K activation in full-growth medium (10% FBS), but a patient-derived mutant (L64P) protein does not. p-NF2 (S518), NF2 protein phosphorylated at S518. (C) Merlin-deficient meningioma [merlin(−) MN] cells were transduced with vector only or NF2 protein (MOI, 50) and subjected to serum starvation (0% FBS) overnight. NF2 protein-infected cells exhibit the inhibition of S6 phosphorylation compared to that in vector-infected cells. (D) HEK293T cells were cotransfected with HA-S6K and vector only or FLAG-NF2 protein, TSC2 siRNA (TSC2 si), or FLAG-NF2 protein and TSC2 siRNAs together (NF2 + TSC si). Immunoblot analyses of HA immunoprecipitations (IP) using an anti-HA antibody indicate that S6K phosphorylation is not inhibited by NF2 protein in TSC2 siRNA-expressing cells in full-growth medium. The decrease in levels of S6K phosphorylated at T389 in the NF2 + TSC2 si lane compared to those in the TSC2 si lane is due to reduced levels of HA-S6K expression. (E) $Tsc2^{-/-}$ MEFs were infected with vector (MOI, 50) or NF2 protein (MOIs, 50 and 100) as described in the legend to panel C. NF2 protein does not inhibit S6K or S6 phosphorylation compared to that in vector-infected cells. Lanes −, untransfected cells. (F) Among HEK293T cells transfected as described in the legend to panel D, those coexpressing NF2 protein and Rheb show no inhibition of S6K phosphorylation.

boxy-terminal regions corresponding to the exons (16 and 17) that distinguish the two isoforms. Although the MAPK/ERK signaling pathway is aberrantly regulated in merlin-deficient cells, we found that mTORC1 activation due to merlin loss is independent of either PI3K-Akt or MAPK/ERK signaling.

The results of our studies suggest that merlin may function upstream of the TSC1-TSC2 complex, the major mTORC1 regulator in the cell. Our data are consistent with the model that merlin signals through TSC-Rheb to regulate mTORC1. Merlin-deficient cells resemble TSC null cells in that the loss of either tumor suppressor results in high levels of constitutive mTORC1 signaling, a definitive absence of Akt phosphoryla-
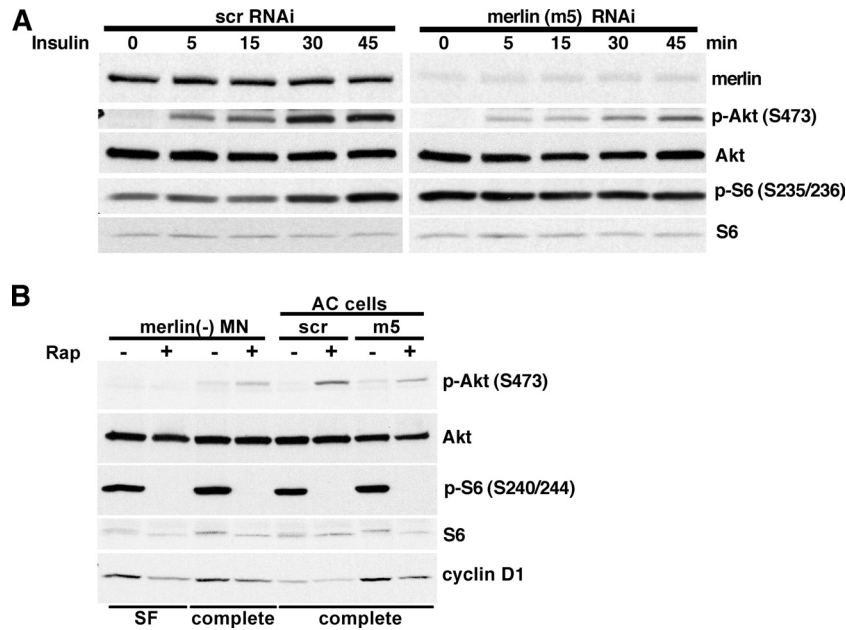
FIG. 6. Akt signaling is attenuated in cells lacking NF2 protein due to mTORC1-mediated negative feedback inhibition. (A) Control (scr RNAi) and merlin knockdown (m5 RNAi) arachnoidal cells were deprived of serum (in 0.2% FBS) overnight and stimulated with insulin (100 nM) for the times indicated. scr RNAi arachnoidal cells exhibit a time-dependent increase in activated Akt (S473) and S6 (S235/236) phosphorylation, in contrast to merlin knockdown arachnoidal cells, which exhibit impaired Akt phosphorylation and constitutive S6 activation that is insensitive to insulin stimulation. (B) Merlin-deficient meningioma [merlin(−) MN] cells and control (scr) and merlin (m5) RNAi arachnoidal cells were incubated with (+) and without (−) rapamycin (Rap; 10 nM) for 24 h. Untreated cells in complete medium (15% FBS) exhibit S6 phosphorylation (at S240/244) and the attenuation of Akt phosphorylation (at S473), resulting from constitutive activation of an mTORC1-S6K negative feedback loop. In the presence of rapamycin, mTORC1-S6K signaling is blocked, preventing S6 phosphorylation, and Akt phosphorylation is restored by relief from negative feedback. Under serum-free (SF; 0% FBS) conditions, merlin-deficient meningioma cells do not exhibit the restoration of Akt signaling. In addition, cyclin D1 levels are reduced in rapamycin-treated cells, suggesting that expression is, in part, mTORC1 dependent.

tion at S473, and enlarged cells. Merlin deficiency in human cells, similar to TSC deficiency, results in lower proliferation rates than those of their WT counterparts under growth conditions with full serum, perhaps due to their decreased responsiveness to serum (19, 48). Although the mechanism by which merlin regulates TSC1-TSC2 is unclear, hamartin was shown previously to interact with the ERM family proteins and to weakly bind merlin (13, 24). The TSC1-TSC2 protein complex has also been shown to modulate actin cytoskeleton processes,

focal adhesions, and cell migration (9), cellular activities that are also regulated by merlin/ERM proteins (3, 27). A recent study suggested that TSC2 is inhibited by Akt-mediated phosphorylation by being sequestered in the cytoplasm, away from its membrane-bound partners hamartin and Rheb (5). Further investigations are necessary to understand whether merlin deficiency results in mTORC1 activation by altering the localization of TSC1 or TSC2, by causing the dissociation of the TSC1-TSC2 complex, and/or by affecting the Rheb–GTPase-
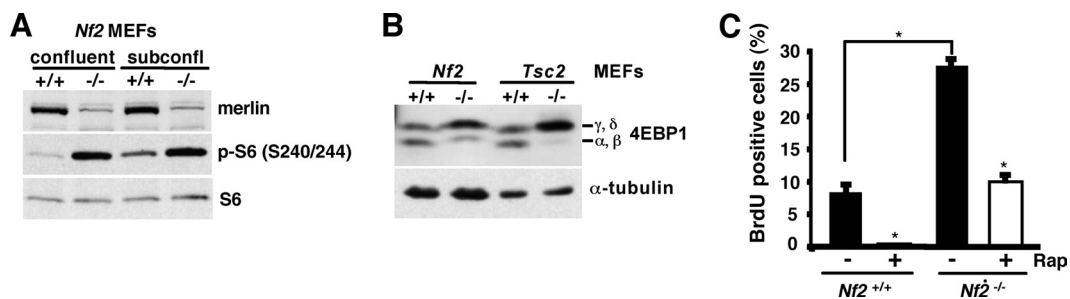

FIG. 7. Nf2-deficient ($Nf2^{-/-}$) MEFs exhibit constitutive mTORC1 activation compared to Nf2 WT ($Nf2^{+/+}$) MEFs. (A) $Nf2^{-/-}$ MEFs, plated confluently and subconfluently (subconfl), exhibit constitutive S6 activation, in contrast to $Nf2^{+/+}$ MEFs, under conditions of serum starvation (0% FBS). (B) $Nf2^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs exhibit increased accumulation of phosphorylated species of 4EBP1 compared to that in control $Nf2^{+/+}$ and $Tsc2^{+/+}$ MEFs, respectively, under conditions of serum starvation (0% FBS). 4EBP1 phosphorylation (detected by anti-4EBP1 antibody) is reflected by 4EBP1 electrophoretic mobility results. (C) Rapamycin (Rap) suppresses the proliferation of $Nf2^{-/-}$ MEF cells. $Nf2^{+/+}$ and $Nf2^{-/-}$ MEFs incubated with 20 nM rapamycin or vehicle (DMSO) for 24 h were examined for S-phase entry by using BrdU incorporation as detailed in Materials and Methods. Data are presented as mean percentages of DAPI-stained cells ± standard errors of the means. *, $P < 0.01$ for DMSO-treated control versus rapamycin-treated groups or $Nf2^{+/+}$ versus $Nf2^{-/-}$ MEF cells ($n = 5$); +, present; −, absent.

activating protein activity of tuberin. However, we cannot rule out the possibility that merlin may signal through a parallel pathway (independent of TSC) to regulate mTORC1. It is also tempting to speculate that as shown recently for TSC proteins (16), merlin may also regulate mTORC2, which may explain the cytoskeletal defects commonly observed in NF2 protein-deficient cells.

Our data identify merlin as yet another tumor suppressor protein that restricts mTORC1 signaling. It has been suggested that the limited malignancy potential of some tumors associated with other tumor suppressor syndromes such as TSC syndrome may be due, in part, to the attenuation of Akt signaling, resulting from the existence of potent mTORC1-dependent feedback loops which strongly suppress signaling upstream of the PI3K pathway. Multiple mechanisms for negative feedback to Akt signaling have been described previously (10, 17), one of which appears to involve S6K1-dependent phosphorylation of insulin receptor substrate proteins, rendering the PI3K-Akt pathway resistant to insulin/IGF1 stimulation in a rapamycin-dependent manner. In support of this possibility, we found that growth factor (insulin)-stimulated phosphorylation of Akt is impaired in cells lacking merlin and that prolonged (24-h) rapamycin treatment can restore Akt phosphorylation under growth conditions with full serum. However, it remains to be established whether the state of this mTOR-dependent feedback mechanism is what delineates the malignancy of these tumors.

Our findings identify mTORC1 as a critical regulator of meningioma and schwannoma growth in vitro and in vivo, suggesting that mTOR inhibition may provide new avenues for targeted therapies to arrest the growth of these tumors. Rapamycin and rapamycin analogs have shown antitumor activity across a variety of human cancers in clinical trials, and early successes in the treatment of benign hamartoma syndromes like TSC have been reported (2, 8). However, the existence of a strong negative feedback loop from S6K1 to Akt signaling presents a potential therapeutic problem, as losing this feedback inhibition may promote cell survival and chemoresistance. Therefore, dual inhibition of the PI3K pathway and mTOR may be an effective strategy. Interestingly, in some cell types, prolonged rapamycin treatment inhibited mTORC2 assembly and, consequently, Akt phosphorylation, suggesting that clinical responses to rapamycin may result from the inhibition of both mTORCs (34). Given our findings, we believe that rapamycin merits consideration for clinical studies as a therapeutic agent, either alone or in combination with PI3K pathway inhibitors, against NF2-associated tumors.

ACKNOWLEDGMENTS

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