Award Number: W81XWH-11-1-0264

TITLE: Role of merlin/NF2 in mTOR signaling and meningioma growth

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REPORT DATE: April 2012

TYPE OF REPORT: Annual

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Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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The scope of this research project is to mechanistically define how merlin regulates mTORC1 signaling, to examine signaling downstream of mTORC2 and to validate the efficacy of mTOR inhibitors in both in vitro and in vivo preclinical models. The results obtained during assay development of an unbiased kinome screening clearly establish that Rheb is required for mTORC1 activation mediated by NF2 loss, supporting our hypothesis that NF2 may function through TSC1-TSC2 protein complex. Similar to TSC proteins, merlin negatively regulates mTORC1 and positively regulates mTORC2. However, contrary to activation of mTORC1, the attenuated mTORC2 signaling profiles exhibited by normal arachnoid and Schwann cells in response to acute merlin loss are not consistently reflected in NF2-deficient meningiomas and schwannomas, suggesting that additional genetic events may have been acquired in tumors after initial merlin loss. Our results show that mTOR kinase inhibitor such as Torin1 is more effective in blocking signaling and inhibiting proliferation of benign (WHO grade 1) and atypical (WHO grade 2) meningioma cells. A manuscript describing these results is now in press and expected to be published soon. We have shown that implantation of benign meningioma cells form tumors and may serve as valuable preclinical model for NF2.

14. ABSTRACT

The scope of this research project is to mechanistically define how merlin regulates mTORC1 signaling, to examine signaling downstream of mTORC2 and to validate the efficacy of mTOR inhibitors in both in vitro and in vivo preclinical models. The results obtained during assay development of an unbiased kinome screening clearly establish that Rheb is required for mTORC1 activation mediated by NF2 loss, supporting our hypothesis that NF2 may function through TSC1-TSC2 protein complex. Similar to TSC proteins, merlin negatively regulates mTORC1 and positively regulates mTORC2. However, contrary to activation of mTORC1, the attenuated mTORC2 signaling profiles exhibited by normal arachnoid and Schwann cells in response to acute merlin loss are not consistently reflected in NF2-deficient meningiomas and schwannomas, suggesting that additional genetic events may have been acquired in tumors after initial merlin loss. Our results show that mTOR kinase inhibitor such as Torin1 is more effective in blocking signaling and inhibiting proliferation of benign (WHO grade 1) and atypical (WHO grade 2) meningioma cells. A manuscript describing these results is now in press and expected to be published soon. We have shown that implantation of benign meningioma cells form tumors and may serve as valuable preclinical model for NF2.
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Role of Merlin/NF2 in mTOR Signaling and Meningioma Growth

P.I. Vijaya Ramesh

Introduction

Neurofibromatosis 2 (NF2) is a dominantly inherited disorder characterized by multiple benign nervous system tumors, including schwannomas and meningiomas. Although merlin is implicated in a wide range of cellular activities, the precise growth inhibitory mechanism in human arachnoidal and Schwann cells, and how its loss results in tumor formation from these specific cell types in NF2 remains poorly understood. We believe that elucidating the cell/context-dependent functions of merlin will be critical in understanding the growth mechanisms of NF2-associated meningiomas and schwannomas. The tumor suppressor product of NF2 encodes merlin, a member of the ezrin-radixin-moesin (ERM) protein family that functions to link membrane proteins to the cortical actin cytoskeleton.

Employing human-derived, merlin-deficient meningiomas and merlin-suppressed arachnoidal cells, the non-neoplastic cell counterpart of meningiomas, we recently identified merlin as a novel negative regulator of mammalian target of rapamycin complex 1 (mTORC1) signaling. We believe that merlin functions upstream of the Tuberous Sclerosis Complex (TSC) proteins to regulate mTORC1. However, unlike in NF1, it does not regulate mTORC1 via the established PI3K/Akt- or MAPK/ERK-mediated TSC2 inactivation and may instead regulate TSC/mTORC1 signaling in a novel fashion (James et al., 2009). The scope of this research is to mechanistically define how merlin regulates mTORC1 signaling, to obtain insights as to whether aberrant activation of mTORC1 signaling and signaling downstream of mTORC2 can explain the benign nature of tumors in NF2, and to validate the efficacy of mTOR inhibitors in both in vitro and in vivo preclinical models.

Body

Research Accomplishments

Task 1: To define the mechanism(s) by which merlin regulates mTORC1

Hypothesis: NF2 regulation of TORC1 is dependent on an intact TSC complex. Absence of merlin in meningiomas could result in degradation/decrease of TSC complex activity through novel phosphorylation sites in TSC1 and/or TSC2, which are mediated by as yet unidentified kinases in merlin-deficient cells.

Loss-of-function kinome screen

We are in the final stages of assay development for performing a high-throughput, loss-of-function kinome screen to identify kinases involved in NF2-regulated mTORC2 signaling. A library of specific, short-hairpin RNAs (shRNAs), developed by The RNAi Consortium (TRC; Broad Institute/MIT), will be used to suppress ~800 known kinases in NF2-deficient arachnoidal (AC) and meningioma cells. For this screen, distinct shRNAs that target each kinase are introduced into NF2-suppressed arachnoidal cells (ACs) or a patient-derived, NF2-deficient benign meningioma cell line (BenMen1) by lentiviral infection in a 384-well format. In order to avoid false-positive effects, at least 5 distinct shRNAs are used for each individual target kinase, followed by immunofluorescence-based analysis to look for decrease of mTORC1 pathway activation. A twofold decrease in mTORC1 signaling observed with at least 2 of the 5 shRNAs would be scored as positive. Specific kinases that lead to decreased pathway activation when suppressed will be considered as strong candidates for causing constitutive activation of mTORC1 in response to NF2
loss. A standard fluorescent staining readout used to define the activation state of the mTORC1 pathway is phosphorylated S6 (P-S6$^{\text{Ser240/244}}$). In addition, DAPI staining is also used to tag each cell nucleus in order to quantitate the level of P-S6 staining/cell.

**NF2 regulates the mTORC1 pathway in a Rheb-dependent manner**

During the assay development phase, we carried out RNAi-mediated suppression of four established mTORC1 pathway targets (5 shRNAs/target) in NF2-suppressed ACs under serum-deprived conditions. These targets included mTORC1 complex components mTOR and Raptor; p70S6K, a direct target of mTORC1 and upstream kinase for phosphorylating S6 at Ser240/244 and Rheb, a small GTPase that activates mTORC1 in response to inactivation of TSC1-TSC2 complex.

Immunofluorescence screening and quantitation was performed using the Acumen eX3 laser scanning image cytometer. As predicted, decreased P-S6$^{\text{Ser240/244}}$ staining was observed using shRNAs for mTOR (2/5 shRNAs), Raptor (3/5 shRNAs), and S6K (2/5 shRNAs) compared to a null control shRNA. Importantly, hairpins targeting the mTORC1 regulator Rheb (3/5 shRNAs) also demonstrated downregulation of P-S6 (Figures 1 and 2). These results suggest that the NF2-mediated regulation of mTORC1 is Rheb-dependent, further supporting the likelihood that NF2 functions upstream of TSC1-TSC2, and loss of NF2 may lead to inactivation of the TSC complex.

**Figure 1.** Representative figure shows DAPI staining (blue) of individual cell nuclei along with P-S6 (green) used as an mTORC1 pathway readout. Left panel shows NF2-suppressed ACs infected with a null control shRNA, which retain the constitutive P-S6 activation. Right panel shows NF2-suppressed ACs infected with a Rheb-specific shRNA where abnormal P-S6 activation is reversed (decreased green signal).

**Figure 2.** Preliminary results show downregulation of P-S6 staining following shRNA-mediated suppression of several mTORC1-related pathway targets (using 5 shRNAs/target) including mTOR, Rheb, p70S6K and Raptor compared with null control shRNA.
Task 2: To define the mechanisms that limit the malignancy potential of benign meningiomas and vestibular schwannomas in NF2 when mTORC1 is activated

Hypothesis: mTORC1-dependent feedback mechanisms regulating IRS-1 and PDGFR functions, as well as impaired mTORC2 activity, may be responsible for the observed attenuation of Akt activation upon merlin loss in arachnoidal cells. The lack of Akt activation will affect the survival properties of merlin negative meningioma cells, which will be examined by treating benign and atypical meningioma cells with compounds that inhibit mTORC1 and Akt.

Regulation of mTORC2 signaling in NF2-deficient target cell types

Previously, we demonstrated that merlin loss results in hyperactivation of mTORC1 in vitro and in vivo and that Akt signaling is impaired in response to insulin stimulation through an mTORC1-mediated negative feedback loop (James et al., 2009). Deregulation of mTORC1 signaling in merlin-deficient arachnoid cells is reminiscent of TSC deficiency in cells/tumors suggesting that growth control mechanisms may be overlapping in TSC and NF2 tumor suppressor syndromes. The TSC1-TSC2 complex, in addition to its role in inhibiting mTORC1, was shown to interact with mTORC2 and positively regulates its kinase activity (Huang et al., 2008). We have examined the regulation of mTORC2 signaling by merlin in NF2 target cell types and tumors. Our results show that merlin positively regulates the kinase activity of mTORC2, a second functionally distinct mTOR complex, and that downstream phosphorylation of mTORC2 substrates, including Akt, is reduced upon acute merlin deficiency in cells. In response to general growth factor stimulation, Akt signaling is attenuated in merlin-suppressed human arachnoid and Schwann cells through mechanisms mediated by hyperactive mTORC1 and impaired mTORC2. Moreover, Akt signaling is impaired differentially in a cell type-dependent manner in response to distinct growth factor stimuli (Figure 1 and 2 in the appended paper). However, contrary to activation of mTORC1, the attenuated mTORC2 signaling profiles exhibited by normal arachnoid and Schwann cells in response to acute merlin loss were not consistently reflected in NF2-deficient meningiomas and schwannomas with chronic merlin loss, suggesting additional genetic events may have been acquired in tumors after initial merlin loss (Figure 4 and Table 1 in the appended paper). This finding contrasts with TSC, which exhibits attenuated mTORC2 signaling profiles in both cells and tumors. Furthermore, we tested the efficacy of mTOR pathway inhibitors including the mTORC1 inhibitor rapamycin; an ATP-competitive mTOR inhibitor Torin1 that potently inhibits both mTORC1 and mTORC2 complex; and the dual PI3K/mTOR inhibitor PI-103 on primary benign and atypical meningioma cells. We observed that Torin1 was more effective in blocking mTORC1 and Akt activation in meningioma cells in vitro than rapamycin and PI-103, and more effective than rapamycin in inhibiting cell proliferation (Figure 5 and 6 in the appended paper). A manuscript detailing these results is published and is included in the Appendix of this progress report.

Task 3: To test mTOR/PI3K inhibitors in a preclinical model of NF2-associated benign meningiomas

Hypothesis: Intracranial implantation in mice of hTERT-immortalized, merlin-deficient human arachnoidal cells and patient-derived meningioma cells with aberrant mTORC1 activation will result in benign meningiomas resembling human NF2, which will be an appropriate in vivo preclinical model for biological and therapeutic studies.

We have proposed the development of a relevant in vivo preclinical meningioma mouse model for use in determining the efficacy of mTOR and dual mTOR/PI3K inhibitors as potential therapeutics for NF2. To establish an appropriate NF2 meningioma tumor model we have utilized human
immortalized normal arachnoid and patient-derived meningioma cells that we have engineered to stably express GFP and firefly luciferase by lentiviral-mediated infection (LV-GFP-Fluc) for use in monitoring tumor progression. For intracranial implantation experiments in nude mice, immortalized normal arachnoidal cells were used as control cells, and merlin-deficient arachnoidal cells or immortalized benign meningioma cells (BenMen1) cells were used for developing suitable tumor models. In preliminary studies, we observed by bioluminescence imaging (BLI) that merlin-deficient cells implanted at the cerebral convexity was optimal to the skull base location in sustaining the growth of implanted cells. In a pilot study of 20 mice, 10 each of nu/nu or NOD/SCID immunodeficient mice, we determined that both mice strains were equally sufficient to support the development of meningioma tumors. In initial studies, in collaboration with Dr. H. Wakimoto of MGH Neurosurgery, we have implanted approximately $0.75 \times 10^6$ BenMen1 cells in a 5 µl volume at the cerebral convexity and followed progression of tumor development by BLI through 7 months. Representative BLI and H&E staining images of a mouse brain sacrificed 6.5 months post implantation demonstrate meningioma development (Figure 3).

**Key Research Accomplishments**

- Rheb is required for NF2 loss to activate mTORC1 signaling, strengthening the idea that NF2 functions through Rheb-TSC1-TSC2 complex.

- NF2 positively regulates mTORC2 signaling and acute loss of merlin in human arachnoidal and Schwann cells results in decrease in phosphorylation of mTORC2 targets.

- NF2-associated meningiomas with chronic loss of merlin do not completely resemble acute loss of merlin in human arachnoidal cells, suggesting that either compensatory mechanisms or additional genetic events occur in meningiomas subsequent to initial merlin loss.
• Torin1, an ATP-competitive mTOR kinase inhibitor is more effective than rapamycin in inhibiting signaling and proliferation of benign and atypical meningioma cells.

Reportable Outcome

Marianne F. James, Elizabeth Stivison, Roberta Beauchamp, Sangyeul Han, James F. Gusella, Margaret R. Wallace, Anat Stemmer-Rachamimov, and Vijaya Ramesh. Signaling events downstream of mammalian target of rapamycin complex 2 (mTORC2) in NF2-deficient target cell types. (2011) Abstract presented at the Children’s Tumor Foundation meeting.


Conclusion

We are in the final stages of assay development for the unbiased large-scale kinome screen that we will be undertaking in NF2-deficient arachnoidal and meningioma cells (Task1). The results obtained during assay development clearly establish that Rheb is required for mTORC1 activation mediated by NF2 loss, supporting our hypothesis that NF2 may function through the TSC1-TSC2 protein complex. As a complementary strategy, we will examine a set of kinase inhibitors for blocking mTORC1 activation mediated by NF2 loss. Most of the experiments proposed under Task 2 are completed and a manuscript describing these results is now in press and expected to be published soon. Our results show that mTOR kinase inhibitor such as Torin1 is more effective than rapamycin in blocking signaling and inhibiting proliferation of benign (WHO grade 1) and atypical (WHO grade 2) meningioma cells. We have shown that implantation of benign meningioma cells in mice form tumors and may serve as a valuable preclinical model for NF2 (Task 3). In addition to taking approaches to improve this model we would consider other relevant models, which may become available.

References


Regulation of mTOR Complex 2 Signaling in Neurofibromatosis 2–Deficient Target Cell Types

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Abstract

Inactivating mutations in the neurofibromatosis 2 (NF2) tumor suppressor gene results in the development of schwannomas and meningiomas. Using NF2-deficient meningioma cells and tumors, together with the normal cellular counterparts that meningiomas derive, arachnoid cells, we identified merlin as a novel negative regulator of mTOR complex 1 (mTORC1). We now show that merlin positively regulates the kinase activity of mTORC2, a second functionally distinct mTOR complex, and that downstream phosphorylation of mTORC2 substrates, including Akt, is reduced upon acute merlin deficiency in cells. In response to general growth factor stimulation, Akt signaling is attenuated in merlin RNA interference-suppressed human arachnoid and Schwann cells by mechanisms mediated by hyperactive mTORC1 and impaired mTORC2. Moreover, Akt signaling is impaired differentially in a cell type–dependent manner in response to distinct growth factor stimuli. However, contrary to activation of mTORC1, the attenuated mTORC2 signaling profiles exhibited by normal arachnoid and Schwann cells in response to acute merlin loss were not consistently reflected in NF2-deficient meningiomas and schwannomas, suggesting additional genetic events may have been acquired in tumors after initial merlin loss. This finding contrasts with another benign tumor disorder, tuberous sclerosis complex, which exhibits attenuated mTORC2 signaling profiles in both cells and tumors. Finally, we examined rapamycin, as well as the mTOR kinase inhibitor, Torin1, targeting both mTOR complexes to identify the most efficacious class of compounds for blocking mTOR-mediated signaling and proliferation in merlin-deficient meningioma cells. These studies may ultimately aid in the development of suitable therapeutics for NF2-associated tumors. Mol Cancer Res; 10(5); 649–59. ©2012 AACR.

Introduction

Germline mutations of the neurofibromatosis 2 (NF2) gene are associated with NF2, a severe, inherited tumor syndrome characterized by bilateral vestibular schwannomas, often in combination with other cranial and spinal schwannomas and meningiomas (1). Biallelic inactivation of NF2 is also the initiating event in the development of the majority (~60%) of sporadic meningiomas and almost all schwannomas, tumors that arise from cell types of neural crest origin. Specifically, meningiomas and schwannomas develop respectively from arachnoid cells of the meninges covering the brain and spinal chord, and from Schwann cells that ensheathe and myelinate peripheral nerves. Meningiomas comprise approximately 30% of all brain neoplasms and although some can often be effectively treated with surgery and radiation, an important subset remains inoperable or has recurrence rates of up to 20% over 10 years. To date, most medical therapies have generally been ineffective indicating that improved understanding of the molecular pathogenesis of these tumors is needed to benefit the development of new treatments (1).

NF2 encodes the tumor suppressor product, merlin, a member of the ezrin, radixin, moesin (ERM) family of membrane-cytoskeletal linker proteins (2, 3), which regulates membrane organization and numerous actin cytoskeletal-based cellular processes including cell adhesion, cell–cell contact, membrane transport, and signal transduction pathways (4). Although merlin overlaps functionally in part with ERM proteins, merlin is distinguished by its tumor suppressor activity. Merlin is suggested to control cell proliferation by mediating contact inhibition of growth through mechanisms that include formation of stable adherens junctions or promoting efficient activity, expression, and/or transport of specific growth factor receptors (5, 6). Consequently, merlin loss induces signaling of numerous...
mitogenic pathways including Ras/mitogen-activated protein kinase (MAPK), Rac, phosphoinositide 3-kinase (PI3K), Hippo/Mst1 (7–12), as well as E3 ubiquitin ligase activity (13) in multiple cell types. Although Merlin is implicated in a wide range of cellular activities in different cell types, it is unclear which of these functions are essential for inhibiting cell proliferation and tumor growth in meningiomas and Schwann cells.

To investigate the mechanism(s) by which Merlin loss results in tumor growth, we have developed in vitro NF2 model systems using human meningioma- and Schwann-derived cells to more accurately reflect the environment of NF2 tumorigenesis. Using primary human Merlin-deficient meningioma and Merlin RNA interference (RNAi)-suppressed arachnoid cells, we recently identified Merlin as a novel negative regulator of the mTOR complex 1 (mTORC1; ref. 14), a large multisubunit protein complex that integrates signals from growth factors, nutrients, and energy to coordinate many cellular processes including growth (cell mass gain), and proliferation. mTOR, an evolutionarily conserved Ser/Thr kinase exists in one of 2 distinct functional complexes, mTORC1 and mTORC2, which consists of discrete sets of proteins and appears to carry out nonoverlapping functions (15). mTORC1 regulates cell growth and proliferation by promoting increased translation and protein synthesis through phosphorylation of effector proteins, S6 kinase (S6K), and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; ref. 16). mTORC1 is potently inhibited by the tuberous sclerosis complex (TSC) tumor suppressors TSC1 and TSC2 that together regulate the GTP-loading state of Rheb (Ras homologue enriched in brain), a key upstream activator of mTORC1 (15, 17). Rapamycin acutely and specifically inhibits mTORC1, whereas its effects on mTORC2 are more variable and generally requires prolonged treatment. In addition to their differential sensitivity to rapamycin, mTORC1 and mTORC2 are activated in different ways and possess distinct substrate specificity (18).

Although much less is known about the regulation and function of mTORC2, its best characterized function is the phosphorylation of Akt on S473 that lies within a hydrophobic motif (T450 on Akt), is also dependent on mTORC2. Although the kinase activity of mTORC2 can be stimulated by growth factors, perhaps downstream of PI3K, some functions of mTORC2, such as the phosphorylation of PKCa S657 or Akt T450, are independent of growth factor signaling (19).

Activation of mTORC1 has been found to negatively impact Akt phosphorylation in response to insulin or insulin-like growth factor (IGF)1 through negative feedback loops at multiple levels. Inhibition of PK3 signaling by mTORC1 can be attributed to the phosphorylation and degradation of insulin receptor substrate 1 (IRS1) by active S6K or by inhibition of platelet-derived growth factor (PDGF) receptors through an unknown mechanism (22–24). In addition, very recent phosphoproteome studies have identified Grb10 as an mTORC1 substrate that mediates feedback inhibition of PI3K and ERK-MAPK pathways (25, 26). The TSC1-TSC2 complex, in addition to its role in inhibiting mTORC1, was shown to interact with mTORC2 and positively regulates its kinase activity (17). These findings help to explain the enhanced Akt inhibition as well as reduced activation of mTORC2 in TSC1-TSC2 deficiency (27, 28).

Previously, we showed that Merlin loss results in hyper-activation of mTORC1 in vitro and in vivo and that Akt signaling is impaired in response to insulin stimulation through an mTORC1-mediated negative feedback loop (14). Deregulation of mTORC1 signaling in Merlin-deficient arachnoid cells is reminiscent of TSC deficiency in cells/tumors suggesting that growth control mechanisms may be overlapping in TSC and NF2 tumor suppressor syndromes. Here, we examined the regulation of mTORC2 signaling by Merlin in NF2 target cell types and tumors. We identify both parallels and distinctions in mTORC1/2 downstream signaling events and in negative feedback regulation to Akt between NF2-deficient Schwann and arachnoid cells. Our data also indicate that acute Merlin suppression in arachnoid cells may not completely reflect the landscape of NF2-associated meningiomas. In addition, we evaluated the efficacy in vitro of mTOR and dual PI3K/mTOR inhibitors in blocking activated mTOR signaling and proliferation/survival.

Materials and Methods
Antibodies and reagents
Antibodies to phospho-S6 (S240/244), S6, p70 S6K, phospho-p70 S6K (T389), phospho-Akt (T450; S473), Akt, phospho-p44/42 MAPK (ERK1/2) (T202/Y204), p44/42 MAPK (ERK1/2), phospho-NDRG1 (T346), PKCa, preimmune rabbit immunoglobulin G (IgG) were obtained from Cell Signaling Technology. An antibody to Rictor was obtained from Bethyl Laboratories; phospho-PKCα (S657) from Upstate; NDRG1 from Abcam; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Sigma. Merlin rabbit polyclonal antibody (C26) was described previously (29). Growth factors were from Sigma (insulin, EGF, PDGF-BB) and Austral Biologicals (IGF1). Rapamycin and PI-103 were purchased from Calbiochem. Torin1 was kindly provided by Dr. David Sabatini (Whitehead Institute/MIT, Cambridge, MA). Commercial inhibitors were reconstituted in dimethyl sulfoxide (DMSO) as per manufacturer’s recommendations.

Sample collection and cell culture
Fresh tissues were collected at the time of clinically indicated surgery for tumor resection (excess discarded tissues) or from patients who underwent autopsy by the
MGH neurooncology tumor repository in accordance with an Institutional Review Board–approved protocol. Informed consent was obtained from all study subjects. Tissue was flash frozen in liquid nitrogen or fixed in formalin to use for histology and immunohistochemical analyses. NF2-deficient tumors were classified as sporadic tumors or NF2-associated tumors based on diagnoses of referring clinicians. Unless otherwise specified, meningiomas and meningioma cells described in text refer to benign or World Health Organization (WHO) grade I meningioma tumors and cells. Atypical meningioma cells were established from atypical (WHO grade II) meningiomas.

Cultures of primary meningioma and normal arachnoid cells from fresh tissues were established as described previously (29). Primary meningioma cells and the immortalized arachnoid cell line, AC007-hTERT (14), were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with a 4.5 g/L glucose solution containing 15% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin (full serum medium). Primary normal arachnoid cells were maintained in a mixture of improved minimum essential medium with l-glutamine (Richter’s Modified Medium (Cellgro; Mediatech, Inc.)) and 100 U/mL penicillin and 100 μg/mL streptomycin (full serum medium). Cultures of primary meningioma and normal arachnoid cells were established by lentiviral infections at a multiplicity of infection of 10, and harvested for cell lysates 7 to 10 days postinfection.

Cell lysis and immunoblotting

Protein lysates were harvested from subconfluent cultures because many growth factor–mediated signaling events are inhibited upon cell confluence. Control (scr) and NF2/merlin (m5) knockdown arachnoid and pn02.3 Schwann cell lysates were prepared from subconfluent (80%–90%) cultures in NP-40 lysis buffer (20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl2, 1% Nonidet p-40 (NP-40) containing calyculin (50 mmol/L; Cell Signaling Technology), ×1 HALT phosphatase inhibitor cocktail (Thermo Scientific) and ×1 Complete protease inhibitor cocktail (Roche Diagnostics). Subconfluent primary merlin-deficient meningioma cell cultures were harvested in RIPA buffer for protein lysates as described previously (14). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and subjected to immunoblot analysis using indicated antibodies, horseradish peroxidase– conjugated secondary antibodies (Cell Signaling Technology), and the enhanced chemiluminescence (ECL) detection systems (Amersham Pharmacia Biotechnology).

mTORC2 kinase assays

Kinase assays on endogenous mTORC2 were conducted as described previously with minor modifications (17, 32). Near-confluent, 150-mm plates of control (scr) or NF2/merlin knockdown (m5 RNAi) arachnoid cells were stimulated with EGF (5 ng/mL) for 30 minutes before lysis in 1 mL mTORC lysis buffer (40 mmol/L HEPES (pH 7.5), 120 mmol/L NaCl, 1 mmol/L EDTA, 0.3% CHAPS [3-[(3-chloramidopropyl)- dimethylammonio]-1-propanesulfonate] containing phosphatase and protease inhibitors as described above, and immunoprecipitations conducted with 1.5 μg Rictor or control IgG antibodies. The soluble fraction of the lysates was precleared for 1 hour with nonspecific IgG antibodies with rotation at 4°C and then incubated with the precipitating antibody for 2 hours. Immunoprecipitates were captured with protein A/G-agarose (Amersham) for 1.5 hours and then washed 3 times with mTORC lysis buffer and twice with the Rictor-mTOR kinase buffer (25 mmol/L HEPES, pH 7.5, 100 mmol/L potassium acetate, 2 mmol/L MgCl2). For mTORC2 kinase reactions, immunoprecipitates were incubated in a final volume of 15 μL for 20

www.aacjrournals.org Mol Cancer Res; 10(5) May 2012 651

Published OnlineFirst March 16, 2012; DOI:10.1158/1541-7786.MCR-11-0425-T

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minutes at 32°C in the Rictor-mTORC kinase buffer containing 500 ng inactivate Akt1/PKB1 (Upstate Biotechnology) as the substrate and 500 μmol/L ATP (32). The reaction was stopped by placing samples on ice and immediately adding SDS sample buffer. The supernatant was removed from protein A/G agarose and analyzed by SDS-PAGE and immunoblotting as indicated.

**Immunohistochemistry**

Five benign NF2-deficient meningiomas and 4 vestibular schwannomas were formalin and paraffin embedded (27). Antigen unmasking was achieved by microwaving in 10 mmol/L sodium citrate (pH 6.0) followed by immersion in 3% hydrogen peroxide for 20 minutes and 5% goat serum for 1 hour to block endogenous peroxidase and nonspecific antibody binding, respectively. After incubation with the appropriate primary antibodies overnight at 4°C, secondary antibodies and avidin-biotin-peroxidase complex were applied according to the manufacturer’s protocol (ABC Elite Staining; Vector Labs). Visualization was achieved by incubating with 3,3’-diaminobenzidine tetra-chloride (Pierce), and the sections were counterstained with hematoxylin. Control tissues for antibodies include TSC-associated kidney angiomyolipomas (AML) (negative control).

**Cell proliferation/viability assays**

Cell proliferation/viability was assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) that determines the number of viable cells based on quantitation of ATP present. On day 0, 96-well plates were seeded with 1,000 cells per well in triplicate and grown overnight. On days 1 and 4, cells were treated with the appropriate compounds and viability assays were conducted on days indicated. Plates were incubated with the CellTiter-Glo reagent according to manufacturer’s suggestions, shaken on an orbital shaker for 2 minutes, and incubated 10 minutes further in darkness at room temperature to stabilize the luminescent signal. Luminescence was detected on a Micro-LumatPlus LB 96V Luminometer (Berthold Technologies) and average values depicted as relative luminescent units (RLU).

**Results**

**General attenuation of Akt phosphorylation in merlin-deficient arachnoid and Schwann cells**

We previously reported that arachnoid cells deficient for merlin show constitutive phosphorylation of S6, a marker of mTORC1 activity, but impaired Akt S473 phosphorylation in response to insulin stimulation (14). Attenuated Akt S473 phosphorylation in merlin knockdown arachnoid cells is consistent with a negative feedback mechanism mediated by mTORC1/S6K phosphorylation of IRS proteins and consequent inhibition of PI3K-Akt signaling (15, 33). To determine whether merlin knockdown arachnoid cells show defective Akt signaling in response to stimulation by additional growth factors, we examined Akt S473 phosphorylation in response to IGF1, EGF, PDGF-BB, and FBS stimulation. Akt S473 phosphorylation is attenuated in response to IGF1 but not PDGF stimulation. Akt S473 phosphorylation is attenuated in response to IGF1, EGF, PDGF, but not FBS. Total protein and/or phosphorylation levels for merlin, Akt, phospho-S6 (pS6 S240/244), S6, phosphory-Erk (pERK T202/Y204), ERK, and GAPDH as a loading control, were determined by immunoblotting with indicated antibodies. B, control and merlin-suppressed immortalized Schwann cells (pno2.3) were stimulated as described in (A), substituting 10% FBS for 15% FBS stimulation. Akt S473 phosphorylation is attenuated in response to IGF1 but not PDGF or FBS stimulation. ERK1/2 phosphorylation (T202/Y204) was elevated in serum deprived, IGF, and PDGF-stimulated pno2.3 immortalized Schwann cells.
NF2 tumorogenesis. Consistent with arachnoid cells, merlin deficiency by RNAi in an immortalized human Schwann cell line (pno2.3) resulted in elevated mTORC1 signaling as indicated by constitutive S6 phosphorylation. In further agreement, Akt S473 phosphorylation was not observed in merlin knockdown Schwann cells under serum-deprived conditions compared with control cells. In response to growth factor stimulation by IGF1 and EGF, but not PDGF or FBS, merlin knockdown Schwann cells exhibited impaired Akt S473 phosphorylation relative to control cells (Fig. 1B). The lack of attenuated Akt S473 phosphorylation in response to PDGF stimulation in merlin-suppressed Schwann cells is in sharp contrast to merlin knockdown arachnoid cells. Our data are in agreement with a general loss of Akt stimulation observed characteristically in TSC-null cells in response to a variety of growth factors including those that signal independent of IRS proteins (27). These findings indicate that in addition to mTORC1/S6K-dependent feedback inhibition of IRS, other mechanism(s) may be functional in merlin-deficient NF2 target cells to dampen Akt signaling.

Loss of merlin leads to a decrease in mTORC2 signaling and kinase activity

To determine whether decreased mTORC2 signaling contributes to general Akt attenuation in response to growth factors in merlin-deficient arachnoid and Schwann cells, we examined phosphorylation sites of several mTORC2-dependent substrates of the AGC family of kinases including Akt (S473 and T450), PKCα (S657), and SGK1 (S422). Because mTORC2 phosphorylates the hydrophobic motif of Akt S473 and SGK1 S422 in a PI3K-dependent manner, and Akt T450 and PKCα S657 sites independent of growth factor signaling, we examined the phosphorylation of mTORC2 substrates in the absence and presence of full serum growth conditions.

Consistent with our earlier findings, Akt S473 phosphorylation was not observed under serum-deprived conditions in either control or merlin-suppressed arachnoid and Schwann cells. Although no difference in Akt phosphorylation was detected in NF2-deficient cells in response to acute serum stimulation (Fig. 1), steady-state levels of Akt phosphorylation were reduced in these cells grown in full serum (Fig. 2A and B). Prolonged rapamycin treatment (18 hours) had varying effects on the 2 cell types, increasing Akt-S473 phosphorylation in arachnoid cells but decreasing it in Schwann cells. Surprisingly, we detected increased phosphorylation of N-Myc downstream regulated gene 1 (NDRG1) T346, a specific marker for SGK1 signaling (19, 21), in both merlin knockdown arachnoid and Schwann cells compared with controls. In a growth factor–independent manner, we observed reduced levels of Akt T450 and PKCα S657 phosphorylation upon merlin suppression in arachnoid and Schwann cells compared with controls. In addition, PKCα total protein levels were reduced in merlin knockdown cells reflecting the significance of this residue to PKCα stability (19). Collectively, these findings indicate that merlin regulates mTORC1 as well as mTORC2 signaling.

To more fully understand whether the attenuated Akt phosphorylation in merlin-deficient cells is due to mTORC1 as well as mTORC2 signaling, we treated NF2 knockdown arachnoidal and Schwann cells with rapamycin for short-term (1 hour) and long-term (24 hours) time periods, which specifically blocks mTORC1 signaling (1 hour), or mTORC1 and mTORC2 signaling (24 hours). In response to short-term rapamycin treatment, arachnoidal cells show an increase in Akt S473 phosphorylation indicating relief of the negative feedback regulation from mTORC1-S6K signaling (Fig. 2C). An increase in Akt S473 phosphorylation was not observed in Schwann cells indicating cell type–dependent differences in response to rapamycin (Fig. 2D). In response to long-term exposure to rapamycin, Schwann cells, unlike arachnoidal cells, exhibited a further decrease in Akt S473 phosphorylation indicating that mTORC2 assembly may be affected in Schwann cells and not in arachnoid cells (Fig. 2C and D). Our data are in agreement with the established notion that long-term rapamycin treatment does not always lead to the total loss of mTORC2 activity, and that mTORC2 assembly is not completely blocked in all cell types (18).

To determine whether the attenuation of mTORC2 signaling observed in merlin knockdown arachnoid cells and Schwann cells could be due to diminished mTORC2 kinase activity, we directly assayed mTORC2 kinase activity. Endogenous mTORC2 was isolated from control and merlin knockdown arachnoid cell lysates by immunoprecipitating Rictor, and its kinase activity was assayed using an exogenous Akt1 substrate. Similar amounts of mTOR were immunoprecipitated with Rictor in control and merlin-deficient cells stimulated with EGF, however, mTORC2
kinase activity was greatly impaired in merlin knockdown arachnoid cells indicating that merlin loss inhibits mTORC2 kinase activity (Fig. 3A). Akt S473 phosphorylation levels for control and NF2 RNAi samples from 2 independent mTORC2 kinase assays were quantified. The average decrease in EGF-stimulated mTORC2 kinase activity in merlin knockdown arachnoidal cells compared with control cells was approximately 50% (Fig. 3B).

**Heterogeneous expression of PKCα and Akt S473 phosphorylation in merlin-deficient meningiomas and schwannomas**

Our data indicate that acute merlin downregulation by RNAi in NF2 target cell types such as normal arachnoid and Schwann cells results in elevated mTORC1 signaling and decreased mTORC2 signaling. We have shown earlier that mTORC1 signaling is activated in NF2-deficient meningiomas and schwannomas (14). To determine whether NF2 tumors exhibit reduced mTORC2 signaling, we examined PKCα and phospho-Akt S473 expression levels in merlin-deficient meningioma and schwannoma tumors compared with normal arachnoid tissue by immunohistochemical staining. We detected heterogeneity in both PKCα and phospho-Akt S473 expression levels across multiple benign meningioma (n = 5) and schwannoma (n = 4) samples compared with TSC AML (Fig. 4 and Table 1). Previous studies showed that TSC AMLs display high phospho-S6 levels but do not express detectable levels of PKCα or phospho-Akt (27). PKCα immunoreactivity was moderate to strongly positive in normal arachnoid tissue as well as benign meningiomas and schwannomas. Weak to moderate levels of phospho-Akt S473 positivity were detected in both normal arachnoid and benign meningioma samples relative to TSC AML tissue. In schwannomas, however, phospho-Akt S473 staining was uniformly reduced across tumor samples relative to normal arachnoid and meningioma tissues, and more comparable with TSC AMLs. The lack of detectable Akt S473 phosphorylation...
in schwannoma tissue by immunohistochemistry is in agreement with the study of Ammoun and colleagues (2010), which reports that Akt phosphorylation was not observed in vestibular schwannoma specimens (n = 10) by phosphokinase profiling array analysis (37). However, other earlier studies showed positive Akt S473 phosphorylation in vestibular schwannoma samples and/or increased Akt protein levels (38, 39). These differences could be due to tumor heterogeneity or variations in sensitivity of the methods/antibodies employed. Our data show that reduced signaling events downstream of mTORC2 in response to acute merlin suppression in arachnoid and Schwann cells are not necessarily reflective of mTORC2 signaling in merlin-deficient meningiomas and schwannomas with chronic merlin loss.

mTOR signaling and proliferation in merlin-deficient meningioma cells in response to mTOR and dual PI3K/mTOR inhibitors

Treatments directed against mTOR signaling pathways have shown promise in the management of solid tumors and therefore may constitute a potential target for the treatment of NF2 meningiomas. However, rapamycin and its analogs effectively inhibit S6K phosphorylation, but not 4E-BP1 phosphorylation and therefore incompletely inhibit mTORC1-dependent protein synthesis (40). In addition, a major concern is that rapalogs also cause activation of prosurvival and oncogenic pathways such as PI3K/Akt. These trepidations have fueled the development of mTOR kinase inhibitors and PI3K/mTOR kinase inhibitors. Because merlin-deficient meningiomas exhibit activation of mTORC2 as detected by Akt S473 phosphorylation (Fig. 4), we believe that these cells might be more sensitive to mTOR kinase inhibitors, which are efficient in inhibiting both mTORC1 and mTORC2 complexes. Therefore, in addition to rapamycin, we have evaluated the effectiveness of the dual PI3K/Akt–mTOR inhibitor, PI-103, as well as the ATP-competitive inhibitor, Torin1, targeting both mTOR complexes (41), in blocking mTOR-dependent signaling and proliferation/prosurvival pathways in merlin-deficient meningioma cells. In the first set of experiments, we evaluated the effect of short (1 hour) and prolonged exposures (24 hours) of mTOR or PI3K/mTOR inhibition on primary benign and atypical meningioma cells by western analysis. Inhibition of S6 (S240/244) or S6K (T389) phosphorylation was observed in merlin-deficient benign or WHO grade I meningioma cells (Fig. 5A and C), and atypical (WHO grade II; Fig. 5B and D) meningioma cells in response to low doses (50 nmol/L; ref. 41) of rapamycin at both time points, showing that rapamycin effectively suppresses the mTORC1 pathway in these cells in vitro. As expected, blocking mTORC1 with rapamycin resulted in elevated Akt S473 phosphorylation above levels of untreated (vehicle) cells under short (Fig. 5A and B) and long (Fig. 5C and D) treatment periods. Torin1, used at a concentration (250 nmol/L) reported to overcome hyperactivated PI3K due to the negative feedback between mTORC1 and PI3K under conditions of prolonged mTORC1 inhibition (>5 hours; ref. 41), inhibited S6 or S6K phosphorylation at both treatment times, similar to rapamycin treatment (Fig. 5A–D). However, in contrast to rapamycin, Torin1, effectively blocked Akt S473 phosphorylation at both treatment periods.

Similar to rapamycin and Torin1, PI-103 (500 nmol/L) inhibited S6 or S6K phosphorylation after short exposure periods (Fig. 5A and B), but in contrast, failed to sufficiently inhibit S6 or S6K phosphorylation after prolonged time periods in meningioma cells regardless of tumor grade (Fig. 5C and D). Likewise, PI-103 efficiently blocked Akt S473 phosphorylation after short exposure times in all cells examined, but was ineffective in inhibiting Akt phosphorylation after a prolonged exposure period. The general ineffectiveness of PI-103 in blocking
phosphorylation of Akt and S6K/S6 after 24 hours may be, at least in part, due to the short half-life and rapid metabolism of this drug that has been reported in vivo (42).

We next evaluated the effect of rapamycin or Torin1 on cell proliferation/viability of merlin-deficient meningioma cells (n = 4) treated over a period of 6 days. In comparison with untreated cells, we observed that Torin1 (250 nmol/L) was most effective in inhibiting proliferation of all meningioma cells tested, and rapamycin (50 nmol/L) significantly reduced the rate of proliferation. These data are provided in a representative growth curve of a benign meningioma (Fig. 6A). When we compared the average proliferative rates of 2 independent cultures of benign meningioma or atypical meningioma cells at one time point (day 7), we observed that Torin1 elicited the greatest inhibitory response in all cell lines (Fig. 6B).

Discussion

Our previous work showed that merlin deficiency in NF2 target cells results in aberrant activation of mTORC1-mediated signaling as well as attenuation of Akt S473 phosphorylation upon insulin treatment (14), consistent with negative feedback regulation of IRS1 by activated mTORC1 (22, 23, 43). In this study, we show that Akt S473 phosphorylation is impaired in merlin-deficient human arachnoid cells in response to stimulation with growth factors such as EGF and PDGF, and in merlin-deficient human Schwann cells, in response to EGF, but not PDGF. These results clearly show that merlin-deficient arachnoid and Schwann cells may differ from each other in PDGF-mediated signaling. Attenuation of Akt S473 phosphorylation by growth factors other than insulin/IGF suggest that in NF2, similar to TSC, more than one mechanism could be operative to downregulate Akt activation. Furthermore, recent studies have shown that mTORC1 activation, functioning through S6K phosphorylates Rictor to inhibit mTORC2 (44, 45).

Here, we examined signaling downstream of mTORC2, including phosphorylation of Akt (S473, T450) as well as other well-characterized substrates of mTORC2 such as PKCα (S657) and NDRG1, the latter being a specific readout for mTORC2 and SGK1 activation (21). The observed decrease in phospho-AKT (S473, T450) and phospho-PKCα (S657) in merlin-suppressed arachnoid and Schwann cells resemble TSC1- or TSC2-deficient cells (27).

Table 1. Summary of results from immunostaining of PKCα and phospho-Akt S473 in 5 benign (WHO grade I) merlin-deficient meningiomas and 4 vestibular schwannomas

<table>
<thead>
<tr>
<th>Case no.</th>
<th>PKCα</th>
<th>Phospho-Akt (S473)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN 1034</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MN 2805</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>MN 2896</td>
<td>++</td>
<td>+/+++</td>
</tr>
<tr>
<td>MN 3633</td>
<td>+/++</td>
<td>+</td>
</tr>
<tr>
<td>MN 1670</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>VS 3924</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>VS 4206</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>VS 4231</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>VS 4787</td>
<td>++</td>
<td>-/+</td>
</tr>
<tr>
<td>Arachnoid</td>
<td>++</td>
<td>+/+</td>
</tr>
<tr>
<td>TSC-AML</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: Staining was scored semiquantitatively: -, negative; ++, weak; ++, medium; +/+, strong.
Abbreviations: MN, merlin-deficient meningiomas; VS, vestibular schwannomas.
The decrease in mTORC2 kinase activity observed in merlin knockdown cells is consistent with growth factor–independent targets of mTORC2 (Akt T450, PKCα S657) being defective in these cells. Future studies are necessary to understand the precise molecular mechanisms of mTORC2 regulation by NF2 and whether this regulation is dependent or independent of TSC proteins. It is intriguing that contrary to other mTORC2 targets, NDRG1 is aberrantly activated (independent of growth factors) in merlin knockdown arachnoid and Schwann cells. Interestingly, a very recent study has shown that knockout of Protor-1, an interactor of Rictor results in a decrease in phosphorylation of SGK and its physiologic substrate NDRG1, without influencing the phosphorylation of Akt and PKCα at their hydrophobic or turn motifs (46). It is therefore tempting to speculate whether Protor-1 is involved in mediating NDRG1 activation in merlin deficiency, and further studies are necessary to understand the mechanism and consequences of NDRG1 activation in merlin-deficient cells.

Our results suggest that primary meningioma cells and meningioma tumors with long-term (chronic) loss of merlin may exhibit heterogeneity in mTORC2 signaling when compared with acute loss of merlin achieved by RNAi in control normal arachnoid and Schwann cells in vitro. Phosphorylation of Akt S473 is not dramatically reduced in meningiomas when compared with normal arachnoid tissue.

Figure 5. Torin1 blocks mTORC1 and mTORC2 signaling in benign and atypical merlin-deficient meningioma cells. Immunoblot analysis of phospho-S6 (pS6 S240/244) and phospho-Akt S473 (pAkt S473) in primary cell cultures of benign (A and C) and atypical (B and D) meningioma cells treated with PT-103 (P; 500 nmol/L), rapamycin (R, 50 nmol/L), Torin1 (T; 250 nmol/L), or vehicle (-; DMSO) for 1 hour (A and B) or 24 hours (C and D). Arrow indicates phospho-p70 S6K (T389) signal.

Figure 6. Torin1 effectively inhibits proliferation of merlin-deficient meningioma cells. A and B, mTOR inhibition by Torin1 prevents the proliferation of merlin-deficient meningioma cells more effectively than rapamycin at concentrations tested. A, representative growth curve of a benign merlin-deficient meningioma cell line (MN1) grown in the presence of vehicle, 50 nmol/L rapamycin, or 250 nmol/L Torin1 for 6 days. Cell proliferation was measured in triplicate at indicated time points using the CellTiterGlo viability assay. B, cell proliferation of benign and atypical meningioma cells are inhibited most effectively in response to Torin1. Bar graphs represent the average RLU values of 2 benign and 2 atypical meningiomas treated for 6 days with indicated compounds as described in (A). Data are presented as average RLU values ± SE from 2 individual cultures treated in triplicate.
However, interestingly, phospho-Akt S473 staining in schwannomas is dramatically reduced, more closely resembling the staining pattern of TSC tumors than meningiomas. The lack of strong attenuation of mTORC2 signaling, particularly Akt S473 phosphorylation, which is a driver of cell proliferation and survival in primary meningiomas, suggests that either compensation mechanisms exist or additional genetic events cooperate with merlin loss in meningiomas. This is in agreement with a recent study, which elegantly documents frequent chromosome alternations in NF2-associated grade 1 meningiomas (47). We also speculate that similar to TSC lesions, the lack of Akt S473 phosphorylation in schwannomas may explain the benign nature of these tumors as well as the difficulty in establishing cells from them in culture. On the contrary, the atypical features commonly seen in benign meningiomas may be elicited by additional genetic events and/or Akt activation.

Clinical trials with the allosteric inhibitor, rapamycin and its analogs, known as rapalogs, are shown to be effective for various TSC-associated benign tumors including subependymal giant cell astrocytomas (48, 49). However, the feedback activation of oncogenic pathways by rapamycin and rapamycin-resistant phosphorylation of 4E-BP1 has led to the development of kinase inhibitors of mTOR as well as dual PI3K/mTOR inhibitors (50). We tested the efficacy of mTORC1 inhibitor rapamycin; an ATP-competitive mTOR inhibitor Torin1, which is shown to be a potent inhibitor of both mTORC1 and mTORC2 complex; and the dual PI3K/mTOR inhibitor PI-103 on primary benign and atypical meningioma cells. We conclude that Torin1 is more effective in blocking mTORC1 and Akt activation in meningioma cells in vitro than rapamycin and PI-103 and more effective than rapamycin in inhibiting cell proliferation. Although benign in nature, we believe that heterogeneity exists in NF2-associated tumors adding complexity to signaling events. Therefore, therapeutic strategies employing Torin1 or equivalent mTOR kinase inhibitors in combination with other pathway inhibitors may be promising for treating NF2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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 Analysis and interpretation of data: M.F. James, R. Beauchamp, A.O. Stemmer-Rachamimov, V. Ramesh.
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Acknowledgments
The authors thank Brendan D. Manning and Christian C. Diblee (Harvard School of Public Health/Brighton and Women’s Hospitals) for valuable discussions; Sun Kim for technical assistance, and Stephen Raveney, and James C. Kim for valuable assistance in obtaining tissue samples.

Grant Support
This work was supported by the NIH grants NS024279, Department of Defense (DOD) Neurofibromatosis Research Program, S. Sydney De Young Foundation, Neurofibromatosis, Inc., New England, and Children’s Tumor Foundation Drug Discovery Initiative.

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Received September 7, 2011; revised March 5, 2012; accepted March 5, 2012; published OnlineFirst March 16, 2012.

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