The neurofibromatosis 2 protein, merlin, regulates glial cell growth in an ErbB2- and Src-dependent manner

S. Sean Houshmandi  
*Washington University School of Medicine*

Ryan J. Emnett  
*Washington University School of Medicine*

Marco Giovannini  
*House Ear Institute, Los Angeles*

David H. Gutmann  
*Washington University School of Medicine*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)  
Please let us know how this document benefits you.

**Recommended Citation**  
[https://digitalcommons.wustl.edu/open_access_pubs/2293](https://digitalcommons.wustl.edu/open_access_pubs/2293)
The Neurofibromatosis 2 Protein, Merlin, Regulates Glial Cell Growth in an ErbB2- and Src-Dependent Manner

S. Sean Houshmandi,¹ Ryan J. Emnett,¹ Marco Giovannini,² and David H. Gutmann¹*

Department of Neurology, Washington University School of Medicine, St. Louis, Missouri,¹ and House Ear Institute, Los Angeles, California²

Received 4 September 2008/Returned for modification 16 October 2008/Accepted 13 December 2008

Individuals with the inherited cancer predisposition syndrome neurofibromatosis 2 (NF2) develop several central nervous system (CNS) malignancies, including glial cell neoplasms (ependymomas). Recent studies have suggested that the NF2 protein, merlin (or schwannomin), may regulate receptor tyrosine kinase signaling, intracellular mitogenic growth control pathways, or adherens junction organization in non-nervous-system cell types. For this report, we used glial fibrillary acidic protein conditional knockout mice and derivative glia to determine how merlin regulates CNS glial cell proliferation. We show that the loss of merlin in glial cells results in increased proliferation in vitro and in vivo. Merlin regulation of glial cell growth reflects deregulated Src activity, such that pharmacologic or genetic inhibition of Src activation reduces NF2−/− glial cell growth to wild-type levels. We further show that Src regulates NF2−/− glial cell growth by sequentially regulating FAK and paxillin phosphorylation/activity. Next, we demonstrate that Src activation results from merlin regulation of ErbB2 activation and that genetic or pharmacologic ErbB2 inhibition reduces NF2−/− glial cell growth to wild-type levels. Lastly, we show that merlin competes with Src for direct binding to ErbB2 and present a novel molecular mechanism for merlin regulation of ErbB2-dependent Src signaling and growth control.

In order to identify the key signaling pathways regulated by the merlin tumor suppressor protein, previous studies have focused on merlin growth regulation in fibroblasts, primary Schwann cell and human schwannoma cell cultures, meningioma and schwannoma tumor cell lines, and other non-central nervous system (non-CNS) cell types. These investigations have resulted in the identification of a large number of non-intersecting growth control pathways regulated by merlin in different cell types. In this regard, merlin has been implicated in epidermal growth factor receptor (EGFR) (9), β1-integrin (15), and CD44 (1, 35, 48) function as well as in Ras (25, 59), Rac1 (34, 52), phosphatidylinositol 3-kinase (44), mitogen-activated protein kinase (MAPK) (7, 30), and STAT (51) intracellular signaling. While each of these pathways is involved in growth control in the brain, it is not known which of these intracellular signaling pathways are deregulated in NF2-deficient CNS cell types.

To gain insights into the role of the NF2 gene in glial cell growth control relevant to the development of targeted therapies for NF2-associated glial cell malignancies, we studied the consequence of merlin loss on the growth of primary brain glial cells (astrocytes) in vitro and in vivo, using NF2 conditional knockout genetically engineered mice (GEM). We demonstrate for the first time that merlin regulates brain glial cell growth by controlling the phosphorylation/activity of Src and its downstream effectors, FAK and paxillin. Furthermore, we show that merlin regulation of Src phosphorylation/activity is modulated by ErbB2 phosphorylation/activation and ErbB2-Src binding. Finally, we show that merlin competitively inhibits Src binding to ErbB2 and, in this manner, prevents ErbB2-mediated Src phosphorylation and downstream mitogenic signaling. Based on these findings, we propose a novel mechanism for merlin growth regulation in CNS glia.

Neurofibromatosis type 2 (NF2) is an autosomal dominant inherited cancer syndrome in which affected individuals develop nervous system tumors, including peripheral nerve tumors (schwannomas), leptomeningeal tumors (meningiomas), and glial fibrillary acidic protein (GFAP)-immunoreactive glial cell tumors (spinal ependymomas). NF2 results from a germ line mutation in the NF2 tumor suppressor gene, located on chromosome 22q (46, 60). Tumors in this disorder arise following somatic inactivation of the one remaining wild-type (WT) NF2 allele in specific cell types. In this regard, NF2-associated schwannomas, meningiomas, and ependymomas all exhibit biallelic NF2 gene inactivation (33, 47, 61). In addition, NF2 gene inactivation is also observed in 50 to 78% of sporadic schwannomas, 32 to 84% of sporadic meningiomas, and 37% of sporadic ependymomas (21, 29), suggesting that this gene is also a key growth regulator in nonhereditary nervous system cancers.

The NF2 gene was identified in 1993 and found to code for a 595-amino-acid protein, termed merlin or schwannomin (46, 60). Analysis of the predicted protein sequence revealed striking sequence similarity between merlin and a family of protein 4.1 family members that link the actin cytoskeleton to cell surface glycoproteins (55). In particular, merlin most closely resembles the ezrin/radixin/moesin (ERM) subfamily and has been shown to bind actin as well as to associate with several cell surface glycoproteins, including CD44 and β1-integrin (5, 32, 48). However, unlike the ERM proteins, merlin is unique in its capacity to function as a nervous system tumor suppressor gene.
**Materials and Methods**

**Cell culture.** Forebrain glial cell cultures from postnatal day 3 N2flox/flox mice (18) were generated as previously described (24, 49). Briefly, forebrains were isolated and enzymatically digested with 0.25% trypsin for 10 min at 37°C. Cells were then placed in modified Eagle’s medium with 10% fetal bovine serum and grown for 2 weeks to generate cultures composed of >97% GFAP-immunoreactive cells (glia). Adenovirus type 5 (Ad5) viruses, namely, Ad5-LacZ and Ad5-Cre (University of Iowa Gene Transfer Core, Iowa City, IA), were used to produce control (WT) and N2flox/flox (N2fl) glia, respectively, according to protocols described previously in our laboratory (10, 49). Merlin loss was confirmed by immunoblot analysis using a rabbit polyclonal NF2 antibody (NF2-C18; Santa Cruz Biotechnology, Santa Cruz, CA) (1:1,000 dilution) or the laboratory-generated WA30 antibody (50) (1:2,000 dilution).

**Pharmacological inhibitors.** Glial cell cultures were treated with the following experimentally determined concentrations of inhibitors for 24 h prior to all analyses: Src inhibitor PP2, 0.5 µM (Calbiochem, San Diego, CA); ErbB2 inhibitor AG825, 4.0 µM (Calbiochem); FAK/paxillin inhibitor echistatin, 2.0 µM (Sigma, St. Louis, MO).

**shRNA constructs and lentiviral delivery.** Mouse gene-specific lentiviral plasmids for Src (GenBank accession no. NM_009271; TRCN0000023595 and TRCN0000025398), nF2 (NM_109715) small hairpin RNAs (shRNAs) were commercially purchased (Sigma). Corresponding lentiviruses were produced as previously described (54), and the most effective silencing construct was selected for further study. Briefly, 293T cells were co-transfected with Src-shRNA, shRNA, and pLKO1-GFP plasmids (Sigma). The following day, conditioned medium containing ErbB2-shRNA lentiviral plasmids or a control vector (pLKO1-GFP plasmid; courtesy of Sheila Stewart, Washington University), using Fugene HD (Roche, Mannheim, Germany). Lentiviruses were harvested at 48 and 72 h posttransfection. WT and N2fl/fl glia were infected for a total of 48 h. WT and N2fl/fl cells were serum starved for 24 h prior to each experiment.

**Thymidine incorporation assay.** Glielial cells were seeded at 5 x 10^4 cells per well for 24 h of serum starvation. [³H]thymidine (1 Ci per well) was added to serum-free medium and the number of counts per minute was determined after 24 h.

**Cell attachment, apoptosis, and cell adhesion.** Cell attachment was measured by seeding 5 x 10^5 N2fl/fl glielial cells treated with Ad5-LacZ (WT) and Ad5-Cre (N2fl/fl) to 96-well plates precoated with 10 µg/ml fibronectin (Gibco) following 24 h in serum-free medium. After 4 hours, the wells were washed with 1% phosphate-buffered saline (PBS) and incubated in 0.5% crystal violet for 30 min. The number of attached cells was determined by extraction in 1% sodium dodecyl sulfate (SDS) overnight and spectrophotometric analysis at 540 nm.

**Apoptosis** was measured by seeding WT and N2fl/fl glielial cells into 24-well plates, followed by preincubation in serum-free medium in the presence and absence of 5 nM staurosporine (Sigma) for 24 h. Cells were then immunostained with cleaved caspase-3 antibody (1:500 dilution; Cell Signaling Technology) and NF2 fragments containing residues 300 to 557 (NF2.300-557), and WT Src (Src) in the presence and absence of 10 µM ATP and 1,250 mM DTT. Western blots were run using antibodies against ACK1 (Cell Signaling Technology) (1:1,000 dilution), phosphotyrosine monoclonal antibody [MAb] P-Tyr-100 (1:500 dilution) (Promega, Madison, WI) was used as previously described (17).

**Rac1 activation assay.** Rac activation in WT and N2fl/fl glielial cells were determined following incubation of cell lysates with agarose-conjugated monoclonal anti-phosphotyrosine (PY20) antibody (Sigma) according to the manufacturer’s protocols. Following washes, proteins were eluted by boiling in gel loading buffer and detected by immunoblotting using antibodies against Ack1 (Cell Signaling Technology) (1:1,000 dilution), p130CAS (Sigma) (1:1,000 dilution), CASPR (Santa Cruz Biotechnology) (1:500 dilution), ErbB2 (Cell Signaling Technology) (1:1,000 dilution), and ErbB4 (Cell Signaling Technology) (1:1,000 dilution).

**Serrc and ErbB2 kinase activity assays.** Primary glial cells were lysed by sonication in precipitation buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 10 mM dithiothreitol [DTT]) with protease inhibitors (leupeptin, benzamidine, aprotinin, and phenylmethylsulfonyl fluoride). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) prior to detection with antibodies against phosphorylated Src (Y416, FAK-Y576/577) (Cell Signaling Technology, Beverly, MA) (1:1,000 dilution), ErbB2-Y877 (Abcam, Cambridge, MA) (1:1,000 dilution), and paxillin-Y118 (Cell Signaling Technology, Beverly, MA) (1:1,000 dilution). Antibodies against total Src, ErbB2, and paxillin (from the same manufacturers and at the same dilutions as the phospho-specific antibodies), as well as α-tubulin (Sigma) (1:5,000 dilution), were used controls for equal protein loading and quantitation. Antibodies against phosphorylated MAPK p44/p42, AKT-3T6, and EGFR-8345 as well as total MAPK p44/p42, AKT, and EGFR were purchased from Cell Signaling Technology and used at a 1:1,000 dilution. Densitometric analysis was performed using Gel-Pro Analyzer 4.0 software (MediaCybernetics, Silver Spring, MD) with anti-tubulin or non-phospho-Src, -paxillin, -FAK, and -ErbB2 antibodies as internal loading controls.

**Immunoprecipitation.** Primary glia were lysed by sonication in precipitation buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Anti-bodies against Src (1 µg/ml), FAK (1 µg/ml), and ErbB2 (Upstate, Temecula, CA) (1:100 dilution) were added to the lysates and incubated with gentle rocking overnight at 4°C. Protein A-agarose beads (Calbiochem) were then added to the mixture and rotated for 4 hours. Beads were washed with precipitation buffer and resuspended in 2 x Laemmli buffer. Each sample was then boiled and resolved by SDS-PAGE, followed by immunoblot analysis as described above.

**Tissue phosphate photodetection** was performed following incubation of cell lysates with agarose-conjugated monoclonal anti-phosphotyrosine (PY20) antibody (Sigma) according to the manufacturer’s protocols. Following washes, proteins were eluted by boiling in gel loading buffer and detected by immunoblotting using antibodies against Ack1 (Cell Signaling Technology) (1:1,000 dilution), p130CAS (Sigma) (1:1,000 dilution), CASPR (Santa Cruz Biotechnology) (1:500 dilution), ErbB2 (Cell Signaling Technology) (1:1,000 dilution), and ErbB4 (Cell Signaling Technology) (1:1,000 dilution).

**Serrc and ErbB2 kinase activity assays.** Primary glial cells were lysed by sonication in precipitation buffer. Antibodies against Src (Cell Signaling) (1:50 dilution) or ErbB2 (Cell Signaling) (1:50 dilution) were added to the lysates and incubated with gentle rocking overnight at 4°C. Protein G agarose beads (Calbiochem) were then added to the mixture and rotated overnight. Beads were washed with 1 x Laemmli buffer and resuspended in 1 x kinase assay buffer containing ATP, 0.5 µM Tyr160-biotinylated peptide substrate (1.5 µM; Cell Signaling), 20 µM ATP, and 1,250 mM DTT for Src or FLT3 Tyr589-biotinylated peptide (1.5 µM; Cell Signaling), 20 µM ATP, and 1,250 µM DTT for ErbB2. After 30 min, each reaction was stopped by the addition of EDTA. Twenty-five microliters of each reaction mix was added to a 96-well streptavidin-coated plate containing 75 µl distilled H₂O/well and incubated at room temperature for 60 min. After the plates were washed with PBS-Tween 20 (PBS-T, 100 µl of diluted detection antibody (phosphotyrosine monoclonal antibody [MAb] P-Tyr-100 [1,000 dilution]) in PBS-T with 1% bovine serum albumin) was added to each well for 2 h at 37°C. Next, 100 µl of horseradish peroxidase-labeled mouse secondary antibody (1:500 in PBS-T with 1% bovine serum albumin; Cell Signaling) was added, and the plates were incubated at room temperature for 30 min, washed with PBS-T, and developed in 100 µl TMB solution (Cell Signaling) for 10 min at 37°C. Following the addition of stop solution (Cell Signaling), the absorbance was read at 450 nm.

**Rac1 activation assay.** Rac activation in WT and N2fl/fl glial cell lysates was determined using a Rac1 activation assay kit (Upstate Biotechnologies, Temecula, CA) according to the manufacturer’s instructions, as previously described (17).

**In vitro binding assays.** A commercially available TrkF quick coupled transcription/translation system (Promega, Madison, WI) was used as previously described (20) to transcribe and translate WT merlin (NF2.WT), mutant merlin (NF2.L64P), a C-terminal merlin fragment containing residues 300 to 595 (NF2.C-term), an N-terminal merlin fragment containing residues 1 to 300 (NF2.N-term), a merlin fragment containing residues 300 to 557 (NF2.300-557), and WT Src (Src) in the

---

**Vol. 29, 2009 MERLIN GROWTH CONTROL REQUIRES ErbB2 AND Src 1473**

Downloaded from http://mcb.asm.org/ on January 11, 2014 by guest
presence of 2 μCi [3H]thymidine (1,000 Ci/mmol) at 10 μCi/ml. Perkin-Elmer, Waltham, MA). The first set of experiments were direct binding experiments in which 75% of each TnT preparation containing radiolabeled merlin or Src was incubated with 1.0 μg recombinant His-tagged ErbB2 or Src protein (Invitrogen, Carlsbad, CA) overnight at 4°C. The nickel affinity gel was washed and eluted with 2X Laemmli buffer. Bound proteins along with the remaining original 25% of each TnT preparation were then separated by SDS-PAGE and analyzed by autoradiography. Densitometric analysis was performed using Gel-Pro Analyzer 4.0 software (MediaCybernetics), with 25% of each TnT preparation serving as the input control (Sigma) for 4 h at 4°C. The nickel affinity gel was washed and eluted with 2X Laemmli buffer. Bound proteins along with the remaining original 25% of each TnT preparation were then separated by SDS-PAGE and analyzed by autoradiography. Densitometric analysis was performed using Gel-Pro Analyzer 4.0 software (MediaCybernetics), with 25% of each TnT preparation serving as the input control (Sigma) for 4 h at 4°C. The nickel affinity gel was washed and eluted with 2X Laemmli buffer. Bound proteins along with the remaining original 25% of each TnT preparation were then separated by SDS-PAGE and analyzed by autoradiography. Densitometric analysis was performed using Gel-Pro Analyzer 4.0 software (MediaCybernetics), with 25% of each TnT preparation containing radiolabeled merlin or Src was incubated with His-Select nickel affinity gel, followed by SDS-PAGE, autoradiography, and densitometric analysis.

In vivo Src inhibition. N2FGFAPCKO mouse brains were generated by crossing N2F mice with GFAP-Cre mice, and merlin expression in the brain was detected by Western blotting using merlin antibodies (NF2 C-18; 1:1,000 dilution). Ten-day-old N2Flox (WT) and N2FloxGFAPCKO (N2Flox) mice (n = 4 per experimental group) received 2 mg/kg of body weight of PP2 (Cal-

FIG. 2. Merlin regulates glial cell growth in vivo. (A) Immunoblot analysis of merlin expression in N2Flox and N2FloxGFAPCKO mouse brain lysates also demonstrates a >90% reduction in merlin expression in N2FloxGFAPCKO mice, as assessed by scanning densitometry. α-Tub, α-tubulin. (B) Representative immunofluorescence photomicrographs of hippocampi from BrdU-injected N2Flox and N2FloxGFAPCKO mice labeled with both BrdU (green) and GFAP (red) antibodies. N2FloxGFAPCKO mouse brains had a 2.2-fold more BrdU-positive cells than N2Flox mouse brains. *, P < 0.001 (unpaired t test). (C) Representative photomicrographs of hippocampi from N2Flox and N2FloxGFAPCKO mice labeled with GFAP (red) antibodies. N2FloxGFAPCKO mouse brains had a 2.4-fold increase in the number of GFAP-expressing cells compared to N2Flox mouse brains. *, P = 0.001 (unpaired t test). (D) Representative photomicrographs of in vivo TUNEL staining of hippocampi from N2Flox and N2FloxGFAPCKO mouse brains, revealing no significant differences in the number of apoptotic cells between N2FloxGFAPCKO and N2Flox mouse brains.
RESULTS

Merlin regulates glial cell growth in vitro and in vivo. To study merlin growth regulation in CNS glia, we employed primary neonatal forebrain glial cultures from Nf2 conditional knockout (Nf2floxfloxflox) mice, in which the expression of merlin is eliminated by adeno viral delivery of Cre recombinase (Ad-Cre). Following Cre recombinase transduction, Nf2−/− (Nf2floxfloxflox + Ad-Cre) glia consistently demonstrated a >95% decrease in merlin expression compared to Nf2floxfloxflox glia transduced with Ad-LacZ virus (WT) (Fig. 1A). As previously reported for other cell types, Nf2-deficient glia were indistinguishable from WT glia with respect to cell attachment (Fig. 1C), apoptosis (Fig. 1D), and adherens junctions (Fig. 1E).

To determine whether our findings using Nf2-deficient glia in vitro were also observed in the intact brain in vivo, we analyzed glial cell proliferation in Nf2floxfloXCKO mouse. Nf2floxfloXCKO mice were generated by the successive intercrossing of Nf2floxfloxflox mice with Nf2floxfloxcre, GFAP-Cre mice to yield mice with Nf2 loss in GFAP-expressing cells (glia). Nf2floxfloXCKO mice were born at the expected Mendelian frequencies, were phenotypically normal, and demonstrated a decrease in merlin expression compared to Nf2flox/flox (data not shown). In contrast, Nf2flox/flox glia exhibited a 4.7-fold increase in Src compared to WT glia in vitro. Total Rac1, MAPK, and AKT expression levels were used as internal controls for equal protein loading, α-Tub, α-tubulin.

Immunohistochemistry. To analyze proliferation in vivo, 7- to 10-day-old Nf2floxfloxFK62 and Nf2flox/YAPCKO mice (n = 3) were injected with 5-bromo-2-deoxyuridine (BrdU; Sigma) (50 μg/g of body weight) as previously described (24). After 2 hours, mice were euthanized and their brains were removed. A quarter of each brain was separated for immunoblot analyses, and the remainder of each brain was fixed in 4% paraformaldehyde and paraffin sections prepared. Brain sections were immunolabeled with BrdU (Abcam, Cambridge, MA) (1:100 dilution) and GFAP (Sigma) (1:200 dilution) antibodies followed by Alexa 488 (BrdU; Abcam) (1:200 dilution) and Cy3 (GFAP; Sigma) (1:500 dilution) conjugated secondary antibodies. All sections were photographed with a digital camera (Optronics) attached to an inverted microscope (Nikon). The number of BrdU-positive cells in the hippocampus for each set of three littersmates was determined by direct cell counting.

To quantify glial cell numbers in vivo, GFAP immunohistochemistry (Sigma) (1:200 dilution) and GFAP (Sigma) (1:200 dilution) antibodies followed by Alexa 488 conjugated secondary antibodies. All sections were photographed with a digital camera (Optronics) attached to an inverted microscope (Nikon). The number of GFAP-positive cells in the hippocampus for each set of three littersmates was determined by direct cell counting.
>90% decrease in merlin expression in the brain compared to Nf2\textsuperscript{fox/fox} mice (Fig. 2A). Despite efficient Nf2 inactivation in glia throughout the neuroaxis, these mice did not develop nervous system tumors, even after 15 months of age (n = 15 mice to date).

To measure glial cell proliferation in vivo, postnatal day 10 Nf2\textsuperscript{GFAP}CKO pups were injected with BrdU, and the numbers of BrdU- and GFAP-positive glia were quantitated. Consistent with our findings in vitro, we observed a 2-fold increase in the numbers of BrdU-positive cells and GFAP-expressing cells in Nf2\textsuperscript{GFAP}CKO mice compared to those in Nf2\textsuperscript{fox/fox} controls (mean 2.2- and 2.4-fold increases, respectively) (Fig. 2B and C). No in vivo changes in apoptosis were observed in the hippocampus for Nf2\textsuperscript{GFAP}CKO mice compared to Nf2\textsuperscript{fox/fox} controls (Fig. 2D).

To demonstrate that the observed increase in cell proliferation was directly related to merlin expression, we restored merlin expression in Nf2-deficient glia by MSCV retroviral transduction. For these experiments, we reintroduced either WT merlin (NF2) or an inactive merlin protein containing a nonfunctional patient missense mutation (NF2.L64P) (4) into WT and Nf2\textsuperscript{−/−} glia. Following transduction, the levels of merlin expression in Nf2\textsuperscript{−/−} glia were similar to those observed in WT glia (Fig. 3A). Consistent with the notion that merlin directly regulates cell growth in glia, the reintroduction of WT but not mutant merlin reduced the proliferation of Nf2\textsuperscript{−/−} glia to the levels observed in WT glia (Fig. 3B). No effect of merlin expression was observed on WT glial cell proliferation. Collectively, these results demonstrate that merlin is a direct regulator of glial cell growth in vitro and in vivo.

Merlin regulates glial cell growth in a Src-dependent fashion. Previous studies using different primary cell types and established cell lines have shown that merlin regulates several distinct signaling pathways. To identify the signaling pathway responsible for merlin growth regulation in glia, we employed activity assays and activation (phospho-)specific antibodies to determine which of these previously implicated NF2-associated signaling pathways were deregulated in Nf2\textsuperscript{−/−} glia. In contrast to previous reports using non-CNS cell types, no changes in Rac1, MAPK, and AKT activation were observed in Nf2\textsuperscript{−/−} compared to WT glia (Fig. 4A). However, we found that Nf2\textsuperscript{−/−} glia exhibited hyperphosphorylation of the Src nonreceptor tyrosine kinase oncoprotein on tyrosine 416, a residue located within the Src kinase domain activation loop (45) (Fig. 4B). No changes in Src phosphorylation were seen on another common activation site (tyrosine 215) or on the deactivation site (tyrosine 527). We also examined Src phosphorylation in lysates from Nf2\textsuperscript{GFAP}CKO mice and found that Src tyrosine 416 phosphorylation was likewise increased in Nf2\textsuperscript{GFAP}CKO mice relative to that in control mice in vivo (Fig. 4C). To directly demonstrate that Src activity was increased in Nf2\textsuperscript{−/−} glia, we assayed Src activity following Src immunoprecipitation. Similar to the results obtained with the Src Y416 phospho-antibody, we observed a 4.7-fold increase in Src activity in Nf2\textsuperscript{−/−} glia relative to that in their WT counterparts (Fig. 4D). Finally, to demonstrate that merlin directly regulates
Src activity, we assessed Src Y416 phosphorylation following the reexpression of merlin. In these experiments, WT but not mutant (NF2.L64P) merlin reexpression in Nf2−/− glia resulted in a reduction of Src Y416 phosphorylation to the levels observed in WT glia (Fig. 4E).

To determine whether Src hyperactivation resulting from merlin loss was responsible for the increased proliferation seen in Nf2-deficient glia, we treated WT and Nf2−/− cells with PP2, a potent inhibitor of the Src family of kinases (23). PP2 treatment resulted in a >90% reduction in Src activity, as assessed using Src phospho-specific antibodies (Fig. 5A), and was sufficient to reduce the proliferation of Nf2−/− glia to WT levels (Fig. 5B). No effect of PP2 treatment on WT glial cell proliferation was observed. To complement these findings, we utilized shRNA interference to silence Src expression. Nf2−/− glia infected with Src.shRNA lentivirus exhibited a >90% reduction in Src expression, as assessed using total and Src activation-specific antibodies (Fig. 5C), and similar to the case for PP2 treatment, the shRNA decreased Nf2−/− glial cell proliferation to WT levels (Fig. 5D). Similar results were obtained using a second, independent Src.shRNA lentivirus (data not shown). In all cases, no effect of Src.shRNA on WT glial cell proliferation was observed. Together, these results demonstrate that merlin regulation of glial cell growth requires Src Y416 phosphorylation and activation.

Src-mediated merlin regulation of glial cell growth requires FAK and paxillin. To determine how Src regulates glial cell growth, we next examined the activation of known Src downstream effector molecules, including FAK, paxillin, p130CAS, Ack1, and CASPR. While we found no change in the phosphorylation status of p130CAS, Ack1, and CASPR in Nf2−/− versus WT glia (Fig. 6A), we found increased FAK tyrosine 576/577 and paxillin tyrosine 118 phosphorylation in Nf2−/− glia relative to their WT counterparts (Fig. 6B, left panels). Similarly, immunoblot analysis of Nf2GAP-CKO mouse brain lysates demonstrated increased FAK and paxillin phosphorylation relative to that in control littermates (Fig. 6B, right panels).

To demonstrate that merlin loss and Src activation were directly responsible for the increases in FAK and paxillin phosphorylation, we transduced WT and Nf2−/− glia with WT or mutant (L64P) merlin, using MSCV retroviral delivery. In these experiments, reexpression of WT but not mutant merlin in Nf2−/− glia reduced FAK and paxillin phosphorylation to WT levels (Fig. 6C). To demonstrate that Src activation was responsible for FAK and paxillin activation in Nf2−/− glial cells, we employed PP2 pharmacologic Src inhibition and shRNA Src silencing (Src.shRNA) against p130CAS, Ack1, and CASPR in Nf2−/− glia. Both PP2 (left panels) and Src.shRNA (right panels) inhibition reduced FAK and paxillin phosphorylation in Nf2−/− glial cells to WT levels (Fig. 6D). No effect of merlin expression, PP2 treatment, or Src.shRNA lentiviral infection was observed on FAK and paxillin phosphorylation in WT glia.

To determine whether FAK and paxillin were responsible for the increased proliferation observed in Nf2-deficient glia, we initially used a known FAK/paxillin pharmacological inhibitor, echistatin (11). Treatment of Nf2−/− glia with echistatin resulted in inhibition of FAK and paxillin phosphorylation but had no effect on Src phosphorylation (Fig. 7A, upper panel). Furthermore, echistatin treatment reduced the proliferation of Nf2−/− glia to WT levels (Fig. 7A, lower panel). No effect of echistatin treatment on WT glial cell proliferation was observed. We next performed complementary experiments using specific FAK (FAK.shRNA) and paxillin (Pxn.shRNA) shRNA reagents to circumvent the problems inherent in using relatively nonselective pharmacological inhibitors. In these experiments, lentiviral FAK.shRNA expression in Nf2−/− glia inhibited paxillin phosphorylation but had no effect on Src phosphorylation (Fig. 7B, upper panel). FAK shRNA inhibition in Nf2−/− glia also reduced the proliferation to levels observed in WT glia (Fig. 7B, lower panel). In addition, we inhibited paxillin expression in Nf2−/− glia with lentiviral Pxn.shRNA and observed no effect on either Src or FAK phosphorylation (Fig. 7C, upper panel); however, this reduced the proliferation of Nf2−/− glia to WT levels (Fig. 7C, lower panel). In both cases, no effects of FAK and paxillin inhibition on WT glial cell proliferation were observed. These experiments demonstrate that merlin negatively regulates glial cell proliferation in a Src-, FAK-, and paxillin-dependent manner.
and place paxillin downstream of Src and FAK in the merlin glial cell growth control pathway.

Merlin regulates glial cell proliferation through Src signaling in vivo. To demonstrate that merlin regulates glial cell proliferation in a Src-dependent manner in vivo, postnatal day 10 \(N^{2}/^{2}\)GFAPCKO pups were injected with either the Src inhibitor PP2 or vehicle three times a week for 2 weeks (14). \(N^{2}/^{2}\)GFAPCKO pups were then injected with BrdU, and the numbers of BrdU- and GFAP-positive glia were quantitated as previously reported (3, 24). Consistent with our findings in vitro, PP2 treatment of \(N^{2}/^{2}\)GFAP mice resulted in reductions in the numbers of BrdU- and GFAP-positive glia as quantitated previously reported (3, 24). Consistent with our findings in vitro, PP2 treatment of \(N^{2}/^{2}\)GFAP mice resulted in reductions in the numbers of BrdU- and GFAP-positive glia as quantitated previously reported (3, 24). Consistent with our findings in vitro, PP2 treatment of \(N^{2}/^{2}\)GFAP mice resulted in reductions in the numbers of BrdU- and GFAP-positive glia as quantitated previously reported (3, 24). Consistent with our findings in vitro, PP2 treatment of \(N^{2}/^{2}\)GFAP mice resulted in reductions in the numbers of BrdU- and GFAP-positive glia as quantitated previously reported (3, 24). Consistent with our findings in vitro, PP2 treatment of \(N^{2}/^{2}\)GFAP mice resulted in reductions in the numbers of BrdU- and GFAP-positive glia as quantitated previously reported (3, 24).

Merlin regulates Src by modulating ErbB2 activation. Several receptor tyrosine kinase proteins have been shown to regulate Src activity, including ErbB2 (erythroblastic leukemia viral oncogene homolog 2) (36, 53) and EGFR (13, 19). Based on previous studies implicating EGFR in merlin growth regulation in non-nervous-system cells (9), we first examined EGFR in \(N^{2}/^{2}\)glia in vitro and in \(N^{2}/^{2}\)GFAPCKO mice in vivo. We found no changes in EGFR expression or phosphorylation in \(N^{2}/^{2}\)glia relative to that in WT glia (Fig. 9A). Next, we examined the activation status of other members of the EGFR family, including ErbB2, ErbB3, and ErbB4, using phospho-specific antibodies. Whereas we observed no changes in ErbB3 or ErbB4 tyrosine phosphorylation (Fig. 9B), we observed a significant increase in ErbB2 phosphorylation on tyrosine 877 in \(N^{2}/^{2}\)glial cells compared to that in WT glia (Fig. 9C). In contrast, no change in ErbB2 tyrosine 1248 phosphorylation was observed in \(N^{2}/^{2}\)glial cells relative to that in WT glia (Fig. 9C). As expected, we also detected increased ErbB2 tyrosine 877 phosphorylation in \(N^{2}/^{2}\)GFAP mice compared to \(N^{2}/^{2}\)flox/flox mouse brains (Fig. 9D). To directly demonstrate that ErbB2 activity was increased in \(N^{2}/^{2}\)glia, we assayed ErbB2 activity following ErbB2 immunoprecipitation. Similar to the results obtained with the ErbB2 Y877 phospho-antibody, we observed a 3.2-fold increase in ErbB2 activity in \(N^{2}/^{2}\)glia relative to that in their WT counterparts (Fig. 9E).

FIG. 7. Merlin growth regulation requires FAK and paxillin signaling. (A) (Top) Echistatin inhibits the phosphorylation of both FAK and paxillin but has no effect on Src activation. (Bottom) Echistatin treatment eliminates the growth advantage observed in \(N^{2}/^{2}\) glia, as determined by [\(^{3}H\)]thymidine incorporation. *, \(P < 0.001\) (unpaired \(t\) test). (B) (Top) FAK.shRNA inhibits the expression of FAK (>92% reduction) as well as the phosphorylation of both FAK and paxillin. No effect on Src activation was observed. (Bottom) FAK.shRNA treatment eliminates the growth advantage observed in \(N^{2}/^{2}\) glia, as assessed by [\(^{3}H\)]thymidine incorporation. *, \(P < 0.003\) (unpaired \(t\) test). (C) (Top) Pxn.shRNA treatment inhibits the expression of paxillin (>95% reduction) as well as the phosphorylation of paxillin but has no effect on FAK and Src activation. Total tubulin, Src, FAK, and paxillin expression levels were used as internal controls for equal protein loading. (Bottom) Pxn.shRNA treatment eliminates the growth advantage observed in \(N^{2}/^{2}\) glia, as determined by [\(^{3}H\)]thymidine incorporation. *, \(P < 0.001\) (unpaired \(t\) test).
levels (Fig. 9F). No effect of merlin expression on ErbB2 Y877 phosphorylation was observed in WT glia.

Consistent with ErbB2 activation as the initiating signal in the merlin growth control pathway, inhibition of Src or Src effectors in vitro or in vivo had no effect on ErbB2 Y877 phosphorylation. In these studies, no changes in ErbB2 tyrosine 877 phosphorylation were seen following PP2 treatment of Nf2\(^{-/-}\) glia in vitro (Fig. 10A, left panels), PP2 treatment of Nf2GFAP\(^{-/-}\) mouse brains in vivo (Fig. 10A, right panels), inhibition of FAK and paxillin by echistatin (Fig. 10B), or shRNA genetic inhibition of Src (Fig. 10C), FAK (Fig. 10D), and paxillin (Fig. 10E).

To determine whether merlin-dependent ErbB2 activation was responsible for Src-FAK-paxillin pathway activation as well as increased proliferation in Nf2\(^{-/-}\) glia, we initially employed the ErbB2-specific inhibitor tyrphostin (AG825) (39). Following treatment of Nf2\(^{-/-}\) glia in vitro (Fig. 10A, left panels), PP2 treatment of Nf2GFAP\(^{-/-}\) mouse brains in vivo (Fig. 10A, right panels), inhibition of FAK and paxillin by echistatin (Fig. 10B), or shRNA genetic inhibition of Src (Fig. 10C), FAK (Fig. 10D), and paxillin (Fig. 10E).

To determine whether merlin-dependent ErbB2 activation was responsible for Src-FAK-paxillin pathway activation as well as increased proliferation in Nf2\(^{-/-}\) glia, we initially employed the ErbB2-specific inhibitor tyrphostin (AG825) (39). Following treatment of Nf2\(^{-/-}\) glia with AG825, Src, FAK, and paxillin phosphorylation in Nf2\(^{-/-}\) glia to WT levels (Fig. 11A, top panel). As observed following AG825 treatment, ErbB2 shRNA inhibition also decreased Nf2\(^{-/-}\) glial cell growth to WT levels (Fig. 11B, bottom panel). These findings demonstrate that merlin selectively regulates ErbB2 activation, which is responsible for the increased Src signaling and proliferation observed in Nf2\(^{-/-}\) glial cells.

Merlin regulates the interaction between Src and ErbB2. Based on the observation that ErbB2 can interact with both merlin (15) and Src (27, 36, 42), we next sought to determine whether merlin loss is associated with increased ErbB2-Src binding. In these experiments, ErbB2 was precipitated from WT and Nf2\(^{-/-}\) glia using total ErbB2 antibodies. First, we confirmed that merlin binds to ErbB2 in WT glia and demonstrated that merlin loss in glia leads to a dramatic increase in both total and Y416-phosphorylated Src binding to ErbB2 (Fig. 12A). Second, we showed that the reintroduction of WT but not mutant merlin inhibited ErbB2-Src binding (Fig. 12B). Third, we found that inhibition of ErbB2 activation using the AG825 inhibitor blocked the interaction of ErbB2 with Src in Nf2\(^{-/-}\) glia (Fig. 12C), whereas inhibition of Src activation with PP2 had no effect on ErbB2-Src binding (Fig. 12D). These findings demonstrate that only functional merlin can inhibit Src, FAK, and paxillin phosphorylation in Nf2\(^{-/-}\) glia to WT levels (Fig. 11B, top panel). As observed following AG825 treatment, ErbB2 shRNA inhibition also decreased Nf2\(^{-/-}\) glial cell growth to WT levels (Fig. 11B, bottom panel). These findings demonstrate that merlin selectively regulates ErbB2 activation, which is responsible for the increased Src signaling and proliferation observed in Nf2\(^{-/-}\) glial cells.
Src binding to ErbB2 and that the association between Src and ErbB2 is dependent on ErbB2 but not Src activity.

To determine whether ErbB2 binding to merlin and Src represents a direct interaction, we immunoprecipitated radio-labeled in vitro-transcribed/translated merlin and Src proteins with a recombinant ErbB2 protein containing the activation region of ErbB2 known to interact with Src (27). Both merlin and Src directly interacted with ErbB2 (Fig. 12E). Importantly, we observed no binding of mutant merlin (L64P) to ErbB2, consistent with its inability to restore normal growth regulation in Nf2−/− glia. We next sought to determine whether merlin and Src compete for binding to ErbB2. In these experiments, excess amounts of merlin or Src were added to interfere with Src-ErbB2 or merlin-ErbB2 binding, respectively. We show that merlin inhibited ErbB2-Src binding and Src inhibited ErbB2-merlin binding in a concentration-dependent manner (Fig. 12F and G).

To validate the Src-ErbB2 association results, we performed reciprocal immunoprecipitation studies, using Src to detect ErbB2 binding. In these experiments, we found that merlin loss in glia led to a dramatic increase in Src binding to ErbB2 (Fig. 13A) and that reexpression of WT but not mutant merlin inhibited ErbB2-Src binding (Fig. 13B). Moreover, the Src-ErbB2 interaction was dependent only on ErbB2 activation (Fig. 13C) and not Src activity (Fig. 13D). Interestingly, we also noticed that merlin was found in the Src immunoprecipitates from WT glial cells (Fig. 13A). However, unlike ErbB2-Src binding, the interaction between Src and merlin was not dependent on Src or ErbB2 activity.

The finding that merlin can associate with either Src or ErbB2 suggests two non-mutually exclusive models for merlin function. First, merlin may compete with Src for binding to ErbB2, such that merlin-ErbB2 binding precludes an association between Src and ErbB2. In this fashion, merlin loss allows Src to bind to ErbB2, which culminates in Src activation and increased glial cell growth. Second, merlin may bind to Src and...
sequester Src from binding to ErbB2. The loss of merlin would release Src to bind to ErbB2 and initiate Src-dependent signaling to increase glial cell proliferation.

To distinguish between these two possibilities, we sought to define the merlin residues important for Src and ErbB2 binding. In these experiments, we employed an in vitro binding assay using recombinant ErbB2 and Src proteins to determine which regions of merlin were necessary for merlin-ErbB2 and merlin-Src interactions. In vitro-transcribed/translated radiolabeled full-length merlin (WT) as well as merlin fragments containing residues 1 to 300 (NF2.N-term), 300 to 595 (NF2.C-term), and 300 to 557 were immunoprecipitated with recombinant ErbB2 or Src protein. Merlin binding to ErbB2 required C-terminal domain residues 300 to 557 (Fig. 14A), whereas merlin binding to Src required C-terminal domain residues 557 to 595 (Fig. 14B). These findings demonstrate that the residues important for merlin binding to Src and ErbB2 are distinct and separable.

Since the merlin C-terminal fragment containing residues 300 to 557 binds only to ErbB2 and not to Src, we took advantage of this selective binding to discriminate between the two possible models of merlin function. We found that increasing amounts of the merlin C-terminal fragment inhibits Src binding to ErbB2 (Fig. 14C). In contrast, increasing amounts of Src did not inhibit the association between the merlin C-terminal 300-557 fragment and ErbB2 (Fig. 14D). These results indicate that merlin binding to ErbB2 precludes the ability of Src to associate with ErbB2 and that the association between merlin and ErbB2 likely interferes with ErbB2-mediated activation of Src.

**DISCUSSION**

In this report, we use GFAP-positive (glial) cells in vitro and in vivo as a model system to define the mechanism underlying merlin growth regulation in the CNS. First, we show that Nf2-deficient glia exhibit increased proliferation, which is reversed by merlin reexpression. Second, we demonstrate that merlin...
regulation of glial cell growth requires ErbB2 activation, ErbB2-Src binding, and Src pathway activity in vitro and in vivo. Third, based on the direct binding of merlin to Src and ErbB2, we propose a molecular mechanism for merlin function in glia in which merlin competitively inhibits the association between Src and ErbB2. Importantly, although no visible tumors were observed in our Nf2 glial conditional knockout mice, these results identify a critical targetable growth control pathway in glia relevant to human NF2-associated glial cell tumors.

The lack of glial tumors in the Nf2 glial conditional knockout mice may reflect the generally low incidence of tumor formation in Nf2 conditional knockout GEM strains. In this regard, the incidence of nervous system tumor formation in the schwannoma and meningioma Nf2 GEM models ranged between 24% and 29%, with an average latency of 10 to 14 months (18, 26). While we aged the Nf2GFAPCKO mice for 15 months and performed detailed necropsies, it is possible that tumor formation will require larger numbers of mice and longer periods of observation. Alternatively, it is possible that glial cell tumor formation requires stromal Nf2 heterozygosity, as we have previously reported for NF1-associated optic gliomas in Nf1 optic glioma GEM models (2, 3, 64). Future experiments are necessary to explore these possibilities.

Previous studies with other cell types have shown that Src controls cell proliferation and motility by signaling through downstream effectors, including FAK and paxillin (8). In Nf2−/− glia, we found that Src hyperactivation leads to the sequential activation/phosphorylation of FAK and paxillin (8). In Nf2−/− glia, we found that Src hyperactivation leads to the sequential activation/phosphorylation of FAK and paxillin (8).
Src has been implicated in the control of many intracellular signaling pathways, initiated by cell surface receptor activation, which regulate cell differentiation, survival, proliferation, and motility (58). Previous studies have shown that the growth regulatory function of merlin may be mediated in a variety of different cell types by its interaction with several cell surface receptors, including CD44, β1-integrin, ErbB2, and EGFR (9, 15, 35, 38). While all of these receptors have been shown to regulate Src activity in other cell types, only ErbB2 has been shown to form complexes with CD44, EGFR, and β1-integrin. In this regard, ErbB2 represents a logical candidate for a cell surface receptor capable of integrating multiple environmental signals relevant to merlin growth regulation. In this report, we demonstrate for the first time that ErbB2 plays a critical role in regulating Src-mediated proliferation in Nf2−/− glia and that ErbB2 is the main ErbB receptor kinase family member whose activation is deregulated by merlin loss in glial cells.

Consistent with the role of ErbB2 in controlling cell differentiation, survival, and growth, ErbB2 has been shown to interact with several molecules previously implicated in merlin growth regulation in other cell types, including CD44. In this regard, ErbB2 interacts with CD44 in ovarian carcinoma cell lines and has been shown to regulate ovarian carcinoma cell growth (63). In addition, ErbB2 also interacts with EGFR to regulate glioma cell proliferation (28, 40) and motility (41). In the present study, we show that ErbB2 is hyperactivated in Nf2−/− glia in vitro and in vivo and that pharmacologic or genetic inhibition of ErbB2 in vitro reverses the growth phenotype seen in Nf2−/− glia. While the exact mechanism underlying merlin regulation of ErbB2 activation is not known, these findings raise the intriguing possibility that ErbB2 is a central mediator of cell signaling and might integrate growth regulatory messages from other cell membrane receptors. In the absence of merlin, these receptor associations may not form, leading to deregulated ErbB2 activation. In support of this mechanism, preliminary studies in our laboratory have recently shown that the interactions between ErbB2 and CD44, ErbB2 and EGFR, and ErbB2 and another ErbB2 binding partner, ErbB4, are all dramatically attenuated in Nf2−/− glia (Houshmandi and Gutmann, unpublished observations).

Finally, we show that merlin regulates ErbB2-mediated activation of Src. This observation is consistent with previous studies which have shown that ErbB2 interacts with and activates Src in breast cancer cells and that ErbB2-Src binding is specific to the catalytic domain of ErbB2 (27, 36). In Nf2−/− glia, we found increased ErbB2-Src binding, which was blocked by pharmacologic ErbB2 inhibition. It is worth noting that the increase in Src and ErbB2 phosphorylation in Nf2−/− deficient glia is limited to specific sites in the catalytic domain of each protein (Src-Y416 and ErbB2-Y877) which have been predicted to regulate their activation and to mediate their interactions with other proteins (27). In this report, we demonstrate that a recombinant ErbB2 cytoplasmic domain protein which contains the activation region as well as the Src-interacting domain directly interacts with merlin and Src in vitro and that merlin competitively inhibits the interaction between Src and ErbB2 in a concentration-dependent manner. We further show that merlin binding to ErbB2 and Src involves different residues in the C terminus of the merlin protein. We took advan-

![FIG. 13. Merlin interacts with both ErbB2 and Src. (A) Immunoprecipitation of Src from WT and Nf2−/− glia shows that the association between Src and ErbB2 occurs only in Nf2-deficient glia. (B) WT but not mutant merlin expression inhibits Src and ErbB2 interaction in Nf2−/− glia. (C) AG825 treatment inhibits the association between Src and ErbB2 in Nf2-deficient glia. Total Src was used as an internal control for Src precipitations. In WT glia, merlin and Src associate; however, this binding is not affected by SrbB2 or Src activation.](http://mcb.asm.org/Downloaded from http://mcb.asm.org)
tage of this differential merlin binding to demonstrate that merlin binding to ErbB2 blocks Src binding. Based on these findings, we propose a novel mechanism for merlin function (Fig. 14E). In WT cells, merlin binds to the cytoplasmic domain of ErbB2 and precludes an association between Src and ErbB2. However, in the absence of merlin, Src binds to activated ErbB2, which results in increased Src activity, Src effector activation, and increased cell growth. Our results demonstrating a central role for ErbB2 and Src in regulating NF2-deficient glial cell growth suggest that potential therapeutic approaches for treating NF2-associated tumors might involve targeting ErbB2 and/or Src activation. With the recent success employing ErbB2 inhibitors (e.g., trastuzumab) and Src kinase inhibitors (e.g., dasatinib) in clinical studies for the treatment of breast cancer (40, 43) and myelogenous leukemia (16, 31, 37), the future use of these and related inhibitors may prove beneficial for the treatment of human tumors characterized by merlin loss of function.

FIG. 14. Merlin binding to ErbB2 precludes the association of Src with ErbB2. (A) In an in vitro binding assay, [35S]methionine-radiolabeled full-length merlin containing residues 1 to 595 (NF2.WT), NF2.C-term containing residues 300 to 595, and NF2.C-term containing residues 300 to 557, bound to recombinant His-tagged ErbB2 (ErbB2.His). In contrast, no binding to ErbB2 was observed using NF2.N-term containing residues 1 to 300. Input levels for the reactions are shown and were used as normalization controls in scanning densitometric analyses to determine the percentages of bound proteins. Thirty percent of radiolabeled NF2.WT, 25% of NF2.C-term, and 24% of NF2.300-557 bound to recombinant ErbB2 protein, whereas <1% of NF2.N-term bound to ErbB2. (B) In an in vitro binding assay, [35S]methionine-radiolabeled full-length merlin containing residues 1 to 595 (NF2.WT), and NF2.C-term containing residues 300 to 595, bound to recombinant His-tagged ErbB2 (ErbB2.His). In contrast, no binding to ErbB2 was observed using either NF2.C-term containing residues 300 to 557, or NF2.N-term containing residues 1 to 300. Input levels for the reactions are shown and were used as normalization controls in scanning densitometric analyses to determine the percentages of bound proteins. Thirty-one percent of radiolabeled NF2.WT and 19% of NF2.C-term bound to recombinant Src protein, whereas <1% of NF2.N-term and NF2.300-557 bound to Src. (C) (Top) ErbB2-Src binding was decreased 48% in the presence of equal amounts of NF2.300-557, decreased 96% in the presence of a 5-fold excess of NF2.300-557, and completely eliminated in the presence of a 10-fold excess of NF2.300-557. (Bottom) Coomassie staining was used to verify the amount of nonradiolabeled NF2.300-557 added to each reaction mix. (D) (Top) NF2.300-557-ErbB2 binding was not altered by equal or excess amounts of Src. (Bottom) Immunoblotting was used to verify the amount of nonradiolabeled Src added to the reaction mixtures. (E) Potential molecular model for merlin growth regulation in glial cells. In WT cells, merlin associates with ErbB2 and precludes Src binding to ErbB2. In the absence of functional merlin, Src binds to activated ErbB2, leading to ErbB2-mediated Src phosphorylation/activation, Src effector protein phosphorylation, and increased glial cell growth.
ACKNOWLEDGMENTS

This work was supported in part by Department of Defense grant DAMD17-04-0266 (to D.H.G.), with a nested postdoctoral fellowship (S.S.H.). We also acknowledge the generous support of the Vincent Buono Research Fund (to D.H.G. Hand M.G.). Additionally, we thank Ron Bose, Sutapa Banerjee, Nicole Hicklin, Scott Gianario, and Cory Lewis for their technical assistance during the preparation of the manuscript.

REFERENCES


