Proteomic analysis reveals GIT1 as a novel mTOR complex component critical for mediating astrocyte survival

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As a critical regulator of cell growth, the mechanistic target of rapamycin (mTOR) protein operates as part of two molecularly and functionally distinct complexes. Herein, we demonstrate that mTOR complex molecular composition varies in different somatic tissues. In astrocytes and neural stem cells, we identified G-protein-coupled receptor kinase-interacting protein 1 (GIT1) as a novel mTOR-binding protein, creating a unique mTOR complex lacking Raptor and Rictor. Moreover, GIT1 binding to mTOR is regulated by AKT activation and is essential for mTOR-mediated astrocyte survival. Together, these data reveal that mTOR complex function is partly dictated by its molecuflar composition in different cell types.

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Eukaryotic cell growth relies on the precise coordination and control by the highly conserved serine/threonine protein kinase mechanistic target of rapamycin (mTOR). In response to growth factors, amino acids, oxygen levels, and stress, mTOR assembles into two functionally and molecularly distinct multiprotein complexes to regulate a variety of diverse growth-related cellular processes, including mRNA translation (Barbet et al. 1996), ribosomal biogenesis, transcription (Mayer et al. 2004), cell cycle progression (Fingar et al. 2004), survival (Paglin et al. 2005), and cytoskeletal dynamics (Jacinto et al. 2004; Sarbassov et al. 2004).

Based on studies in mouse embryonic fibroblasts and nonneural cell lines (HEK293 cells), mTOR has been shown to interact with either Raptor and the proline-rich AKT substrate of 40 kDa (PRAS40) to form mTOR complex 1 (mTORC1) or Rictor and the mammalian stress-activated map kinase interacting protein 1 (mSIN1) to generate mTORC2 (Kim et al. 2002; Sarbassov et al. 2004; Jacinto et al. 2006; Oshiro et al. 2007). As such, mTORC1 function is critical for the regulation of transcription and protein translation through phosphorylation of p70-S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4-EBP1) (Gingras et al. 1998; Hara et al. 1998). In contrast, mTORC2 controls actin cytoskeleton dynamics and cell survival by signaling to protein kinase C-α (PKCα), protein kinase B (AKT), and serum- and glucocorticoid-induced protein kinase 1 (SGK1). Beyond these protein interactors, other mTOR-binding proteins have been identified, including DEP domain-containing mTOR-interacting protein (Deptor), mammalian lethal with SEC13 protein 8 (mLST8), and protein observed with Rictor (Protor), each with varying capacities to influence mTOR-dependent function (Kim et al. 2003; Peterson et al. 2009). While the roles of these other mTOR complex molecules in cell growth have not been fully elucidated, it is clear that the mTOR complex is a central regulator of normal cell biology.

The importance of mTOR function to normal development and maintenance is underscored by the identification of mutations in genes encoding proteins that negatively regulate mTOR as causative etiologies for several human neurological diseases, including Neurofibromatosis type 1 (NF1), Cowden's syndrome (PTEN), and tuberous sclerosis complex (TSC). In these disorders, loss of function of neurofibromin (NF1), PTEN, and tuberin/hamartin (TSC) lead to mTOR hyperactivation and increased cell growth. While each of these proteins negatively regulates mTOR function, the biological consequences are distinct. First, the mechanisms underlying tuberin/hamartin and neurofibromin mTOR suppression are separable and reflect different modes of mTOR activation (Banerjee et al. 2011). As such, *Nf1* loss in astrocytes leads to increased proliferation, whereas *Tsc1* loss has no effect on astrocyte proliferation but results in increased cell size (Uhlmann et al. 2004). Second, mTOR controls proliferation distinctly in different cell types. While *Tsc1* loss increases mTOR activation and cell growth in fibroblasts, it has no effect on astrocyte proliferation despite increased mTOR activation (Sandsmark et al. 2007; Banerjee et al. 2010). Third, the composition and function of the mTOR complex is partly dictated by tissue-specific constraints. For example, the ability of brainstem, but not cortical, neural stem cells (NSCs) to increase their proliferation and glial cell differentiation following *Nf1* gene inactivation reflects a fivefold increase in Rictor expression in brainstem NSCs relative to their cortical counterparts (Lee da et al. 2010).

In light of these findings, we sought to define the molecular composition and function of the mTOR complex in the brain. Leveraging a combination of proteomic, genetic, and pharmacological approaches, we identified a novel AKT-regulated mTOR complex protein (G-protein-coupled receptor kinase-interacting protein 1 [GIT1]) in brain tissues whose function is essential for mTOR-mediated astrocyte survival.

Results and Discussion

To characterize the mTOR complex in brain cells, we focused on astrocytes, since these cells represent the nonmalignant counterpart of the cancer cells in the most common brain tumor (astrocytoma or glioma). Western

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blotting of whole-cell lysates from wild-type brainstem astrocytes demonstrated cell type differences in the expression of known mTOR-binding proteins (Fig. 1A). While Rictor, Raptor, mSIN1, PRAS40, and mLST8 were expressed in human HEK293 cells, mouse astrocytes, and mouse fibroblasts, there was an absence of both Deptor and Protor-1 expression in wild-type mouse astrocytes and fibroblasts (Supplemental Fig. S1A).

Next, to determine whether the known mTOR-binding proteins were contained in the mTOR complex in nervous system cells, mTOR immunoprecipitation was performed. As observed in other cell types, Rictor, Raptor, mSIN1, PRAS40, and mLST8 were found in mTOR complexes in mouse astrocytes (Fig. 1B), similar to fibroblasts (Fig. 1C). Since there are two functionally distinct mTOR complexes, identified by their expression of Raptor and PRAS40 (mTORC1) or Rictor and mSIN1 (mTORC2), we performed Raptor and Rictor immunoprecipitations. As observed in other cell types, both the mTORC1 complex (Raptor, PRAS40, and mLST8) and the mTORC2 complex (Rictor, mSIN1, and mLST8) were identified in mouse astrocytes (Fig. 1D).

To identify potential novel astrocyte-specific mTOR-binding proteins, we performed proteomic analyses on mTOR immunoprecipitations from wild-type astrocytes. Using a spectral abundance score of ≥10 (and a spectral count for nonspecific rabbit IgG = 0) and excluding known nonspecific binding proteins (Mellacheruvu et al. 2013; Contaminant Repository for Affinity Purification, http://141.214.172.226, version 1.1), three mTOR-binding protein candidates were identified (Fig. 2A). Using independently prepared mTOR immunoprecipitations, only endogenous GIT1–mTOR binding was validated (Fig. 2B). Importantly, mTOR was contained in GIT1 immunoprecipitations (Fig. 2C), and mTOR and GIT1 binding was observed in HEK293T (293T) cells transfected with myc-mTOR and Flag-GIT1 (Fig. 2D). We also identified a known GIT1-binding protein, Rho guanine nucleotide exchange factor 7 (also called PAK-interacting exchange factor (β-PIX) (Bagrodia et al. 1999; Zhao et al. 2000), in

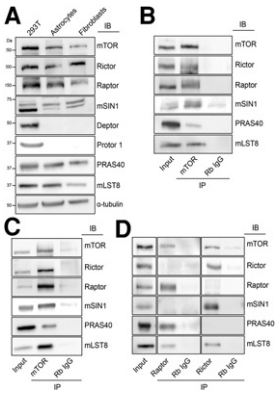

Figure 1. mTOR complex composition is cell type-specific. (*A*) Representative immunoblots demonstrate little Deptor and Protor-1 expression in wild-type mouse astrocytes and fibroblasts relative to HEK293T (293T) cells. Immunoblots from mTOR immunoprecipitations reveal Rictor, Raptor, mSIN1, PRAS40, and mLST8 in wild-type astrocytes (*B*) and fibroblasts (*C*). Rb IgG was included as an internal control for nonspecific binding. (*D*) While Raptor (mTORC1) immunoprecipitations contained mTOR, Raptor, PRAS40, and mLST8 (but not Rictor), Rictor (mTORC2) immunoprecipitations contained mSIN1 and mLST8 but lacked Raptor and PRAS40 in wild-type astrocytes.

mTOR immunoprecipitations (spectral abundance score = 9), which was subsequently confirmed by immunoprecipitation (Supplemental Fig. S2A).

To determine whether the interaction between GIT1 and mTOR was neural cell-specific, GIT1 binding to mTOR was examined in whole-mouse brainstem tissues, mouse brainstem NSCs, mouse *Nf1*$^{-/-}$; *p53*$^{-/-}$ *NPcis* glioma cells, and 293T cells. GIT1 was expressed and binds mTOR in 1-d-old (postnatal day 1 [P1]) and 1-mo-old mouse brainstem tissues (Supplemental Fig. S2B), NSCs, and *NPcis* glioma cells (data not shown) as well as in 293T cells, albeit at 100-fold reduced levels (Supplemental Fig. S2C). In this regard, GIT1 was predominately expressed in the brain (the cortex, cerebellum, and olfactory bulb) relative to nonneural tissues (the liver, kidney, and heart) (Supplemental Fig. S2D).

Next, to ascertain whether GIT1 was contained in the mTORC1 or C2 complex, Raptor (mTORC1) and Rictor (mTORC2) immunoprecipitations were performed. While mTOR and mLST8 were contained in both Raptor and Rictor immunoprecipitations, GIT1 was not found in either mTOR complex (Fig. 2E), and Raptor and Rictor were not found in GIT1 immunoprecipitations. Furthermore, mSIN1 and PRAS40 were also not found in GIT immunoprecipitations (Supplemental Fig. S2E). These data demonstrate that GIT1 is not a component of either of the two established mTOR complexes and likely belongs to a newly identified mTOR complex containing GIT1 and β-PIX (but lacking Raptor, Rictor, mSin1, PRAS40, and mLST8).

To identify GIT1 residues important for mTOR binding, 293T cells were transfected with full-length epitope-tagged mTOR and GIT1 deletion constructs. Following myc-mTOR immunoprecipitation, full-length GIT1 and GIT1 fragments containing residues 250–770 or 420–770 as well as the deletion mutant missing residues 264–430 (delSHD) bound mTOR (Fig. 2F). In contrast, GIT1 containing residues 1–420 did not associate with mTOR, demonstrating that GIT1 binding to mTOR requires GIT1 residues 420–770, encompassing the synaptic localization domain (SLD) and paxillin-binding domain (PBD). Finally, to define the mTOR residues critical for GIT1 binding, 293T cells were transduced with epitope-tagged full-length GIT1 and mTOR deletion fragments. mTOR fragments containing residues 1271–2008 and 1750–2549, but not residues 1–1482, bound GIT1 (Fig. 2G), demonstrating that GIT1 binds mTOR between residues 1482 and 2008 within the FRAP, ATM, and TRAP (FAT) domain.

To determine how GIT1 binding to mTOR is regulated, we focused on one tumor suppressor protein that operates through the mTOR pathway and is essential for glioma pathogenesis. The *NF1* protein neurofibromin suppresses astroglial growth by inhibiting RAS activation of AKT-mediated mTOR signaling (Sandsmark et al. 2007; Banerjee et al. 2011; Kaul et al. 2015) such that neurofibromin loss results in increased (1.7-fold) astrocyte proliferation and S6 (phospho-S6$^{Ser240/244}$; 2.2-fold) activation (Fig. 3A). In *Nf1*$^{-/-}$ astrocytes, there was a 2.5-fold decrease in GIT1 binding to mTOR relative to wild-type astrocytes (Fig. 3A). Since GIT1 binding to mTOR requires residues 420–770, containing a putative phosphorylation residue (Tyr^{544}), GIT1 phosphorylation was examined. No change in GIT1^{Tyr544} phosphorylation was observed following *Nf1* loss (Supplemental Fig. S3A), excluding this residue as the regulatory phosphorylation site responsible for GIT1 binding to mTOR.

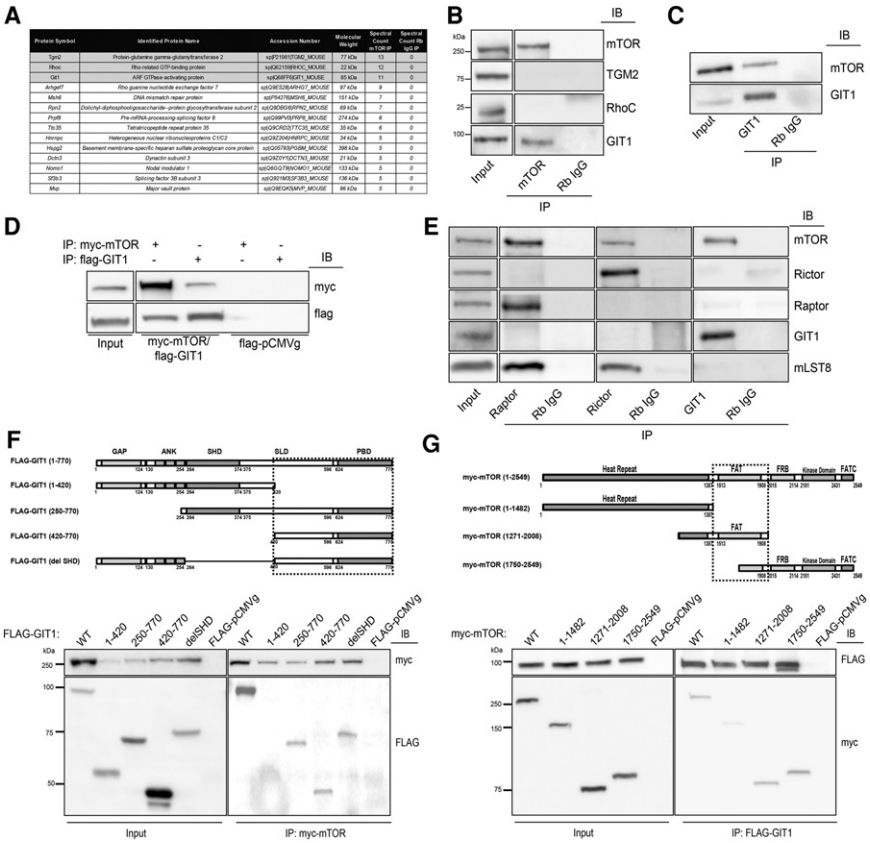

Figure 2. GIT1 is a novel mTOR-binding protein in astrocytes. (*A*) Proteomic analysis of mTOR immunoprecipitations from wild-type astrocytes reveals several potential mTOR-binding proteins. (*B*) Validation of mTOR-binding proteins with spectral counts of ≥10 by immunoblot reveals that endogenous GIT1, but not TGM2 or RhoC, associates with mTOR. Rb IgG was included as an internal control for nonspecific binding. (*C*) GIT1 and mTOR binding is also observed in GIT1 immunoprecipitations from wild-type astrocytes. (*D*) Full-length Flag-tagged GIT1 (Flag-GIT1) and myc-tagged mTOR (myc-mTOR) associate in HEK293 cells. Flag-pCMVg (empty vector) was used as a control. (*E*) GIT1 is not found in Raptor (mTORC1) or Rictor (mTORC2) immunoprecipitations by immunoblot. Raptor, Rictor, and mLST8 are not contained in GIT1 immunoprecipitations. (*F*) Full-length myc-mTOR binding to Flag-GIT1 requires GIT1 residues 420–770 (dotted outline) containing the paxillin-binding domain (PBD) and a portion of the synaptic localization domain (SLD). ANK denotes the ankyrin repeats, while SHD denotes the Spa2 homology domain. (*G*) Full-length Flag-GIT1 binding to myc-mTOR requires mTOR residues 1482–2008, encompassing the mTOR FRAP, ATM, and TRAP (FAT) domains.

Because neurofibromin controls mTOR function in an AKT-dependent manner, the impact of its inhibition on GIT1/mTOR binding was assessed. First, GIT1 binding to mTOR was increased following treatment with 10 nM rapamycin (2.5-fold) (Fig. 3B), suggesting that conformational changes in mTOR resulting from rapamycin/FKBP12 binding to the adjacent FRB domain might be responsible for this effect. Second, pharmacological AKT inhibition (50 nM MK2206) decreased mTOR ($S6^{Ser240/244}$ phosphorylation) and AKT (AKT^{Thr308}, $PRAS40^{Thr246}$, and GSK-$3\beta^{Ser9}$ phosphorylation) activation in *Nf1*$^{-/-}$ astrocytes (Fig. 3C) and also resulted in increased (3.9-fold) GIT1 binding to mTOR, establishing that GIT1 binding to mTOR is mediated by AKT/mTOR activation. This increase in GIT1 binding to mTOR following AKT inhibition was not observed in wild-type astrocytes (Supplemental Fig. S3C). Importantly, no change in GIT1/mTOR binding was observed following MEK inhibition (PD901) (data not shown) in *Nf1*$^{-/-}$ astrocytes. Third, since AKT regulates GIT1 binding to mTOR, we sought to determine whether two mTOR phosphorylation sites (Ser2448 and Ser2481) (Peterson et al. 2000; Sekulic et al. 2000) were regulated by mTOR/AKT activation. No changes in mTOR Ser2448 and Ser2481 phosphorylation were observed in *Nf1*$^{-/-}$ astrocytes relative to their wild-type counterparts (Supplemental Fig. S3B), excluding this potential mechanism. However, it is possible that other phosphorylation sites exist for which antibodies are currently unavailable.

To determine whether GIT1 is required for neurofibromin-mediated astrocyte growth, three independently generated mouse-specific *Git1* shRNA (sh*Git1*) constructs were evaluated, and the two with the greatest knockdown in wild-type (3.1-fold and 3.5-fold reduction) and *Nf1*$^{-/-}$ (2.5-fold and 2.1-fold reduction) astrocytes were selected (Fig. 4A). Following sh*Git1* knockdown, *Nf1*$^{-/-}$ astrocyte growth was reduced to wild-type levels, with no effect on wild-type astrocytes (Fig. 4B; Supplemental Fig. 4A). This reduction in cell growth was the result of increased cell death (apoptosis), as revealed by an increase in cleaved caspase-3 levels (Supplemental Fig. S4B) and the percentage of TUNEL^{+} astrocytes (Fig. 4C). No change in LC3A/B expression (autophagy) was observed (Supplemental Fig. S4B). As before, no change in cleaved caspase-3 levels or the percentage of TUNEL^{+} cells was observed following sh*Git1* knockdown in wild-type astrocytes (Supplemental Fig. S4C).

GIT1 was initially identified as a G-protein-coupled receptor kinase 2-binding protein (Premont et al. 1998) and has been implicated in the control of receptor trafficking, protein complex assembly/transport, focal adhesion turnover, synapse formation, cell motility, and cell growth (Zhao et al. 2000; Manabe et al. 2002; Za et al. 2006). GIT1 can also act as a GTPase-activating protein for the ADP ribosylation factor family of small GTPases (Claing et al. 2000; Vitale et al. 2000), including Rac1 (Zhang et al. 2005; Chang et al. 2015), by binding to the C-terminal region of the Rho guanine nucleotide exchange factor 7 (ARHGEF7 or β-PIX) and promoting the interaction of β-PIX with Rac1 (Bagrodia et al. 1999; Zhang et al. 2005; Fiuza et al. 2013). However, we observed no reproducible changes in Rac1 activity following sh*Git1* knockdown (Supplemental Fig. S4D), and β-PIX knockdown did not alter *Nf1*$^{-/-}$ astrocyte proliferation (data not shown). In addition, GIT1 has been implicated as a Hippo pathway regulator; however, no change in YAP levels or YAP phosphorylation (Ser137) was observed in *Nf1*-deficient astrocytes before or after sh*Git1* knockdown (Supplemental Fig. S4E). Since GIT1 is an mTOR-binding protein, it is possible that reduced

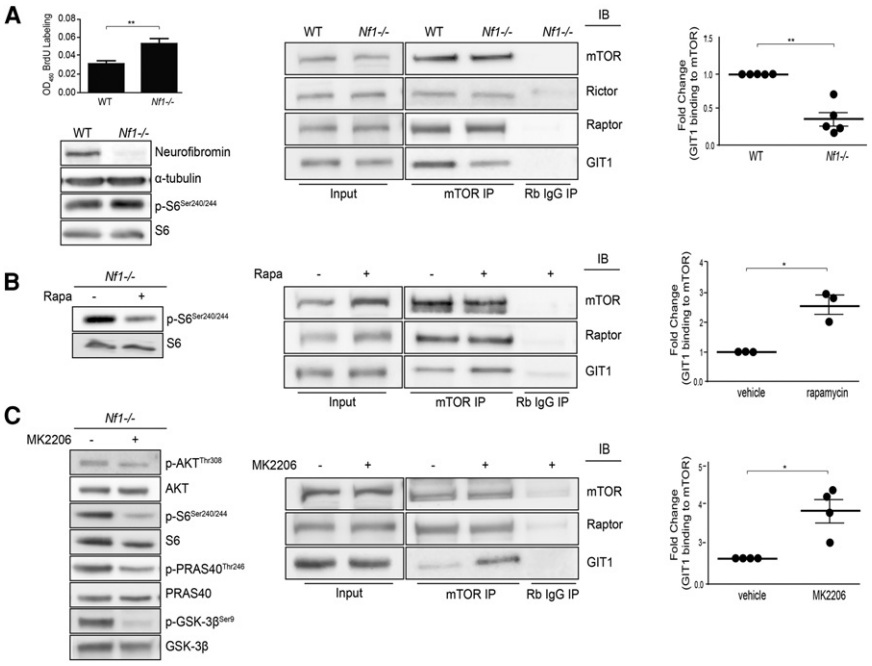

Figure 3. Neurofibromin loss decreases GIT1 binding to mTOR. (*A*) Neurofibromin loss results in increased astrocyte growth and mTOR activation (phospho-S6$^{S240/244}$) relative to wild-type controls. Reduced GIT1 binding to mTOR is observed in *Nf1*$^{-/-}$ relative to wild-type astrocytes. Scatter plots illustrate independently generated mTOR immunoprecipitations. Treatment with the either 10 nM rapamycin (*B*) or 50 nM MK2206 (*C*) increases GIT1 binding to mTOR in *Nf1*$^{-/-}$ astrocytes. mTOR and AKT inhibition were confirmed by decreased S6 (phospho-S6$^{Ser240/244}$), AKT (phospho-AKT^{Thr308}), PRAS40 (phospho-PRAS40^{Thr246}), and GSK-3β (phospho-GSK-3β^{Ser9}) phosphorylation. (*) $P < 0.05$; (**) $P < 0.005$.

GIT1–mTOR association results in either increased Raptor (C1) or Rictor (C2) binding to mTOR or increased mTORC1/C2 effector activation. However, there was no change in Raptor or Rictor binding to mTOR following *Nf1* loss/AKT activation (Fig. 3A), and no changes in the activation of known mTORC1 (4EBP1) or mTORC2 (SGK1 and PKCα/β-II) effectors were observed before or after sh*Git1* knockdown in *Nf1*-deficient astrocytes (Supplemental Fig. S4E). Collectively, the findings reported here suggest that GIT1/mTOR binding likely sequesters GIT1 from activating its currently unidentified downstream effectors to mediate astrocyte survival (Fig. 4E). Future studies will be required to identify these signaling intermediates.

Taken together, the identification of a new GIT1/mTOR complex in this study raises several important points relevant to mTOR composition and function. First, mTOR creates a level of functional diversity by interacting with unique binding partners in different tissue types. In this regard, we reported previously that mTOR complex composition and function in NSCs are dictated by brain region-specific differences in Rictor expression (Lee da et al. 2010). Similarly, while Deptor regulates mTOR kinase activity in HEK293 cells, it is not expressed in wild-type mouse astrocytes and fibroblasts. Loss of Protor-1 expression decreases N-myc downstream-regulated gene-1 (NDRG1; mTORC2 effector) activation in the kidney but not in the brain (Pearce et al. 2011). As such, the differential expression of mTOR-interacting proteins in various tissues and species (Pearce et al. 2011; Yuan et al. 2015) or cell lines (Sarbassov et al. 2004; Foster et al. 2010) may partly explain potentially conflicting results obtained when studying mTOR in different tissue types or cell populations. Second, mTOR function is also dictated by the formation of at least two functional complexes containing distinct binding partners. While two distinct mTOR complexes (mTORC1 and mTORC2) were identified in astrocytes, we describe for the first time the existence of a third mTOR complex composed of GIT1 and β-PIX but not Rictor or Raptor. Support for additional mTOR complexes derives from studies examining oxygen-induced mTOR-mediated translational activation of terminal oligopyrimidine (TOP) mRNA in fibroblasts (Miloslavski et al. 2014), where mTOR regulation of TOP mRNA translation was independent of Rictor or Raptor and occurred in the absence of S6K and 4EBPI activation. Additionally, rapamycin inhibition of mSin1 phosphorylation in human rhabdomyosarcoma cells was mimicked by mTOR or mLST8 silencing but not by Raptor, Rictor, S6K1, or AKT loss (Luo et al. 2015), suggesting the presence of a new mTOR complex containing only mSIN1 and mLST8. Third, mTOR function can be regulated by different upstream modulators. As such, we showed previously that Pten and neurofibromin loss have similar effects on mTOR activation and proliferation in astrocytes, whereas tuberin/hamartin loss activates mTOR through Rheb and does not result in increased astrocyte growth (Banerjee et al. 2011). Moreover, *Nf1*$^{+/-}$ mice with somatic *Nf1* gene inactivation in astroglial progenitors develop optic gliomas, whereas *Nf1*$^{+/-}$ mice with Rheb expression in astroglial progenitors do not develop optic gliomas despite robust mTOR hyperactivation.

In summary, these studies establish that the molecular composition and function of mTOR can be cell type-specific and identify a novel mTOR complex critical for astrocyte survival. These findings have important implications for the interpretation of future mTOR functional studies in distinct tissues relevant to the design and execution of clinical therapeutic trials that target mTOR function in specific organs.

Materials and methods

Mice

Nf1$^{flox/flox}$ (wild-type) mice were generated and maintained as previously described (Zhu et al. 2001; Bajenaru et al. 2002). Mice were used in strict accordance with an approved animal studies protocol at the Washington University School of Medicine.

Cell culture

Wild-type and *Nf1*-deficient (*Nf1*$^{-/-}$) astrocytes were generated from the brainstems of P1–P2 mouse pups, and *Nf1* gene inactivation was performed using adenovirus type 5 (Ad5) (University of Iowa Gene Transfer Vector Core, Iowa City, IA) as previously described (Sandsmark et al. 2007). Fibroblasts were prepared from small ear sections from P1–P2

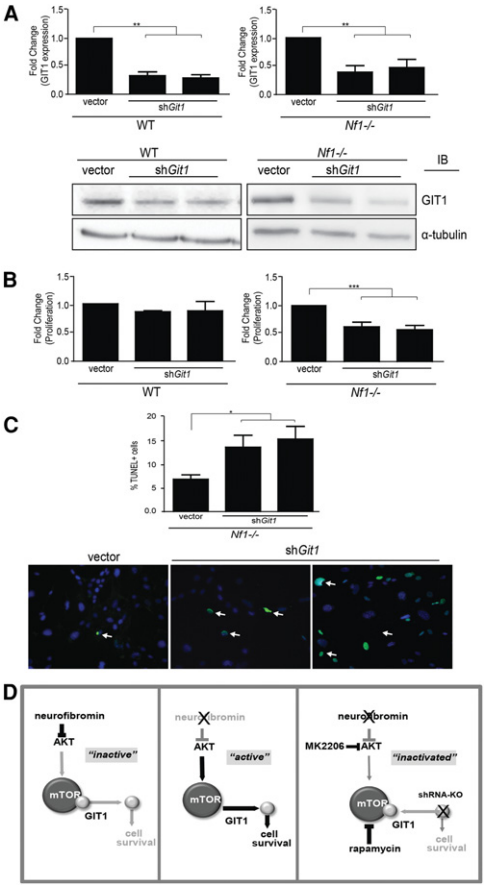

Figure 4. Neurofibromin-mediated astrocyte survival operates in an mTOR/GIT1-dependent manner. (*A*) *Git1* knockdown using two independently generated mouse-specific RNAi (sh*Git1*) lentiviruses reduces GIT1 expression in wild-type and *Nf1*-deficient ($Nf1^{-/-}$) astrocytes. (*B*) sh*Git1* knockdown decreases $Nf1^{-/-}$, but not wild-type, astrocyte proliferation relative to (vector) controls. (*C*) sh*Git1* knockdown increases apoptosis ("% TUNEL$^+$ cells"; arrows denote TUNEL$^+$ cells). (*D*) Proposed mechanism for GIT1-mediated neurofibromin/mTOR-regulated astrocyte survival. While neurofibromin inhibits AKT/mTOR/GIT1-mediated astrocyte growth (*left* panel), neurofibromin loss leads to an AKT/mTOR-dependent reduction in mTOR/GIT1 binding, which results in increased astrocyte survival (*middle* panel). (*Right* panel) Genetic and pharmacological inhibition of AKT, mTOR, or GIT1 reduces astrocyte survival. (*) $P < 0.05$; (**) $P < 0.005$; (***) $P < 0.0005$.

wild-type pups dissociated in collagenase type I-A. All cells were serum-starved for 48 h prior to analysis, and neurofibromin loss was confirmed by Western blot. 293T cells (3×10^6 cells) maintained in complete DMEM were incubated in antibiotic-free DMEM 5 h prior to transfection.

Western blotting

Cells were lysed in 0.3% CHAPS buffer containing 40 mM HEPES (pH 7.4) (Gibco), 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and protease inhibitors. Western blots were performed as described previously (Uhlmann et al. 2004) using primary antibodies (Supplemental Table S1) and horseradish peroxidase-conjugated affinity-purified secondary antibodies (1:5000; Cell Signaling Technology) by enhanced chemiluminescence-based detection (Pierce).

Immunoprecipitation

Precleared protein lysates (200–300 µg; 0.3% CHAPS buffer) were incubated for 16–18 h with primary antibodies (Supplemental Table S1) or normal rabbit IgG (Rb IgG; control) and immunoprecipitated using protein G beads. Bead/protein complexes were washed three times with 0.3% CHAPS buffer containing 120 mM $NaCl_2$ and eluted in 2× Laemmli buffer prior to Western blot analysis. Rapamycin (10 nM) was included during the immunoprecipitation incubation step to inhibit mTOR. For 293T cells, mTOR immunoprecipitations were performed using 600–800 µg of input protein with and without fetal bovine serum (FBS).

Proteomic analysis

Precleared lysates (1.3 mg) from wild-type astrocytes were processed for immunoprecipitation as described above. Flash-frozen elutes were separated by SDS-PAGE on 10% Bis-Tris NuPAGE gels (Invitrogen) and processed by in-gel digestion with trypsin at 37°C for analysis by nanoscale liquid chromatography coupled to tandem mass spectrometry (MS Bioworks). Resulting peptides were identified using Mascot and filtered using a 1% protein and peptide false discovery rate and requiring at least two unique peptides per protein. Peptides are listed by descending spectral counts (protein abundance) with their accession number and molecular weight (Supplemental Table S3).

Viral production

293T cells were transfected with 10 µg of each protein fragment (Supplemental Table S2; Yin et al. 2004) in Opti-MEM (Gibco) using X-tremeGENE 9 (Roche). Mouse-specific *Git1* shRNAi lentiviral constructs (sh*Git1*) (Sigma Mission, TRCN0000346581 and TRC0000346504) were generated following 293T Fugene HD-mediated transfection with 10 µg of sh*Git1*, 5 µg of pMD1, 2.5 µg of Rev, and 3 µg of pCMVg (Supplemental Table S2). Filtered virus was applied to astrocytes in 0.6 µL/mL Polybrene (Millipore). pLKO-anti-GFP lentivirus was used as a control.

Pharmacological inhibitors

Cells were treated in serum-free medium with 10 nM rapamycin (LC Laboratories), 50 nM MK2206 (Selleck), or 1 nM PD901 (Selleck) for 18 h (for proliferation) or 2 h (for Western blot).

Cell proliferation

Astrocytes were serum-starved for 48 h and analyzed following 20 h of exposure to bromodeoxyuridine (BrdU) (Roche). Following the manufacturer's instructions, fixed cells were incubated with peroxidase-conjugated anti-BrdU antibodies and developed in tetramethyl-benzidine. Optical absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

Apoptosis

Astrocytes were permeabilized in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate. Apoptosis was detected using an in situ cell death kit (Roche), with DNase included as positive control. Nuclei were counterstained with 0.005% bis-benzimide (Sigma). Images were captured using a fluorescence microscope (Leica), and apoptotic cells were quantified by direct cell counting using ImageJ.

Statistical analysis

All in vitro studies were repeated at least three times with comparable results. Data were analyzed using parametric Student's *t*-test or one-way ANOVA in GraphPad Prism 5. Significance was set at $P < 0.05$.

Proteomic data will be deposited in Gene Expression Omnibus at the time of publication.

Acknowledgments

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