The Neurofibromatosis Type 1 Tumor Suppressor Controls Cell Growth by Regulating Signal Transducer and Activator of Transcription-3 Activity

In vitro and In vivo

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Abstract

Neurofibromatosis type 1 (NF1) is a common cancer predisposition syndrome in which affected individuals develop benign and malignant nerve tumors. The NF1 gene product neurofibromin negatively regulates Ras and mammalian target of rapamycin (mTOR) signaling, prompting clinical trials to evaluate the ability of Ras and mTOR pathway inhibitors to arrest NF1-associated tumor growth. To discover other downstream targets of neurofibromin, we performed an unbiased cell-based high-throughput chemical library screen using NF1-deficient malignant peripheral nerve sheath tumor (MPNST) cells. We identified the natural product, cucurbitacin-I (JSI-124), which inhibited NF1-deficient cell growth by inducing apoptosis. We further showed that signal transducer and activator of transcription-3 (STAT3), the target of cucurbitacin-I inhibition, was hyperactivated in NF1-deficient primary astrocytes and neural stem cells, mouse glioma cells, and human MPNST cells through Ser727 phosphorylation, leading to increased cyclin D1 expression. STAT3 was regulated downstream of mTOR that mediate neurofibromin tumor suppression. In summary, we used a chemical genetics approach to reveal STAT3 as a novel neurofibromin/mTOR pathway signaling molecule, define its action and regulation, and establish STAT3 as a tractable target for future NF1-associated cancer therapy studies. Cancer Res; 70(4); 1356–66. ©2010 AACR.

Introduction

Neurofibromatosis type 1 (NF1) is one of the most common genetic causes for nervous system tumors, and affected patients develop both benign and malignant tumors involving the brain and peripheral nerves (1). Within the central nervous system, low-grade gliomas of the optic pathway are observed in 15% of children with NF1 (2), whereas adults with NF1 develop high-grade astrocytomas at a 50-fold increased incidence (3). In addition, benign peripheral nerve sheath tumors (neurofibromas) and malignant peripheral nerve sheath tumors (MPNST) are seen in patients with NF1 (4).

With the identification of the NF1 gene in 1990, several investigators found that the NF1 gene product neurofibromin functions primarily as a negative regulator of the RAS protooncogene such that NF1 gene inactivation in tumors is associated with increased RAS pathway activation and cell proliferation (5–7). Further studies showed that inhibition of RAS function reduces NF1-deficient cell and tumor growth in vitro and in vivo (8, 9). These exciting observations led to the initiation of clinical trials using anti-RAS biologically-based therapies, including farnesyltransferase inhibitors (FTI), to treat tumors arising in patients with NF1. Unfortunately, FTI therapy has shown little efficacy in the treatment of NF1-associated plexiform neurofibroma (10).

In light of the limited success of FTI monotherapy in NF1 clinical trials and the fact that RAS activation can mediate cell growth through a multitude of effector proteins, we previously used an unbiased proteomic method to identify neurofibromin/RAS downstream effector proteins that might serve as improved targets for therapeutic drug design. Using this approach, we found that neurofibromin/RAS growth regulation requires mammalian target of rapamycin (mTOR) function (11). Similar findings were also reported by others (12), prompting preclinical studies with rapamycin showing their efficacy in vivo (13–15).

In an effort to more precisely define the signaling effectors downstream of mTOR that mediate neurofibromin tumor
suppression, we have previously shown that mTOR-dependent growth control requires Rac1 activation in NF1-deficient cells (16). Unfortunately, because there are few Rac1-specific pharmaceutical-grade inhibitors suitable for preclinical/clinical study, we next sought to identify additional targets in NF1-deficient cells by means of high-throughput chemical library screening. Using this approach, we identified one previously unrecognized compound (cucurbitacin-I) and found that cucurbitacin-I inhibits human NF1-deficient MPNST and mouse NF1−/− astrocyte growth in vitro. Because cucurbitacin-I is a potent inhibitor of signal transducer and activator of transcription-3 (STAT3) function, we next showed that STAT3 is hyperactivated in NF1−/− primary astrocytes and that STAT3 hyperactivation in NF1-deficient cells results from increased phosphatidylinositol 3-kinase (PI3K)/mTOR/Rac1 pathway signaling. Lastly, we showed that STAT3 inhibition by cucurbitacin-I blocks human MPNST growth in vivo. Collectively, these findings provide novel insights into neurofibromin growth control and identify an exciting novel target for future NF1-associated tumor therapeutic drug design.

Materials and Methods

Mice. Six- to 8-wk-old male nu/nu mice were purchased from Taconic Laboratories. NF1+/- mice were intercrossed to generate NF1+/-/floX/floX pups, whereas NF1+/-/floX/+/floX GFAP-Cre mice were intercrossed to generate mice lacking NF1 gene expression in GFAP+ (glial) cells (NF1−/−/GFAPCKO mice), as previously described (17, 18). All mice were used in accordance with established animal study protocols at the Washington University School of Medicine.

Cell lines. The NF1-deficient ST88-14 MPNST cell line was kindly provided by Dr. Jeff DeClue [National Cancer Institute (NCI), Bethesda, MD]. These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% glutamine (5,000 cells per well)

Cell proliferation. ST88-14 cells were plated (20,000 per well) in 24-well dishes and allowed to adhere for 24 h followed by treatment with cucurbitacin-I for another 18 h. Cells were exposed to [3H]thymidine (1 Ci/mL) for 4 h. Astrocytes (105) were allowed to adhere for 24 h in 24-well dishes and maintained in serum-free DMEM for 24 h before exposure to [3H] thymidine (1 µCi/mL). [3H]thymidine incorporation was determined by scintillation counting (11).

Detection of apoptotic cells. NF1-deficient ST88-14 cells were seeded in 100 µL/well DMEM supplemented with 10% heat-inactivated FBS and 1% glutamine (5,000 cells per well). Plates were maintained in an environmentally-controlled Cytomat incubator until needed for operations, thereby optimizing health and uniform treatment of all plates. Compounds from the NCI 2000 Diversity Set I (in DMSO) were dissolved to 5 µmol/L final concentration in DMSO supplemented with 10% heat-inactivated FBS and 1% glutamine and added to cells 24 h after cell plating by exchanging the medium. Compound addition was performed with a 96-multichannel head on the FX liquid handler. Compounds were laid out in columns 2 to 11 of each plate. Control wells for DMSO (0.25% final concentration) and two concentrations of rapamycin (10 and 100 nmol/L) were placed in columns 1 and 12. Plates were maintained in the Cytomat incubator for 48 h. Cell viability was then determined with resazurin dye (final concentration, 44 µmol/L; Sigma) after a 3-h incubation at 37°C as monitored on a FLUOstar OPTIMA fluorescence reader (excitation, 544 nm; emission, 590 nm; BMG Labtech).

Statistical analysis and “hit” selection. Cell viability data acquired 48 h after drug treatment were normalized by dividing treatment fluorescence values by untreated control (DMSO) values. Data were then averaged over three compound replicates. We used two different statistical methods to analyze the data: first, on a plate-by-plate analysis, because the median absolute deviation (MAD) analysis is more robust for outliers (20). A hit was defined as values (α) less than −4 MAD from the median (20). A secondary criterion involved the application of a percentile analysis. A hit was defined as less than the 7th percentile (Supplementary Fig. S1). Compounds meeting both criteria were selected for further analysis (Supplementary Table S1). Compounds of interest were selected by review of published structures and literature; those chosen for further characterization are shown in Supplementary Table S2.

High-throughput chemical library screening. The high-throughput compound screen was performed with a cell growth and viability assay in black, clear-bottomed, 96-well culture plates (Corning Costar) using a Beckman Coulter Core robotics system, including a FX liquid handler, controlled by the Sagian graphical method development tool (SAMI scheduling software). NF1-deficient ST88-14 cells were seeded in 100 µL/well DMEM supplemented with 10% heat-inactivated FBS and 1% glutamine (5,000 cells per well). Plates were maintained in an environmentally-controlled Cytomat incubator until needed for operations, thereby optimizing health and uniform treatment of all plates. Compounds from the NCI 2000 Diversity Set I (in DMSO) were dissolved to 5 µmol/L final concentration in DMSO supplemented with 10% heat-inactivated FBS and 1% glutamine and added to cells 24 h after cell plating by exchanging the medium. Compound addition was performed with a 96-multichannel head on the FX liquid handler. Compounds were laid out in columns 2 to 11 of each plate. Control wells for DMSO (0.25% final concentration) and two concentrations of rapamycin (10 and 100 nmol/L) were placed in columns 1 and 12. Plates were maintained in the Cytomat incubator for 48 h. Cell viability was then determined with resazurin dye (final concentration, 44 µmol/L; Sigma) after a 3-h incubation at 37°C as monitored on a FLUOstar OPTIMA fluorescence reader (excitation, 544 nm; emission, 590 nm; BMG Labtech).

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of the total number of DAPI-stained cells in the field. At least 250 cells were counted.

**Immunocytochemistry.** WT and Nf1−/− astrocytes (P2) were seeded in 24-well dishes (50,000 cells well) in astrocyte growth medium for 24 h, fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 100% methanol, and blocked for 1 h in 5% goat serum/0.3% Triton X-100 in PBS at room temperature. To identify phospho-STAT3–expressing cells, astrocytes were incubated with a rabbit anti–phospho-STAT3 (Ser272) antibody (1:100 dilution; Cell Signaling Technology) in 1% bovine serum albumin/0.3% PBS–Triton X-100 in PBS overnight at 4°C. Alexa Fluor 488–conjugated anti-rabbit IgG secondary antibody (1:200 dilution; Molecular Probes) was used for detection. Nuclei were counterstained with DAPI. Immunolabeling was visualized using the avidin–biotin conjugation method (1:500 dilution; Vectastain ABC Elite, Vector Labs) and 3,3′-diaminobenzidine tablets (Sigma). Photomicrographs were obtained using a fluorescence Nikon Eclipse TE300 inverted microscope equipped with a digital camera (Optronics).

**Retroviral and lentiviral constructs and viral delivery.** Mouse-specific small hairpin RNA (shRNA) lentiviruses were obtained from Sigma. Raptor (NM_028898; TRCN 0000077472) lentivirus was produced, and the most effective silencing construct was selected for further study as previously reported (21). Raptor silencing in ST88-14 cells was achieved following three infections with lentivirus. Empty pLKO.1 virus was used as a control.

Murine stem cell virus (MSCV) containing mTOR small interfering RNA (siRNA), constitutively active Rac1 (Rac1V12), or dominant-negative Rac1 (Rac1N17) was generated following 293T cell transfection with ω-helper DNA using Fugene HD (Roche; ref. 16). Forty-eight hours later, virus-containing supernatants were filtered through 0.45-μm syringe filters. Cells were infected thrice and harvested 72 h later. MSCV expressing green fluorescent protein was included as a control.

**Western blotting.** Cells were lysed in standard NP40 lysis buffer with protease and phosphatase inhibitors for Western blotting as previously described (16). All antibodies were purchased from Cell Signaling Technology and used at a 1:1000 dilution unless otherwise stated. Primary Rac1 antibody was purchased from Upstate Biotechnology. Following horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) incubation, detection was accomplished by enhanced chemiluminescence (Amersham Biosciences). Densitometry analysis was performed with Gel-Pro Analyzer 4.0 software (Media Cybernetics) using α-tubulin (Sigma) or non–phospho-STAT3, AKT, and S6 antibodies for normalization.

**Rac1 activation assay.** GTP-bound Rac1 was measured using a Rac activation kit (Upstate Biotechnology) according to the manufacturer’s instructions (16). Briefly, ST88-14 cells were lysed, incubated with PAK1-PBD–conjugated agarose beads, washed in lysis buffer, and boiled in 2× Laemml buffer, and bound protein (active Rac1) was separated by SDS-PAGE gels for Western blotting. An aliquot of the lysate was saved for Western blotting to ensure equal protein loading.

**Immunohistochemical analysis of STAT3 phosphorylation.** The human glioma tissue microarray (TMA) slides, containing triplicate cores from 34 patients with sporadic pilocytic astrocytoma (PA), 13 with NF1-associated PA, and 5 cases of normal brain tissue (22), were used in accordance with approved human study protocols. Immunohistochemistry was performed as previously published (18). Phospho-STAT3 (Ser272) antibody was used at a 1:100 dilution, and sections were counterstained with hematoxylin. The slides were examined independently by two investigators blinded to both the clinical and the pathologic data. Tumors were scored as “positive” if they contained >10% immunoreactive cells or “negative” if they contained <10% immunopositive cells. A tumor was only scored as positive or negative if there was concordance between the three cores representing the same tumor on the TMA.

**In vivo tumor implantation.** One million ST88-14 cells in a total volume of 200 μL were injected s.c. into the right flank of 6- to 8-wk-old male athymic nu/nu mice. Tumors were allowed to grow until they became palpable, and then animals were randomly assigned to two groups. Five mice in each group received daily i.p. injections of vehicle (10% ethanol) or drug (1 mg/kg JSI-124 in 10% ethanol) for a total of 5 d. Twice weekly, body weights were recorded and tumor sizes were measured by Vernier caliper. Tumor volume (mm³) was calculated using the following equation: \( V = \frac{4}{3} \pi r^3 \), where \( V \) represents the longest dimension and \( W \) the shortest dimension of the tumor (23). Following 5 d of treatment, mice were euthanized and their tumors were removed. Apoptotic cell death was detected using a terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) kit in post-fixed tumor sections. Statistic significance was evaluated using the Student’s t test.

**Results**

**Cucurbitacin-I is a novel inhibitor of NF1-deficient cell growth.** To identify previously unrecognized downstream targets of neurofibromin relevant to growth regulation, we used high-throughput chemical library screening (Fig. 1A). This high-throughput screening was based on inhibition of NF1-deficient ST88-14 cell proliferation using DMSO (0.25%) and two concentrations of rapamycin (10 and 100 nmol/L) as internal negative and positive controls, respectively. We identified several candidate inhibitors but only one novel chemical, cucurbitacin-I. We therefore chose cucurbitacin-I for more detailed study. To validate the effect of cucurbitacin-I on NF1-deficient cell growth, we used [3H] thymidine and bromodeoxyuridine (BrdUrd) incorporation: cucurbitacin-I (10 nmol/L) reduced ST88-14 proliferation, as measured by either [3H]thymidine incorporation (Fig. 1B) or BrdUrd incorporation (Supplementary Fig. S2A). Moreover, cucurbitacin-I also inhibited ST88-14 anchorage-independent cell growth (Supplementary Fig. S2B).
To extend these findings to other Nf1-deficient cell populations, we showed that cucurbitacin-I (10 nmol/L) inhibited Nf1−/− astrocyte proliferation (Fig. 2B), with minimal effects on WT astrocytes. Similarly, cucurbitacin-I treatment reduced the proliferation of Nf1-deficient mouse low-grade K1861 glioma cells (Supplementary Fig. S3A).

To determine whether this reduced cell growth reflected increased cell death, we measured apoptosis in ST88-14 cells treated with 10 nmol/L cucurbitacin-I using the TUNEL assay. Cucurbitacin-I treatment increased the percentage of TUNEL-positive ST88-14 cells by 8-fold compared with vehicle-treated cells (Fig. 1C). Identical results were also observed...
using K1861 cells (Supplementary Fig. S3B). These findings show that cucurbitacin-I decreases NF1-deficient cell growth by inducing apoptosis.

**Cucurbitacin-I inhibits STAT3 activation in NF1-deficient cells.** One of the mechanisms underlying cucurbitacin-I function is inhibition of STAT3 function (24, 25). Because neurofibromin has not previously been implicated in STAT3 regulation, we first measured STAT3 activation using phospho-specific antibodies. Cucurbitacin-I treatment of ST88-14 cells (Fig. 1D, top) or K1861 mouse glioma cells (Supplementary Fig. S3C) resulted in suppression of STAT3 activation, as reflected by reduced phosphorylation on Ser727. In contrast, no change in STAT3 Tyr705 phosphorylation was observed (Fig. 1D, bottom). Identical results were obtained using a commercial source of cucurbitacin-I (JSI-124; data not shown).

Second, to directly show that neurofibromin regulates STAT3 activation, we examined STAT3 phosphorylation in Nf1−/− and WT astrocytes: STAT3 Ser727 phosphorylation was increased 4-fold in Nf1−/− compared with WT astrocytes (Fig. 2A, bottom). Similar results were observed using brain lysates from Nf1GFAP CKO mice lacking Nf1 expression in GFAP+ astrocytes: Compared with Nf1flox/flox (“WT”) mice, Nf1GFAP CKO mouse brains exhibited 5-fold greater STAT3 (Ser727) phosphorylation *in vivo* (Fig. 2A, bottom). No STAT3 Tyr705 hyperphosphorylation was observed in Nf1−/−
astrocytes compared with WT astrocytes (data not shown). In addition, primary Nf1−/− neocortical neural stem cells likewise exhibited increased STAT3 Ser727 phosphorylation relative to their WT counterparts (Supplementary Fig. S4A). Because STAT3 activation (phosphorylation) is associated with its translocation to the nucleus, we found that Nf1−/− astrocytes had greater nuclear STAT3 immunostaining compared with WT astrocytes (Fig. 2A, right).

Third, to show that cucurbitacin-I inhibits Nf1−/− astrocyte cell proliferation as well as STAT3 activation, we examined the effect of cucurbitacin-I treatment on [3H]thymidine incorporation and STAT3 Ser727 phosphorylation in WT and Nf1−/− astrocytes. We observed a 1.4-fold reduction in cell proliferation (Fig. 2B) and a 3-fold reduction in Ser727 STAT3 phosphorylation (Fig. 2C) in Nf1-deficient astrocytes following cucurbitacin-I treatment. The effects of cucurbitacin-I treatment on Nf1-deficient astrocyte proliferation were similar to those observed following STAT3 shRNAi inhibitor (shRNAi) knockdown (Supplementary Fig. S4B).

Finally, to determine whether STAT3 is also activated in brain tumors from patients with NF1, we examined STAT3 Ser727 phosphorylation by immunohistochemistry in NF1-associated and sporadic PAs: Whereas 41% of NF1-associated PAs were phospho-STAT3 immunopositive, only 7% of the sporadic PAs were phospho-STAT3 immunoreactive (Fig. 2D). No phospho-STAT3 Ser727 expression was detected in the normal brain tissue specimens (n = 5). Collectively, these data show that neurofibromin is a negative regulator of STAT3 activity in vitro and in vivo.

STAT3 activation is regulated by mTOR in Nf1-deficient cells. We next sought to determine how neurofibromin regulates STAT3. Our previous studies showed that neurofibromin

Figure 3. STAT3 activation is regulated by mTOR in Nf1-deficient cells in vitro. A, the PI3K inhibitor LY294002 inhibits ST88-14 STAT3 Ser727 phosphorylation. Phospho-AKT antibodies were used to show PI3K activity. Total AKT and STAT3 served as loading controls. B, left, rapamycin inhibited STAT3 Ser727 phosphorylation and resulted in increased AKT Ser473 phosphorylation. Phospho-S6 was used to show mTOR activity. Total S6, AKT, and STAT3 serve as internal loading controls. Right, GTP-bound Rac1 was immunoprecipitated from ST88-14 cells treated with vehicle (ethanol) or rapamycin (10 and 100 nmol/L) using PAK1-PBD affinity chromatography. Equal protein loading was confirmed by total Rac1 immunoblotting. GTP-bound Rac1 activation was decreased in rapamycin-treated cells. STAT3 Ser727 and S6 phosphorylation were also reduced by rapamycin treatment. C, siRNA-mediated mTOR knockdown inhibited STAT3 Ser727 phosphorylation. Total STAT3 and α-tubulin served as loading controls. D, raptor silencing by shRNAi inhibited ST88-14 STAT3 Ser727 phosphorylation and resulted in increased AKT Ser473 phosphorylation. α-Tubulin, AKT, and STAT3 served as loading controls.
astrocyte growth control is mediated by AKT/mTOR pathway signaling (11, 16). Here, we showed that pharmacologic inhibition of either PI3K (LY294002; Fig. 3A) or mTOR (rapamycin; Fig. 3B, left) blocked STAT3 Ser727 phosphorylation in ST88-14 cells. Second, we showed that rapamycin blocks STAT3 Ser727 phosphorylation (activation) in both human ST88-14 and mouse K1861 cells (Fig. 3B, right; Supplementary Fig. S3D) as well as Rac1 activation in ST88-14 cells (Fig. 3B, right). Third, we genetically silenced mTOR expression using a mTOR-specific siRNA virus in ST88-14 cells and found reduced STAT3 Ser727 phosphorylation following mTOR knockdown (Fig. 3C). Identical results were also observed following rapamycin inhibition (Supplementary Fig. S5A) or mTOR knockdown (Supplementary Fig. S5B) in Nf1−/− astrocytes. Fourth, because rapamycin-sensitive mTOR signaling operates largely through mTOR complex 1 (TORC1), we silenced the major TORC1 protein raptor in ST88-14 cells using shRNAi: Raptor knockdown also reduced STAT3 Ser727 phosphorylation (Fig. 3D). Consistent with TORC1-negative regulation of AKT activity, we also observed increased AKT Ser473 phosphorylation (activation) in ST88-14 cells following both rapamycin (Fig. 3B, left) and raptor shRNAi knockdown (Fig. 3D).

Fifth, based on our studies showing that mTOR regulates Nf1−/− astrocyte growth in a Rac1-dependent manner (16), we examined STAT3 Ser727 phosphorylation in ST88-14 cells following the introduction of a constitutively active Rac1 mutant (Rac1V12) or a dominant-inhibitory Rac1 mutant (Rac1N17): Rac1V12 increased STAT3 Ser727 phosphorylation (Fig. 4A), whereas Rac1N17 attenuated STAT3 Ser727 phosphorylation (Fig. 4B). Similarly, Rac1N17 expression in Nf1−/− astrocytes reduced STAT3 Ser727 phosphorylation (Supplementary Fig. S5C, bottom), whereas Rac1V12 expression in WT astrocytes increased STAT3 Ser727 phosphorylation (Supplementary Fig. S5C, top). Lastly, as we previously reported (16), active Rac1V12 expression increased ST88-14 cell growth (Supplementary Fig. S6A), whereas dominant-inhibitory Rac1N17 expression reduced both ST88-14 (Supplementary

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**Figure 4.** Rac1 regulates STAT3 phosphorylation in Nf1-deficient cells. A, constitutively active Rac1 (Rac1V12) increases STAT3 Ser727 phosphorylation and cyclin D1 expression. B, dominant-inhibitory Rac1 (Rac1N17) suppresses STAT3 Ser727 phosphorylation and cyclin D1 expression. α-Tubulin and STAT3 serve as loading controls. C, cyclin D1 expression was reduced following cucurbitacin-I treatment. α-Tubulin was used as a protein loading control. D, schematic diagram of neurofibromin regulation of cell growth through Akt/mTOR-raptor/Rac1/STAT3 signaling.
Fig. S6B) and Nf1−/− astrocyte cell proliferation (Supplementary Fig. S6C). Together, these data show that mTOR/Rac1 activation regulates STAT3 function in NF1-deficient cells.

**STAT3 regulates cyclin D1 expression in NF1-deficient cells.** To determine whether STAT3 regulates cyclin D1 expression (26), cyclin D1 levels were measured following curcurbitacin-I treatment. Cyclin D1 expression was reduced in ST88-14 cells (Fig. 4C) and Nf1−/− astrocytes (Supplementary Fig. S5D) after curcurbitacin-I treatment. Because cyclin D1 is increased following Rac1V12 expression (Fig. 4A) and decreased following Rac1N17 expression (Fig. 4B), we conclude that STAT3 regulation of cyclin D1 resulted from Rac1 activation in NF1-deficient cells.

**STAT3 inhibition inhibits ST88-14 tumor growth in vivo.** To provide in vivo support for STAT3-mediated NF1 growth control, we used JSI-124 to inhibit STAT3 function in ST88-14 tumor explants. Fifteen days after implantation, male nu/nu mice with visibly growing tumors were randomly assigned to daily i.p. injections of either JSI-124 (1 mg/kg) or vehicle for 5 days. After 5 days, tumors in the JSI-124–treated group were 4-fold smaller than those from the vehicle-treated group (P ≤ 0.0008; Fig. 5A and B). Next, to determine whether the decrease in tumor growth reflected increased cell death, we measured apoptosis in frozen tumor sections and found a 2-fold increase in apoptosis following JSI-124 treatment (P < 0.0001; Fig. 5C). Similar results were also obtained in a second independent study (Supplementary Fig. S7A). No effect of JSI-124 treatment on animal body weight (Supplementary Fig. S7B) or apoptosis in the liver (data not shown) was observed. These results show that STAT3 inhibition results in decreased NF1-deficient cell and tumor growth in vitro and in vivo.

**Discussion**

The identification of the NF1 gene and the subsequent discovery that neurofibromin regulates the RAS/mTOR pathway have ushered in a new era of biologically-targeted therapies for NF1-associated tumors. In this regard, clinical studies using RAS inhibitors have already been initiated and those focused on mTOR blockade have recently commenced. Unfortunately, RAS inhibition using FTIs has not shown efficacy for the treatment of NF1-associated peripheral nerve sheath tumors (10). Based on these studies, subsequent translational research has focused on RAS downstream effectors, including Raf/mitogen-activated protein/extracellular signal-regulated kinase kinase and AKT/mTOR. Several laboratories have recently shown that hyperactivation of the mTOR pathway underlies the growth advantage seen in NF1-deficient glioma and MPNST cells and that mTOR
inhibition, using the macrolide rapamycin, results in decreased optic glioma and MPNST growth in vivo (13–15). However, it is worth noting that rapamycin does not induce apoptosis and does not lead to a durable response following drug cessation (13). In addition, human MPNST cells grown as explants in mice exhibit increased AKT activation following rapamycin treatment as a result of mTOR-mediated AKT phosphorylation (15). Although rapamycin analogues are now in clinical trial for human NF1-associated peripheral nerve sheath tumors, this biologically-based therapy may not result in sustained tumor shrinkage.

For these reasons, we sought to discover new targets using an unbiased high-throughput chemical library screening approach. In this screen, we found several promising compounds. A number of our top candidates were known chemotherapeutic agents (mitomycin, daunorubicin, and topotecan), whereas others were crude inhibitors of major biological processes (bouvardin, tubulosine, and breflate). Only one compound, cucurbitacin-I, was novel and was therefore selected for further study. Additionally, we chose cucurbitacin-I because it is known to inhibit STAT3, a signaling molecule not previously implicated in neurofibromin growth control, and against which inhibitors have been used to treat other cancers (27, 28). Consistent with these other reports, we found that cucurbitacin-I blocked the proliferation of Nf1−/− astrocytes in vitro and Nf1-deficient MPNST cells by suppressing STAT3 activation in vitro and in vivo.

STAT3 is a transcription factor whose activity is regulated by phosphorylation: Tyrosine Tyr705 results in STAT3 dimerization and translocation to the nucleus, whereas transcriptional activation requires phosphorylation on residue Ser727 (29). In Nf1-deficient cells, we observed increased phosphorylation of STAT3 on Ser727 and not Tyr705. This is consistent with previous reports showing that RAS activation increases STAT3 Ser727 phosphorylation (26). Moreover, phosphorylation of STAT3 at Ser727 is sufficient for STAT3 activation in prostate cancer cells, independent of Tyr705 phosphorylation (30), and STAT3 Ser727 phosphorylation is essential for postnatal survival and cell growth in mice, such that mice with a Stat3 allele in which the serine residue has been converted to alanine exhibit increased apoptosis (31).

Because neurofibromin has never been shown to regulate STAT3 activity, we sought to determine whether STAT3 was deregulated in Nf1-deficient cells through previously implicated neurofibromin signaling pathways. We found that STAT3 activity was regulated in Nf1-deficient primary cells and tumor cells in an AKT/mTOR-dependent manner. Although unique to neurofibromin/AKT/mTOR pathway regulation, STAT3 hyperactivation is mediated by PI3K/AKT signaling in other cell types (32). Based on our previous studies showing that AKT/mTOR growth regulation in Nf1-deficient astrocytes results from mTOR-mediated Rac1 activation, we show that STAT3 activation is controlled by Rac1 activation downstream of mTOR in Nf1−/− mouse astrocytes and Nf1-deficient human MPNST cells. The finding that Rac1 controls STAT3 function in Nf1-deficient cells is supported by previous studies showing that Rac1 can bind to STAT3 and regulate STAT3 activation (33–36). Together, our findings establish a more complete model for neurofibromin growth regulation involving mTOR/Rac1/STAT3 signaling (Fig. 4D).

STAT3 plays a central role in regulating oncogenesis by controlling the transcription of several target genes essential for cell cycle progression, apoptosis, and proliferation (37). In this regard, Ser277 STAT3 phosphorylation is associated with increased cyclin D1 expression (26); we found that cyclin D1 expression was increased in Nf1-deficient astrocytes relative to WT astrocytes and was reduced following either Rac1 or STAT3 inhibition. Whereas cyclin D1 may be partly responsible for promoting cell growth in Nf1-deficient cells, other STAT3 target genes, including regulators of apoptosis, are likely involved in facilitating malignant transformation and continued growth following neurofibromin loss. Additional studies will be required to identify and validate these other potential STAT3 transcriptional target genes.

Our finding that neurofibromin regulates STAT3 activation in primary Nf1−/− astrocytes, neural stem cells, and low-grade glioma cells coupled with the observation that STAT3 inhibition blocks Nf1-deficient astrocyte and low-grade glioma cell proliferation has particular relevance to brain tumors in children with NF1. Previously, we have shown that optic glioma growth in Nf1 genetically-engineered mice can be suppressed by treatment with rapamycin; however, this inhibitor is only cytostatic and murine optic glioma growth resumes following the cessation of rapamycin treatment (13). The observation that STAT3 blockade results in apoptosis is exciting, as this antitumor effect might result in a durable response similar to what we have observed in Nf1 optic glioma mice treated with conventional genotoxic therapy (temozolomide). Moreover, the potential use of STAT3 inhibitors for antiglioma therapy is underscored by results from numerous preclinical studies using high-grade glioma cell lines (25, 38–42). Finally, it is conceivable that STAT3 blockade could be combined with currently used brain tumor therapies to improve outcome. In this regard, JSI-124 treatment has been shown to sensitize glioma cells to temozolomide, nitrosourea, and cisplatin in a synergistic manner (43). The ability to use STAT3 inhibitors to reduce the overall dose of alkylating chemotherapy is highly attractive, especially in children whose developing brains are most sensitive to the untoward effects of chemotherapy. Future studies will be required to explore these possibilities and to develop potential strategies to integrate STAT3 adjuvant therapies into our current treatment approach to NF1-associated brain tumors.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts to disclose.

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