 Human Molecular Genetics, 2002, Vol. 11, No. 25 3179–3189

Neurofibromatosis 2 (NF2) tumor suppressor schwannomin and its interacting protein HRS regulate STAT signaling

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Received August 10, 2002; Revised and Accepted October 4, 2002

Mutations in the neurofibromatosis 2 (*NF2*) gene with the resultant loss of expression of the NF2 tumor suppressor schwannomin are one of the most common causes of benign human brain tumors, including schwannomas and meningiomas. Previously we demonstrated that the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) strongly interacts with schwannomin. HRS is a powerful regulator of receptor tyrosine kinase trafficking to the degradation pathway and HRS also binds STAM. Both of these actions for HRS potentially inhibit STAT activation. Therefore, we hypothesized that schwannomin inhibits STAT activation through interaction with HRS. We now show that both schwannomin and HRS inhibit Stat3 activation and that schwannomin suppresses Stat3 activation mediated by IGF-I treatment in the human schwannoma cell line STS26T. We also find that schwannomin inhibits Stat3 and Stat5 phosphorylation in the rat schwannoma cell line RT4. Schwannomin with the pathogenic missense mutation Q538P fails to bind HRS and does not inhibit Stat5 phosphorylation. These data are consistent with the hypothesis that schwannomin requires HRS interaction to be fully functionally active and to inhibit STAT activation.

INTRODUCTION

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder caused by mutations in the *NF2* gene. The inherited disorder is characterized by bilateral vestibular schwannomas and a predisposition to multiple benign tumors of the brain and peripheral nervous system. The tumors seen in NF2 patients occur more frequently as sporadic tumors as a consequence of somatic *NF2* mutation. Virtually all sporadic schwannomas bear *NF2* mutations or deletions and *NF2* gene alterations are also common in meningiomas and ependymomas (1–4).

The *NF2* gene encodes the tumor suppressor protein schwannomin or merlin, which has homology to members of the protein 4.1/ERM family of proteins that link the plasma membrane to the cytoskeleton (5,6). Schwannomin closely resembles the ERM proteins ezrin, radixin and moesin, which interact with themselves and with other ERM proteins (7). Since the identification of the *NF2* gene, significant advances have been made in determining schwannomin function. Schwannomin is a multifunctional protein that binds or complexes with a variety of other proteins, including syntenin (8), SCHIP1 (9), CD44 (10), EBP50/NHERF (11,12), Rho GDP dissociation inhibitor (13), other ERM proteins (14–16), βII-spectrin (17), Paxillin (18) and both polymerized actin and microtubules (19). The multitude of interactions suggest that schwannomin probably participates in a variety of signaling pathways mediated by the plasma membrane and cytoskeleton.

Despite the many known interactions for schwannomin, pathways by which schwannomin suppresses tumor growth remain undefined. Schwannomin may have a role in maintaining normal signaling mediated by the actin cytoskeleton as abnormal stress fiber structure results upon schwannomin loss (20–22). In addition, overexpression of schwannomin results in impairment of cell spreading, attachment and motility, which are mediated by the actin cytoskeleton (23). Schwannomin appears to act in the signaling pathways mediated by the small GTP binding proteins Rho and Rac in human fibroblasts, which

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regulate lamellipodial outgrowth (20). More recently schwannomin was shown to be phosphorylated by p21-activated kinase 2 (PAK2) in response to signals mediated by Rac/Cdc42 (24–26).

We identified hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) as a protein binding to the NF2 tumor suppressor schwannomin (27). Tests of the interaction strengths using a yeast two-hybrid-based assay showed that the interaction between the two proteins was exceptionally strong compared with other schwannomin interacting proteins (27,28). HRS is a FYVE-domain protein that binds endosome-associated phosphatidylinositol (3) phosphate [PtdIns(3)*P*] and possesses a ubiquitin interacting motif (UIM) (29–31). HRS is a powerful regulator of trafficking of ubiquitinated receptor tyrosine kinases (RTKs) to the lysosome (31–33). We previously co-localized schwannomin and HRS to EEA1 positive early endosomes, suggesting that schwannomin might have a role in HRS-mediated receptor trafficking (27). HRS also interacts with and inhibits the signal transducing activator molecule STAM, an activator of Janus kinases and inhibits DNA synthesis in BAF-B03 cells (34,35). We also demonstrated that schwannomin and HRS similarly function to inhibit proliferation of RT4 cells (15). Because HRS probably facilitates the degradation of receptors in the lysosome and inhibits STAM, we hypothesized that schwannomin and HRS can inhibit one common output of both of these events: activation of signal transducers and activators of transcription (STATs). We now show that both schwannomin and HRS are inhibitors of STAT activation in human and mouse schwannoma cell lines. Schwannomin with a naturally occurring *NF2* missense mutation that alters HRS binding abolishes the ability for schwannomin to inhibit STAT activation.

RESULTS

Schwannomin and HRS reduce STAT activity

We measured the effect of HRS and schwannomin overexpression on STAT activity using a STAT-responsive luciferase reporter construct pLucTKSIE containing the *sis* inducible element (SIE) upstream of the TK (thymidine kinase) minimal promoter element. Each assay was controlled with a paired assay using pLucTK, containing only the TK minimal promoter element. The utility of these reporter constructs for the study of STAT activation has been previously validated (36). Each of HRSi1, SCHi1 and SCHi2 inhibited SIE activation in a dosage-dependent manner in STS26T cells (Fig. 1A–C). Under the conditions of our model system, 9 μg of schwannomin isoform 2 reduced SIE activity by 84% compared with the vector control, while Q538 schwannomin isoform II reduced SIE activity by only 42%. However, 3 μg of schwannomin isoform II reduced SIE activity by 30%, in contrast to 3 μg of Q538 schwannomin isoform II, which reduced SIE activity by only 4%. Therefore, in our model system, the Q538P mutation reduces the ability of schwannomin to inhibit SIE activation by 50–88% (Fig. 1B).

We also determined the ability of SCHi2 to inhibit SIE activation in RT4 cells. Schwannomin isoform II inhibited SIE to about the level of the TK control in these cells (Fig. 1D). Because SIE is activated by more than one type of STAT protein, we used an additional construct that specifically detects Stat3 activation via the Stat3 inducible element S3 (36). We showed that both SCHi2 and HRSi1 suppressed the S3 element in a dosage-dependent manner (Fig. 1E). In all experiments, transfection of plasmids encoding SCHi1, SCHi2, HRSi1 and Q538P SCHi2 resulted in strong protein expression in STS26T cells (Fig. 1F).

Overexpression of schwannomin alters Stat3 nuclear translocation

To determine the effect of schwannomin on Stat3 subcellular localization, we overexpressed schwannomin in STS26T cells. We determined the degree of Stat3 nuclear translocation in response to overexpression of schwannomin by counting STS26T cell nuclei labeling with Stat3 antibody after transient transfection of Xpress-epitope tagged schwannomin isoform II (SCHi2). We photographed 107 cells including 23 transfected with Xpress-SCHi2. Nuclear Stat3 labeling was scored with the investigator blinded to the transfection status (0 = not different than cytoplasmic labeling, to 4 = intense nuclear labeling). The average scores were highly significantly different between transfected and untransfected cells (0.70 ± 0.76 for transfected cells, 2.38 ± 0.71 for untransfected cells, Student's t probability <0.001), showing that schwannomin significantly inhibited Stat3 nuclear translocation. Examples are shown in Figure 2A–F. Transfection with GFP did not result in changes of nuclear STAT (data not shown).

To support the data obtained by transient overexpression, we employed an inducible (Tet-on) RT4-*NF2* cell line that overexpressed schwannomin isoform I upon treatment with doxycycline (15). As demonstrated above, induction of schwannomin resulted in reduced nuclear Stat3 labeling compared with uninduced cells by confocal microscopy (Fig. 2G–J).

IGF-I stimulates proliferation of RT4 cells

Previous studies have demonstrated that IGF-I is a Schwann cell mitogen (37,38). In 293T cells and other cell types, IGF-I also stimulates the activation of Stat3 (39). To determine the effect of IGF-I on RT4 cell proliferation, we measured thymidine incorporation after IGF-I treatment and demonstrated increased proliferation in response to 100 ng/ml IGF-I. The increased RT4 cell proliferation was equivalent to that observed with HGF, another potent Schwann cell mitogen (40). Upon induction of schwannomin with doxycycline, we observed a reduction in IGF-1 induced cell proliferation (Fig. 3). These data support the hypothesis that schwannomin inhibits cell proliferation in a STAT-dependent fashion.

Schwannomin inhibits IGF-I-mediated Stat3 activation

Based on the results demonstrating that schwannomin inhibits IGF-I-mediated cell proliferation and Stat3 subcellular localization, we next assessed the ability of schwannomin isoform I to inhibit Stat3 phosphorylation in RT4 and STS26T cells. A decreased abundance of phosphorylated Stat3 relative to total

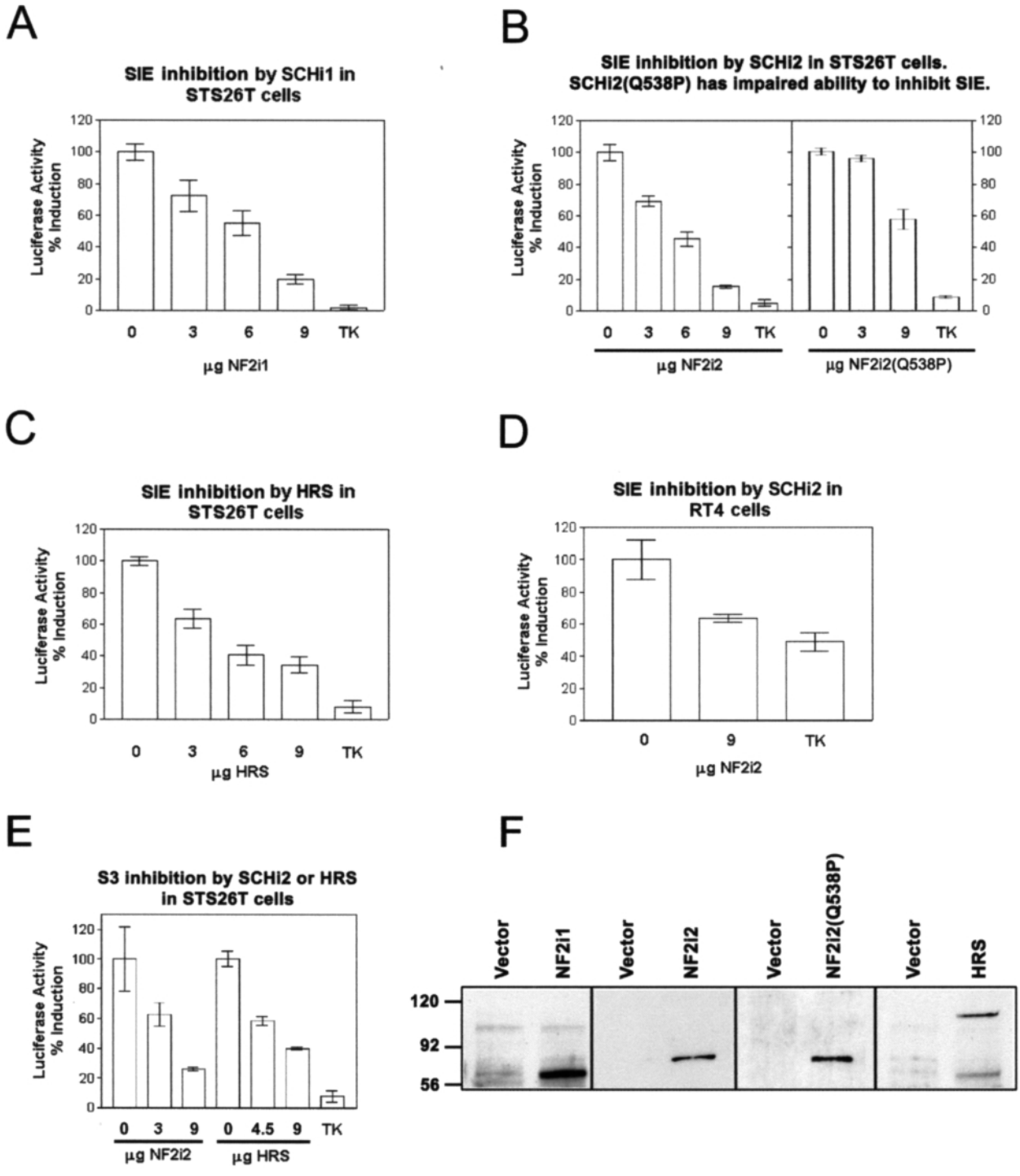


Figure 1. Effects of HRS and schwannomin on inhibition of STAT activation in STS26T and RT4 cells. STS26T cells were transfected with pLukTK or pLukTKSIE and increasing amounts of plasmids that express (**A**) schwannomin isoform I, (**B**) schwannomin isoform II or Q538P schwannomin isoform II, or (**C**) HRS. (**D**) RT4 cells were transfected with pLukTK or pLukTKSIE and increasing amounts of plasmid expressing schwannomin isoform II. (**E**) STS26T cells were transfected with pLukTK or pLukTKS3 and increasing amounts of plasmids that express schwannomin isoform II or HRS. Cells were transiently transfected and luciferase reporter activities were measured as light emission with a luminometer. The results shown are mean ± standard deviation of triplicate measures. (**F**) Immunoblot analysis of expressed proteins. The expression plasmids for schwannomin isoform I and II, HRS and Q538P schwannomin isoform II express proteins with an N-terminal Xpress epitope tag. Proteins of the predicted sizes were detected using the anti-Xpress antibody. Note that Xpress-tagged schwannomin isoform II migrates slower than isoform I with the identical tag. This holds for the GFP tag as well. Proteins expressed by *NF2* cDNAs from these very same constructs without N-terminal tags show an identical electrophoretic migration pattern.

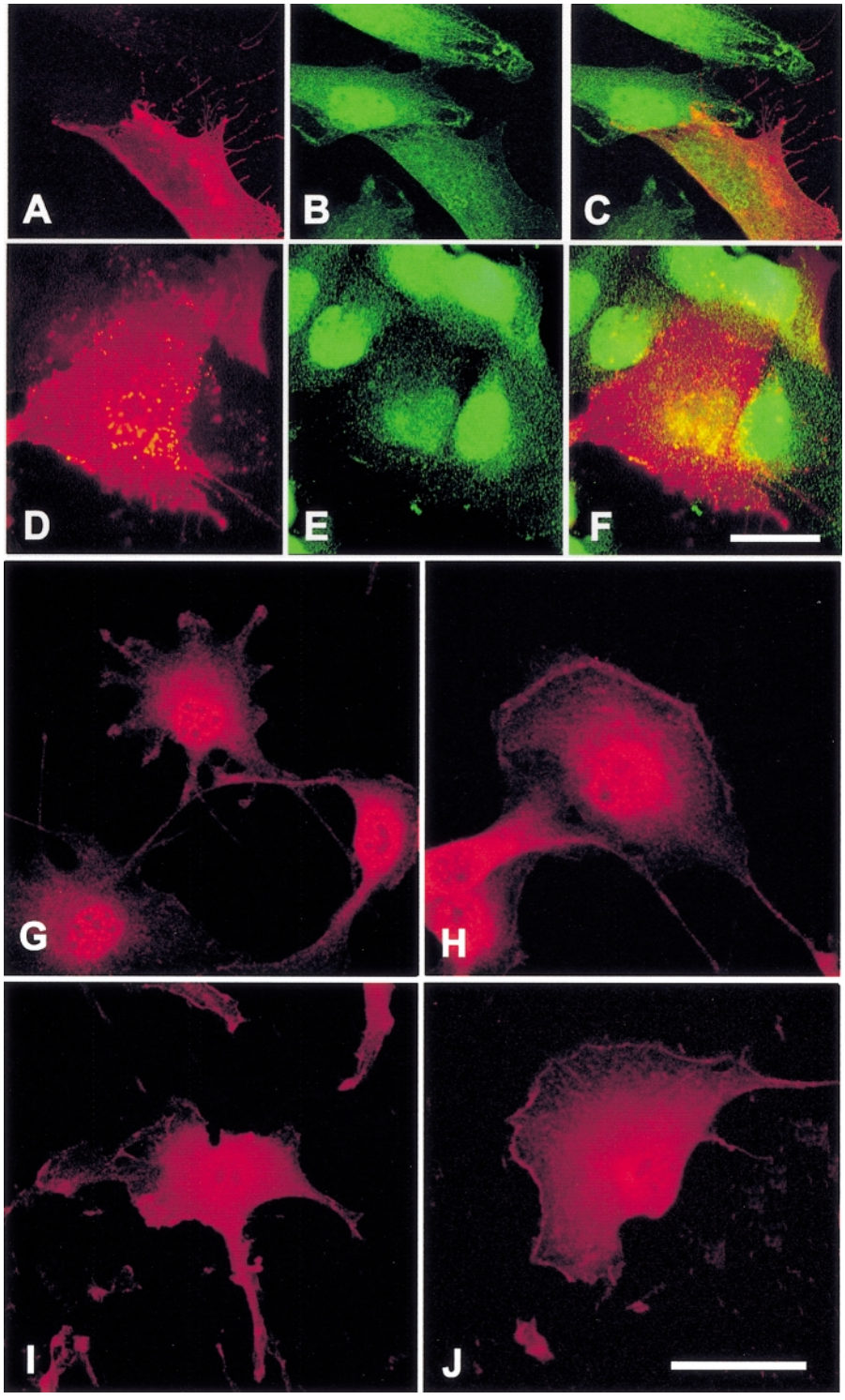


Figure 2. Overexpression of schwannomin alters Stat3 subcellular localization. (**A**–**F**): STS26T cells were transfected with Xpress-epitope tagged schwannomin isoform II, and co-labeled with anti-Xpress and anti-Stat3 antibodies. A transfected cell labeled with anti-Xpress antibody (A) had very little nuclear Stat3 compared with non-transfected cells, demonstrated by labeling the same cells with anti-Stat3 antibody and immunofluorescent confocal microscopy (B). Overlay of A and B (C). Another example of a transfected STS26T cell labeled with anti-Xpress antibody (D) with little nuclear Stat3 compared to non-transfected cells (E). Overlay of D and E (**F**). (**G**–**J**) Stat3 observed in Tet-on RT4 NF2.17 without doxycycline, detected by immunofluorescent confocal microscopy, was more strongly nuclear (G and H) than in these cells grown at the same time and under identical conditions but with doxycycline (I and J). These cells were photographed with equal exposures and digital contrast. Bar = 40 μm.

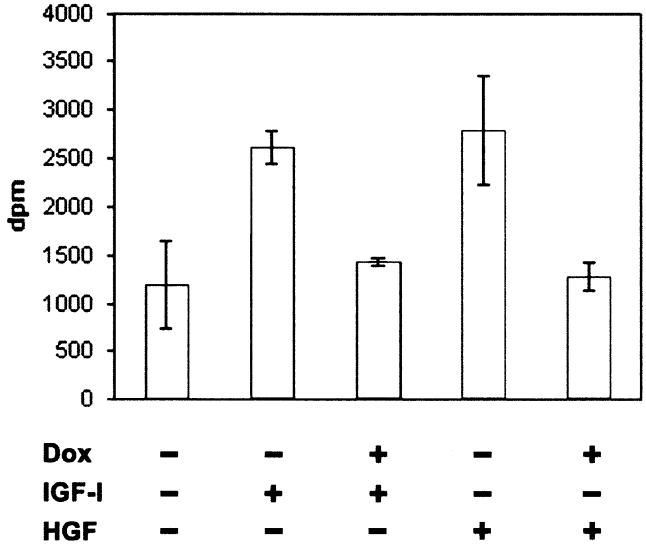


Figure 3. Schwannomin inhibits RT4 schwannoma proliferation in response to IGF-I and HGF. Ten thousand Tet-on RT4 NF2.17 cells were seeded in quadruplicate 24-well plates and serum-starved for 24 h prior to the addition of 100 ng/ml IGF-I or 200 ng/ml HGF overnight either in presence or absence of doxycycline to induce schwannomin expression. Thymidine incorporation was measured after 4 h. Mean and SD are shown for each condition.

Stat3 was observed after 24 h of induced schwannomin isoform I expression in Tet-on RT4 NF2.17 cells (Fig. 4A). We also showed that IGF-I phosphorylates Stat3 in Tet-off STS26T NF2i1 cells and that schwannomin isoform I expression in these cells inhibited Stat3 phosphorylation to the level observed in untreated cells (Fig. 4B). We then tested whether IGF-I-mediated SIE activity could be inhibited by schwannomin in STS26T cells. In these experiments, SIE activation increased in response to increasing IGF-I concentrations (0, 20 and 40 ng/ml) in the absence of schwannomin, but when schwannomin was expressed no increased SIE activation was observed (Fig. 5). We conclude that STAT activation by the STAT activator, IGF-I, is inhibited by schwannomin, and that schwannomin lies in a signaling pathway between the IGF-I receptor and Stat3.

Schwannomin inhibits phosphorylation of Stat5

To determine whether schwannomin inhibits only STATs that activate SIE, we also assessed the ability of schwannomin isoform I to inhibit Stat5 phosphorylation in RT4 cells. For these experiments, we employed RT4 cells that inducibly express schwannomin isoform I in response to doxycycline treatment. Increased expression of schwannomin isoform I resulted in decreased abundance of phosphorylated Stat5 (Fig. 6A, left). However, when we used a matched Tet-on RT4 inducible cell line expressing schwannomin isoform I containing the Q538P mutation, we did not observe any decrease in Stat5 phosphorylation (Fig. 6A, right). We tested the ability of this schwannomin isoform I mutant to bind HRS and found that the Q538P mutation abolished the HRS interaction (Fig. 6B).

DISCUSSION

The *NF2* gene is one of the most commonly mutated genes in human benign brain tumors, including schwannomas and meningiomas. Despite the identification of several proteins that interact with the NF2 tumor suppressor schwannomin, a single schwannomin-regulated pathway altered by *NF2* mutation to cause tumorigenesis has not been defined.

In previous studies we identified HRS as a schwannomin interacting protein (27). Two intriguing features about HRS suggested that HRS could regulate pathways that lead to STAT activation and, being a schwannomin interactor, that schwannomin might regulate these HRS actions. Firstly, HRS facilitates trafficking of ubiquitinated RTKs to the degradation pathway (31–33) and, secondly, HRS interacts with the signal transduction adapter molecule STAM, which is a ubiquitous protein involved in cytokine-mediated signal transduction (34,35). While the action of STAM on STATs has not been studied, it has been shown that STAM interaction with Janus kinases causes elevated c-*myc* induction and DNA synthesis and that HRS can inhibit STAM activation (34,35). Like HRS, STAM also possesses a ubiquitin-interacting motif (UIM) and may participate in HRS-mediated trafficking of RTKs (41). Since STAT proteins are often found overexpressed in a variety of tumors types, including ovary and breast tumor cells (42–44) and Stat1 and Stat3 are overexpressed in meningiomas (45), we investigated schwannomin and HRS ability to inhibit STAT signaling in human and rat schwannoma cell lines.

The STAT proteins comprise a family of proteins that are activated upon ligand binding by cytokine receptors or RTKs, sometimes requiring Janus kinases (Jaks) (46). Once phosphorylated, STATs then homo- or heterodimerize and translocate to the nucleus where they function as transcription factors, interacting with specific inducible elements. We chose to investigate the ability of schwannomin to inhibit the activation of the *sis* inducible element SIE, which is activated by Stat1 and Stat3, and the S3 inducible element, which is activated by Stat3. We now show that both schwannomin and HRS inhibit SIE and S3 activation.

Overexpression of increasing dosages of schwannomin isoforms or HRS in STS26T cells strongly inhibited SIE and S3 activation. Inducible expression of schwannomin in RT4 cells and transiently expressed schwannomin in STS26T cells decreased the abundance of nuclear Stat3. The abundance of phosphorylated Stat3 was decreased when we inducibly overexpressed schwannomin in RT4 or STS26T cells. The Q538P schwannomin mutation impaired the ability of schwannomin isoform II to inhibit SIE activation. To determine whether schwannomin might inhibit other STATs that do not activate SIE, we also investigated the effect of schwannomin on Stat5. Induced schwannomin expression inhibited Stat5 phosphorylation. In addition, Q538P-mutated schwannomin isoform I, which does not interact with HRS, did not inhibit the phosphorylation of Stat5, when inducibly expressed. These findings are consistent with a requirement for HRS interaction by schwannomin for schwannomin to inhibit STAT activation.

IGF-I is established as an activator of Stat3 in a variety of cell types, and stimulates proliferation of Schwann cells (37–39). We verified that both IGF-I and HGF induce proliferation of RT4 cells and demonstrated that schwannomin overexpression

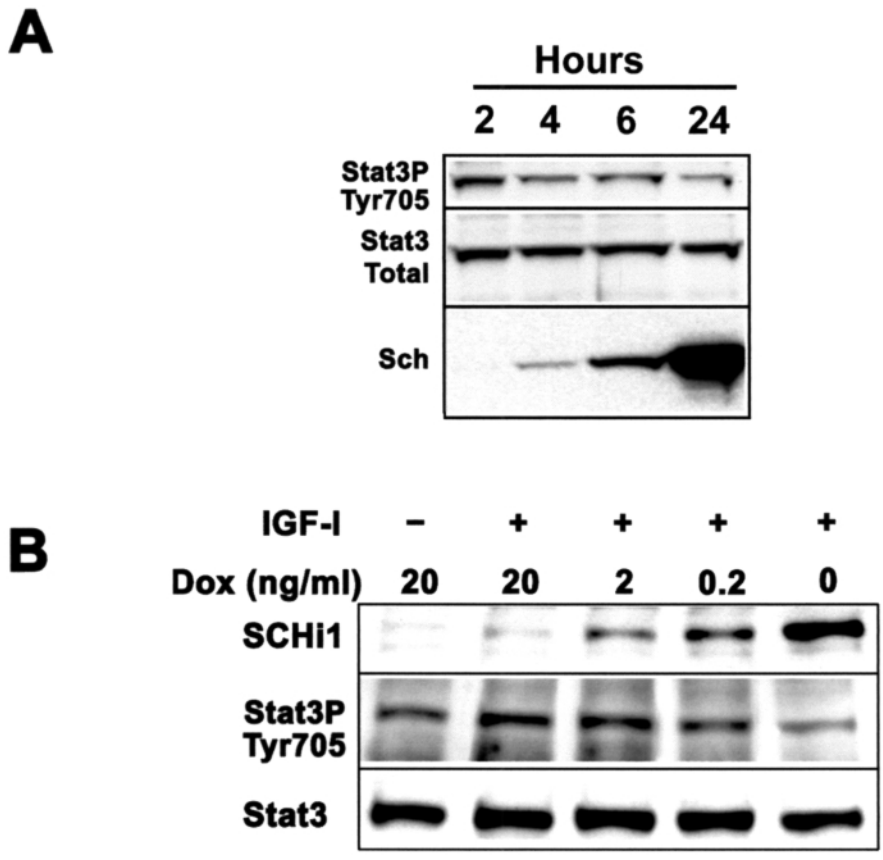


Figure 4. Schwannomin inhibits Stat3 phosphorylation in RT4 and STS26T cells. (**A**) Induction of schwannomin results in decreased Stat3 activity. Subconfluent schwannomin-inducible Tet-on RT4 NF2.17 cell cultures were treated with doxycycline for 2, 4, 6 and 24 h prior to harvest. Protein extracts were subjected to immunoblot analysis using antibodies that recognize Stat3 phosphorylated on tyrosine 705 (Stat3p) or total Stat3. At 24 h, decreased Stat3 activity in response to schwannomin induction was indicated by lower abundance of phosphorylated Stat3, while total Stat3 remained unchanged. (**B**) Schwannomin-inducible Tet-off STS26T NF2i1 cell cultures were treated with the indicated amounts of doxycycline for 2 days. Cells were serum-starved overnight (same dox conditions) and then treated with 50 ng/ml IGF-I (or diluent) for 10 min before extraction. The addition of IGF-I increased the abundance of phosphorylated Stat3. In cells expressing the greatest amount of schwannomin, the level of phosphorylated Stat3 was reduced to amounts seen in cells that were not stimulated with IGF-I. Schwannomin was detected in (A) using antibody WA30 and in (B) using antibody ab2781.

can inhibit SIE activation stimulated by IGF-I treatment. Both schwannomin isoforms I and II were able to inhibit STAT activation. Previous studies using model systems to compare the schwannomin isoforms functionally have demonstrated that, unlike schwannomin isoform I, schwannomin isoform II had no ability to alter Schwann cell proliferation, tumor growth or cytoskeletal reorganization (21,23,47). The schwannomin isoforms are highly conserved evolutionarily, including the splice signals, suggesting that both isoforms have important cellular functions (48,49). STATs are powerful mediators of signal transduction and while when previously used *in vitro* systems they did not establish a link between isoform II and tumorigenesis, both schwannomin isoforms may have some significant role in the regulation of proliferative pathways at the organismal level. Alternatively, it is possible that the ability of schwannomin and HRS to inhibit STAT activation reflects another function of these proteins not related to the inhibition of cell proliferation.

A variety of receptors that activate Stat3 also activate Stat5. The ability of schwannomin to inhibit both Stat3 and Stat5 activation is consistent with the ability of schwannomin to function as a growth regulator by inhibiting a wide variety of signals that activate multiple STATs. The actions by schwannomin on STATs may involve HRS and its ability to traffic STAT-activating cell surface receptors to the lysosome (32,33). HRS overexpression in RT4 cells strongly alters the localization of EGF receptor in RT4 cells (Scoles, Gutmann and Pulst, manuscript in preparation). Recently, we have shown that schwannomin acts upstream of HRS and that HRS is required for schwannomin-mediated inhibition of proliferation in mouse embryo fibroblasts (50). Consistent with this finding, we could show no synergistic effect on STAT inhibition caused by the expression of both HRS and schwannomin (data not shown). This finding is also consistent with the inability of Q538 schwannomin to inhibit STAT activation caused by loss of HRS interaction. However, we have not yet proven that STAT inhibition by schwannomin involves HRS-mediated receptor trafficking. In an effort to demonstrate a schwannomin link to STAM, we investigated the ability of schwannomin to inhibit c-fos expression, but we were unable to reproducibly show that

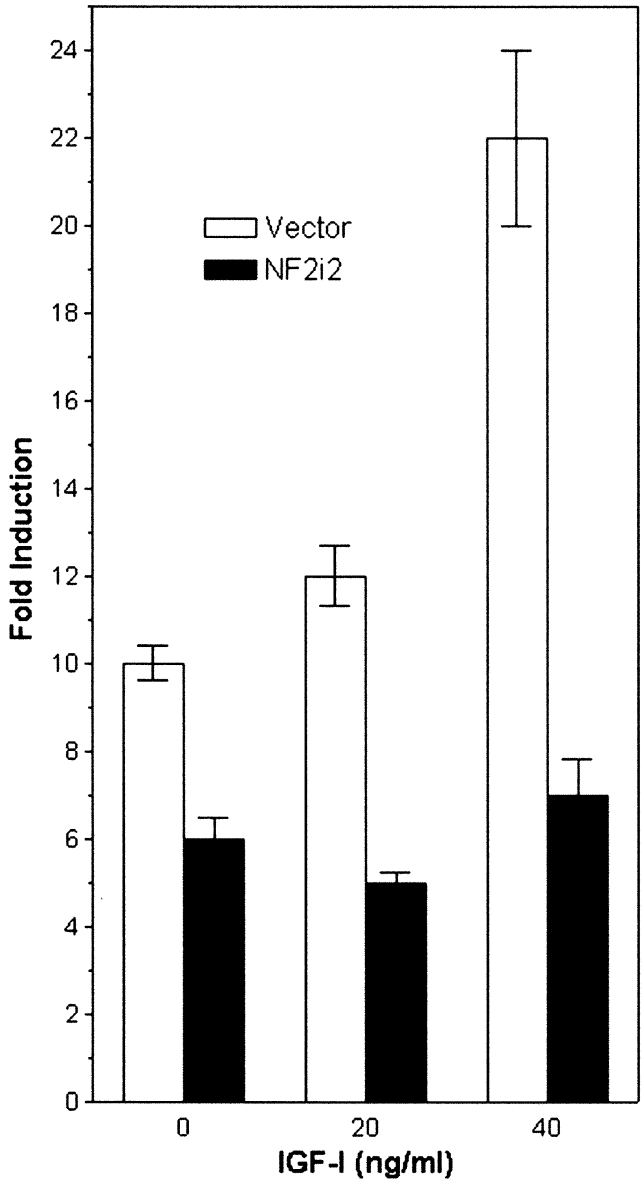


Figure 5. Schwannomin inhibits IGF-I mediated activation of the SIE promoter element for STAT signaling. STS26T cells were transfected with pLukTKSIE and 9 μg expression vector with no insert, or pLukTKSIE and 9 μg plasmid expressing schwannomin isoform II. Cells were treated with the indicated amounts of IGF-I before assaying for luciferase activity. Fold induction is relative to control experiments detecting pLukTK activation. For this experiment, STS26T cells were transiently transfected and luciferase reporter activities were measured as light emission with a luminometer. The results shown are mean ± SD of triplicate measures.

schwannomin overexpression affected c-fos abundance. Further work will be required to elucidate the mechanism by which schwannomin inhibits STAT activation.

Regulation of Stat3 by schwannomin may be related to Rac signaling. Recent studies have shown that schwannomin can be phosphorylated by p21-activated kinase (PAK2) in response to activated Rac1 (24–26). In Schwann cells, dominant inhibitory Rac1 blocks IGF-I-mediated motility (51) and constitutively active Rac1 can directly activate Stat3, leading to Stat3 nuclear translocation (52,53). Our results demonstrating schwannomin suppression of Stat3 are consistent with a relationship to Rac signaling and suggest one possible mechanism by which schwannomin can inhibit Rac-mediated Stat3 activation. The finding that schwannomin and its interacting protein HRS both strongly suppress Stat3 signaling is significant to potential treatment strategies for tumors in NF2, since constitutive activation of Stat3 by mutation is oncogenic (54). Treatments that target Stat3 and other STAT proteins may prove to be highly effective at inhibiting the growth of NF2-related tumors.

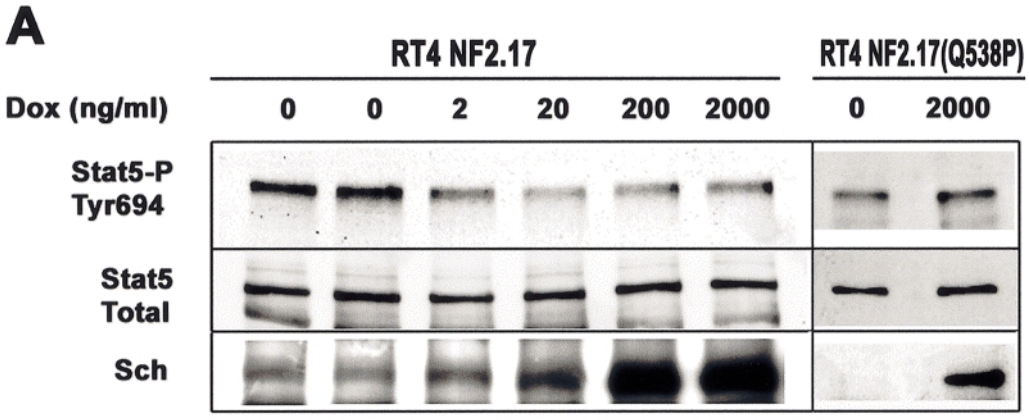
MATERIALS AND METHODS

Antibodies

The Xpress epitope tag encoded on pcDNA3.1his was detected with anti-Xpress antibody (R91025, Invitrogen). Polyclonal chicken anti-schwannomin antibody ab2781 was raised against schwannomin residues 528–541 (YMEKSKHLQEQLNE) and affinity purified. Polyclonal rabbit anti-merlin antibody WA30 was raised against schwannomin residues 192–209 (YAEHRGRARDEAEMEYLK) as previously described (55). Phosphorylated Stat3 was detected on immunoblots using monoclonal anti-phospho-Stat3 (Tyr705) antibody (9131, Cell Signaling Technology). Total Stat3 was detected on immunoblots using a rabbit polyclonal Stat3 antibody (9132, New England Biolabs) and was detected for immunofluorescent localization using a rabbit polyclonal Stat3 antibody (sc482, Santa Cruz). Phosphorylated Stat5 was detected using monoclonal anti-phospho-Stat5 (Tyr694) antibody (9351, New England Biolabs) and total Stat5 was detected using a monoclonal anti-Stat5 antibody (S21520, Transduction Laboratories). All secondary antibodies used in the study were purchased from Jackson ImmunoResearch Laboratories.

Constructs

The pcDNA3.1hisB-NF2i2 construct was generated by excising the NF2i2 fragment from pGBT9-NF2i2 [previously described (22)] with *Sma*I and *Sal*I and ligating it with pcDNA3.1hisB (Invitrogen) digested with *Eco*RV and *Xho*I. The pcDNA3.1hisB–NF2i1 construct was made by removing the C-terminal half of the NF2i2 gene from pcDNA3.1hisB–NF2i2 with *Xho*I and *Xba*I and ligating in its place the C-terminal fragment of the NF2i1 gene removed from pGBT9–NF2i1 with the same two restriction enzymes. To construct pcDNA3.1hisB–NF2i2 (Q538P), the full-length mutant NF2 gene was removed from the pGBT9–NF2i2 (Q538P) [previously described (22)] with *Sma*I and *Sal*I and ligated with pcDNA3.1hisB digested with *Eco*RV and *Xho*I. pcDNA3.1hisB–HRSi1 was made by excising the HRSi1 fragment from pGAD10–HRSi1 [previously described (22)] with *Sal*I and ligating in the *Xho*I site of pcDNA3.1hisB. The plasmids pGBT9–NF2i1 and pGAD10–HRS were described previously (22). To construct pGBT9–NF2i1 (Q538P), a portion of the mutant NF2 gene containing Q538P was removed from pGBT9–NF2i2 (Q538P) with *Hpa*I and *Stu*I and ligated in place of the corresponding portion removed from



B

Schwannomin isofrom I with mutation Q538P does not bind HRS

Interaction			Plate Assay	β-Galactosidase Units
pGBT9		pGAD10		
NF2i1	*vs*	HRS		214.3 ± 4.1
NF2i1(Q538P)	*vs*	HRS		1.19 ± 0.093
NF2i1	*vs*	Vector		0.17 ± 0.069
NF2i1(Q538P)	*vs*	Vector		0.28 ± 0.023
Vector	*vs*	HRS		0.36 ± 0.023

Figure 6. Schwannomin inhibits STAT5 phosphorylation in Tet-on RT4 NF2.17 cells. (**A**) Left panel: seven identical cultures of Tet-on RT4 NF2.17 cells were treated with the indicated concentrations of doxycycline (ng/ml) for 24 h. Protein extracts were subjected to immunoblot analysis using antibodies that recognize Stat5 phosphorylated on tyrosine 694 (Stat5p), total Stat5 or schwannomin detected with antibody ab2781. A reduced abundance of Stat5p was observed for all treatments when doxycycline was used. Note that the treatment without doxycycline is replicated. Right panel: no reduction of Stat5 phosphorylation was observed upon expression of schwannomin mutated at position Q538P. (**B**) Yeast two-hybrid tests of interaction demonstrated that schwannomin isoform I with the mutation Q538P has considerably reduced HRS binding affinity compared with wild-type schwannomin isoform I.

pGBT9–NF2i1 with the same restriction enzymes. The pRevTet-Off–NF2i1 construct was made by excising the NF2i1 insert from pcDNA3.1hisB–NF2i1 with *Bam*HI and ligating in to the *Bam*HI site of pRevTet-Off (Clontech).

Luciferase assays

STS26T or RT4 cells were grown in DMEM containing 10% FBS. Transfections were conducted using Superfect as recommended by the vendor (Qiagen). Total transfected DNA was 16.7 μg on a 100 mm dish, including 7.1 μg of luciferase reporter plasmid (pLucTK, pLukTKS3 or pLucTKSIE), 0.6 μg pcDNA3.1his–LacZ internal control plasmid (Invitrogen) and 9 μg total expression plasmid [pcDNA3.1his, pcDNA3.1his–NF2i1, pcDNA3.1his–NF2i2, pcDNA3.1his–NF2i2 (L46R), pcDNA3.1his–NF2i2 (Q538P), pcDNA3.1his–NF2N (1-305), pcDNA3.1his–NF2i1C (299–595), pcDNA3.1his-HRSi1]. After 48 h of transfection, cells were washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma), removed with 0.5% trypsin, 5.3 mM EDTA and trypsin was neutralized with growth media and cells were pelleted. When cells were stimulated with IGF-I, twice the cell volume was used for each treatment (2 × 100 mm dishes). After transfection cells were recovered in media containing 10% serum overnight, then were serum-starved for 24 h before being stimulated with IGF-I at the indicated concentrations for an additional 6 h. Cytosolic extracts were used for luciferase assays (Luciferase Assay System, Promega). β-Galactosidase activity was determined by the liquid assay method as described below for yeast extracts, with chloroform omitted. Luciferase activity = [counts

luciferase/OD_{420}]. The luciferase reporter system was a gift from Richard Jove, University of South Florida.

Yeast two-hybrid tests of interaction

The methods for testing protein–protein interactions were described previously (22,27,28). The yeast strain Y190 was co-transformed with pGBT9 and pGAD10 constructs as indicated and grown on SC media with leucine and tryptophan dropped out and with 2% glucose. β-Galactosidase production was assayed by incubating freeze-fractured colonies on nitrocellulose in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 0.03 mM β-mercaptoethanol and 2.5 μM X-gal) at 37°C. Liquid assays for β-galactosidase were conducted by incubating yeast extracted in Z-buffer and 5% chloroform with 0.6 mg/ml *o*-nitrophenyl-beta-D-galactopyranoside for 1 h. Color intensity depends on the amount of β-galactosidase present and is measured spectrophotometrically at 420 nm. β-Galactosidase units = $1000 \times [OD_{420}/(OD_{600} \times \text{time} \times \text{volume})]$.

Cell lines

The Schwann-like STS26T cell line is derived from a human malignant schwannoma (56). The schwannomin-inducible Tet-off RT4 NF2.17 cell line was described previously (15). The schwannomin-inducible Tet-off STS26T cell line was generated by using the RevTet-Off vector system (Clontech) as follows: STS26T cells were infected with retrovirus made from packaging cell line PT67 stably transfected with pRevTet-Off (G418, Invitrogen). Resistant Tet-Off STS26T cells were then trypsinized, diluted 40 000 times and plated in three 96-well plates (these dilution conditions allowed for an average of one cell per well). After cells reached confluency, each plate was seeded in triplicate [one pair of plates for dox/no dox comparisons, grown in black opaque tissue culture plates (Greiner Bio-One) and a third for propagation of lines]. Each pair of plates was transiently transfected (Superfect, Qiagen) with pRevTRE-Luc and then one of each pair was treated with doxycycline and the other with no doxycycline. Luciferase was detected in the plates using a 6-detector 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer) with coincidence counting deactivated and Luciferase Assay Reagent (Promega). Of the <288 lines screened, we propagated 24 of the best lines and we assayed for induction of transiently transfected pRevTRE-Luc activation using a luminometer. The line best inducing luciferase with minimal detected reporter leak was then infected by retrovirus made from PT67 stably selected with RevTRE-NF2i1 and cells were placed under neomycin/hygromycin B selection (Invitrogen). Forty-eight colonies of Tet-off STS26T NF2i1 cells were collected by ring-cloning and tested for doxycycline inducible schwannomin expression by immunoblotting with antibody ab2781. The line used in our study is Tet-off STS26T NF2i1.F9.3 and is one of seven retained.

Immunofluorescence

STS26T cells (30 000 cells per well in four-well slides) were grown in DMEM with 10% FBS overnight. Cells were labeled for immunofluorescence as previously described (22,57). The primary antibody dilutions were 5 μg/ml anti-Stat3, 5 μg/ml anti-schwannomin ab1781, or 1 : 500 dilution anti-Xpress. Primary antibodies were incubated 60 min at 37°C. For the co-detection of Xpress-schwannomin and Stat3, cells were incubated with FITC-conjugated affinity purified goat anti-rabbit IgG or rhodamine-conjugated affinity purified goat anti-mouse IgG for 1 h at room temperature. For the detection of Stat3 in RT4 NF2.17 cells, cells were plated on poly-lysine-coated cover glasses and detected using rhodamine-conjugated affinity purified goat anti-rabbit IgG. After antibody incubations, cells were washed six times in cold DPBS and mounted. For Figure 2A–F, fluorescent confocal microscopy was performed using a Zeiss LSM 310 confocal microscope. Fluorescein was visualized with a BP485/20/BP520–560 excitation/emission filter set (Zeiss filter set 17) and scanning was done with a 488 nm argon laser and a BP520–560 barrier filter. Rhodamine visualization was done with a BP515–560/LP590 excitation/emission filter set (Zeiss filter set 15) and scanning performed with a 543 nm HeNe laser and an LP590 nm barrier filter. For Figure 2G–J, fluorescent confocal microscopy was performed using a Leica TCS SP confocal microscope. Rhodamine was excited with a HeNe laser at 568 nm with emission set to a range of 596–671 nm.

Proliferation assays

Proliferation experiments were performed after an overnight induction in 1 μg/ml doxycycline after a 24 h serum starvation period. IGF-I (100 ng/ml) or HGF (200 ng/ml) was added for the 4 h thymidine incorporation period. Thymidine incorporation was performed as previously described (47). Each condition was performed in six duplicate wells.

Stat phosphorylation assay

For the analysis of Stat3 phosphorylation in Tet-off STS26T NF2il cells, the cells were equally-plated in DMEM containing 10% FBS prior to the addition of 100 μl of a 100x solution of doxycycline (to maintain the diluent background, the dilution series was made prior to the addition). After two days induction cells were serum-starved overnight then treated with 50 ng/ml IGF-I for 10 min. For the analysis of Stat3 phosphorylation in Tet-on RT4 NF2.17 cells, the cells were equally-plated in DMEM containing 10% fetal bovine serum (FBS) prior to the addition of 1 μg/ml doxycycline for 2, 4, 6 and 24 h. For the analysis of Stat5 in Tet-on RT4–NF2.17, or RT4–NF2.17(Q538P) cells, the cells were equally-plated in DMEM containing 10% FBS prior to the addition of 100 μl of a 100× solution of doxycycline and incubated for 24 h. Cells were harvested on ice in Cell Lysis Buffer (Cell Signaling Technology) and equal amounts of protein were loaded onto 4–15% SDS–PAGE gels and immunoblotting was performed.

ACKNOWLEDGEMENTS

We thank Richard Jove (University of South Florida) for providing luciferase reporter plasmids and Matt Schibler (UCLA Brain Institute), Kolja Wawrowski and Kamlesh Asotra (Cedars-Sinai Medical Center) for assistance with confocal microscopy. We thank Richard Deem for assistance

on luciferase detections in the microtiter plate format. This work was supported by the Carmen and Louis Warschaw Endowment Fund and FRIENDs of Neurology. Support was also provided by grants NS01428-01A1 from the National Institutes of Health (NIH) and DAMD17-99-1-9548 from the Department of Defense (DoD) to S.M.P., NS35848 from NIH to D.H.G., NS10524-02 from NIH and DAMD17-00-1-0553 from DoD to D.R.S. and a shared equipment grant no. 1S10-RR13717 from NIH.

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