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14. ABSTRACT

We find that the NF2 gene product, merlin, regulates p75NTR expression levels and signaling. This depends on the phosphorylation state of merlin. We also find that activation of p75NTR fails to induce apoptosis in SCs and schwannoma cells that lack functional merlin expression, in contrast to normal SCs. Further, activation of p75NTR in human VS cells protects the cells from some forms of cell death. Nevertheless, VS cells in vitro and in vivo remain sensitive to JNK inhibitors in the absence of p75NTR ligands. Indeed, the JNK inhibitor, AS602801, induces regression of human schwannoma xenografts in nude mice.

15. SUBJECT TERMS
vestibular schwannoma, neurofibromatosis type II, $p75^{NTR}$, c-Jun N-terminal kinase, radiation

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1. Introduction

Neurofibromatosis type II (NF2) results from mutation in the *NF2* gene leading to the development of multiple intracranial and spinal tumors including schwannomas. Our overall objective is to identify the fundamental differences between non-tumorous Schwann cells (SCs) and schwannoma cells and to determine the efficacy of therapies that target these differences in reducing schwannoma cell growth in culture and in animal models of human schwannoma disease. We find that the *NF2* gene product, merlin, regulates $p75^{NTR}$ expression levels and signaling. This depends on the phosphorylation state of merlin. We also find that activation of $p75^{NTR}$ fails to induce apoptosis in SCs and schwannoma cells that lack functional merlin expression, in contrast to normal SCs. Further, activation of $p75^{NTR}$ in human VS cells protects the cells from some forms of cell death, including in response to c- Jun N-terminal kinase inhibitors. Nevertheless, VS cells *in vitro* and *in vivo* remain sensitive to JNK inhibitors in the absence of $p75^{NTR}$ ligands. Indeed, the JNK inhibitor, AS602801, induces regression of human schwannoma xenografts in nude mice. Finally, we find that human schwannoma cells are highly resistant to ionizing radiation likely due to their low proliferative capacity and their ability to mitigate oxidative stress.

2. Keywords

vestibular schwannoma, neurofibromatosis type II, $p75^{NTR}$, c-Jun N-terminal kinase, radiation

3. Accomplishments

Task 1- Obtain animal care approval and IRB exemption from University of Iowa and Department of Defense regulatory agencies

Animal care approval and IRB exemptions from the University of Iowa and Dept. of Defense have been obtained.

Task 2- Demonstrate that $p75^{NTR}$ expression, cleavage, and signaling depend on merlin status.

Treatment of Schwann cell (SC) cultures derived from ROSA-CreER;$Nf2^{f/f}$ mice with tamoxifen (Tx) results in deletion of *Nf2* and reduction in merlin expression. $p75^{NTR}$ levels are significantly elevated in SCs lacking merlin expression compared to SCs with retained merlin expression. Further, sciatic nerves from mice that harbor a dominant negative mutant isoform of *Nf2* (P0SchΔ39-121) demonstrate elevated $p75^{NTR}$ levels compared to nerves from wild-type mice. Finally, replacement of merlin into human vestibular schwannoma (VS) cultures reduces $p75^{NTR}$ expression. Taken together, these data demonstrate that merlin suppresses $p75^{NTR}$ expression in SCs and VSs (appendix, Ahmad, et al. *Neurobiol Dis*, 2015).

Increased $p75^{NTR}$ expression in normal SCs following nerve injury correlates with phosphorylation of merlin. Further, transduction of human VSs with a phosphomimetic merlin isoform fails to suppress $p75^{NTR}$ expression (appendix, Ahmad, et al. *Neurobiol Dis*, 2015), suggesting that the phosphorylation status of merlin determines its ability to suppress $p75^{NTR}$ expression.

Lack of merlin expression leads to increased c-Jun N-terminal kinase (JNK) and NF-κB activity in human VS cultures and $p75^{NTR}$ ligands activate JNK and NF-κB in human VS cultures (appendix, Ahmad, et al. *Glia*, 2014).

To examine the influence of $p75^{NTR}$ cleavage on VS cell proliferation, we transduced human VS cultures with adenoviral vectors expressing the intracellular domain (ICD) of $p75^{NTR}$ with or without nuclear export or localization signals. Our preliminary results did not find any significant differences in VS cell proliferation (as determined by EdU uptake) in cultures that over-express the $p75^{NTR}$ ICD isoforms (Fig. 1).

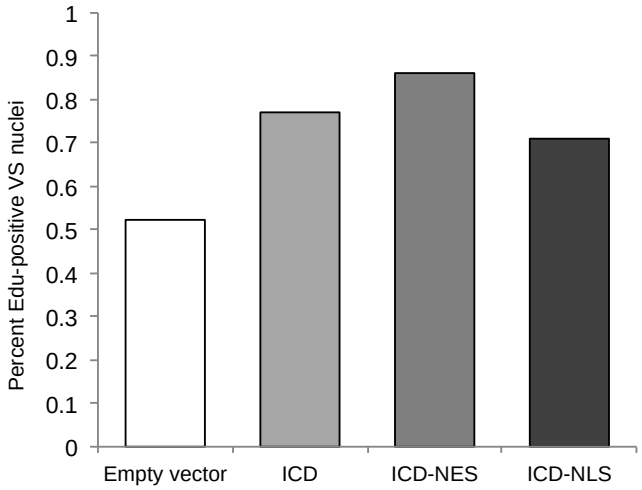

Figure 1. Overexpression of $p75^{NTR}$ intracellular domain (ICD) does not increase VS cell proliferation. Human VS cultures were transduced with adenoviruses that express the ICD p75NTR without a targeting sequence (ICD) or with a nuclear export signal (ICD-NES) or nuclear localization sequence (ICD-NLS). The number of VS nuclei that had incorporated Edu was determined.

Task 3- Determine the cytoprotective effects of $p75^{NTR}$ signaling in VS cells treated with JNK or mTOR inhibitors.

Activation of $p75^{NTR}$ signaling protects human VS cells from apoptosis induced by inhibition of JNK. This prosurvival effect is due, at least in part, to the ability of $p75^{NTR}$ to activate NF-κB (appendix, Ahmad, et al. *Glia*, 2014). mTOR inhibitors did not induce VS cell death singly or in combination with the $p75^{NTR}$ inhibitory peptide, TAT-Pep5 (data not shown).

Task 4- Determine the cytoprotective effects of $p75^{NTR}$ signaling in VS cells treated irradiation.

We find that human VS cells are highly resistant to radiation in vitro. By MTT assay, we did not find any significant reduction in overall VS cell viability with single doses of radiation up to 70 Gy seven days following radiation (Fig. 2). Further, treatment of cultures with proNGF, a $p75^{NTR}$ ligand, did not alter VS cell sensitivity to radiation at any dose (Fig. 2). Further, treatment of VS cell cultures with the TAT-Pep5 peptide did not alter VS cell radiosensitivity. We find that VS cells continue to survive and grow up to seven years following radiation despite evidence of ongoing oxidative stress (3-nitrotyrosine labeling) in 3 of 4 irradiated tumors (Fig. 3). Taken together these data indicate that VS cells are relative radioresistant due, at least in part, to their low proliferative capacity and their ability to mitigate oxidative stress.

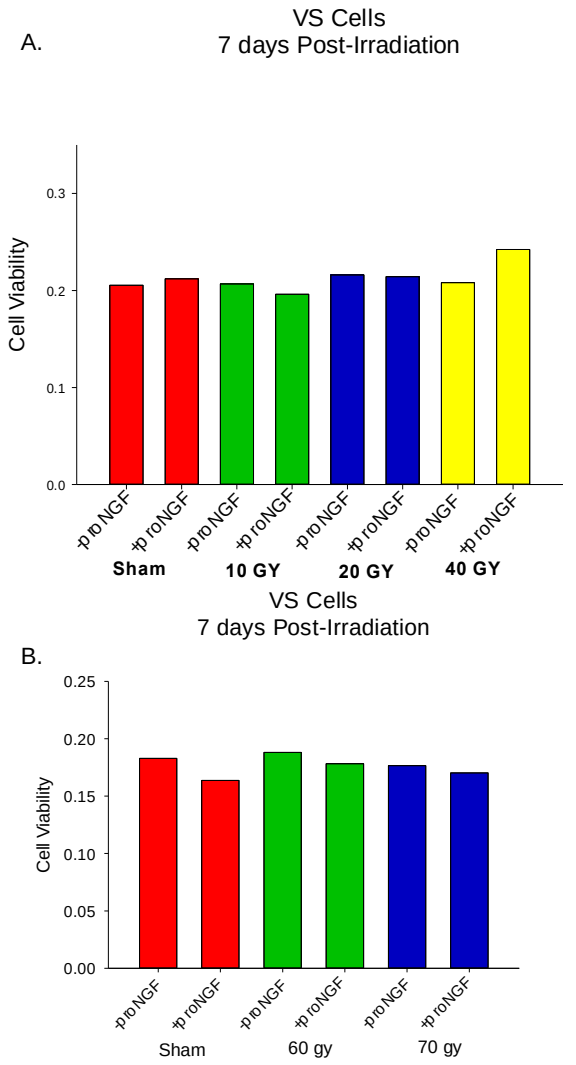

Figure 2. Human VS cultures are resistant to radiation in the absence or presence of proNGF (3 nM), a $p75^{NTR}$ ligand. Human VS cultures were treated with sham or 10-40 Gy (A) or 60-70 Gy (B) of ionizing radiation. Cell viability was measured by the MTT assay seven days following radiation exposure.

Task 5- Define the ability of simultaneous inhibition of $p75^{NTR}$ and mTOR or JNK to suppress human schwannoma xenograft growth.

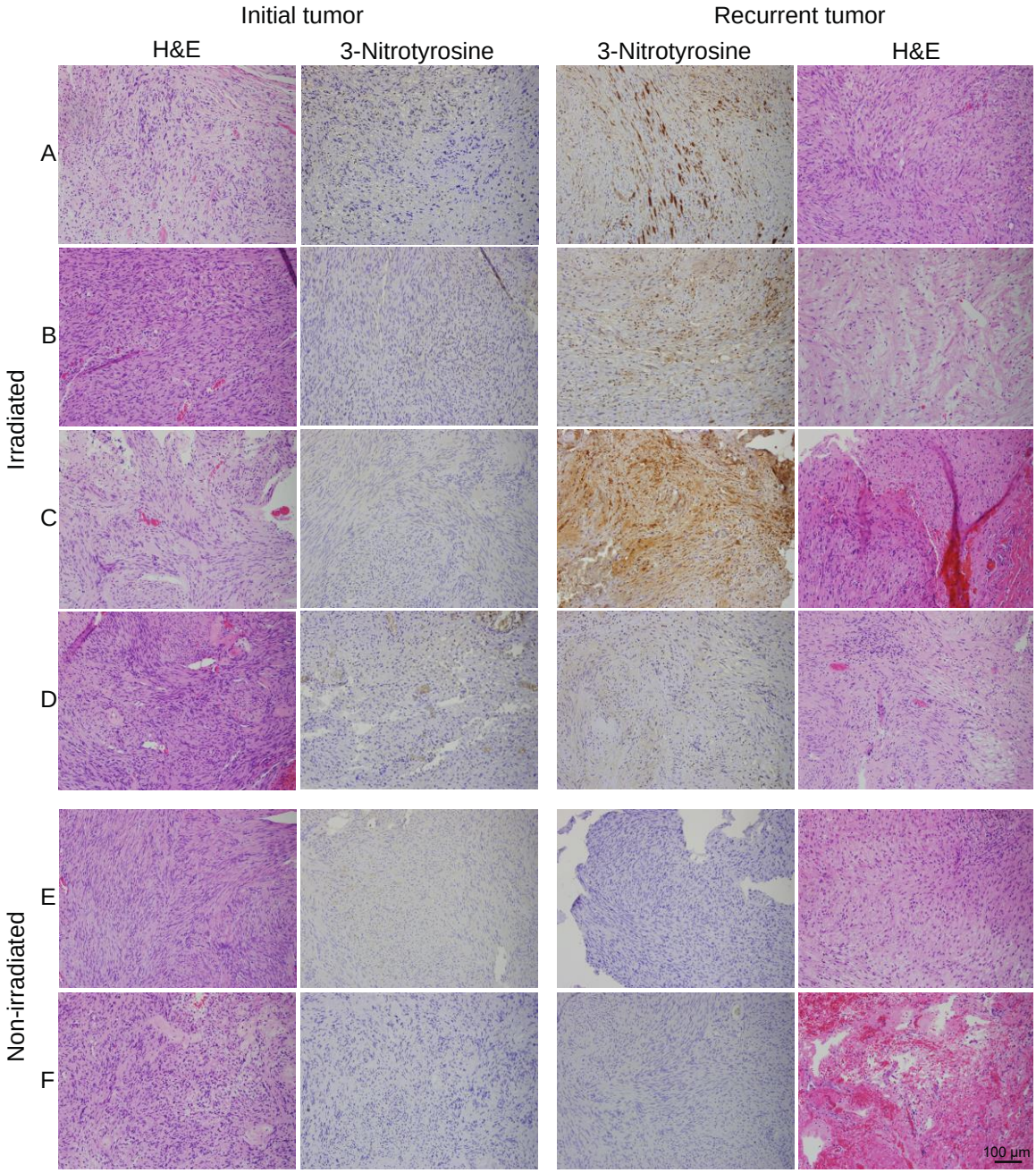

Figure 3. Radiation increases 3-nitrotyrosine (3-NT) immunostaining in vestibular schwannomas (VSs). Sections of VSs were immunostained with an antibody that detects 3-NT. For four VSs (A-D) sections were derived from the initial tumor resection and from the recurrent tumor that had previously been irradiated. For two VSs (E,F) sections were derived from the initial tumor resection and from the recurrent tumor that had not previously been irradiated. Representative hematoxylin and eosin (H&E) stains are shown for each tumor. Scale bar=100 µm.

Although activation of $p75^{NTR}$ protects VS cells from cell death to JNK inhibitors, we did not find any additive benefit of inhibiting $p75^{NTR}$ with JNK inhibitors on

cultured VS cells. Further we did not find any significant increase in VS cell death in cultures treated with mTOR inhibitors, either singly or in combination with p75^{NTR} inhibitory peptides. Given these results we focused our in vivo studies on JNK inhibitors. We have treated 2 separate cohorts of nude mice (n=18-20 mice/cohort) bearing human VS xenografts with the JNK inhibitor AS602801 or control vehicle for 75 days. We measured changes in tumor volume with magnetic resonance imaging and cell proliferation with EdU uptake. We find that AS602801 induced regression of VS xenografts and decreased cell proliferation (Fig. 4). We are currently performing TUNEL labeling to quantify the degree of cell death due to AS602801.

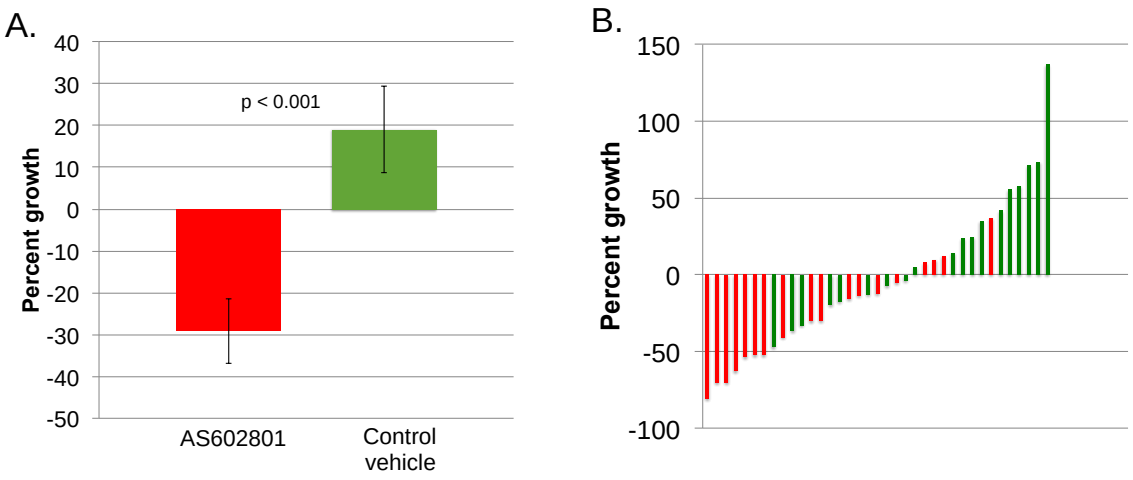

Figure 4. Treatment of human VS xenografts in nude mice with AS602801 (red) induces regression of tumor volume compared to control vehicle (green). A. Average change in tumor volume over the treatment period. B. Waterfall plots of change in tumor volume for each tumor.

Task 6- Define the ability of simultaneous inhibition of p75^{NTR} and mTOR or JNK to suppress schwannoma growth in periostinCre;Nf2$^{f/f}$ mice.

As above, we focused these *in vivo* studies on JNK inhibitors, since these were the most effective *in vitro*. In collaboration with Dr. Yates in Indiana, we treated periostinCre;Nf2$^{f/f}$ mice with AS602801 or control vehicle for 75 days with serial audiometric assessment by measuring auditory brainstem response thresholds. We find that AS602801 significantly reduced growth of human VS xenografts in nude mice (Fig. 4) and reduced proliferation of schwannoma cells in periostinCre;Nf2$^{f/f}$ mice (manuscript in preparation).

Training and professional development- Nothing to report.

Dissemination of results to communities of interest- Three manuscripts have been published (see appendix), a fourth has been submitted and is under review, and a fifth is in preparation. This final manuscript will provide a full dataset for the in vivo experiments demonstrating the ability of JNK inhibitors to reduce VS growth in xenograft and NF2 mouse models.

4. Impact

Impact on the development of the principal discipline(s) of the project

Our results have demonstrated that merlin, the protein product of the *NF2* gene, profoundly influences $p75^{NTR}$ expression and signaling. The results help explain the ability of human vestibular schwannoma (VS) cells to survive and grow in the absence of axonal contact and to resist cell death. The results provide a novel therapeutic target for the treatment of vestibular schwannomas, especially in patients with neurofibromatosis type II.

The results also inform normal neural development and regeneration processes and help explain the role of merlin in normal Schwann cell homeostasis and response to injury.

The results help explain mechanisms that render VS cells resistant to radiation therapy

The results identify AS602801 as a potential novel therapeutic agent to treat VSs.

Impact on other disciplines- Nothing to report.

Impact on other technology transfer- Nothing to report.

Impact on other society beyond science and technology- Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change-
We modified our radiation experiments to include higher doses and longer times to assay cell death since our preliminary experiments indicate that human VS cells are highly resistant to radiation. Further, given the lack of efficacy of the TAT-Pep5 peptide and mTOR inhibitors in culture, we have not used these compounds in animal models.

Changes that had a significant impact on expenditures- None.

Significant changes in use or care of human subjects- None.

Significant changes in use or care of vertebrate animals- None.

Significant changes in use of biohazards and/or select agents- None.

6. Products

Publications -

1- Ahmad, I, Yue, WY, Fernando, A, Clark, JJ, Woodson, EA, Hansen, MR. $p75^{NTR}$ is highly expressed in vestibular schwannomas and promotes cell survival by activating NF-κB. *Glia,* 2014, 62(10):1699-712.*cover

2- Ahmad, I, Fernando, A, Gurgel, R, Clark, JJ, Xu, L, Hansen, MR. Merlin status regulates $p75^{NTR}$ expression and apoptotic signaling in Schwann cells following nerve injury. *Neurobiol Disease*, 2015 6;82:114-122.

3- Cheng, E, Hansen, MR. Schwannomas provide insight into the role of $p75^{NTR}$ and merlin in Schwann cells following nerve injury and during regeneration. Neural Regen Res. 2016 11(1):73-4.

4- Robinett, ZN, Bathla, G, Wu, A, Clark, JJ, Sibenaller, ZA, Wilson, T, Kirby, P, Allen, BG, Hansen, MR. Persistent oxidative stress in vestibular schwannomas that grow after stereotactic radiation therapy. Otol&Neurotol, 2018, 39(9):1184-1190.

5- Kersigo, J, Gu, L, Xu, L, Pan, N, Vijayakuma, S, Jones, T, Shibata, S, Fritzsch, B, Hansen, MR. Neurod1 expression reduces mouse and human schwannoma cell proliferation. Laryngoscope, submitted.

Website(s) or other Internet site(s)- Nothing to report

Technologies or techniques- Nothing to report

Inventions, patent applications, and/or licenses- Nothing to report

Other products- Nothing to report

7. Participants and Other Collaborating Organizations

Name	Marlan R. Hansen
Project Role	Principal investigator
Nearest person month worked	1
Contribution to project	Helped design, execute, and interpret experiments; preparation of data for publication and writing.
Funding support	NIH, Clinical and departmental funds, endowed professorship

Name	Frank Canady
Project Role	Pre-doctoral scholar
Nearest person month worked	10
Contribution to project	design, performance, and interpretation of experiments; preparation of data for publication and writing.
Funding support	University of Iowa Carver College of Medicine and departmental funds

Name	Jed Rasmussen
Project Role	Post-doctoral fellow
Nearest person month worked	5
Contribution to project	Designed and performed experiments, cell signaling and protein trafficking, cell culture
Funding support	

Name	J. Jason Clark
Project Role	Research Assistant
Nearest person month worked	7
Contribution to project	Designed and performed experiments, radiation, maintenance of mouse colonies, cell culture
Funding support	NIH P30, Departmental funds

Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period- Nothing to Report

Other organizations were involved as partners-

Organization Name: Indiana University
Location of Organization: Indianapolis, IN
Partner's contribution to the project- Drs. Wade Clapp and Charles Yates are collaborators on the grant to provide the periostinCre; $Nf2^{f/f}$ mice.

8. Special reporting requirements

Nothing to report

9. Appendices

1- Ahmad, I, Yue, WY, Fernando, A, Clark, JJ, Woodson, EA, Hansen, MR. $p75^{NTR}$ is highly expressed in vestibular schwannomas and promotes cell survival by activating NF-κB. *Glia,* 2014, 62(10):1699-712.*cover

2- Ahmad, I, Fernando, A, Gurgel, R, Clark, JJ, Xu, L, Hansen, MR. Merlin status regulates $p75^{NTR}$ expression and apoptotic signaling in Schwann cells following nerve injury. *Neurobiol Disease*, 2015 6;82:114-122.

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4- Robinett, ZN, Bathla, G, Wu, A, Clark, JJ, Sibenaller, ZA, Wilson, T, Kirby, P, Allen, BG, Hansen, MR. Persistent oxidative stress in vestibular schwannomas that grow after stereotactic radiation therapy. Otol&Neurotol, 2018, 39(9):1184-1190.

RESEARCH ARTICLE

p75^{NTR} is Highly Expressed in Vestibular Schwannomas and Promotes Cell Survival by Activating Nuclear Transcription Factor κB

Iram Ahmad, Wei Ying Yue, Augusta Fernando, J. Jason Clark, Erika A. Woodson, and Marlan R. Hansen

Vestibular schwannomas (VSs) arise from Schwann cells (SCs) and result from the loss of function of merlin, the protein product of the *NF2* tumor suppressor gene. In contrast to non-neoplastic SCs, VS cells survive long-term in the absence of axons. We find that p75^{NTR} is overexpressed in VSs compared with normal nerves, both at the transcript and protein level, similar to the response of non-neoplastic SCs following axotomy. Despite elevated p75^{NTR} expression, VS cells are resistant to apoptosis due to treatment with proNGF, a high affinity ligand for p75^{NTR}. Furthermore, treatment with proNGF protects VS cells from apoptosis due to c-Jun N-terminal kinase (JNK) inhibition indicating that p75^{NTR} promotes VS cell survival. Treatment of VS cells with proNGF activated NF-κB while inhibition of JNK with SP600125 or siRNA-mediated knockdown reduced NF-κB activity. Significantly, proNGF also activated NF-κB in cultures treated with JNK inhibitors. Thus, JNK activity appears to be required for basal levels of NF-κB activity but not for proNGF-induced NF-κB activity. To confirm that the increase in NF-κB activity contributes to the prosurvival effect of proNGF, we infected VS cultures with Ad.IκB.SerS32/36A virus, which inhibits NF-κB activation. Compared with control virus, Ad.IκB.SerS32/36A significantly increased apoptosis including in VS cells treated with proNGF. Thus, in contrast to non-neoplastic SCs, p75^{NTR} signaling provides a prosurvival response in VS cells by activating NF-κB independent of JNK. Such differences may contribute to the ability of VS cells to survive long-term in the absence of axons.

GLIA 2014;62:1699–1712

Key words: acoustic neuroma, apoptosis, merlin, Schwann cell, neurotrophin

Introduction

Vestibular schwannomas (VS) originate from Schwann cells (SCs) of the vestibular nerve and typically occur as either unilateral, sporadic tumors, or as bilateral tumors in patients with neurofibromatosis type 2 (NF2) (Evans 2009; RoosLi et al., 2012). Both sporadic and NF2-associated VSs result from loss of function of the *NF2* tumor suppressor gene (Rouleau et al., 1993; Stemmer-Rachamimov et al., 1997; Trofatter et al., 1993). Merlin, the protein product of the *NF2* gene, regulates several signaling events that control tumor growth (Xiao et al., 2003; Zhou and Hanemann, 2012). Merlin appears to associate transmembrane and signaling molecules with cytoskeletal actin thereby affecting cell–cell attachments, cell motility, and the subcellular localization and activity of transmembrane receptors and signaling molecules in response to cell contact inhibition (McClatchey and Giovannini, 2005; Scoles, 2008; Welling et al., 2007; Xiao et al., 2003).

Recent evidence suggests that merlin suppresses mitogenic signaling at the cell membrane and in the nucleus (Li et al., 2012; Zhou and Hanemann, 2012). At the membrane, merlin inhibits signaling by integrins and tyrosine receptor kinases (RTKs) and the activation of downstream pathways, including the Ras/Raf/MEK/ERK, FAK/Src, PI3K/AKT, Rac/PAK/JNK, mTORC1, and Wnt/β-catenin pathways (Bosco et al., 2010; Chadee and Kyriakis, 2004; Chadee et al., 2006; Flaiz et al., 2009; Fraenzer et al., 2003; Houshmandi et al., 2009; James et al., 2009, 2012; Kaempchen et al., 2003; Kissil et al., 2003; Lim et al., 2003; Lopez-Lago et al., 2009; Rong et al., 2004; Yi et al., 2008; Zhou et al., 2011). Merlin

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also acts upstream of the Hippo pathway to suppress the function of Yes-associated protein 1 (YAP1), an oncogene implicated in meningioma tumor growth (Baia et al., 2012; Hamaratoglu et al., 2006; Striedinger et al., 2008; Zhang et al., 2010). In the nucleus, merlin suppresses the E3 ubiquitin ligase CRL4 (DCAF1) to inhibit proliferation (Li et al., 2010).

p75^{NTR}

p75^{NTR} is the founding member of the tumor necrosis family (TNF) receptor superfamily and was the first identified nerve growth factor receptor (Bothwell, 1995). p75^{NTR} binds mature neurotrophins with low affinity, while proneurotrophins bind avidly to p75^{NTR} (Chao, 2003; Lee et al., 2001). Neurotrophins also signal through Trk receptors to promote cell survival, which are capable of forming high affinity binding sites with p75^{NTR} (Hempstead et al., 1991).

Activation of p75^{NTR} elicits a variety of responses, including apoptosis or cell survival, depending on the cellular context. In the absence of Trk receptors, p75^{NTR} activates nuclear transcription factor κB (NF-κB), the sphingomyelin cycle, and c-Jun N-terminal kinase (JNK) (Dobrowski et al., 1994; Gentry et al., 2000; Harrington et al., 2002; Roux and Barker, 2002). Consistent with the notion that p75^{NTR} signaling initiates cell death, pro-nerve growth factor (NGF) and pro-brain derived neurotrophic factor (BDNF) induce apoptosis in cells expressing p75^{NTR} (Clewes et al., 2008; Koshimizu et al., 2010; Masoudi et al., 2009; Provenzano et al., 2011). This proapoptotic function of p75^{NTR} requires binding of the co-receptor sortilin as well as γ-secretase-dependent intramembranous cleavage and release of the intracellular domain (Jansen et al., 2007; Kenchappa et al., 2006; Parkhurst et al., 2010; Skeldal et al., 2012). In other cells, p75^{NTR} signaling promotes cell survival. What determines whether p75^{NTR} activation leads to cell death or survival remains unknown. However, p75^{NTR} activation of the NF-κB has been implicated in the prosurvival response (Gentry et al., 2000), whereas activation of JNK is required for the prodeath signal (Friedman, 2000; Harrington et al., 2002; Koshimizu et al., 2010; Yoon et al., 1998).

p75^{NTR} and JNK Signaling in SCs

Following axotomy, SCs upregulate p75^{NTR} expression (Provenzano et al., 2008; Taniuchi et al., 1986). In the absence of reinnervation, denervated SCs ultimately undergo p75^{NTR}-mediated apoptosis (Ferri and Bisby, 1999; Petratos, 2003; Syroid et al., 2000). Consistent with a proapoptotic function of p75^{NTR} and JNK in SCs (Parkinson et al., 2001), pro and mature isoforms of NGF activate JNK and induce apoptosis in SCs (Hirata et al., 2001; Provenzano et al., 2011; Soilu-Hanninen et al., 1999; Yeiser et al., 2004). As VSs arise from cells of the SC lineage, they express p75^{NTR} similar to denervated SCs (Bonetti et al., 1997; Laskin et al., 2005; Miettinen et al., 2001). In contrast to non-neoplastic SCs, VS cells have the ability to survive long-term in the absence of axonal contact. Recent reports indicate that JNK is persistently active in human VS cells due to the lack of merlin expression (Hilton et al., 2009; Kaempchen et al., 2003; Yue et al., 2011). Significantly, this elevated JNK activity contributes to VS cell proliferation and survival (Yue et al., 2011).

To better understand the factors that contribute to the ability of VS cells to survive in the absence of axonal contact, we investigated the expression level of p75^{NTR} in primary VS specimens and the responses of primary cultures derived from acutely resected human VSs to high affinity p75^{NTR} ligands. We find that VS cells fail to die in the presence of proNGF unlike their non-neoplastic SC counterparts. Further, proNGF rescues VS cells with suppressed JNK signaling suggesting that, in contrast to its role in normal SCs, p75^{NTR} promotes VS cell survival. We also find that proNGF activates NF-κB to promote survival.

Materials and Methods

VS and Nerve Collection and Primary VS Cultures

The institutional review board of the University of Iowa approved the study protocol. VS were collected from patients undergoing microsurgery for removal of sporadic VS and immediately placed in ice-cold Hank's balanced salt solution until used for cultures or snap frozen in liquid nitrogen until used for RNA or protein extracts. None of the specimens were derived from NF2-associated tumors. Histological analysis confirmed typical schwannoma in each instance. Greater auricular nerve (GAN) and vestibular nerve (VN) specimens were collected after surgical removal from separate patients undergoing neck dissection or vestibular nerve section, respectively, and immediately snap frozen in liquid nitrogen until used for RNA (VN) or protein (GAN) extraction.

Primary Human VS Cultures

Primary human VS cultures were prepared from acutely resected tumors as previously described (Hansen et al., 2006; Yue et al., 2011). The cultures were not passaged prior to experimental manipulation. Adenoviral-mediated gene transfer was also performed as previously described with Ad5.empty vector (EV), Ad5.merlin, or Ad5.IκBαS (gift from Dr. Isaac Samuel), each at 2×10^8 pfu/mL (Yue et al., 2011). To knockdown JNK expression, cultures were transfected using RNAiMax (Life Technologies, Invitrogen, Carlsbad, CA) with small interfering ribonucleic acid (siRNA) oligonucleotides targeting JNK1 and JNK2 (Cell Signaling, #6232, Beverly, MA), as previously described (Yue et al., 2011). I-JIP (30–100 μM) and SP600125 (20 μM) (both from EMD Millipore, Billerica, MA) were used as JNK inhibitors, as before (Yue et al., 2011). At the conclusion of the experiments, the cultures were fixed for 10 min with 4% paraformaldehyde and immunolabeled with anti-S100 (Sigma, St. Louis, MO), p75^{NTR} (kindly provided by Dr. Moses

Chao) and/or sortilin (Abcam, Cambridge, MA) antibodies followed by secondary detection with Alexa 488, 568, or 647-conjugated secondary antibodies (Life Technologies).

Non-neoplastic Schwann Cell Cultures

Non-neoplastic Schwann cell cultures were prepared from neonatal rat or mouse sciatic nerve as previously described (Provenzano et al., 2008, 2011). Briefly, sciatic nerves were dissected from P5 pups, washed in ice-cold PBS, and enzymatically digested in 0.125% typsin with EDTA (Life Technology) and 0.2% collagenase (Sigma) in Hank's balanced salt solution without calcium and magnesium (Life Technology), for 20 min in 37°C. Fetal bovine serum at 10% final concentration (Life Technology) was used to quench the trypsin, the tissue was washed twice in DMEM (Life Technology) and then suspended in Dulbecco's modified Eagle's medium (DMEM) with N2 supplements (Life Technology) and 10 μg/mL insulin (Sigma). The cells were gently dissociated by titration through fire-polished narrow bored glass pipettes and cultured on laminin-coated eight-well plastic culture slides (Labtek, Campbell, CA) or 35 mm tissue culture dishes. Cultures were maintained in serum-free N2 medium until the cells were 70–90% confluent containing over 98% SCs as determined by S100 immunolabeling.

Schwann Cell and Schwannoma Cell Apoptosis

Primary human VS cultures and mouse sciatic nerve Schwann cells maintained in the presence or absence I-JIP or SP600125 (JNK inhibitors) were treated with cleavage resistant proNGF (Alomone Labs, Jerusalem, Israel). After 24 h, the cultures were fixed and immunolabeled for S100. Apoptotic nuclei were detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method as previously described (Hansen et al., 2008; Yue et al., 2011). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Criteria for scoring an apoptotic cell included: S100-positive, TUNEL-positive nucleus, and condensed or fragmented nucleus. The percent of TUNEL-positive VS cells was scored from 10 randomly selected 20× fields for each well, as described elsewhere (Hansen et al., 2008; Yue et al., 2011). Only S100-positive cells were scored. Given the variability in basal apoptotic rates for each primary tumor, the percent of TUNEL-positive VS cells was expressed as a percentage of the control condition, defined as 100%. Apoptosis was confirmed in a subset of cultures by immunoblotting for cleaved caspase 3. Each condition was performed in duplicate and was repeated on $\geq$3 VS cultures derived from separate tumors. Statistical significance of differences in the average percent of apoptotic cells among the various treatment conditions was determined by one-way ANOVA followed by Dunn's method for normally distributed data or by Kruskal-Wallis one-way ANOVA on ranks for nonparametric data using SigmaStat software (Systat Software, Richmond, CA).

Real-time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from five VSs and four VNs, each treated as a separate specimen. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed with the TaqMan and 7500 Real-Time PCR systems (Life Technologies, Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions using 6-carboxyfluorescein (FAM)-labeled probes. We used primer pairs #4331182 for human p75^{NTR} and #4326317E for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Life Technologies, Applied Biosystems). Quantification of gene expression was performed using the ddCt method according to the manufacturer's instructions and expressed as the ratio p75^{NTR} to GAPDH transcript levels. A student's non-paired, two-tailed *t*-test was used to determine statistical differences of transcript levels.

Western Blots

Western blots of protein extracts prepared from VS or GAN tissue or culture lysates were performed as described previously (Brown and Hansen, 2008; Hansen et al., 2006; Yue et al., 2011). The primary antibodies used were anti-p75^{NTR} (kindly provided by Dr. Moses Chao), phosphorylated JNK (pJNK, Cell Signaling), JNK (Cell Signaling), phosphorylated JUN (pJUN, Cell Signaling), merlin (Santa Cruz), cleaved caspase-3 (Cell Signaling), receptor-interacting protein 2 (RIP2) (Enzo Lifesciences, Farmingdale, NY), sortilin (Abcam), β-actin (Sigma), and Rho-GDI (Cell Signaling). Secondary antibodies (dilution, 1:5,000–50,000; Santa Cruz) were conjugated with horseradish peroxidase. Blots were developed using Super Signal West Femto kit (Thermo Fisher Scientific, Rockford, IL) and exposed to film (Amersham Hyperfilm TM ECL; GE Healthcare, Piscataway, NJ). As needed, membranes were stripped and reprobed with other antibody combinations. Densitometry to quantify protein levels was performed as previously described, and statistical significance was determined with a student's nonpaired, two-tailed *t*-test.

NF-κB Assay

Protein lysates were prepared 2 h following treatment of primary VS cultures with proNGF or sham. JNK inhibitors were added 30 min prior to treatment with or without proNGF. To determine NF-κB activity, we used a chemiluminescent capture assay (Thermo Fisher Scientific, Pierce, Rockford, IL, #89858) to capture activated NF-κB bound to the consensus DNA binding site according to the manufacturer's protocol. Activated NF-κB binds to the DNA sequence coating the wells, and the bound NF-κB is detected with anti-p50 antibody followed by peroxidase conjugated secondary antibody and chemiluminescent quantification with a luminometer. Results are expressed as a fold change in activity relative to the control condition for each repetition. Each condition was performed in duplicate and repeated on $\geq$3 cultures derived from separate tumors. Statistical significance of differences among the treatment conditions was determined by one way ANOVA followed by Dunn's method.

Results

Vestibular Schwannoma Cells Express High Levels of p75^{NTR}

SCs upregulate p75^{NTR} expression following denervation by axotomy and in the absence of reinnervation, ultimately undergo p75^{NTR}-mediated apoptosis (Ferri and Bisby, 1999; Petratos, 2003; Provenzano et al., 2008; Syroid et al., 2000; Taniuchi et al., 1986). Since VS cells lack axonal contact, we compared the expression level of p75^{NTR} in VSs with normal nerve. We used real-time RT-PCR to compare the

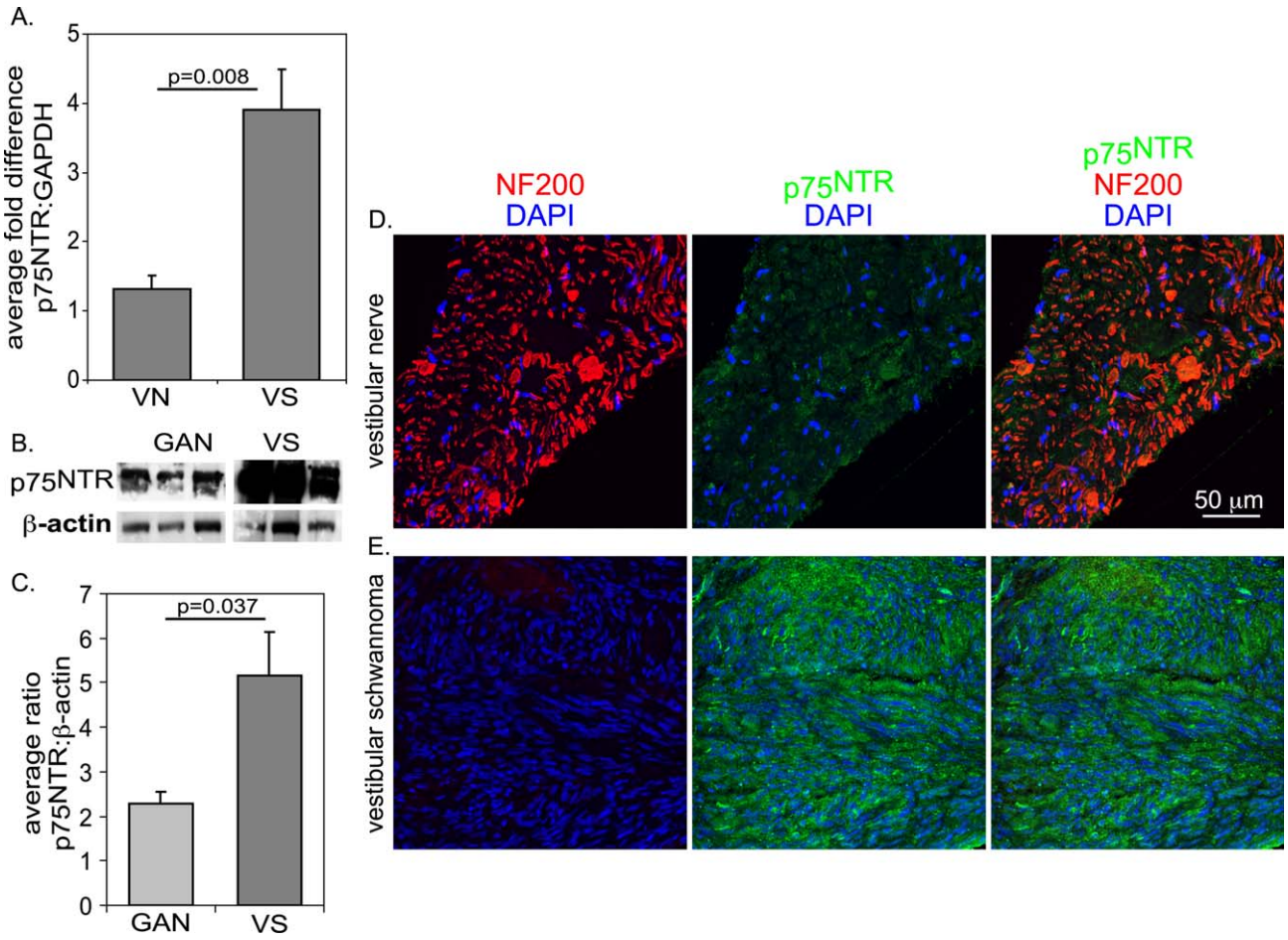


FIGURE 1: Vestibular schwannomas express high levels of p75^{NTR}. (A) Real-time RT-PCR comparing p75^{NTR} expression to GAPDH in normal human VN ($n = 4$) and in human VS ($n = 5$) specimens. (B, C). Western blot and comparison of anti-p75^{NTR} band intensity relative to anti-β actin band intensity in protein lysates from normal human GAN ($n = 4$) and human VS ($n = 5$). The differences in relative p75^{NTR} expression levels in (A) and (C) were statistically significant by Student's unpaired *t*-test. (D, E) Immunostaining of frozen sections of normal human VN (D) and human VS (E) with anti-NF200 (red) and anti-p75^{NTR} (green) antibodies. Nuclei were labeled with DAPI (blue).

ratio of p75^{NTR} to GAPDH transcript levels in five VS tumors and four normal human vestibular nerves. As shown in Fig. 1A, there was a less than threefold increase in the mean ratio of p75^{NTR} to GAPDH transcript levels in VSs compared with normal vestibular nerve. To confirm increased p75^{NTR} protein expression in VSs compared with normal nerves, we quantified p75^{NTR} levels in immunoblots of protein lysates from five VS and four human GAN specimens by densitometry. The blots were reprobed with anti-β-actin to assess protein loading levels. The mean ratio of p75^{NTR}/β-actin band intensity was significantly increased in lysates from VSs compared with GAN (Fig. 1B,C). We also immunolabeled frozen sections of VSs and normal vestibular nerves with antineurofilament 200 (NF200, neuronal/axon marker) and anti-p75^{NTR} antibodies. p75^{NTR} immunolabeling was relatively weak in the SCs and neuronal structures in normal vestibular nerves and was more intense in VS tissue, which lacked NF200 labeling (Fig. 1D,E). These results confirm that VSs express higher levels of p75^{NTR} compared with normal nerve.

ProNGF Fails to Induce Apoptosis in VS Cells

Proisoforms of neurotrophins, such as proNGF and proBDNF, are high affinity ligands for p75^{NTR} and induce apoptosis in SCs in vitro and in vivo (Petratos, 2003; Provenzano et al., 2008, 2011). To determine whether VS cells are likewise susceptible to proNGF-mediated apoptosis, we treated primary VS cultures with escalating doses of cleavage-resistant proNGF. After 24 h the cultures were fixed, immunolabeled with anti-S100 antibodies, and apoptotic nuclei were detected with the TUNEL method. The percent of TUNEL-positive VS cell nuclei was determined. Treatment of cultures of primary human VS cells with proNGF (0.1–3 nM) failed to significantly increase the percent of TUNEL-positive VS cell nuclei (Fig. 2). We confirmed that proNGF (0.1 nM) induces apoptosis in non-neoplastic mouse SCs

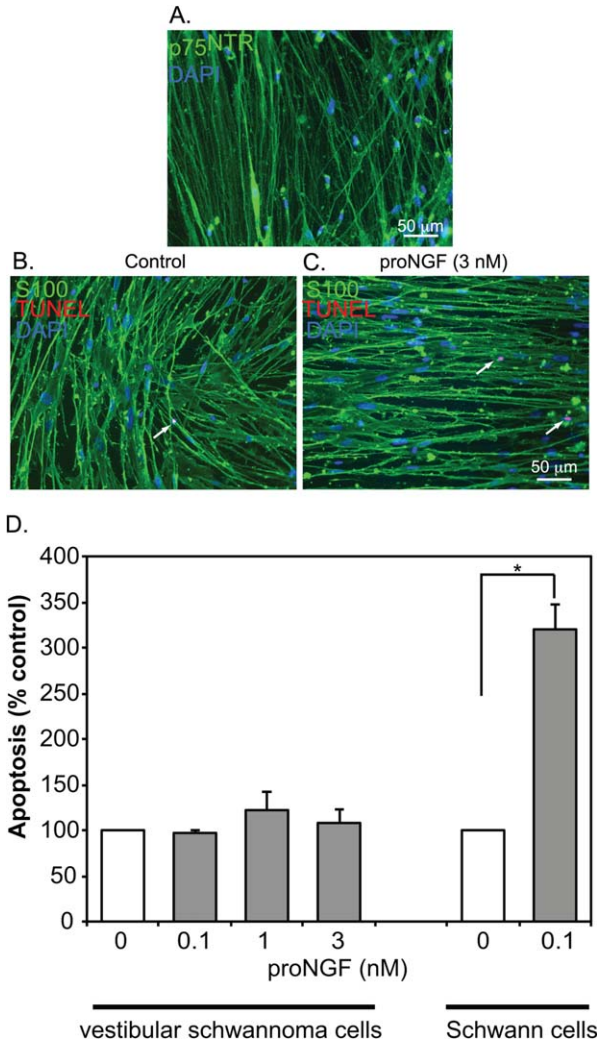


FIGURE 2: ProNGF fails to induce apoptosis in primary VS cultures. (A). Primary VS cultures immunostained with anti-p75^{NTR} antibody (green). Nuclei are labeled with DAPI (blue). (B, C) Primary human VS cultures were treated with escalating doses of proNGF, fixed, immunostained with anti-S100 (green) antibodies and labeled with TUNEL (red) and DAPI (blue). (D) The average percent of TUNEL-positive, S100-positive condensed nuclei from primary human vestibular schwannoma cultures or non-neoplastic mouse Schwann cell cultures were scored and plotted relative to cultures not treated with proNGF (control). Error bars present standard error of the mean. VS data are from cultures derived from six separate patients while data for non-neoplastic Schwann cells were derived from three separate cultures. There was no significant difference between treatment conditions for the vestibular schwannoma cultures by one-way ANOVA. *, $P < 0.01$ by Student's two tailed *t*-test.

(Fig. 2D). These results suggest that VS cells are resistant to proNGF-mediated apoptosis despite high levels of p75^{NTR} expression.

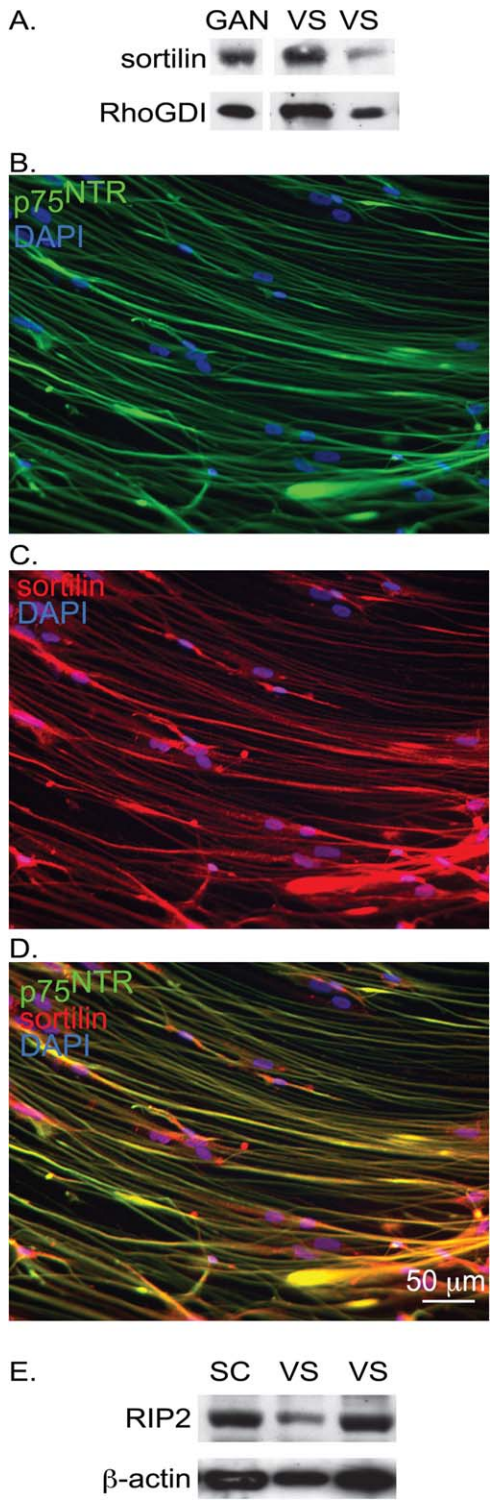
Sortilin functions a p75^{NTR} coreceptor critical for proNGF-mediated apoptosis (Jansen et al., 2007; Skeldal et al., 2012). Immunoblots and immunostaining confirmed that cultured VS cells express sortilin (Fig. 3A–D) Likewise, we confirmed that cultured VS cells and normal rat sciatic nerve SC cultures express RIP2 (Fig. 3E), an adaptor protein with a carboxy-terminal caspase activation and recruitment domain that is necessary for p75^{NTR}-mediated SC apoptosis (Khursigara et al., 2001). These results indicate that cultured VS cells express the necessary coreceptor and RIP2 for proNGF-mediated apoptosis.

ProNGF Protects VS Cells from Apoptosis due to Inhibition of JNK

Because ProNGF induces apoptosis in sympathetic neurons and SCs by activating JNK whereas JNK activity promotes VS cell survival (Linggi et al., 2005; Yue et al., 2011), we next sought to characterize the interaction of p75^{NTR} and JNK signaling on VS cell survival. Primary human VS cultures were treated with I-JIP, a peptide inhibitor that blocks JNK activation by disrupting binding to the JNK scaffolding protein, JIP, or SP600125, a small molecule JNK inhibitor that competitively blocks kinase activity. We have previously shown that these inhibitors effectively and specifically reduce JNK signaling in VS cultures (Yue et al., 2011). A subset of cultures were simultaneously treated with proNGF. As previously reported, I-JIP (30 or 100 μM) and SP600125 (20 μM), significantly increased VS cell apoptosis (Fig. 4A–D). We confirmed apoptosis in cultures by immunoblotting protein lysates from the cultures with antiactive caspase 3 antibody (Fig. 4E). Treatment with proNGF significantly reduced the percent of TUNEL-positive VS cells and the extent of caspase 3 activation in the presence of the JNK inhibitors (Fig. 4A–E). To further verify that p75^{NTR} ligands protect VS cells from apoptosis we treated cultures maintained in the presence of SP600125 with proBNDF. As with proNGF, proBDNF failed to induce VS cell apoptosis and protected the cells from apoptosis due to SP600125 (Fig. 4D). We have previously confirmed the ability of proBDNF to induce apoptosis in non-neoplastic SCs (Provenzano et al., 2008, 2011). To determine whether other potential survival factors could likewise prevent VS cell apoptosis due to JNK inhibition we treated cultures with 3 nM neuregulin β-1, a high affinity ErbB2/B3 ligand and potent growth factor for SC and VS cells (Hansen et al., 2006, 2008; Yue et al., 2011). In contrast to proNGF, neuregulin β-1 significantly increased the percent of apoptotic VS cells (Fig. 4H).

To confirm that proNGF rescues VS cells from apoptosis due to loss of JNK activity, we transfected VS cultures with siRNA oligonucleotides targeting JNK1 and JNK2 and maintained the cultures in the presence or absence of proNGF. We verified that these oligonucleotides effectively and specifically reduce JNK1/2 expression in VS cells (Fig. 4G) as previously shown (Yue et al., 2011). Control cultures were transfected with a scrambled oligonucleotide. As before,

treatment with proNGF significantly reduced the percent of VS cells undergoing apoptosis due to inhibition of JNK signaling (Fig. 4F). We considered the possibility that proNGF could be signaling through Trk receptors, the high affinity receptor tyrosine kinases for mature neurotrophins such as NGF, to promote VS cell survival. However, Trks were not detected in immunoblots of protein lysates from VS cultures using an anti-panTrk antibody capable of detecting all Trk isoforms including TrkA, TrkB, and TrkC (Fig. 4I). The anti-panTrk did detect Trk expression in protein lysates from the rat brain (cerebral cortex). Taken together, these results suggest that $p75^{NTR}$, but not Erb2, signaling promotes survival of VS cells with suppressed JNK activity.



ProNGF Activates NF-κB Independent of JNK

In neuroblastoma cells, $p75^{NTR}$ signaling activates the transcription factor, NF-κB, in a JNK dependent fashion (Costantini et al., 2005). As previously reported (Yue et al., 2011), we confirmed that JNK activity is persistently high in VS cultures due to lack of functional merlin by immunoblotting protein lysates of VS cultures, treated with Ad5.EV or Ad5.merlin, with antibodies that detect phosphorylated JNK (Fig. 5). Treatment with proNGF led to an increase in JNK activity in VS cultures that had not been transduced with adenoviral vectors, reflected by a slight increase in JNK phosphorylation and a further increase in c-Jun phosphorylation (Fig. 5B). We next assayed NF-κB activity in VS cultures. Treatment with proNGF increased NF-κB activity by twofold in VS cells (Fig. 5C) whereas the JNK inhibitor SP600125 decreased NF-κB activity by twofold (Fig. 5C). Significantly, the increase in NF-κB activity induced by proNGF was not attenuated by SP600125. Similarly, transfection of VS cultures with siRNA oligonucleotides targeting JNK1/2 decreased basal levels of NF-κB activity but did not reduce NF-κB activation by proNGF (Fig. 5D). These results suggest that basal levels of NF-κB activity depend on JNK in VS cells whereas proNGF can activate NF-κB independent of JNK signaling.

NF-κB is Required for the Prosurvival Effects of $p75^{NTR}$ Signaling

We next sought to determine whether the activation of NF-κB by proNGF contributes to the ability of $p75^{NTR}$ signaling to promote VS cell survival. To inhibit NF-κB function, we transduced VS cultures with an adenoviral vector that expresses

FIGURE 3: Primary VS cultures express sortilin and RIP2. (A) Western blot of protein lysates from human GAN and human VS specimens probed with anti-sortilin antibodies. The blots were stripped and reprobed with anti-Rho-GDI antibodies to compare protein loading. Blots present the lysates with the highest and lowest sortilin expression compared with Rho-GDI. Bots for lysates from 3 additional tumors demonstrated similar expression levels (not shown). (B–D) Immunostaining of primary VS cultures with anti-$p75^{NTR}$ (B, green) and anti-sortilin (C, red) antibodies (D, combined). Nuclei were labeled with DAPI (blue). (E) Western blot of protein lysates from rat SC and primary human VS cultures probed with anti-RIP2 antibodies. Blots present the lysates with the highest and lowest sortilin expression compared with β-actin. Blots for lysates from two additional cultures from separate tumors demonstrated similar expression levels (not shown).

a mutant isoform of IκBα (Ad5.IκBαS). This isoform is not able to be phosphorylated on S32 and S36 and thus blocks NF-κB activation by preventing the dissociation of IκBα from NF-κB subunits and their subsequent translocation to the nucleus. We have previously shown that Ad5 vectors transduce 85% of VS cells at the titer used here (Yue et al., 2011). Transfection of VS cultures with Ad5.IκBαS effectively inhibited activation of NF-κB by proNGF and reduced basal levels of NF-κB activity (Fig. 6). The fold increase in NF-κB by proNGF was greater in cultures treated with Ad5.EV (Fig. 6)

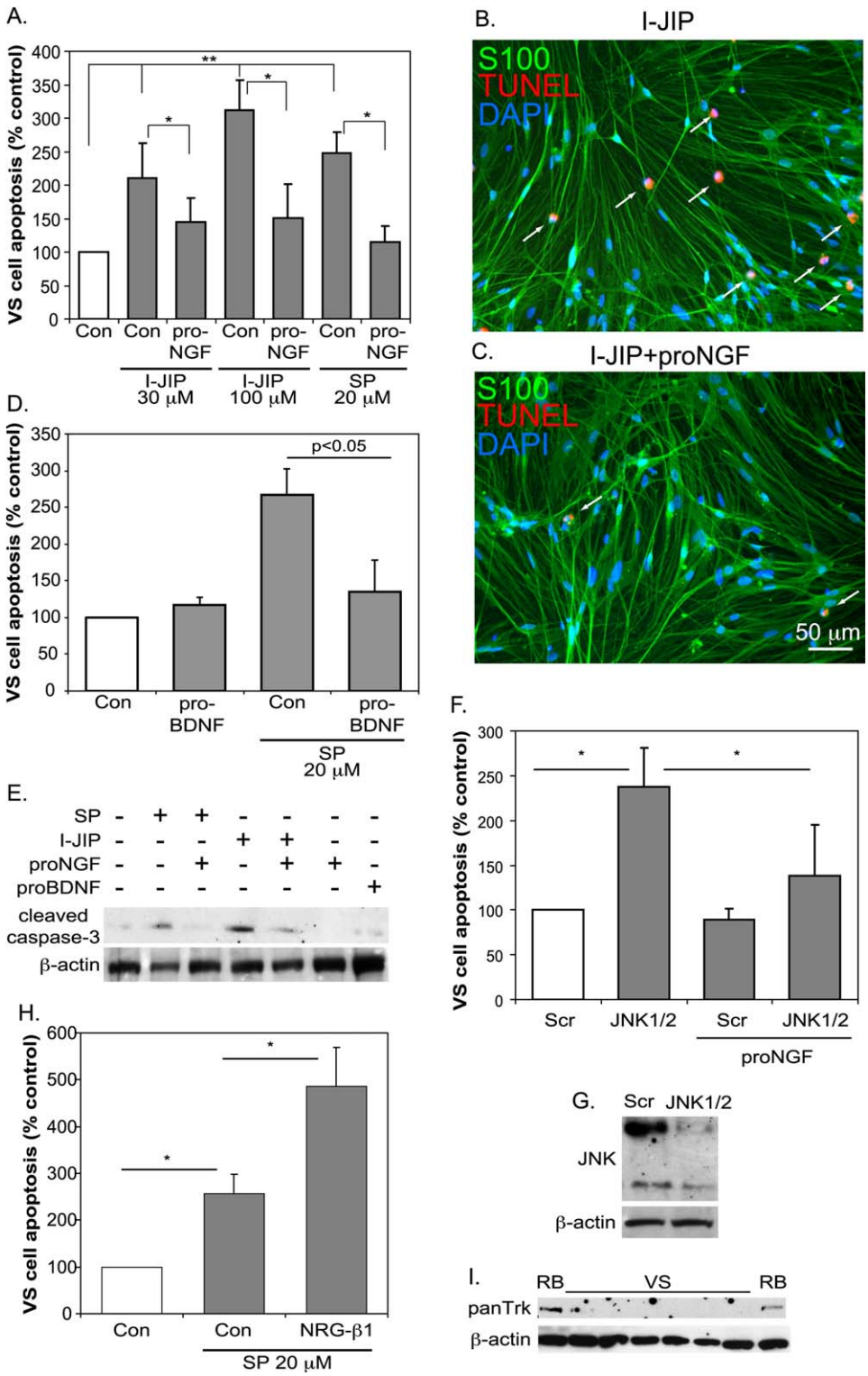


FIGURE 4

compared with cultures maintained without viral vectors (Fig. 5B) raising the possibility that adenoviral vectors sensitize VS cell cultures to upstream activators of NF-κB. In cultures transduced with an Ad5.EV and maintained in SP600125, proNGF significantly reduced the percent of TUNEL-positive VS cells (Fig. 7). The percent of TUNEL-positive VS cells was significantly increased in cultures transduced with Ad5.IκBαS compared with cultures transduced with Ad5.EV even in the presence of proNGF (Fig. 7). Thus, inhibition of NF-κB abolishes the ability of proNGF to rescue VS cells indicating that activation of NF-κB is necessary for the prosurvival effect of $p75^{NTR}$ on VS cells.

Discussion

Limited access to primary tissue has hampered the investigation of human schwannoma tumorigenesis. Transgenic mouse models and transformed cell lines provide useful tools to investigate merlin-dependent tumorigenesis; however, they fail to fully recapitulate human disease including VS formation (Giovannini et al., 1999, 2000; Gutmann and Giovannini, 2002; Hung et al., 2002). Here we used primary tissue derived from acutely resected VSs to investigate the contribution of $p75^{NTR}$ to VS cell responses. Although primary cultures limit some of the analyses that can be performed, they provide a more realistic model of human disease compared with transformed cell lines (e.g. HEI193 cells). We find that $p75^{NTR}$ expression is elevated in VS cells. This elevated expression represents an increase both in transcript and protein levels. Non-neoplastic SCs likewise increase $p75^{NTR}$ expression following axotomy and this increase in $p75^{NTR}$ contributes to SC apoptosis following loss of axonal contact (Ferri and Bisby, 1999; Petratos, 2003; Provenzano et al., 2008; Syroid et al., 2000; Taniuchi et al., 1986). By contrast, activation of $p75^{NTR}$ fails to induce VS cell apoptosis. Further, $p75^{NTR}$ provides an antiapoptotic response in VS cells in contrast to its proapoptotic function in non-neoplastic SCs. This key difference in the response of VS cells to $p75^{NTR}$ likely contributes to the ability of VS cells to proliferate and survive in the absence of axons, whereas non-neoplastic SCs are eventually lost following nerve injury.

Merlin has previously been shown to decrease the expression and, in some cases, alter the subcellular localization of receptor tyrosine kinases such as ErbB1, ErbB2, and platelet-derived growth factor receptors in schwannoma cells and non-neoplastic SCs (Ammoun et al., 2008, 2010; Brown and Hansen, 2008; Doherty et al., 2008; Fernandez-Valle et al., 2002; Fraenzer et al., 2003; Hansen and Linthicum, 2004; Hansen et al., 2006; Houshmandi et al., 2009; Lallemand et al., 2009; Stonecypher et al., 2006; Torres-Martin et al., 2013; Wickremesekera et al., 2007). Thus, ErbB2 expression remains high in VS cells and contributes to cell proliferation and possibly cell survival and tumor growth (Ahmad et al., 2011; Ammoun et al., 2010; Bush et al., 2012; Clark et al., 2008; Doherty et al., 2008; Hansen et al., 2006; Lallemand et al., 2009; Stonecypher et al., 2006; Torres-Martin et al., 2013). In addition to receptor tyrosine kinases, merlin reduces the expression of other transmembrane receptors such as CD44 (Ahmad et al., 2010). The increased $p75^{NTR}$ expression in VS tissue compared with normal nerve raises the possibility that merlin likewise regulates $p75^{NTR}$ expression levels.

In addition to regulating the expression of transmembrane receptors, merlin also suppresses cell motility and proliferation by inhibiting the activity of several intracellular kinase signaling cascades including Ras-Raf-MEK-ERK, PI3-K-Akt, JNK, and mTORC1 (Bosco et al., 2010; Chadee and Kyriakis, 2004; Chadee et al., 2006; Flaiz et al., 2009; Fraenzer et al., 2003; Houshmandi et al., 2009; Jacob et al., 2008; James et al., 2009, 2012; Kaempchen et al., 2003; Kissil et al., 2003; Lim et al., 2003; Lopez-Lago et al., 2009; Rong et al., 2004; Yi et al., 2008; Zhou et al., 2011). Of these, the Ras-Raf-MEK-ERK and PI3-K-Akt and mTORC1 cascades appear to principally promote proliferation in schwannoma cells such that MEK, Akt, and mTORC1 inhibitors provide a cytostatic response, but do not consistently lead to cell death. However, treatment of VS cells with OSU-03012, a celecoxib-derived small-molecule inhibitor of phosphoinositide-dependent kinase-1 and Akt, increased cell

FIGURE 4: ProNGF protects VS cells from apoptosis due to JNK inhibition. (A–D) Primary VS cultures were maintained in the presence of the JNK inhibitors, I-JIP or SP600125, with or without treatment with proNGF (3 nM) (A) of proBDNF (3 nM) (D). Cultures were immunostained with anti-S100 (green) and labeled with TUNEL (red). Nuclei were labeled with DAPI (blue). The average percent of TUNEL-positive, S100-positive condensed nuclei were scored and plotted relative to cultures not treated with JNK inhibitors or proNGF (control). Data are from cultures derived from six separate patients. *, $P < 0.05$, **, $P < 0.01$ by one-way ANOVA followed by Dunn's method. (E) Western blot of protein lysates from primary VS cultures treated with the indicated reagents probed with anticleaved caspase-3 antibodies. Blots were stripped and reprobed with anti-β-actin antibodies. (F) Average percent apoptosis in primary VS cultures transfected with scrambled or anti-JNK1/2 siRNA oligonucleotides and maintained in the presence or absence of proNGF. Comparison for differences among conditions by one-way ANOVA followed by Dunn's method. (G) Western blot of protein lysates from primary VS cultures transfected with scrambled (Scr) or JNK1/2 targeted siRNA oligonucleotides and probed with anti-JNK antibodies. Blots were stripped and reprobed with anti-β-actin antibodies. (H) Average percent apoptosis in primary VS cultures treated with or without neuregulin (NRG) β-1 and maintained in the presence or absence of proNGF. Comparison for differences among conditions by Kruskal-Wallis one-way ANOVA on ranks. (I) Western blot of protein lysates from primary VS and rat brain (RB) specimens with an anti-panTrk antibody. The blots were stripped and reprobed with anti-β actin antibodies.

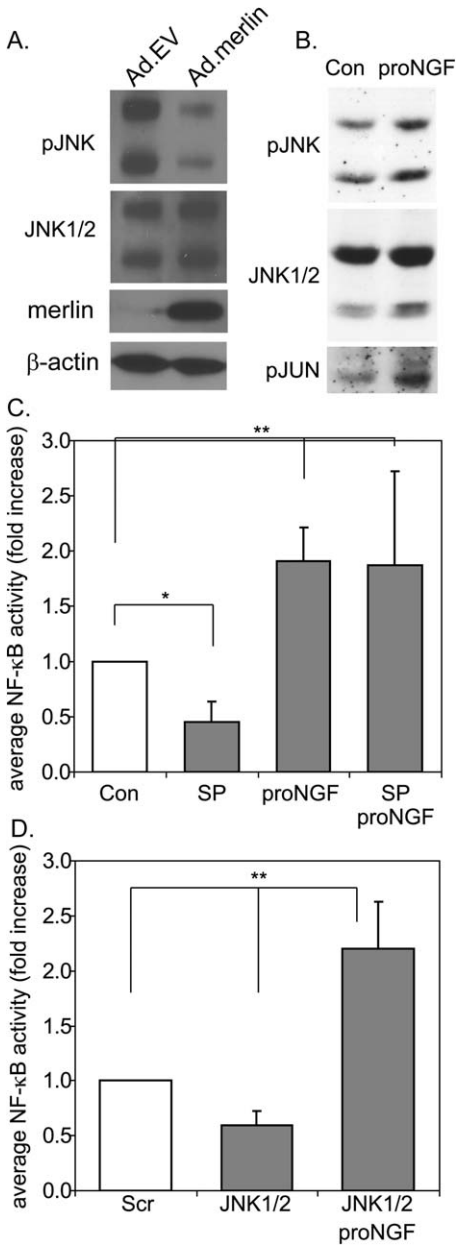


FIGURE 5: ProNGF activates JNK and NF-κB in primary VS cultures. (**A**) Western blots of protein lysates from primary VS cultures treated Ad5.EV or Ad.merlin and probed with anti-phosphorylated JNK antibodies. The blots were stripped and reprobed with non-phospho-specific anti-JNK. Parallel blots were probed with anti-merlin and subsequently with anti-β-actin antibodies. (**B**) Western blots of protein lysates from primary VS cultures treated with proNGF and probed with anti-phosphorylated JNK. The blots were stripped and reprobed with non-phospho-specific anti-JNK and subsequently with anti-phosphorylated c-JUN antibodies. (**C, D**) Average NF-κB activity, relative to control, in primary VS cultures treated with SP600125 (SP) or proNGF or transfected with scrambled or JNK1/2 specific siRNA oligonucleotides. Data in (B) are from cultures derived from six separate patients and in (C) from four separate patients. *, *P* 0.05, **, *P* 0.01 by one-way ANOVA followed by Dunn's method.

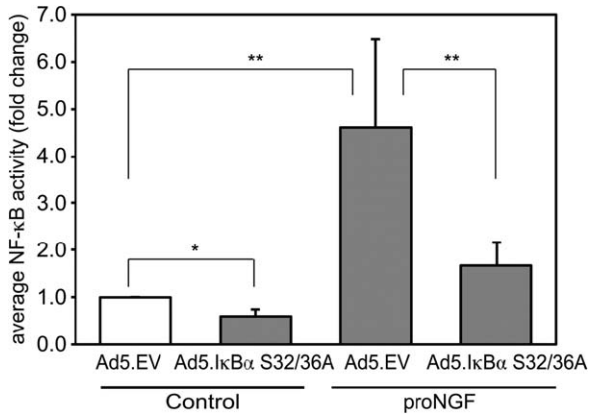


FIGURE 6: Expression of IκBαS32/36A inhibits NF-κB in primary VS cultures. Average NF-κB activity in primary VS cultures transduced with Ad5.EV or Ad5.IκBαS32/36A and treated with proNGF or without proNGF. Activity is expressed as fold change relative to control (Ad5.EV without proNGF). Data are from cultures derived from four separate patients. *, *P* 0.05; **, *P* 0.01 by one-way ANOVA followed by Holm Sidak post hoc method.

death (James et al., 2009; Lee et al., 2009; Yue et al., 2011). Thus, whether Akt promotes VS cell survival in addition to proliferation requires confirmation with more specific,

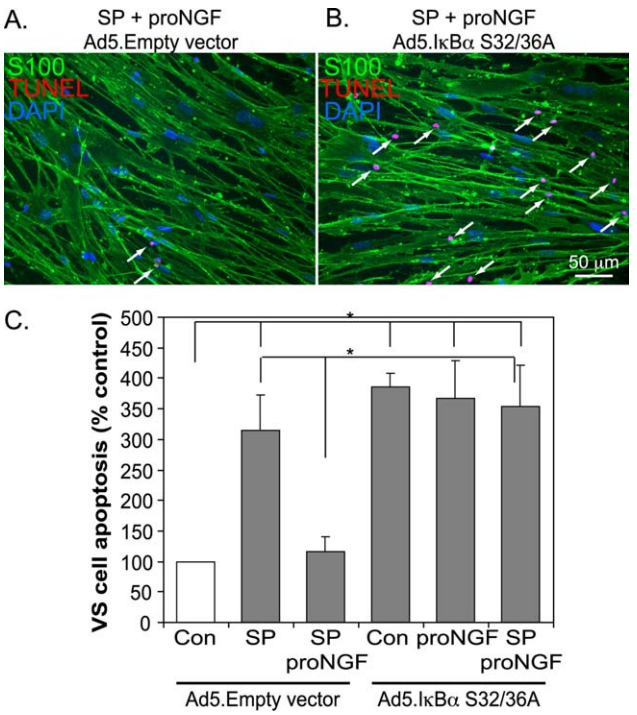


FIGURE 7: Inhibition of NF-κB limits the ability of proNGF to rescue of VS cell from apoptosis. Primary VS cultures were transduced with Ad5.EV or Ad5.IκBαS32/36A and maintained in the presence or absence of SP600125 (SP, 20 μM) with or without treatment with proNGF (3 nM). (**A, B**) Cultures were immunostained with anti-S100 (green) and labeled with TUNEL (red). Nuclei were labeled with DAPI (blue). (**C**) The average percent of TUNEL-positive, S100-positive condensed nuclei were scored and plotted relative to control (Con) cultures. Data are from cultures derived from four separate patients. *, *P* 0.05 by one-way ANOVA followed by Dunn's method.

nonpharmacological methods to suppress Akt activity. Merlin also suppresses proliferation by inhibiting the function of YAP1 and, in the nucleus, CRL4DCAF1 E3 ubiquitin ligase (Baia et al., 2012; Hamaratoglu et al., 2006; Li et al., 2010; Striedinger et al., 2008; Zhang et al., 2010).

Recent reports confirm that JNK is activated in VS cells in a merlin-dependent fashion and contributes to cell proliferation and survival (Hilton et al., 2009; Kaempchen et al., 2003; Yue et al., 2011). Suppression of mitochondrial superoxide accumulation represents one mechanism by which JNK reduces VS cell apoptosis (Yue et al., 2011). Here we demonstrate a requirement for JNK activity to sustain basal levels of NF-κB in VS cells and this likely also contributes to the necessity of JNK activity to support VS cell survival.

VS Cells are Resistant to the Proapoptotic Effect of p75^{NTR} Signaling

The response of cells to p75^{NTR} depends on several factors including expression of coreceptors and the differentiation state of the cell (Casaccia-Bonnefil et al., 1999). In neurons and some carcinoma cells (e.g., breast) that express Trk receptors, activation of p75^{NTR} inhibits apoptosis (Descamps et al., 2001; Hondermarck, 2012; Koshimizu et al., 2010; Lu et al., 2005). In these cases, p75^{NTR} appears to function by facilitating neurotrophin binding to the Trk receptors and Trk signaling (Chao et al., 1998). In cells that lack Trk receptors, including non-neoplastic SCs, mature and pro isoforms of NTs induce apoptosis (Kuchler et al., 2011; Provenzano et al., 2011; Truzzi et al., 2011). In these cells p75^{NTR} appears to activate JNK to induce apoptosis and requires the co-receptor, sortilin (Bhakar et al., 2003; Linggi et al., 2005; Skeldal et al., 2012; Truzzi et al., 2011; Yoon et al., 1998). By contrast, proNGF, which increases JNK activity in VS cells, fails to induce apoptosis in VS cells. One key difference between non-neoplastic SCs and VS cells is that JNK activity induces apoptosis in the former but promotes cell proliferation and survival in the later (Yue et al., 2011). This ability of VS cells to survive in the presence of increased JNK activity may explain, at least in part, the failure of proNGF to induce VS cell apoptosis. Importantly, we confirmed that VS cells express the p75^{NTR} co-receptor, sortilin, and adaptor protein, RIP2, suggesting that the failure of proNGF to induce VS cell apoptosis is not due to the lack of expression of these proteins essential for p75^{NTR}-mediated apoptosis (Charalampopoulos et al., 2012; Khursigara et al., 2001).

p75^{NTR} Mediates an Antiapoptotic Response in VS Cells

The data presented here suggest that proNGF/p75^{NTR} signaling elicits an antiapoptotic response in VS cells and thereby may contribute to tumorigenesis. In addition to their effects on neuronal development, plasticity and injury, there has been a recent recognition that neurotrophins and their receptors contribute to tumorigenesis. For example proNGF stimulates invasion of melanoma cells through a mechanism involving p75^{NTR} and sortilin (Truzzi et al., 2008). In this study, proNGF did not influence melanoma cell survival or proliferation but did promote melanoma cell migration. On the other hand, inhibition of Trks, all of which are expressed in melanoma cells, led to apoptosis in addition to decreasing migration and proliferation in response to neurotrophins. Similarly, p75^{NTR} has been implicated in glioma cell invasion (Johnston et al., 2007; Wang et al., 2010). In breast carcinoma, p75^{NTR} appears to promote cell migration and invasion and, at least in some cell lines, exerts antiapoptotic effects via activation of NF-κB and p21(waf1) (Descamps et al., 2001; Hondermarck, 2012; Verbeke et al., 2010). By contrast, p75^{NTR} inhibits the invasive and metastatic abilities of gastric cancer cells, at least in part, by NF-κB-dependent upregulation of tissue inhibitor of matrix metalloproteinase-1 (Jin et al., 2007).

proNGF Activates NF-κb Independent of JNK to Promote VS Cell Survival

NF-κb plays a critical role in immune responses. It is also widely recognized as a key mediator of tumorigenesis (Baldwin, 2012). Generally, NF-κb promotes tumor formation and growth by supporting cell survival although it has been found to have proapoptotic functions in some circumstances. Likewise, in the nervous system, NF-κb appears to exert either proapoptotic or antiapoptotic effects, depending on the cell type and context (Maggirwar et al., 1998; Mattson et al., 1997; Schneider et al., 1999).

In addition to its role in immune responses and tumorigenesis, NF-κB appears to contribute to normal SC development and response to injury. For example, activation of NF-κB is essential for driving immature SCs into a promyelinating phenotype in dorsal root ganglion-SC co-cultures (Limpert and Carter, 2010; Nickols et al., 2003; Yoon et al., 2008). Peripheral nerve injury significantly increases NF-κB activity and inhibition of NF-κB activation in SCs transiently delays axonal regeneration and compact remyelination (Fernyhough et al., 2005; Fu et al., 2010; Ma and Bisby, 1998; Morton et al., 2012; Pollock et al., 2005). Further, activation of p75^{NTR} by NGF treatment increases NF-κB activity in SCs in a TRAF6 and RIP2-dependent fashion (Carter et al., 1996; Khursigara et al., 1999, 2001; Yeiser et al., 2004). The extent to which the increase of NF-κB activity in SCs following nerve injury results from the concurrent upregulation of p75^{NTR} and activation of JNK remains unknown.

Here we find that p75^{NTR} activates JNK and NF-κB in primary VS cells. Typically, NF-κB activation by p75^{NTR} is

TRAF6 and JNK dependent (Costantini et al., 2005). By contrast, p75^{NTR} activates JNK and promotes apoptosis in keratinocytes but suppresses NF-κB activity (Truzzi et al., 2011). The effect of NF-κB activation by p75^{NTR} on cell survival appears to depend on the cell type. For example, p75^{NTR} activates JNK and NF-κB to induce apoptosis in neuroblastoma cells (Bai et al., 2008; Costantini et al., 2005; Kuner and Hertel, 1998). By contrast, NGF activates JNK and NF-κB in the RN22 schwannoma cell line and induces apoptosis in cells with suppressed NF-κB activity (Gentry et al., 2000). Our results indicate that basal NF-κB activity in primary VS cells depends on JNK activity as both SP600125 and siRNA mediated JNK knock-down reduced NF-κB activity. However, we find that p75^{NTR} can activate NF-κB independent of JNK in VS cells. This activation of NF-κB, even in cells with suppressed JNK activity, appears to contribute to ability of p75^{NTR} to prevent VS cell apoptosis.

NF-κB activation promotes survival of cells treated with many different classes of chemotherapeutics or ionizing radiation and inhibition of NF-κB potently enhances chemo- and radiotoxicity (Wang et al., 1996, 1999). JNK inhibitors represent one class of chemotherapeutics that reduce VS cell proliferation and survival (Yue et al., 2011). The ability of p75^{NTR} to activate NF-κB independent of JNK appears to provide a cytoprotective effect in VS cells treated with JNK inhibitors. Parallel targeting of the p75^{NTR}/NF-κB signaling axis offers the prospect of enhancing the response of VS cells to JNK inhibitors and perhaps to other chemotherapeutics and radiation therapy. One advantage of therapeutically targeting p75^{NTR} function in VS cells is that it would represent a fairly specific target since inhibition of p75^{NTR} signaling supports the survival of non-neoplastic SCs.

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References

Ahmad Z, Brown CM, Patel AK, Ryan AF, Ongkeko R, Doherty JK. 2010. Merlin knockdown in human Schwann cells: Clues to vestibular schwannoma tumorigenesis. Otol Neurotol 31:467–472.

Ahmad ZK, Brown CM, Cueva RA, Ryan AF, Doherty JK. 2011. ErbB expression, activation, and inhibition with lapatinib and tyrphostin (AG825) in human vestibular schwannomas. Otol Neurotol 32:841–847.

Ammoun S, Cunliffe CH, Allen JC, Chiriboga L, Giancotti FG, Zagzag D, Hanemann CO, Karajannis MA. 2010. ErbB/HER receptor activation and preclinical efficacy of lapatinib in vestibular schwannoma. Neuro-oncology 12: 834–843.

Ammoun S, Flaiz C, Ristic N, Schuldt J, Hanemann CO. 2008. Dissecting and targeting the growth factor-dependent and growth factor-independent extracellular signal-regulated kinase pathway in human schwannoma. Cancer Res 68:5236–5245.

Bai Y, Li Q, Yang J, Zhou X, Yin X, Zhao D. 2008. p75(NTR) activation of NF-kappaB is involved in PrP106–126-induced apoptosis in mouse neuroblastoma cells. Neurosci Res 62:9–14.

Baia GS, Caballero OL, Orr BA, Lal A, Ho JS, Cowdrey C, Tihan T, Mawrin C, Riggins GJ. 2012. Yes-associated protein 1 is activated and functions as an oncogene in meningiomas. Mol Cancer Res 10:904–913.

Baldwin AS. 2012. Regulation of cell death and autophagy by IKK and NF-kappaB: Critical mechanisms in immune function and cancer. Immunol Rev 246:327–345.

Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EB, Said F, Bonni A, Barker PA. 2003. Apoptosis induced by p75NTR overexpression requires Jun kinase-dependent phosphorylation of Bad. J Neurosci 23:11373–11381.

Bonetti B, Panzeri L, Carner M, Zamboni G, Rizzuto N, Moretto G. 1997. Human neoplastic Schwann cells: Changes in the expression of neurotrophins and their low-affinity receptor p75. Neuropathol Appl Neurobiol 23:380–386.

Bosco EE, Nakai Y, Hennigan RF, Ratner N, Zheng Y. 2010. NF2-deficient cells depend on the Rac1-canonical Wnt signaling pathway to promote the loss of contact inhibition of proliferation. Oncogene 29:2540–2549.

Bothwell M. 1995. Functional interactions of neurotrophins and neurotrophin receptors. Ann Rev Neurosci 18:223–253.

Brown KD, Hansen MR. 2008. Lipid raft localization of erbB2 in vestibular schwannoma and Schwann cells. Otol Neurotol 29:79–85.

Bush ML, Burns SS, Oblinger J, Davletova S, Chang LS, Welling DB, Jacob A. 2012. Treatment of vestibular schwannoma cells with ErbB inhibitors. Otol Neurotol 33:244–257.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA, Barde YA. 1996. Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. Science 272:542–545.

Casaccia-Bonnefil P, Gu C, Chao MV. 1999. Neurotrophins in cell survival/death decisions. Adv Exp Med Biol 468:275–282.

Chadee DN, Kyriakis JM. 2004. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. Nat Cell Biol 6:770–776.

Chadee DN, Xu D, Hung G, Andalibi A, Lim DJ, Luo Z, Gutmann DH, Kyriakis JM. 2006. Mixed-lineage kinase 3 regulates B-Raf through maintenance of the B-Raf/Raf-1 complex and inhibition by the NF2 tumor suppressor protein. Proc Natl Acad Sci USA 103:4463–4468.

Chao MV. 2003. Neurotrophins and their receptors: A convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309.

Chao M, Casaccia-Bonnefil P, Carter B, Chittka A, Kong H, Yoon SO. 1998. Neurotrophin receptors: Mediators of life and death. Brain Res Brain Res Rev 26:295–301.

Charalampopoulos I, Vicario A, Pediaditakis I, Gravanis A, Simi A, Ibanez CF. 2012. Genetic dissection of neurotrophin signaling through the p75 neurotrophin receptor. Cell Rep 2:1563–1570.

Clark JJ, Provenzano M, Diggelmann HR, Xu N, Hansen SS, Hansen MR. 2008. The ErbB inhibitors trastuzumab and erlotinib inhibit growth of vestibular schwannoma xenografts in nude mice: A preliminary study. Otol Neurotol 29:846–853.

Clewes O, Fahey MS, Tyler SJ, Watson JJ, Seok H, Catania C, Cho K, Dawbarn D, Allen SJ. 2008. Human ProNGF: Biological effects and binding profiles at TrkA, P75NTR and sortilin. J Neurochem 107:1124–1135.

Costantini C, Rossi F, Formaggio E, Bernardoni R, Cecconi D, Della-Bianca V. 2005. Characterization of the signaling pathway downstream p75 neurotrophin receptor involved in beta-amyloid peptide-dependent cell death. J Mol Neurosci 25:141–156.

Descamps S, Toillon RA, Adriaenssens E, Pawlowski V, Cool SM, Nurcombe V, Le Bourhis X, Boilly B, Peyrat JP, Hondermarck H. 2001. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. J Biol Chem 276:17864–17870.

Dobrowski RT, Werner MH, Castellino AM, Chao MV, Hannun YA. 1994. Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596–1599.

Doherty JK, Ongkeko W, Crawley B, Andalibi A, Ryan AF. 2008. ErbB and Nrg: Potential molecular targets for vestibular schwannoma pharmacotherapy. Otol Neurotol 29:50–57.

Evans DG. 2009. Neurofibromatosis 2 [Bilateral acoustic neurofibromatosis, central neurofibromatosis, NF2, neurofibromatosis type II]. Genet Med 11: 599–610.

Fernandez-Valle C, Tang Y, Ricard J, Rodenas-Ruano A, Taylor A, Hackler E, Biggerstaff J, Iacovelli J. 2002. Paxillin binds schwannomin and regulates its density-dependent localization and effect on cell morphology. Nat Genet 31: 354–362.

Fernyhough P, Smith DR, Schapansky J, Van Der Ploeg R, Gardiner NJ, Tweed CW, Kontos A, Freeman L, Purves-Tyson TD, Glazner GW. 2005. Activation of nuclear factor-kappaB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons. J Neurosci 25:1682–1690.

Ferri CC, Bisby MA. 1999. Improved survival of injured sciatic nerve Schwann cells in mice lacking the p75 receptor. Neurosci Lett 272:191–194.

Flaiz C, Chernoff J, Ammoun S, Peterson JR, Hanemann CO. 2009. PAK kinase regulates Rac GTPase and is a potential target in human schwannomas. Exp Neurol 218:137–144.

Fraenzer JT, Pan H, Minimo L, Jr., Smith GM, Knauer D, Hung G. 2003. Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation. Int J Oncol 23:1493–1500.

Friedman WJ. 2000. Neurotrophins induce death of hippocampal neurons via the p75 receptor. J Neurosci 20:6340–6346.

Fu ES, Zhang YP, Sagen J, Candiotti KA, Morton PD, Liebl DJ, Bethea JR, Brambilla R. 2010. Transgenic inhibition of glial NF-kappa B reduces pain behavior and inflammation after peripheral nerve injury. Pain 148:509–518.

Gentry JJ, Casaccia-Bonnefil P, Carter BD. 2000. Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells. J Biol Chem 275:7558–7565.

Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, van der Valk M, Woodruff JM, Goutebroze L, Merel P, Berns A, Thomas G. 1999. Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. Genes Dev 13:978–986.

Giovannini M, Robanus-Maandag E, van der Valk M, Niwa-Kawakita M, Abramowski V, Goutebroze L, Woodruff JM, Berns A, Thomas G. 2000. Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. Genes Dev 14:1617–1630.

Gutmann DH, Giovannini M. 2002. Mouse models of neurofibromatosis 1 and 2. Neoplasia 4:279–290.

Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, Jafar-Nejad H, Halder G. 2006. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat Cell Biol 8:27–36.

Hansen MR, Clark JJ, Gantz BJ, Goswami PC. 2008. Effects of ErbB2 signaling on the response of vestibular schwannoma cells to gamma-irradiation. Laryngoscope 118:1023–1030.

Hansen MR, Linthicum FH, Jr. 2004. Expression of neuregulin and activation of erbB receptors in vestibular schwannomas: Possible autocrine loop stimulation. Otol Neurotol 25:155–159.

Hansen MR, Roehm PC, Chatterjee P, Green SH. 2006. Constitutive neuregulin-1/ErbB signaling contributes to human vestibular schwannoma proliferation. Glia 53:593–600.

Harrington AW, Kim JY, Yoon SO. 2002. Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22: 156–166.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678–683.

Hilton DA, Ristic N, Hanemann CO. 2009. Activation of ERK, AKT and JNK signalling pathways in human schwannomas in situ. Histopathology 55:744–749.

Hirata H, Hibasami H, Yoshida T, Ogawa M, Matsumoto M, Morita A, Uchida A. 2001. Nerve growth factor signaling of p75 induces differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves. Glia 36:245–58.

Hondermarck H. 2012. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev 23:357–365.

Houshmandi SS, Emnett RJ, Giovannini M, Gutmann DH. 2009. The neurofibromatosis 2 protein, merlin, regulates glial cell growth in an ErbB2- and Src-dependent manner. Mol Cell Biol 29:1472–1486.

Hung G, Li X, Faudoa R, Xeu Z, Kluwe L, Rhim JS, Slattery W, Lim D. 2002. Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2. Int J Oncol 20:475–482.

Jacob A, Lee TX, Neff BA, Miller S, Welling B, Chang LS. 2008. Phosphatidylinositol 3-kinase/AKT pathway activation in human vestibular schwannoma. Otol Neurotol 29:58–68.

James MF, Han S, Polizzano C, Plotkin SR, Manning BD, Stemmer-Rachamimov AO, Gusella JF, Ramesh V. 2009. NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. Mol Cell Biol 29:4250–4261.

James MF, Stivison E, Beauchamp R, Han S, Li H, Wallace MR, Gusella JF, Stemmer-Rachamimov AO, Ramesh V. 2012. Regulation of mTOR complex 2 signaling in neurofibromatosis 2-deficient target cell types. Mol Cancer Res : MCR 10:649–659.

Jansen P, Giehl K, Nyengaard JR, Teng K, Lioubinski O, Sjoegaard SS, Breiderhoff T, Gotthardt M, Lin F, Eilers A, et al. 2007. Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. Nat Neurosci 10:1449–1457.

Jin H, Pan Y, He L, Zhai H, Li X, Zhao L, Sun L, Liu J, Hong L, Song J, et al. 2007. p75 neurotrophin receptor inhibits invasion and metastasis of gastric cancer. Mol Cancer Res 5:423–433.

Johnston AL, Lun X, Rahn JJ, Liacini A, Wang L, Hamilton MG, Parney IF, Hempstead BL, Robbins SM, Forsyth PA, et al. 2007. The p75 neurotrophin receptor is a central regulator of glioma invasion. PLoS Biol 5:e212.

Kaempchen K, Mielke K, Utermark T, Langmesser S, Hanemann CO. 2003. Upregulation of the Rac1/JNK signaling pathway in primary human schwannoma cells. Hum Mol Genet 12:1211–1221.

Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD. 2006. Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219–322.

Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS, Chao MV. 2001. A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. J Neurosci 21:5854–5863.

Khursigara G, Orlinick JR, Chao MV. 1999. Association of the p75 neurotrophin receptor with TRAF6. J Biol Chem 274:2597–2600.

Kissil JL, Wilker EW, Johnson KC, Eckman MS, Yaffe MB, Jacks T. 2003. Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1. Mol Cell 12:841–849.

Koshimizu H, Hazama S, Hara T, Ogura A, Kojima M. 2010. Distinct signaling pathways of precursor BDNF and mature BDNF in cultured cerebellar granule neurons. Neurosci Lett 473:229–232.

Kuchler J, Hartmann W, Waha A, Koch A, Endl E, Wurst P, Kindler D, Mikeska T, Goodyer CG, Buttner R, et al. 2011. p75(NTR) induces apoptosis in medulloblastoma cells. International journal of cancer Journal Int du cancer 128:1804–1812.

Kuner P, Hertel C. 1998. NGF induces apoptosis in a human neuroblastoma cell line expressing the neurotrophin receptor p75NTR. J Neurosci Res 54: 465–474.

Lallemand D, Manent J, Couvelard A, Watilliaux A, Siena M, Chareyre F, Lampin A, Niwa-Kawakita M, Kalamarides M, Giovannini M. 2009. Merlin regulates transmembrane receptor accumulation and signaling at the plasma

membrane in primary mouse Schwann cells and in human schwannomas. Oncogene 28:854–865.

Laskin WB, Fetsch JF, Lasota J, Miettinen M. 2005. Benign epithelioid peripheral nerve sheath tumors of the soft tissues: Clinicopathologic spectrum of 33 cases. Am J Surg Pathol 29:39–51.

Lee R, Kermani P, Teng KK, Hempstead BL. 2001. Regulation of cell survival by secreted proneurotrophins. Science 294:1945–1958.

Lee TX, Packer MD, Huang J, Akhmametyeva EM, Kulp SK, Chen CS, Giovannini M, Jacob A, Welling DB, Chang LS. 2009. Growth inhibitory and anti-tumour activities of OSU-03012, a novel PDK-1 inhibitor, on vestibular schwannoma and malignant schwannoma cells. Eur J Cancer 45:1709–1720.

Li W, Cooper J, Karajannis MA, Giancotti FG. 2012. Merlin: A tumour suppressor with functions at the cell cortex and in the nucleus. EMBO Rep.

Li W, You L, Cooper J, Schiavon G, Pepe-Caprio A, Zhou L, Ishii R, Giovannini M, Hanemann CO, Long SB, et al. 2010. Merlin/NF2 suppresses tumorigenesis by inhibiting the E3 ubiquitin ligase CRL4(DCAF1) in the nucleus. Cell 140:477–490.

Lim JY, Kim H, Kim YH, Kim SW, Huh PW, Lee KH, Jeun SS, Rha HK, Kang J. 2003. Merlin suppresses the SRE-dependent transcription by inhibiting the activation of Ras-ERK pathway. Biochem Biophys Res Commun 302:238–245.

Limpert AS, Carter BD. 2010. Axonal neuregulin 1 type III activates NF-kappaB in Schwann cells during myelin formation. J Biol Chem 285:16614–16622.

Linggi MS, Burke TL, Williams BB, Harrington A, Kraemer R, Hempstead BL, Yoon SO, Carter BD. 2005. Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. J Biol Chem 280:13801–13808.

Lopez-Lago MA, Okada T, Murillo MM, Socci N, Giancotti FG. 2009. Loss of the tumor suppressor gene NF2, encoding merlin, constitutively activates integrin-dependent mTORC1 signaling. Mol Cell Biol 29:4235–49.

Lu B, Pang PT, Woo NH. 2005. The yin and yang of neurotrophin action. Nat Rev Neurosci 6:603–614.

Ma W, Bisby MA. 1998. Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Res 797:243–254.

Maggirwar SB, Sarmiere PD, Dewhurst S, Freeman RS. 1998. Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. J Neurosci 18:10356–10365.

Masoudi R, Ioannou MS, Coughlin MD, Pagadala P, Neet KE, Clewes O, Allen SJ, Dawbarn D, Fahnestock M. 2009. Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. J Biol Chem 284:18424–18433.

Mattson MP, Goodman Y, Luo H, Fu W, Furukawa K. 1997. Activation of NF-kappaB protects hippocampal neurons against oxidative stress-induced apoptosis: Evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. J Neurosci Res 49:681–697.

McClatchey AI, Giovannini M. 2005. Membrane organization and tumorigenesis—The NF2 tumor suppressor, Merlin. Genes Dev 19:2265–2277.

Miettinen M, Shekitka KM, Sobin LH. 2001. Schwannomas in the colon and rectum: A clinicopathologic and immunohistochemical study of 20 cases. Am J Surg Pathol 25:846–855.

Morton PD, Johnstone JT, Ramos AY, Liebl DJ, Bunge MB, Bethea JR. 2012. Nuclear factor-kappaB activation in Schwann cells regulates regeneration and remyelination. Glia 60:639–650.

Nickols JC, Valentine W, Kanwal S, Carter BD. 2003. Activation of the transcription factor NF-kappaB in Schwann cells is6 required for peripheral myelin formation. Nat Neurosci 6:161–7.

Parkhurst CN, Zampieri N, Chao MV. 2010. Nuclear localization of the p75 neurotrophin receptor intracellular domain. J Biol Chem 285:5361–5368.

Parkinson DB, Dong Z, Bunting H, Whitfield J, Meier C, Marie H, Mirsky R, Jessen KR. 2001. Transforming growth factor beta (TGFbeta) mediates Schwann cell death in vitro and in vivo: Examination of c-Jun activation, interactions with survival signals, and the relationship of TGFbeta-mediated death to Schwann cell differentiation. J Neurosci 21:8572–8585.

Petratos S BH, Shipham K, Cooper H, Bucci T, Reid K, Lopez E, Emery B, Cheema SS, Kilpatrick TJ. 2003. Schwann cell apoptosis in the postnatal axotomized sciatic nerve is mediated via NGF through the low-affinity neurotrophin receptor. J Neuropathol Exp Neurol 62:398–411.

Pollock G, Pennypacker KR, Memet S, Israel A, Saporta S. 2005. Activation of NF-kappaB in the mouse spinal cord following sciatic nerve transection. Exp Brain Res 165:470–477.

Provenzano MJ, Minner SA, Zander K, Clark JJ, Kane CJ, Green SH, Hansen MR. 2011. p75(NTR) expression and nuclear localization of p75(NTR) intracellular domain in spiral ganglion Schwann cells following deafness correlate with cell proliferation. Mol Cell Neurosci 47:306–315.

Provenzano MJ, Xu N, Ver Meer MR, Clark JJ, Hansen MR. 2008. p75NTR and sortilin increase after facial nerve injury. The Laryngoscope 118:87–93.

Rong R, Tang X, Gutmann DH, Ye K. 2004. Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. Proc Natl Acad Sci USA 101:18200–18205.

Roosli C, Linthicum FH Jr., Cureoglu S, Merchant SN. 2012. What is the site of origin of cochleovestibular schwannomas? Audiol Neuro-otol 17:121–125.

Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B, et al. 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 363:515–521.

Roux PP, Barker PA. 2002. Neurotrophin signaling through the p75 neurotrophin receptor. Progr Neurobiol 67:203–233.

Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. 1999. NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med 5:554–559.

Scoles DR. 2008. The merlin interacting proteins reveal multiple targets for NF2 therapy. Biochim Biophys Acta 1785:32–54.

Skeldal S, Sykes AM, Glerup S, Matusica D, Palstra N, Autio H, Boskovic Z, Madsen P, Castren E, Nykjaer A, et al. 2012. Mapping of the interaction site between sortilin and the p75 neurotrophin receptor reveals a regulatory role for the sortilin intracellular domain in p75 neurotrophin receptor shedding and apoptosis. J Biol Chem 287:43798–43809.

Soilu-Hanninen M, Ekert P, Bucci T, Syroid D, Bartlett PF, Kilpatrick TJ. 1999. Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. J Neurosci 19:4828–4838.

Stemmer-Rachamimov AO, Xu L, Gonzalez-Agosti C, Burwick JA, Pinney D, Beauchamp R, Jacoby LB, Gusella JF, Ramesh V, Louis DN. 1997. Universal absence of merlin, but not other ERM family members, in schwannomas. Am J Pathol 151:1649–1654.

Stonecypher MS, Chaudhury AR, Byer SJ, Carroll SL. 2006. Neuregulin growth factors and their ErbB receptors form a potential signaling network for schwannoma tumorigenesis. J Neuropathol Exp Neurol 65:162–175.

Striedinger K, VandenBerg SR, Baia GS, McDermott MW, Gutmann DH, Lal A. 2008. The neurofibromatosis 2 tumor suppressor gene product, merlin, regulates human meningioma cell growth by signaling through YAP. Neoplasia 10:1204–1212.

Syroid DE, Maycox PJ, Soilu-Hanninen M, Petratos S, Bucci T, Burrola P, Murray S, Cheema S, Lee KF, Lemke G, et al. 2000. Induction of postnatal Schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy. J Neurosci 20:5741–5747.

Taniuchi M, Clark HB, Johnson EM, Jr. 1986. Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc Natl Acad Sci USA 83:4094–4098.

Torres-Martin M, Lassaletta L, San-Roman-Montero J, De Campos JM, Isla A, Gavilan J, Melendez B, Pinto GR, Burbano RR, Castresana JS, et al. 2013. Microarray analysis of gene expression in vestibular schwannomas reveals SPP1/MET signaling pathway and androgen receptor deregulation. Int J Oncol 42:848–862.

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K, et al. 1993. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 72:791–800.

Truzzi F, Marconi A, Atzei P, Panza MC, Lotti R, Dallaglio K, Tiberio R, Palazzo E, Vaschieri C, Pincelli C. 2011. p75 neurotrophin receptor mediates apoptosis in transit-amplifying cells and its overexpression restores cell death in psoriatic keratinocytes. Cell Death Differ 18:948–958.

Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, Pincelli C. 2008. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. J Investig Dermatol 128:2031–2040.

Verbeke S, Meignan S, Lagadec C, Germain E, Hondermarck H, Adriaenssens E, Le Bourhis X. 2010. Overexpression of p75(NTR) increases survival of breast cancer cells through p21(waf1). Cell Signal 22:1864–1873.

Wang CY, Cusack JC, Jr., Liu R, Baldwin AS, Jr. 1999. Control of inducible chemoresistance: Enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med 5:412–417.

Wang CY, Mayo MW, Baldwin AS, Jr. 1996. TNF- and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kappaB. Science 274: 784–787.

Wang X, Cui M, Wang L, Chen X, Xin P. 2010. Inhibition of neurotrophin receptor p75 intramembran proteolysis by gamma-secretase inhibitor reduces medulloblastoma spinal metastasis. Biochem Biophys Res Commun 403:264–269.

Welling DB, Packer MD, Chang LS. 2007. Molecular studies of vestibular schwannomas: A review. Curr Opin Otolaryngol Head Neck Surg 15:341–346.

Wickremesekera A, Hovens CM, Kaye AH. 2007. Expression of ErbB-1 and 2 in vestibular schwannomas. J Clin Neurosci 14:1199–206.

Xiao GH, Chernoff J, Testa JR. 2003. NF2: The wizardry of merlin. Genes Chromosomes Cancer 38:389–399.

Yeiser EC, Rutkoski NJ, Naito A, Inoue J, Carter BD. 2004. Neurotrophin signaling through the p75 receptor is deficient in traf6−/− mice. J Neurosci 24: 10521–10529.

Yi C, Wilker EW, Yaffe MB, Stemmer-Rachamimov A, Kissil JL. 2008. Validation of the p21-activated kinases as targets for inhibition in neurofibromatosis type 2. Cancer Res 68:7932–7937.

Yoon C, Korade Z, Carter BD. 2008. Protein kinase A-induced phosphorylation of the p65 subunit of nuclear factor-kappaB promotes Schwann cell differentiation into a myelinating phenotype. J Neurosci 28:3738–3746.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV. 1998. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 18:3273–3281.

Yue WY, Clark JJ, Fernando A, Domann F, Hansen MR. 2011. Contribution of persistent C-Jun N-terminal kinase activity to the survival of human vestibular schwannoma cells by suppression of accumulation of mitochondrial superoxides. Neuro-oncology 13:961–973.

Zhang N, Bai H, David KK, Dong J, Zheng Y, Cai J, Giovannini M, Liu P, Anders RA, Pan D. 2010. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Dev cell 19: 27–38.

Zhou L, Ercolano E, Ammoun S, Schmid MC, Barczyk MA, Hanemann CO. 2011. Merlin-deficient human tumors show loss of contact inhibition and activation of Wnt/beta-catenin signaling linked to the PDGFR/Src and Rac/PAK pathways. Neoplasia 13:1101–1112.

Zhou L, Hanemann CO. 2012. Merlin, a multi-suppressor from cell membrane to the nucleus. FEBS Lett 586:1403–1408.

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Merlin status regulates p75^{NTR} expression and apoptotic signaling in Schwann cells following nerve injury☆

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ABSTRACT

After nerve injury, Schwann cells (SCs) dedifferentiate, proliferate, and support axon regrowth. If axons fail to regenerate, denervated SCs eventually undergo apoptosis due, in part, to increased expression of the low-affinity neurotrophin receptor, p75^{NTR}. Merlin is the protein product of the *NF2* tumor suppressor gene implicated in SC tumorigenesis. Here we explore the contribution of merlin to SC responses to nerve injury. We find that merlin becomes phosphorylated (growth permissive) in SCs following acute axotomy and following gradual neural degeneration in a deafness model, temporally correlated with increased p75^{NTR} expression. p75^{NTR} levels are elevated in P0SchΔ39-121 transgenic mice that harbor an *Nf2* mutation in SCs relative to wild-type mice before axotomy and remain elevated for a longer period of time following injury. Replacement of wild-type, but not phospho-mimetic (S518D), merlin isoforms suppresses p75^{NTR} expression in primary human schwannoma cultures which otherwise lack functional merlin. Despite elevated levels of p75^{NTR}, SC apoptosis following axotomy is blunted in P0SchΔ39-121 mice relative to wild-type mice suggesting that loss of functional merlin contributes to SC resistance to apoptosis. Further, cultured SCs from mice with a tamoxifen-inducible knock-out of *Nf2* confirm that SCs lacking functional merlin are less sensitive to p75^{NTR}-mediated cell death. Taken together these results point to a model whereby loss of axonal contact following nerve injury results in merlin phosphorylation leading to increased p75^{NTR} expression. Further, they demonstrate that merlin facilitates p75^{NTR}-mediated apoptosis in SCs helping to explain how neoplastic SCs that lack functional merlin survive long-term in the absence of axonal contact.

Introduction

Peripheral nerve injury results in axon degeneration distal to the site of injury (Wallerian degeneration) (Stoll and Muller, 1999; Lorenzetto et al., 2008). Following loss of axonal contact, denervated Schwann cells (SCs) undergo a series of events, including dedifferentiation and proliferation, and provide support for eventual axonal regrowth (Chen et al., 2007). They then redifferentiate and remyelinate regenerated axons as part of the repair process (Chen et al., 2007). However, SCs that remain isolated from neural elements following nerve injury eventually die. This SC loss, among other factors, complicates attempts to restore neural function after injury (Hoffman, 1992). Following denervation, SCs dramatically increase expression of the low-affinity neurotrophin receptor, p75^{NTR}, which promotes SC apoptosis (Taniuchi et al., 1986; Ferri and Bisby, 1999).

p75^{NTR} promotes SC apoptosis following denervation

p75^{NTR} is a single pass transmembrane receptor implicated in a wide variety of cellular responses including differentiation, growth, apoptosis and survival depending on the context and co-receptors (Parkhurst et al., 2010). In neurons, it frequently functions as a co-receptor with Trks to bind mature neurotrophins and promote neuronal survival (Chao and Hempstead, 1995). However, in the absence of Trk receptors, p75^{NTR} often interacts with other co-receptors, including sortilin or Nogo, to mediate cell death (Bandtlow and Dechant, 2004; Barker, 2004). Although p75^{NTR} binds with relative low affinity to mature neurotrophins in the absence of Trk receptors, it binds proforms of neurotrophins with high affinity (Barker, 2004). Following ligand binding, p75^{NTR} undergoes intramembrane cleavage by γ-secretase to generate an intracellular domain (ICD) fragment (Jung et al., 2003; Kanning et al., 2003; Kenchappa et al., 2006). The ICD contains a death domain that functions as docking site necessary for the activation of TNF and Fas ligand and leads to c-Jun N-terminal kinase (JNK) activation

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(Haase et al., 2008). Further, γ-secretase-mediated cleavage results in nuclear translocation of NRIF, a DNA binding protein essential for p75-mediated apoptosis (Kenchappa et al., 2006). Recent data indicate that schwannoma cells express high levels of $p75^{NTR}$ yet, in contrast to non-neoplastic SCs, are resistant to $p75^{NTR}$-mediated apoptosis (Ahmad et al., 2014).

The tumor suppressor, merlin, regulates SC proliferation and neoplasia

Merlin is the protein product of the *NF2* tumor suppressor gene. Loss of *NF2* gene function underlies development of neurofibromatosis type 2 (NF2)-associated and sporadic schwannomas (Rouleau et al., 1993; Trofatter et al., 1993; Irving et al., 1994). Merlin mediates cell–cell contact to suppress cell proliferation. The N- and C-termini of merlin interact with each other as merlin alternates between growth permissive and growth suppressive conformations depending on the phosphorylation of serine residues. For example, S518 phosphorylation leads to a conformation that facilitates cell growth (Gutmann et al., 1999). The tumor suppressor function becomes active after S518 dephosphorylation (Okada et al., 2007). Merlin regulates a wide variety of signaling events to suppress cell growth (Li et al., 2010). However, the function of merlin in normal SCs and their response to injury remains largely unknown. Here we explore the possibility that merlin plays a fundamental role in $p75^{NTR}$-mediated SCs responses to loss of axonal contact. We find that merlin suppresses $p75^{NTR}$ expression in a phosphorylation dependent fashion and that merlin facilitates SC cell death in response to $p75^{NTR}$ ligands.

Materials and methods

Mice strains

P0SchΔ(39–121) and $Nf2^{f/f}$ mice were obtained from Riken BioResource Center (Tsukuba, Japan) (Giovannini et al., 1999, 2000) and RosaCRE-ER^{T2} and FVB mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The RosaCRE-ER^{T2} mice contain a tamoxifen-inducible Cre recombinase system. We crossed the $Nf2^{f/f}$ mice with RosaCRE-ER^{T2} mice, producing an F1 generation of mice carrying a Cre-inducible *loxP* sequence that is site specific for the *Nf2* gene. Animals of either sex were used for all experiments. All animal work was approved by the University of Iowa Institutional Animal Care and Use Committee.

Sciatic nerve axotomies and protein lysates

Sciatic nerve (SN) axotomies were performed in adult rats and adult FVB control (WT) and P0SchΔ39-121 mice as previously described (Brown and Hansen, 2008). In short, a small horizontal skin incision was made over the quadriceps muscle. The muscle was then bluntly dissected and the SN was identified. The SN was dissected proximally and transected approximately 1 cm from the spinal cord and the cut ends of the nerve were displaced into separate planes of tissue to prevent reattachment. The contralateral SN remained uncut as the control. The distal portion of the SN and the contralateral intact nerve were excised and on post-axotomy (PA) days 7, 21, and 180. These time points were chosen to correlate with periods of increased $p75^{NTR}$ expression and cellular proliferation (PA7-21) as well as a late phase when SCs are no longer being replenished (see Figs. 3, 5, and 6). Three nerve samples at each time point were pooled and immediately placed in a modified RIPA lysis buffer solution on ice. A sterilized ground glass mortar and pestle was used to crush the nerve. The crude lysate was sonicated (1 s pulses for 30 s) and then incubated on ice for 30 min. The homogenate was cleared by centrifugation at 18,000 × *g* for 10 min at 4 °C. The lysate was then aliquoted and stored at −80 °C.

Kanamycin deafening

Sprague Dawley rats were obtained from Charles River. Deafening was performed as previously described by injecting kanamycin, which is toxic to hair cells, from P8–16 (Provenzano et al., 2011). Deafness was confirmed by elevated auditory brainstem response thresholds in a subset of animals and by a lack of MyoVIIA-positive hair cells in all animals.

Human vestibular schwannoma and mouse Schwann cell cultures

Primary human VS cultures were prepared from acutely resected tumors as previously described (Hansen et al., 2006; Yue et al., 2011). None of the cultures were derived from neurofibromatosis type II-associated tumors. The cultures were not passaged prior to experimental manipulation. Adenoviral-mediated gene transfer was also performed as previously described with Ad5.Emptyvector-GFP, Ad5-wild-type merlin-GFP, Ad5-merlinS518A-GFP, and Ad5-merlinS518D-GFP (Yue et al., 2011; Ahmad et al., 2014). Live cultures were monitored for GFP fluorescence to ensure that over 80% of the cells had been successfully transduced. After 48 h, protein lysates were prepared and immunoblotted.

SC cultures from the sciatic nerves of P3–4 neonatal RosaCre:$NF2^{f/f}$ mice were prepared as previously described (Provenzano et al., 2008). Once the cultures were 70–80% confluent, they were treated with tamoxifen (500 nM, Sigma-Aldrich, St. Louis, MO) or vehicle. The cultures were washed and the tamoxifen was removed with a media change after 72 h. Cultures were then maintained for another 24 h with or without proNGF (3 nM) and then fixed for 10 min with 4% paraformaldehyde.

Western blot

Western blots from the nerve lysates or culture lysates were performed as previously described (Hansen et al., 2006). The blot was probed with anti-$p75^{NTR}$ antibody (generous gift of Dr. Moses Chao) and stripped and reprobed with anti-β-actin antibodies (Sigma-Aldrich) to confirm equal protein loading and to determine relative $p75^{NTR}$ levels. Parallel blots were probed with anti-phosphomerlin (p-merlin) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and stripped and reprobed with non phosphospecific anti-merlin antibodies (Santa Cruz Biotechnology) to determine the relative phosphorylation level of merlin. The experiment was repeated at least 4 times (total of 12 animals) for each time point. Western blots were quantified as previously described using ImageJ software (NIH) (Yue et al., 2011; Ahmad et al., 2014).

Immunohistochemistry

Following axotomy a small portion of each nerve was resected and placed in 4% paraformaldehyde, cryoprotected in a serial sucrose gradient, embedded and frozen in OCT, and finally cryosectioned as previously described (Provenzano et al., 2008). We performed immunostaining of frozen sections (~10 μm thick) with anti-$p75^{NTR}$ and anti-phosphomerlin antibodies to determine the spatial distribution of SCs that express $p75^{NTR}$ and phosphorylated merlin. Subsets of sections were immunostained with anti-neurofilament 200 (NF200) antibodies (Sigma, St. Louis, MO) to confirm loss of axons and correlate $p75^{NTR}$ and phosphorylated merlin expression with axonal contact. Sections were mounted with Prolong Gold + DAPI (Life Technologies, Carlsbad, CA) prior to coverslipping. Images were captured on a Leica DMIRE 2 microscope (Leica Microsystems, Bannockburn, IL) equipped with epifluorescent filters and a cooled CCD camera using Metamorph software (Molecular Devices, LLC, Downington, PA) or with a Leica TCS SP5 confocal microscope (Leica Microsystems, Bannockburn, IL).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Frozen nerve sections and SC cultures were labeled with dUTP for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect apoptotic nuclei as previously described (Provenzano et al., 2008, 2011). All samples were counterlabeled with S100 and nuclei were labeled with DAPI prior to coverslipping. Criteria for scoring apoptotic cells included: S100-positive, TUNEL-positive nucleus, and a condensed or fragmented nucleus. The percent of TUNEL-positive SCs was scored from 10 randomly selected 20× fields as previously described for each culture condition (Hansen et al., 2008; Yue et al., 2011). For cultures, the percent of TUNEL-positive cells was expressed as a percentage of the control condition, defined as 100%. Each condition was performed in duplicate and was repeated on ≥3 cultures. Protein lysates were prepared from parallel cultures and immunoblotted with anti-cleaved caspase 3 antibodies (Cell Signaling) to confirm apoptosis. For nerve sections a total of 10 microscopic fields per section and 6 sections per nerve were counted. Nerves were derived from 4 animals per group. Statistical significance of differences in the average percent of apoptotic cells among the various conditions was determined by one way ANOVA followed by Holm–Sidak method using SigmaStat software (Systat Software Inc, Richmond, CA).

EdU labeling

Starting 24 h prior to euthanasia, mice were injected intraperitoneally 4 times at regular intervals with EdU (10 μM, Life Technologies, Carlsbad, CA) in order to label cells undergoing karyokinesis. SNs were dissected and prepared as described above. EdU was detected in SN nuclei of WT and P0SchΔ(39–121) using the Click-IT reaction per manufacturer's instructions (Life Technologies). Sections were counterstained with S100, mounted with Prolong Gold + DAPI (Life Technologies), and coverslipped. Percentage of SN cells undergoing nuclear division was determined by dividing the total number of SN nuclei (S100-positive + DAPI-positive) by the number of EdU-positive SN nuclei (S100-positive cells + EdU-positive nuclei). A total of 10 microscopic fields per section and 6 sections per nerve were counted. Nerves were derived from 4 animals per group. Statistical significance of differences in the average percent of EdU-positive cells among the various conditions was determined by one way ANOVA followed by Holm–Sidak method using SigmaStat software (Systat Software Inc, Richmond, CA).

Results

Merlin is phosphorylated in SCs following axotomy

To examine the effect of nerve injury on merlin phosphorylation in SCs, we immunoblotted protein lysates from cut and uncut rat sciatic nerves with anti-phospho-merlin (p-merlin) antibodies (Fig. 1A,B). Blots were stripped and reprobed with non-phospho-specific anti-merlin antibodies. Densitometry was performed to quantify the level of p-merlin expression relative to overall merlin levels. Axotomy resulted in a significant increase of merlin phosphorylation. Immunolabeling of frozen nerve sections with anti-p-merlin and anti-NF200 antibodies demonstrated punctate labeling of p-merlin that was confined to the NF200-positive axons prior to nerve injury with no significant labeling in SCs. Following axotomy, there was evidence of axonal degeneration evidenced by loss of intact NF200-positive nerve fibers with a parallel increase in p-merlin labeling in the SCs that remain following axotomy (Fig. 1C). We also examined merlin phosphorylation in a different model of nerve injury. Kanamycin treatment causes hair cell loss leading to a gradual degeneration of spiral ganglion distal axons and denervation of the SCs in the osseous spiral lamina (Alam et al., 2007). We have previously shown that these SCs have undergone a gradual denervation increase of $p75^{NTR}$ expression and re-enter the cell cycle similar to SCs following acute axotomy (Provenzano et al., 2011). Frozen sections from cochleae were immunolabeled with anti-p-merlin and anti-NF200 antibodies. Parallel sections were immunolabeled with anti-myosin VII (MyoVII) to verify loss of hair cells in kanamycin-treated animals. Kanamycin treatment resulted in loss of the NF200-positive peripheral axons in the osseous spiral lamina leading to the organ of Corti and increased p-merlin labeling in the SCs that had lost axonal contact and remain in the osseous spiral lamina (Fig. 2) (Provenzano et al., 2011). These data demonstrate that merlin becomes phosphorylated in SCs following acute or gradual loss of axonal contact.

Merlin suppresses $p75^{NTR}$ expression in Schwann cells

Denervated SCs with phosphorylated merlin and VS cells that lack functional merlin express high levels of $p75^{NTR}$ raising the possibility that merlin status regulates $p75^{NTR}$ expression in SCs. To test this possibility, we performed sciatic nerve axotomies in wild-type (WT) and P0SchΔ39-121 mice. P0SchΔ39-121 mice harbor a dominant negative *Nf2* mutation restricted to SCs (Giovannini et al., 1999). Protein lysates from cut and uncut nerves, collected 7, 21, and 180 days following axotomy, were immunoblotted with anti-$p75^{NTR}$ antibodies (Fig. 3A). The blots were probed with anti-β-actin antibodies to verify protein loading. Densitometry was performed to quantify the level of $p75^{NTR}$ expression relative to β-actin (Fig. 3B). As expected, axotomy led to a significant increase in $p75^{NTR}$ levels. Consistent with the notion that merlin regulates $p75^{NTR}$ expression, $p75^{NTR}$ levels were elevated in uncut nerves from P0SchΔ39-121 mice compared to WT mice. Further, comparison of $p75^{NTR}$ levels following axotomy demonstrates that $p75^{NTR}$ levels were significantly elevated in P0SchΔ39-121 mice compared to WT mice at 7 and 21 days post-axotomy (PA) (Fig. 3B). This difference was no longer statistically significant 180 days PA ($p = 0.228$). Thus, loss of merlin function increases $p75^{NTR}$ expression, even in SCs that remain in contact with axons.

Merlin status regulates $p75^{NTR}$ expression in SCs

To further explore the relationship between $p75^{NTR}$ expression levels and merlin status, we cultured sciatic nerve SCs derived from a transgenic mouse line with floxed *Nf2* and a tamoxifen (Tx)-inducible Cre (RosaCre:$Nf2^{f/f}$) (Giovannini et al., 2000), allowing for conditional knock-out of *Nf2*. The estrogen receptor T2 (ERT2) moiety fused to Cre retains the recombinase in the cytosol until Tx administration releases this inhibition, thus permitting inducible recombination of *LoxP* sites. Sciatic nerve cultures were prepared from these mice. Treatment of cultures with Tx (500 nM) reduced merlin and significantly elevated $p75^{NTR}$ expression levels consistent with the notion that merlin suppresses $p75^{NTR}$ expression in SCs (Fig. 4A, B).

The fact that elevated $p75^{NTR}$ expression in SCs following axotomy correlates with merlin phosphorylation raises the possibility that merlin phosphorylation hinders its ability to suppress $p75^{NTR}$ expression. To address this possibility we performed merlin replacement experiments using primary VS schwannoma cultures. These cultures lack functional merlin expression thereby allowing us to introduce merlin isoforms with specified serine 518 (S518) phosphorylation status (Yue et al., 2011; Ahmad et al., 2014). Cultures were transduced with an adenoviral vector that expresses wild-type merlin, S518-mutated merlin isoforms, or an empty, control vector. The S518A mutation renders merlin unable to be phosphorylated on this residue while the S518D functions as a phospho-mimetic. Protein lysates from the cultures were immunoblotted with anti-$p75^{NTR}$ antibodies followed by anti-merlin and then anti-β-actin antibodies. Replacement of merlin reduced $p75^{NTR}$ expression in primary VS cultures (Fig. 4C). Further, $p75^{NTR}$ expression was suppressed in cultures transduced with S518A merlin isoform whereas transduction with the S518D isoform resulted in increase in $p75^{NTR}$ expression. Taken together these results indicate that S518 phosphorylation of merlin reduces its ability to suppress $p75^{NTR}$ expression.

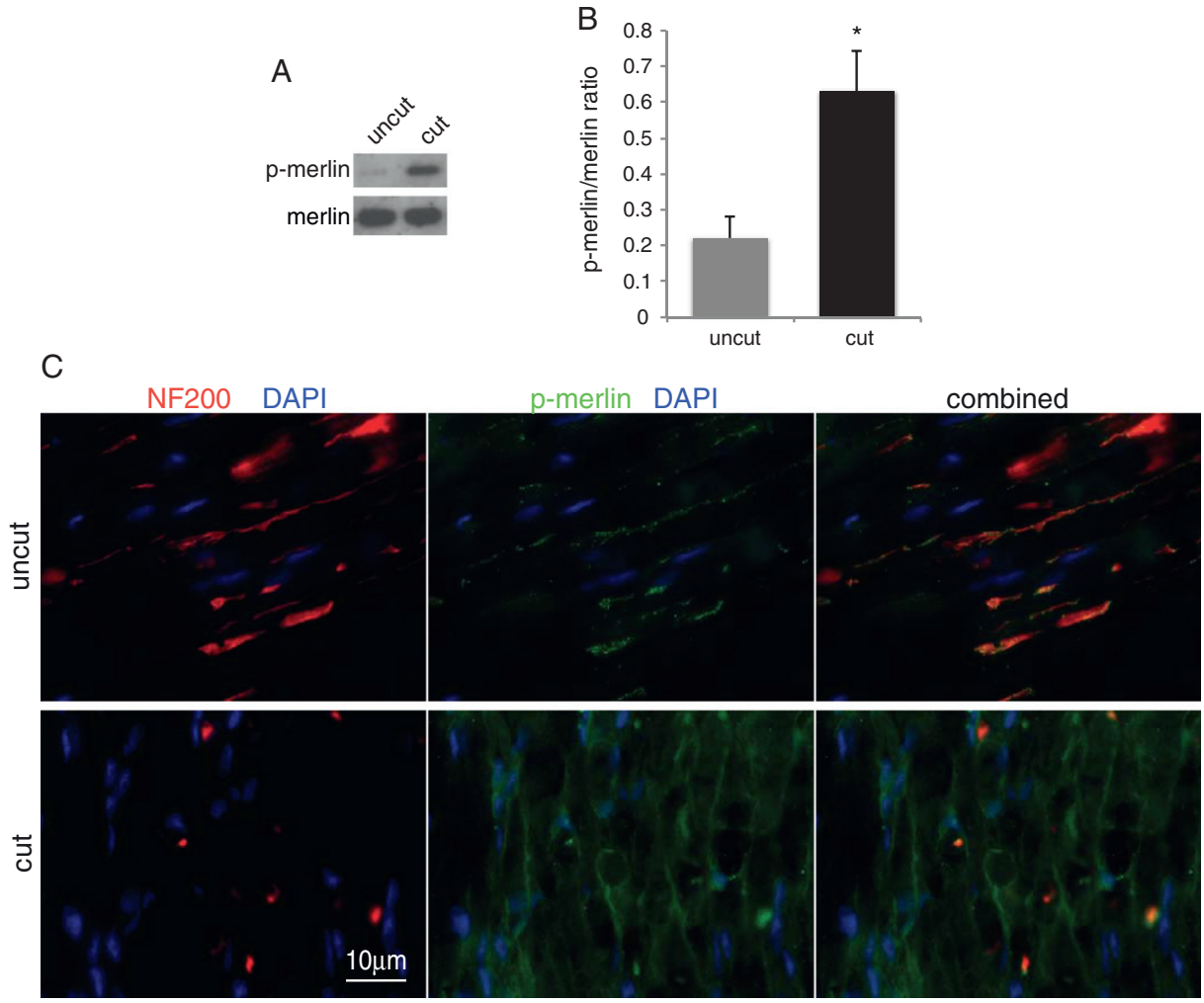

Fig. 1. Merlin is phosphorylated in Schwann cells following nerve injury. A. Immunoblots of protein lysate from cut and uncut rat sciatic nerves probed with anti-phospho-merlin (p-merlin) and merlin antibodies. B. Average p-merlin/merlin levels based on densitometry based on samples from 3 nerves pooled for each condition and averaged from 4 separate repetitions. Error bars present SEM. $^*p = 0.0165$, Student's unpaired *t*-test. C. Frozen sections of cut and uncut sciatic nerves were immunolabeled with anti-neurofilament 200 (NF200, red) and anti-p-merlin (green) antibodies. Nuclei were labeled with DAPI (blue). Right column demonstrates combined image with superimposed staining showing presence of p-merlin confined to the axons in uncut nerve and increased diffuse p-merlin labeling of denervated SCs following axotomy. Scale bar = 10 μm.

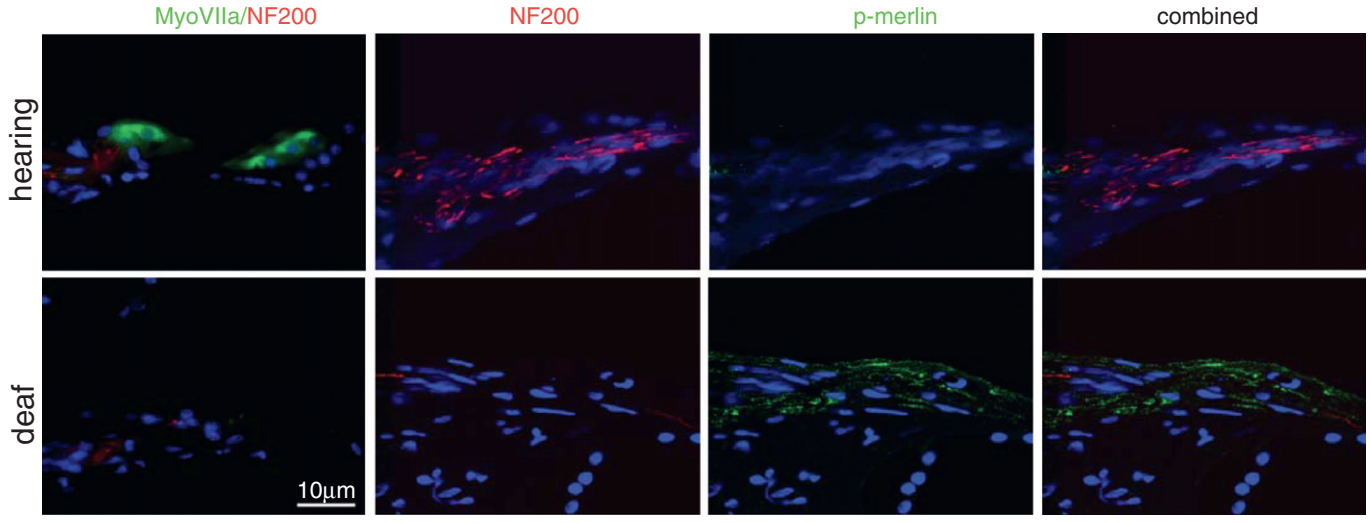

Fig. 2. Merlin is phosphorylated in spiral ganglion Schwann cells after kanamycin-induced hair cell loss. Cochlear frozen sections were labeled with anti-myosin VII (green), anti-NF200 (red), and phospho-merlin (p-merlin, green) antibodies.

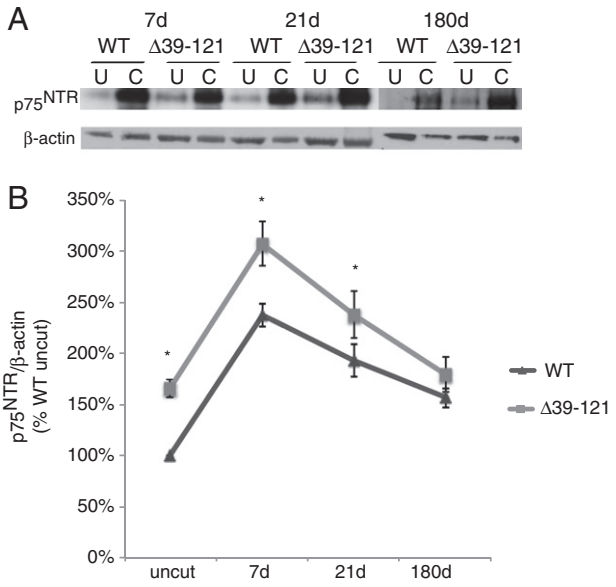

Fig. 3. Merlin status regulates p75^{NTR} expression. A. Protein lysates from cut and uncut sciatic nerves were collected 7, 21, and 180 days following unilateral axotomy in wild-type and P0SchΔ39-121 mice. Blots were probed with anti-p75^{NTR}antibody and then stripped and reprobed with anti-β-actin antibodies. B. Average p75^{NTR}/β-actin levels based on densitometry. Blots are from 3 nerve samples pooled for each condition and averaged from 4 separate repetitions for each time point. Error bars present SEM. *$p < 0.05$ by two-tailed Student's *t*-test.

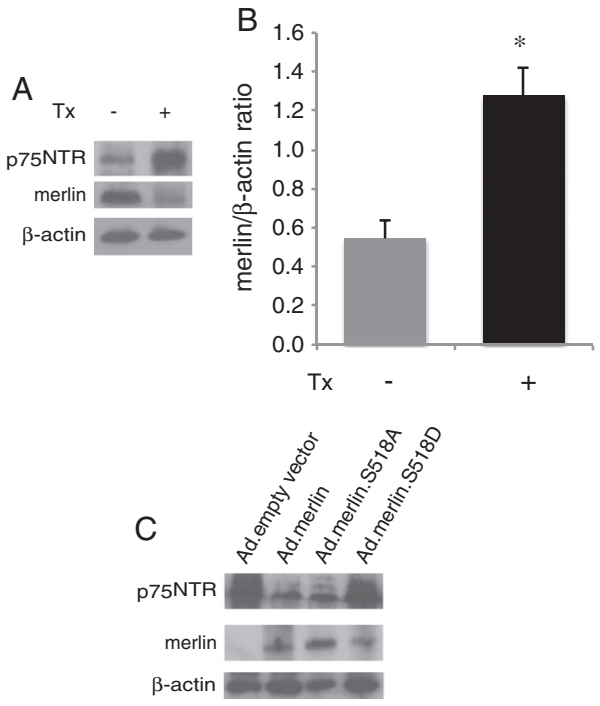

Fig. 4. Merlin regulates p75^{NTR} expression in cultured Schwann and schwannoma cells. A. SC cultures from RosaCre:Nf2$^{f/f}$ mice were treated with (Tx+) or without (Tx−) tamoxifen. Protein lysates were probed with anti-p75^{NTR} and merlin antibodies. The blots were stripped and reprobed with anti-β-actin antibodies. B. Average p75^{NTR}/β-actin levels based on densitometry based on blots from 3 separate repetitions. Error bars present SEM. *$p = 0.012$ by two-tailed Student's *t*-test. C. Primary VS cultures were transduced with Ad-empty vector, Ad-merlin (wild-type), Ad-merlin S518A (unphosphorylatable), or Ad-merlin S518D (phospho-mimetic). Protein lysates were probed with anti-p75^{NTR} and merlin antibodies. The blots were stripped and reprobed with anti-β-actin antibodies.

Merlin mutation decreases Schwann cell proliferation following axotomy

Next we sought to determine the effect of merlin on SC survival and proliferation following axotomy. Sciatic nerve axotomies were performed in FVB control and P0SchΔ39-121 mice and the mice were treated with EdU to label proliferating nuclei. Frozen sections of the portion of the nerves distal to the axotomy were labeled with the Click-IT reaction to identify EdU-positive nuclei and EdU-positive SC nuclei were counted as before (Provenzano et al., 2008, 2011). Axotomy resulted in a significant increase in the percent of EdU-positive SC nuclei 7 days following injury. The percent of EdU-positive SCs diminished by 180 days after axotomy but remained elevated above the level in uncut nerves. Remarkably, the percent of EdU-positive SC nuclei was significantly less in nerves from P0SchΔ39-121 mice 7 and 180 days following injury compared to wild-type mice (Fig. 5A,B). Further, there was no difference in the percent of EdU-positive SC nuclei in uncut nerves from P0SchΔ39-121 and wild-type mice. Thus, merlin mutation blunts the proliferative capacity of SCs following injury.

Merlin mutation reduces SC apoptosis following axotomy

To determine the effect of merlin on SC death following axotomy frozen sections from the portion of the nerve distal to the axotomy were labeled with TUNEL and the number of apoptotic SC nuclei was counted as before (Provenzano et al., 2008, 2011). Axotomy resulted in a significant increase in the percent of TUNEL-positive SC nuclei 7 days following injury. This apoptotic response diminished over time, remaining elevated above baseline at 21 days and returning to baseline by 180 days following axotomy (Fig. 6). The percent of TUNEL-positive SC nuclei was significantly less in nerves from P0SchΔ39-121 mice 7 and 21 days following injury as well as in the uncut nerves (Fig. 6). Thus, merlin mutation renders SCs less sensitive to apoptosis following nerve injury.

Merlin is necessary for p75^{NTR}-induced Schwann cell apoptosis

SCs from P0SchΔ39-121 mice that lack functional merlin express high levels of p75^{NTR} yet are resistant to apoptosis before and following axotomy (Fig. 6). This observation raises the possibility that functional merlin is necessary for p75^{NTR}-induced SC apoptosis. To test this possibility we cultured SCs from RosaCre:Nf2$^{f/f}$ mice. Cultures were either exposed to or not exposed to Tx as above. Subsets of cultures were treated with the p75^{NTR} ligand, proNGF (3 nM) to induce apoptosis. The number of TUNEL-positive SC nuclei was scored as before. proNGF induced apoptosis in SCs that were not exposed to Tx (merlin present) reflected as a significant increase in the percent of TUNEL-positive SC nuclei. This apoptotic response was significantly blunted in SCs that had been exposed to Tx (Fig. 7A,B). To confirm that the increase in TUNEL-positive nuclei represented an apoptotic response, we blotted protein lysates from the cultures with anti-cleaved caspase 3 antibody. Consistent with the TUNEL results, there was an increase in cleaved caspase 3 in cultures treated with proNGF that was diminished by pretreatment with Tx (Fig. 7C). These results confirm that SCs lacking functional merlin are less sensitive to apoptosis, particularly cell death initiated by activation of p75^{NTR}.

Discussion

In a normal nerve, SCs are in contact with axons and remain in a quiescent state. Following axon degeneration due to nerve injury SCs lose contact with axons and initially dedifferentiate, proliferate and provide axonal support. Loss of axonal contact results in a rapid and significant increase in p75^{NTR} expression (Taniuchi et al., 1986, 1988); the

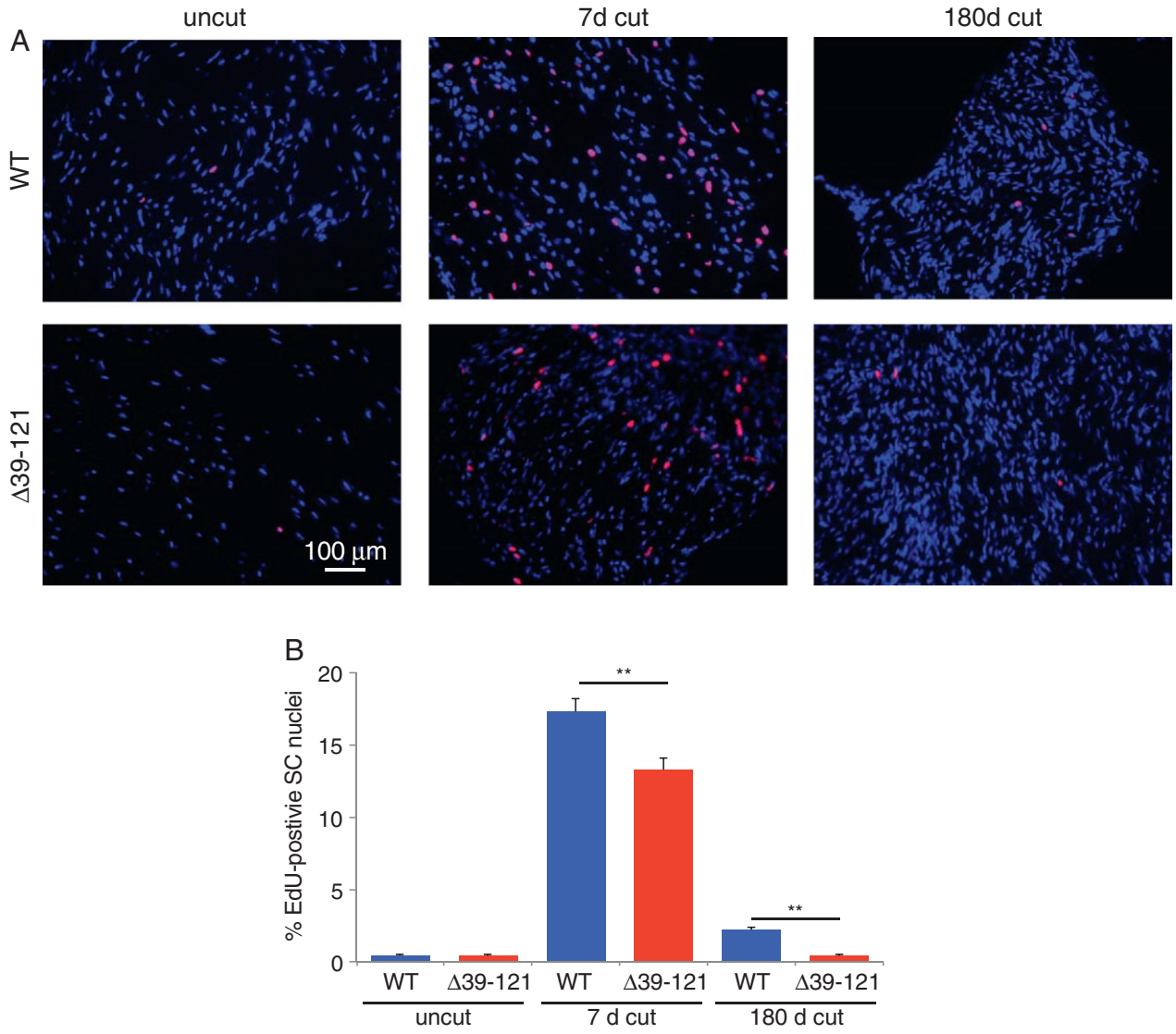

Fig. 5. Lack of functional merlin results in decreased Schwann cell proliferation following axotomy. A. Cut and uncut sciatic nerves from wild-type (WT) and P0SchΔ39-121 mice treated with EdU were collected at 7 and 180 days following unilateral axotomy and frozen sections were labeled for EdU using the Click-IT reaction (red). Nuclei are labeled with DAPI (blue). Scale bar = 100 μm. B. The number of EdU-positive SC nuclei was scored. Counts represent the mean from 4 animals per group. Error bars present SEM. One way ANOVA with post hoc Holm–Sidak was used to test for significance of differences. $^{*}p < 0.05$, $^{**}p < 0.001$.

mechanisms leading to this elevated expression have remained unknown. Eventually in the absence of reinnervation, denervated SCs undergo $p75^{NTR}$-mediated apoptosis (Ferri and Bisby, 1999). SCs are essential for axon regeneration and loss of supportive SCs is a barrier to late neural regeneration (Hoffman, 1992). The data presented here implicate the tumor suppressor merlin as a key mediator of $p75^{NTR}$ expression and apoptotic signaling in SCs following nerve injury.

Merlin phosphorylation after axotomy

Merlin's molecular conformation is altered by its phosphorylation status and this determines its ability to bind other proteins and regulate cell growth (Gutmann et al., 1999; Rong et al., 2004; Ye, 2007; Sher et al., 2012). Phosphorylation of S518 by PKA, p21-activated kinases 1 and 2 (PAK1/2), or Akt, leads to a growth permissive (inactive) conformation (Gutmann et al., 1999; Kissil et al., 2002; Alfthan et al., 2004; Okada et al., 2007; Thaxton et al., 2007; Sher et al., 2012). Besides S518, other residues including S10, S66, T230 and S315 are also targets for phosphorylation; the extent to which phosphorylation of these residues regulates merlin ability to interact with other proteins and control cell growth has not been firmly established. However, Akt-mediated T230 and S315 phosphorylation results in ubiquitination of merlin marking it for degradation (Tang et al., 2007). Using models of both acute, primary (axotomy) and gradual, secondary (deafening by aminoglycosides) neural degeneration, we demonstrate that merlin is phosphorylated in SCs following axon degeneration. Coincident with this phosphorylation, the denervated SCs dedifferentiate and re-enter the cell cycle. These data fit a general model of merlin functioning as a molecular switch responsive to cell–cell contact cues that is able to suppress cell proliferation when it remains in a dephosphorylated conformation (Sher et al., 2012). In this model, merlin phosphorylation in SCs that have lost axonal contact relieves this inhibition allowing cells to re-enter the cell cycle.

Merlin status regulates $p75^{NTR}$ expression following nerve injury

Merlin has been shown to suppress the expression of transmembrane receptors in cultured cells, particularly the ErbB and PDGFR, receptor tyrosine kinase (RTK) families that promote SC proliferation (Lallemand et al., 2009b; Zhou et al., 2011). The data presented here demonstrate that loss of merlin function results in increased $p75^{NTR}$ expression in SCs. Nerve lysates from P0SchΔ39-121 mice prior to injury and following axotomy revealed an increase in $p75^{NTR}$ levels in

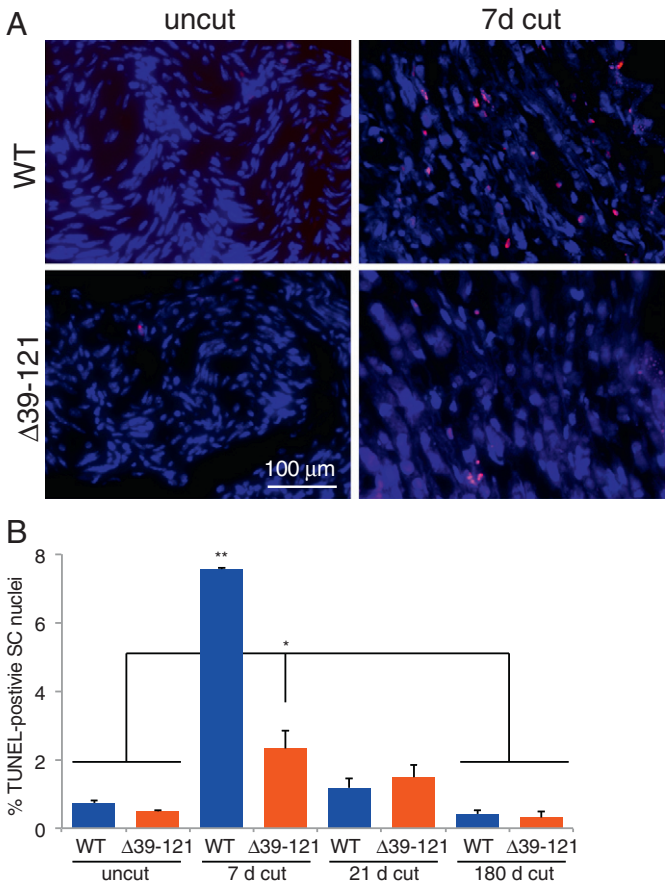

Fig. 6. Lack of functional merlin blunts Schwann cell apoptosis following axotomy. A. Cut and uncut sciatic nerves from wild-type (WT) and P0SchΔ39-121 mice were collected at 7, 21, and 180 days following unilateral axotomy and frozen sections were labeled with TUNEL (red). Nuclei are labeled with DAPI (blue). Scale bar = 100 μm. B. The average number of TUNEL-positive SC nuclei was scored. Counts represent the mean from 4 animals per group. Error bars present SEM. One way ANOVA with post hoc Holm–Sidak was used to test significance of differences. $^{*}p < 0.05$. The greatest difference between wild-type and mutant mice occurred at post-axotomy day 7 ($p < 0.001$).

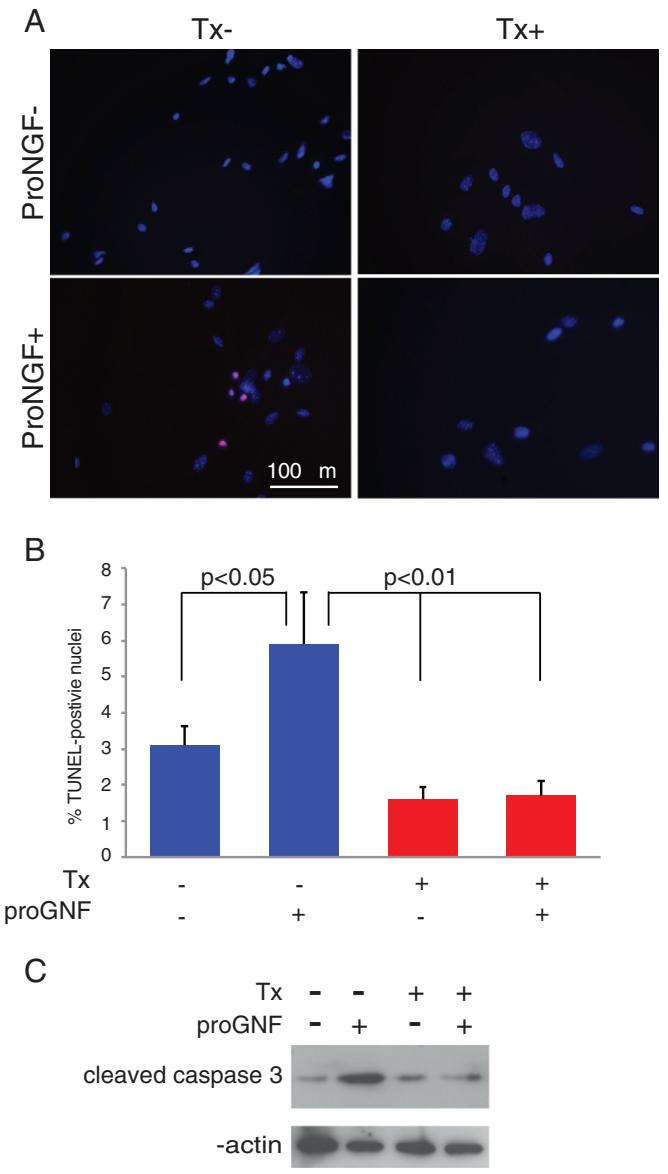

Fig. 7. Merlin is necessary for p75^{NTR}-mediated Schwann cell apoptosis. A. SC cultures from RosaCre:Nf2$^{f/f}$ mice were treated with tamoxifen (Tx+) or carrier (Tx−) and subsequently maintained in the presence or absence of proNGF (3 nM). Cultures were labeled with TUNEL and the number of TUNEL-positive SC nuclei was scored. B. Average number of TUNEL-positive SC nuclei from 3 separate cultures for each condition. Error bars present SEM. One way ANOVA with post hoc Holm–Sidak was used to test for significance of differences. Scale bar = 100 μm. C. Protein lysates from cultures were immunoblotted wth anti-cleaved caspase3 antibody. Blots were stripped and reprobed with anti-β-actin antibody.

comparison to wild-type mice. Further, suppression of merlin expression in cultured SCs increased p75^{NTR} expression while replacement of wild-type merlin reduced p75^{NTR} expression in primary VS cells. Taken together these data confirm that merlin suppresses p75^{NTR} levels in SCs *in vitro* and *in vivo* and are consistent with the observation of elevated p75^{NTR} levels in neoplastic VS cells (Ahmad et al., 2014). While p75^{NTR} levels were elevated in nerve lysates from P0SchΔ39-121 mice compared to wild-type mice prior to injury, there was a significant increase in p75^{NTR} levels in nerves from P0SchΔ39-121 following axotomy indicating that other factors, in addition to merlin status, contribute to the increase in p75^{NTR} expression following nerve injury.

Following nerve injury, merlin becomes phosphorylated which is temporally correlated with an increase in p75^{NTR} expression. To determine whether merlin phosphorylation promotes p75^{NTR} expression we used a merlin replacement strategy in primary VS cells that allowed us to define the status of the S518 residue. The S518A mutation is unable to be phosphorylated and results in suppressed p75^{NTR} expression. By contrast, the S518D mutation functions as a phospho-mimetic and failed to suppress p75^{NTR} expression. Taken together, these data suggest that merlin inactivation by phosphorylation facilitates increased p75^{NTR} expression in SCs following nerve injury.

Merlin is necessary for p75^{NTR}-induced SC apoptosis

Activation of p75^{NTR} leads to apoptosis of SCs *in vivo* following nerve injury and *in vitro* (Ferri and Bisby, 1999; Provenzano et al., 2011). However, neoplastic VS cells, which lack functional merlin, express high levels of p75^{NTR} and resist apoptosis in response to the p75^{NTR} ligands, proNGF and proBNDF (Ahmad et al., 2014). Further, the data presented here demonstrate that SCs from P0SchΔ39-121 mice are less sensitive to apoptosis after nerve injury, despite elevated levels of p75^{NTR}. These observations raise the possibility that merlin facilitates p75^{NTR} apoptotic signaling in SCs. To test this possibility we suppressed merlin expression in cultured SCs using an inducible knock-out gene system. Indeed, SCs with suppressed merlin expression were less sensitive to apoptosis in response to proNGF, confirming that merlin facilitates pro-apoptotic p75^{NTR} signaling in SCs. The observation that SCs lacking functional merlin are less sensitive to p75^{NTR}-apoptosis may explain, at least in part, the ability of neoplastic schwannoma cells to grow and survive long-term in the absence of axonal contact. This is in contrast to the eventual death of non-neoplastic SCs following denervation.

Thus, $p75^{NTR}$ represents a potential therapeutic target that would specifically target neoplastic schwannoma cells, but not their non-neoplastic counterparts.

Although $p75^{NTR}$ signaling is often associated with apoptosis, activation of $p75^{NTR}$ has also been shown to promote survival of several other cell types, particularly neurons that co-express Trk receptors and some breast, melanoma, and glioma cells (Chao and Hempstead, 1995). In the case of breast cancer cells, the prosurvival response is linked to the carboxyl-terminal fragment of $p75^{NTR}$ (Verbeke et al., 2013). Other malignant cell lines (e.g. colorectal) retain sensitivity to $p75^{NTR}$-mediated apoptosis (Yang et al., 2014). What determines whether $p75^{NTR}$ activation leads to cell death or survival remains unknown; however, $p75^{NTR}$ activation of the nuclear transcription factor κB (NF-κB) has been implicated in the prosurvival response (Gentry et al., 2000; Ahmad et al., 2014), whereas activation of JNK is required for the pro-death signal (Yoon et al., 1998; Friedman, 2000; Harrington et al., 2002). The data here suggest that $p75^{NTR}$-mediated apoptosis in SCs depends, at least in part, on functional merlin. Whether sensitivity to $p75^{NTR}$-mediated apoptosis is likewise merlin-dependent in these other cell types remains to be determined.

Beyond failing to induce apoptosis, $p75^{NTR}$ ligands appear to provide prosurvival signaling in neoplastic schwannoma cells (Ahmad et al., 2014). This prosurvival effect is not due to co-expression of Trk receptors and involves activation of c-Jun N-terminal kinase (JNK) and the transcription factor, NF-κB (Ahmad et al., 2014). Thus, proNGF and proBDNF reduce apoptosis in VS cells treated with JNK inhibitors (Ahmad et al., 2014). Those observations coupled with the data from this study raise the possibility that activation of $p75^{NTR}$ represents a mechanism whereby VSs are resistant to chemotherapeutics that target kinase signaling (Karajannis et al., 2012, 2014; Ahmad et al., 2014).

Loss of functional merlin reduces peripheral nerve SC proliferation in vivo

Several studies confirm that merlin suppresses cell proliferation *in vitro* by inhibiting signaling pathways at the cell membrane and in the nucleus (Cooper and Giancotti, 2014). As noted above, merlin regulates the expression, subcellular localization, and activity of RTKs, including ErbB2 and PDGFR (Fraenzer et al., 2003; Brown and Hansen, 2008; Lallemand et al., 2009b; Schulz et al., 2014). It likewise suppresses the activity of several downstream pro-growth signaling cascades including Ras, Rac1/Cdc42, Raf, PAK1/2, extracellular regulated kinase/mitogen activated protein kinase (ERK/MAPK), phosphatidyl-inositol 3-kinase (PI3-K)/Akt/mTORC, and JNK (Guo et al., 2012; Zhou and Hanemann, 2012). Merlin also has an impact on intranuclear signaling by entering the nucleus and inhibiting the E3 ubiquitin ligase $CRL4^{DCAF1}$ to suppress proliferation (Li et al., 2014).

Experiments using SC cultures from mice with an *Nf2* deletion confirm that merlin functions to suppress SC proliferation *in vitro*, particularly when the cells are in contact inhibition (Lallemand et al., 2009a). Interestingly, EdU-uptake was not significantly greater in normal, uninjured SCs from P0SchΔ39-121 mice compared with wild-type mice suggesting that loss of merlin function alone is not sufficient to drive peripheral nerve SCs into a significant hyperplastic response. Indeed, immmunolabeling and ultrastructural studies identified myelination abnormalities in sciatic nerves from P0SchΔ39-121 mice but did not reveal significant SC hyperplasia within the peripheral nerve (Denisenko et al., 2008). Rather, SC hyperplasia in P0SchΔ39-121 mice is found in foci near or within sensory ganglia and skeletal muscle (Giovannini et al., 2014), consistent with the observation that most schwannomas arise within sensory ganglia (Tryggvason et al., 2012), and suggesting that intraganglionic SCs are particularly sensitive to merlin status. Further, SC proliferation in peripheral nerves following axotomy was reduced in P0SchΔ39-121 mice compared with wild-type mice. These data suggest that merlin paradoxically facilitates peripheral nerve SC proliferation *in vivo* following nerve injury, similar to the pro-growth function of other members of the ERM family of proteins (Bretscher et al., 2002). As demonstrated here, merlin is phosphorylated following nerve injury suggesting that phosphorylated merlin not only allows for, but also facilitates, SC proliferation. These data are consistent with the observation that primary neoplastic schwannoma cells, which lack functional merlin, proliferate very slowly *in vitro* particularly in the absence of exogenous mitogens and are also consistent with the slow growth rate of most schwannomas *in vivo* (Stangerup et al., 2006; Hansen et al., 2008).

In summary, the results of these studies demonstrate that the tumor suppressor, merlin, decreases $p75^{NTR}$ expression and apoptotic signaling in peripheral nerve SCs. The ability to decrease $p75^{NTR}$ expression depends on the phosphorylation state of merlin and points to a model whereby loss of axonal contact following nerve injury results in merlin phosphorylation leading to increased $p75^{NTR}$ expression. Interestingly, loss of functional merlin also decreases peripheral nerve SC proliferation. Taken together, these data suggest that loss of merlin function reduces SC sensitivity to apoptosis allowing for long-term survival in the absence of axonal contact consistent with the biology of neoplastic schwannoma cells which are very slow-growing and are able maintain long-term survival in the absence of axons.

References

Ahmad, I., Yue, W.Y., Fernando, A., Clark, J.J., Woodson, E.A., Hansen, M.R., 2014. p75NTR is highly expressed in vestibular schwannomas and promotes cell survival by activating nuclear transcription factor kappaB. Glia 62, 1699–1712.

Alam, S.A., Robinson, B.K., Huang, J., Green, S.H., 2007. Prosurvival and proapoptotic intracellular signaling in rat spiral ganglion neurons in vivo after the loss of hair cells. J. Comp. Neurol. 503, 832–852.

Alfthan, K., Heiska, L., Gronholm, M., Renkema, G.H., Carpen, O., 2004. Cyclic AMP-dependent protein kinase phosphorylates merlin at serine 518 independently of p21-activated kinase and promotes merlin-ezrin heterodimerization. J. Biol. Chem. 279, 18559–18566.

Bandtlow, C., Dechant, G., 2004. From cell death to neuronal regeneration, effects of the p75 neurotrophin receptor depend on interactions with partner subunits. Sci. STKE pe24.

Barker, P.A., 2004. p75NTR is positively promiscuous: novel partners and new insights. Neuron 42, 529–533.

Bretscher, A., Edwards, K., Fehon, R.G., 2002. ERM proteins and merlin: integrators at the cell cortex. Nat. Rev. Mol. Cell Biol. 3, 586–599.

Brown, K.D., Hansen, M.R., 2008. Lipid raft localization of erbB2 in vestibular schwannoma and Schwann cells. Otol. Neurotol. 29, 79–85.

Chao, M.V., Hempstead, B.L., 1995. p75 and Trk: a two-receptor system. Trends Neurosci. 18, 321–326.

Chen, Z.L., Yu, W.M., Strickland, S., 2007. Peripheral regeneration. Annu. Rev. Neurosci. 30, 209–233.

Cooper, J., Giancotti, F.G., 2014. Molecular insights into NF2/Merlin tumor suppressor function. FEBS Lett. 588, 2743–2752.

Denisenko, N., Cifuentes-Diaz, C., Irinopoulou, T., Carnaud, M., Benoit, E., Niwa-Kawakita, M., Chareyre, F., Giovannini, M., Girault, J.A., Goutebroze, L., 2008. Tumor suppressor schwannomin/merlin is critical for the organization of Schwann cell contacts in peripheral nerves. J. Neurosci. 28, 10472–10481.

Ferri, C.C., Bisby, M.A., 1999. Improved survival of injured sciatic nerve Schwann cells in mice lacking the p75 receptor. Neurosci. Lett. 272, 191–194.

Fraenzer, J.T., Pan, H., Minimo Jr., L., Smith, G.M., Knauer, D., Hung, G., 2003. Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation. Int. J. Oncol. 23, 1493–1500.

Friedman, W.J., 2000. Neurotrophins induce death of hippocampal neurons via the p75 receptor. J. Neurosci. 20, 6340–6346.

Gentry, J.J., Casaccia-Bonnefil, P., Carter, B.D., 2000. Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells. J. Biol. Chem. 275, 7558–7565.

Giovannini, M., Robanus-Maandag, E., Niwa-Kawakita, M., van der Valk, M., Woodruff, J.M., Goutebroze, L., Merel, P., Berns, A., Thomas, G., 1999. Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. Genes Dev. 13, 978–986.

Giovannini, M., Robanus-Maandag, E., van der Valk, M., Niwa-Kawakita, M., Abramowski, V., Goutebroze, L., Woodruff, J.M., Berns, A., Thomas, G., 2000. Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. Genes Dev. 14, 1617–1630.

Giovannini, M., Bonne, N.X., Vitte, J., Chareyre, F., Tanaka, K., Adams, R., Fisher, L.M., Valeyrie-Allanore, L., Wolkenstein, P., Goutagny, S., Kalamarides, M., 2014. mTORC1 inhibition delays growth of neurofibromatosis type 2 schwannoma. Neuro-Oncology 16, 493–504.

Guo, L., Moon, C., Niehaus, K., Zheng, Y., Ratner, N., 2012. Rac1 controls Schwann cell myelination through cAMP and NF2/merlin. J. Neurosci. 32, 17251–17261.

Gutmann, D.H., Haipek, C.A., Hoang Lu, K., 1999. Neurofibromatosis 2 tumor suppressor protein, merlin, forms two functionally important intramolecular associations. J. Neurosci. Res. 58, 706–716.

Haase, G., Pettmann, B., Raoul, C., Henderson, C.E., 2008. Signaling by death receptors in the nervous system. Curr. Opin. Neurobiol. 18, 284–291.
Hansen, M.R., Roehm, P.C., Chatterjee, P., Green, S.H., 2006. Constitutive neuregulin-1/ErbB signaling contributes to human vestibular schwannoma proliferation. Glia 53, 593–600.
Hansen, M.R., Clark, J.J., Gantz, B.J., Goswami, P.C., 2008. Effects of ErbB2 signaling on the response of vestibular schwannoma cells to gamma-irradiation. Laryngoscope 118, 1023–1030.
Harrington, A.W., Kim, J.Y., Yoon, S.O., 2002. Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J. Neurosci. 22, 156–166.
Hoffman, W.Y., 1992. Reanimation of the paralyzed face. Otolaryngol. Clin. N. Am. 25, 649–667.
Irving, R.M., Moffat, D.A., Hardy, D.G., Barton, D.E., Xuereb, J.H., Maher, E.R., 1994. Somatic NF2 gene mutations in familial and non-familial vestibular schwannoma. Hum. Mol. Genet. 3, 347–350.
Jung, K.M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P.K., Kim, D.S., Ryu, S.H., Chao, M.V., Kim, T.W., 2003. Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J. Biol. Chem. 278, 42161–42169.
Kanning, K.C., Hudson, M., Amieux, P.S., Wiley, J.C., Bothwell, M., Schecterson, L.C., 2003. Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J. Neurosci. 23, 5425–5436.
Karajannis, M.A., Legault, G., Hagiwara, M., Ballas, M.S., Brown, K., Nusbaum, A.O., Hochman, T., Goldberg, J.D., Koch, K.M., Golfinos, J.G., Roland, J.T., Allen, J.C., 2012. Phase II trial of lapatinib in adult and pediatric patients with neurofibromatosis type 2 and progressive vestibular schwannomas. Neuro-Oncology 14, 1163–1170.
Karajannis, M.A., Legault, G., Hagiwara, M., Giancotti, F.G., Filatov, A., Derman, A., Hochman, T., Goldberg, J.D., Vega, E., Wisoff, J.H., Golfinos, J.G., Merkelson, A., Roland, J.T., Allen, J.C., 2014. Phase II study of everolimus in children and adults with neurofibromatosis type 2 and progressive vestibular schwannomas. Neuro-Oncology 16, 292–297.
Kenchappa, R.S., Zampieri, N., Chao, M.V., Barker, P.A., Teng, H.K., Hempstead, B.L., Carter, B.D., 2006. Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50, 219–232.
Kissil, J.L., Johnson, K.C., Eckman, M.S., Jacks, T., 2002. Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. J. Biol. Chem. 277, 10394–10399.
Lallemand, D., Saint-Amaux, A.L., Giovannini, M., 2009a. Tumor-suppression functions of merlin are independent of its role as an organizer of the actin cytoskeleton in Schwann cells. J. Cell Sci. 122, 4141–4149.
Lallemand, D., Manent, J., Couvelard, A., Watilliaux, A., Siena, M., Chareyre, F., Lampin, A., Niwa-Kawakita, M., Kalamarides, M., Giovannini, M., 2009b. Merlin regulates transmembrane receptor accumulation and signaling at the plasma membrane in primary mouse Schwann cells and in human schwannomas. Oncogene 28, 854–865.
Li, W., You, L., Cooper, J., Schiavon, G., Pepe-Caprio, A., Zhou, L., Ishii, R., Giovannini, M., Hanemann, C.O., Long, S.B., Erdjument-Bromage, H., Zhou, P., Tempst, P., Giancotti, F.G., 2010. Merlin/NF2 suppresses tumorigenesis by inhibiting the E3 ubiquitin ligase CRL4(DCAF1) in the nucleus. Cell 140, 477–490.
Li, W., Cooper, J., Zhou, L., Yang, C., Erdjument-Bromage, H., Zagzag, D., Snuderl, M., Ladanyi, M., Hanemann, C.O., Zhou, P., Karajannis, M.A., Giancotti, F.G., 2014. Merlin/NF2 loss-driven tumorigenesis linked to CRL4(DCAF1)-mediated inhibition of the hippo pathway kinases Lats1 and 2 in the nucleus. Cancer Cell 26, 48–60.
Lorenzetto, E., Panteri, R., Marino, R., Keller, F., Buffelli, M., 2008. Impaired nerve regeneration in reeler mice after peripheral nerve injury. Eur. J. Neurosci. 27, 12–19.
Okada, T., You, L., Giancotti, F.G., 2007. Shedding light on Merlin's wizardry. Trends Cell Biol. 17, 222–229.
Parkhurst, C.N., Zampieri, N., Chao, M.V., 2010. Nuclear localization of the p75 neurotrophin receptor intracellular domain. J. Biol. Chem. 285, 5361–5368.
Provenzano, M.J., Xu, N., Ver Meer, M.R., Clark, J.J., Hansen, M.R., 2008. p75NTR and sortilin increase after facial nerve injury. Laryngoscope 118, 87–93.
Provenzano, M.J., Minner, S.A., Zander, K., Clark, J.J., Kane, C.J., Green, S.H., Hansen, M.R., 2011. p75(NTR) expression and nuclear localization of p75(NTR) intracellular domain in spiral ganglion Schwann cells following deafness correlate with cell proliferation. Mol. Cell. Neurosci. 47, 306–315.
Rong, R., Surace, E.I., Haipek, C.A., Gutmann, D.H., Ye, K., 2004. Serine 518 phosphorylation modulates merlin intramolecular association and binding to critical effectors important for NF2 growth suppression. Oncogene 23, 8447–8454.
Rouleau, G.A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., et al., 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 363, 515–521.
Schulz, A., Kyselyova, A., Baader, S.L., Jung, M.J., Zoch, A., Mautner, V.F., Hagel, C., Morrison, H., 2014. Neuronal merlin influences ERBB2 receptor expression on Schwann cells through neuregulin 1 type III signalling. Brain 137, 420–432.
Sher, I., Hanemann, C.O., Karplus, P.A., Bretscher, A., 2012. The tumor suppressor merlin controls growth in its open state, and phosphorylation converts it to a less-active more-closed state. Dev. Cell 22, 703–705.
Stangerup, S.E., Caye-Thomasen, P., Tos, M., Thomsen, J., 2006. The natural history of vestibular schwannoma. Otol. Neurotol. 27, 547–552.
Stoll, G., Muller, H.W., 1999. Nerve injury, axonal degeneration and neural regeneration: basic insights. Brain Pathol. 9, 313–325.
Tang, X., Jang, S.W., Wang, X., Liu, Z., Bahr, S.M., Sun, S.Y., Brat, D., Gutmann, D.H., Ye, K., 2007. Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation. Nat. Cell Biol. 9, 1199–1207.
Taniuchi, M., Clark, H.B., Johnson Jr., E.M., 1986. Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc. Natl. Acad. Sci. U. S. A. 83, 4094–4098.
Taniuchi, M., Clark, H.B., Schweitzer, J.B., Johnson Jr., E.M., 1988. Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact, and binding properties. J. Neurosci. 8, 664–681.
Thaxton, C., Lopera, J., Bott, M., Baldwin, M.E., Kalidas, P., Fernandez-Valle, C., 2007. Phosphorylation of the NF2 tumor suppressor in Schwann cells is mediated by Cdc42-Pak and requires paxillin binding. Mol. Cell. Neurosci. 34, 231–242.
Trofatter, J.A., MacCollin, M.M., Rutter, J.L., Murrell, J.R., Duyao, M.P., Parry, D.M., Eldridge, R., Kley, N., Menon, A.G., Pulaski, K., et al., 1993. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 72, 791–800.
Tryggvason, G., Barnett, A., Kim, J., Soken, H., Maley, J., Hansen, M.R., 2012. Radiographic association of schwannomas with sensory ganglia. Otol. Neurotol. 33, 1276–1282.
Verbeke, S., Tomellini, E., Dhamani, F., Meignan, S., Adriaenssens, E., Xuefen le, B., 2013. Extracellular cleavage of the p75 neurotrophin receptor is implicated in its pro-survival effect in breast cancer cells. FEBS Lett. 587, 2591–2596.
Yang, Z., Chen, H., Huo, L., Yang, Z., Bai, Y., Fan, X., Ni, B., Fang, L., Hu, J., Peng, J., Wang, L., Wang, J., 2015. Epigenetic inactivation and tumor suppressor behavior of NGFR in human colorectal cancer. Mol. Cancer Res. MCR 13 (1), 107–119.
Ye, K., 2007. Phosphorylation of merlin regulates its stability and tumor suppressive activity. Cell Adhes. Migr. 1, 196–198.
Yoon, S.O., Casaccia-Bonnefil, P., Carter, B., Chao, M.V., 1998. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J. Neurosci. 18, 3273–3281.
Yue, W.Y., Clark, J.J., Fernando, A., Domann, F., Hansen, M.R., 2011. Contribution of persistent C-Jun N-terminal kinase activity to the survival of human vestibular schwannoma cells by suppression of accumulation of mitochondrial superoxides. Neuro-Oncology 13, 961–973.
Zhou, L., Hanemann, C.O., 2012. Merlin, a multi-suppressor from cell membrane to the nucleus. FEBS Lett. 586, 1403–1408.
Zhou, L., Ercolano, E., Ammoun, S., Schmid, M.C., Barczyk, M.A., Hanemann, C.O., 2011. Merlin-deficient human tumors show loss of contact inhibition and activation of Wnt/beta-catenin signaling linked to the PDGFR/Src and Rac/PAK pathways. Neoplasia 13, 1101–1112.

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● PERSPECTIVE

Schwannomas provide insight into the role of p75^{NTR} and merlin in Schwann cells following nerve injury and during regeneration

Peripheral nerve injury leads to Wallerian degeneration of severed axons, leaving the Schwann cell (SC) sheath behind. Denervated SCs may then either survive and remyelinate a regenerating axon, or they may undergo cell death. Because SCs provide trophic support and guidance cues to regenerating nerve fibers, SC loss severely hampers nerve regeneration (Hall, 1986). Thus, significant work has sought to characterize the molecular mechanisms underlying SC fate following peripheral nerve injury. A deeper understanding of these mechanisms has recently been derived from a somewhat unexpected source; cell survival signaling in benign schwannoma tumors has yielded insight into survival signaling in denervated SCs. This recent evidence implicates a pathway involving the single transmembrane neurotrophin receptor p75^{NTR} and Moesin-ezrin-radixin-like protein (merlin, also known as schwannomin or neurofibromin 2), a membrane-cytoskeleton scaffolding protein linking the cellular membrane to the actin cytoskeleton.

Merlin is the product of the neurofibromatosis type 2 (NF2) gene and functions as a tumor suppressor by mediating contact inhibition. NF2 mutations were first identified in two families affected by hereditary NF2; merlin has since then become recognized for its central role in SC tumorigenesis. Individuals with NF2 accumulate multiple schwannomas over their lifetime including bilateral vestibular schwannomas (VS). NF2 loss-of-function mutations have been identified in the majority of sporadic VSs as well, supporting merlin's central role in schwannoma pathogenesis.

Merlin's tumor suppressor function is controlled by phosphorylation. When it is in the unphosphorylated state, merlin inhibits cell proliferation and when it is in the phosphorylated state it is growth permissive. One of the mechanisms by which merlin suppresses cell growth is by suppressing the cell surface expression of tyrosine kinase receptors for SC growth factors. Interestingly, following nerve injury, merlin becomes phosphorylated which correlates with increased ErbB2 localization in cell membrane lipid rafts that serve as hubs for cell signaling (Brown and Hansen, 2008). The extent to which merlin regulates other, non tyrosine kinase cell membrane receptors involved in SC biology is still being explored.

Recent evidence, however, has emerged that implicates merlin as a key mediator of p75^{NTR} receptor expression and signaling in SCs. p75^{NTR} is a single transmembrane receptor, a member of the Fas/TNF family that when not coupled to any co-receptor only weakly binds mature neurotrophins (in mammals, the four neurotrophins are brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin (NT-3 and NT-4/5) but strongly binds pro-neurotrophins (for example proBDNF, proNGF). A number of co-receptors for p75^{NTR} have been identified including TrkA, TrkB, TrkC, sortilin, and Nogo (Meeker and Williams, 2015). The complexes formed when these co-receptors associate with p75^{NTR} change the affinity profile and elicit differing cellular responses depending on the identity of the co-receptor. In neurons, p75^{NTR}/Trk complexes strongly binds mature neurotrophins to promote neuronal survival, while p75^{NTR}/sortilin binds proneurotrophins to promote neuronal death, and p75^{NTR}/Nogo affects growth cone dynamics and neuronal pathfinding (Teng et al., 2010; Meeker and Williams, 2015). SCs show an increase in cell death following treatment with proneurotrophins (Provenzano et al., 2011; Ahmad et al., 2015). The roles of co-receptors for p75^{NTR} in Schwann cell death (*e.g.*, sortilin), and the mechanisms by which they are regulated, remain to be fully elucidated.

Recent work on signaling upstream of p75^{NTR} demonstrated that merlin regulates p75^{NTR} expression and that p75^{NTR} signaling is dysregulated in merlin-deficient VS cells. In normal SCs, p75^{NTR} expression increases dramatically as the SCs lose axonal contact either following acute axotomy or more gradual nerve degeneration (*e.g.*, auditory nerve degeneration following aminoglycoside induced deafening) (Taniuchi et al., 1986; Provenzano et al., 2011). Merlin becomes phosphorylated following both acute and gradual nerve injury, and temporally correlated with phosphorylation there is an increase in p75^{NTR} expression (Ahmad et al., 2015). However, until recently, it was not clear whether merlin phosphorylation was causally related to p75^{NTR} expression levels. Interestingly VS cells, like denervated SCs, are not in contact with axons; they also lack functional merlin. Therefore perhaps not surprisingly VS cells, like denervated SCs, have elevated p75^{NTR} levels compared to SCs in uninjured nerves (Ahmad et al., 2014). Further, SCs from a transgenic mouse line bearing a dominant-negative merlin mutation (P0ΔSch121-39) also exhibit higher baseline p75^{NTR} levels, even in uninjured nerves. These observations raise the possibility that loss of merlin function, either by mutation or phosphorylation, leads to elevated p75^{NTR} expression. Because VS cells lack endogenous merlin they provide a model to test whether or nor merlin status is involved in p75^{NTR} expression. Replacement of wild-type merlin reduces p75^{NTR} expression in VS cells, confirming that merlin regulates p75^{NTR} expression. Significantly, replacement of a phosphomimetic merlin mutant (S518D) failed to similarly suppress p75^{NTR} expression (Ahmad et al., 2015). These results further establish that merlin phosphorylation is causally linked to the increase in p75^{NTR} expression.

An interesting paradox is that schwannoma cells survive in the absence of axonal contact despite elevated elevated p75^{NTR} levels. Similarly, SCs from nerves in P0ΔSch121-39 mice survive despite elevated p75^{NTR} levels before injury and are resistant apoptosis following nerve injury with even higher levels of p75^{NTR} expression (Ahmad et al., 2015). These observations suggest that merlin not only regulates p75^{NTR} expression levels but also influences the ability of p75^{NTR} to induce apoptosis in SCs. This possibility was confirmed using a transgenic mouse line with an inducible merlin knock-out. Cre mediated Nf2 excision rendered SCs resistant to p75^{NTR}-mediated cell death (Ahmad et al., 2015). Given that p75^{NTR} activation increases cell death in non-neoplastic SCs, what explains the continued survival of schwannoma cells in the face of high p75^{NTR}? Recent evidence suggests that dysregulation in c-Jun terminal kinase (JNK) and nuclear factor kappa B (NF-κB) signaling may explain this unexpected difference in behavior between non-neoplastic SCs and VS cells.

When p75^{NTR} binds pro-neurotrophins, intramembrane cleavage by γ-secretase releases the intracellular domain that then activates JNK and thereby promotes cell death (Kenchappa et al., 2006). However, merlin also suppresses JNK activity; in merlin-deficient VS cells JNK is dysregulated in a way that contributes to cell survival and proliferation (Yue et al., 2011). JNK activity is significantly elevated in VS cells and replacement of merlin suppresses JNK activity. Further, inhibition of JNK with small-molecule or peptide JNK inhibitors as well as suppression of JNK expression with siRNA oligonucleotides reduces VS cell proliferation and increases VS cell death (Yue et al., 2011). Thus, elevated JNK activity in VS cells promotes cell survival in contrast to the pro-death response in non-neoplastic SCs. One mechanism by which JNK promotes VS cell

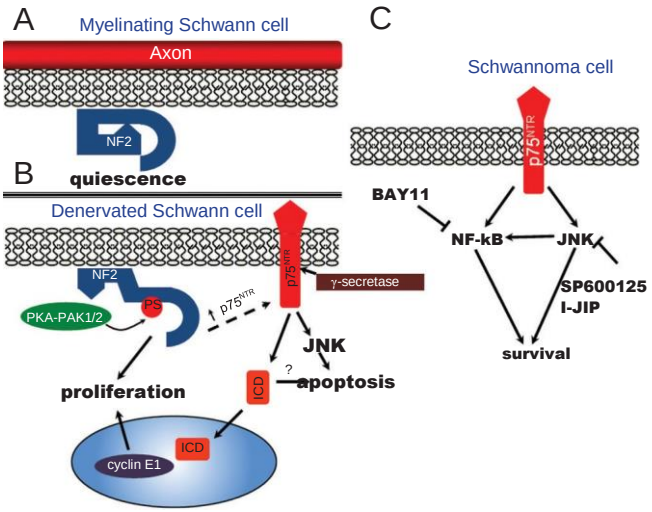

Figure 1 Schematic of merlin and p75^{NTR} signaling in myelinating and demyelinated Schwann cells *versus* schwannoma cells.
(A) Merlin is dephosphorylated in myelinating Schwann cells, which promotes cellular quiescence. (B) When nerve injury occurs, Schwann cells are denervated and merlin is phosphorylated. Denervated Schwann cells may either proliferate or undergo apoptosis. Activation of p75^{NTR} by binding to proneurotrophin promotes apoptosis by gamma secretase cleavage resulting in an intracellular domain that promotes apoptosis. p75^{NTR} also activates the JNK pathway. (C) Signaling downstream of p75^{NTR} is deranged in schwannoma cells. JNK activation by p75^{NTR} promotes survival through a nuclear factor kappaB (NF-κB) dependent pathway and also through an NF-κB independent pathway. NF-κB is also directly activated by p75^{NTR}. Inhibitors of NF-κB, such as BAY11-7082 (BAY11) and of JNK, such as SP600125 or lnhibitor of JNK-based on JNK-interacting protein-1 (I-JIP), have been shown to reduce schwannoma cell survival *in vitro*, confirming that NF-κB and JNK represent potential therapeutic targets.

survival is by suppression of reactive oxygen species in the mitochondria (Yue et al., 2011). Further, JNK inhibition increases oxidative stress in VS cells following γ-irradiation and sensitizes VS cells to radiation though they are otherwise relatively resistant to radiation (Yue et al., 2011). Thus, JNK inhibitors represent excellent potential therapeutic treatments for VSs (**Figure 1**).

Interestingly, treatment of VS cells with proneurotrophins rescues them from apoptosis due to JNK inhibition (Ahmad et al., 2014). This suggests that a pro-survival response is mounted when p75^{NTR} binds proneurotrophins in VS cells in contrast to the pro-death response in non-neoplastic SCs. Indeed, treatment with proneurotrophins activates NF-κB in VS cells (Ahmad et al., 2014). JNK inhibition decreases basal NF-κB activity; however, the increase in NF-κB in response to proneurotrophins occurs even in cells with suppressed JNK (Ahmad et al., 2014). Thus, p75^{NTR} is capable of activating NF-κB *via* a JNK-independent pathway in VS cells. Inhibition of NF-κB by transduction with an adenoviral vector that expresses inhibitor-κB (IκB) overcomes the ability of proneurotrophins to rescue VS cells from apoptosis (Ahmad et al., 2015). Taken together these observations demonstrate that p75^{NTR} activates NF-κB *via* a JNK independent pathway to provide a pro-survival response in VS cells (Gentry et al., 2000). Significantly, network analysis recently implicated aberrant NF-κB activation as a root cause of proliferation in VS cells, and NF-κB inhibitors have been shown to reduce VS cell proliferation *in vitro* further confirming the vital role of this pathway in VS tumorigenesis (Dilwali et al., 2015).

In summary, recent publications have begun to elucidate how merlin regulates responses of SCs to nerve injury and how dysregulation of these responses in the absence of merlin likely contributes to SC tumorigenesis. When SCs lose contact with axons, merlin becomes phosphorylated leading to increased p75^{NTR} expression and ultimately to SC apoptosis and loss. However, in the absence of functional merlin, SCs become resistant to p75^{NTR}-mediated apoptosis. Further, p75^{NTR} signaling elicits a pro-survival response in schwannoma cells, likely contributing to their ability to proliferate and survive in the absence of axons. This pro-survival response may contribute to the relative resistance of VS cells to chemotherapeutic agents such as kinase inhibitors. Thus, simultaneously targeting p75^{NTR} and/or NF-κB may sensitize VS cells to other classes of chemotherapeutic agents. Interestingly, the mechanisms by which VS cells escape cell death also inform our understanding of normal SC behavior following nerve injury.

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References

Ahmad I, Yue WY, Fernando A, Clark JJ, Woodson EA, Hansen MR (2014) p75NTR is highly expressed in vestibular schwannomas and promotes cell survival by activating nuclear transcription factor κB. Glia 62:1699-1712.
Ahmad I, Fernando A, Gurgel R, Jason Clark J, Xu L, Hansen MR (2015) Merlin status regulates p75 expression and apoptotic signaling in Schwann cells following nerve injury. Neurobiol Dis 82:114-122.
Brown KD, Hansen MR (2008) Lipid raft localization of ErbB2 in vestibular schwannoma and schwann cells. Otol Neurotol 29:79-85.
Dilwali S, Briet MC, Kao SY, Fujita T, Landegger LD, Platt MP, Stankovic KM (2015) Preclinical validation of anti-nuclear factor-kappa B therapy to inhibit human vestibular schwannoma growth. Mol Oncol 9:1359-1370.
Gentry JJ, Casaccia-Bonnefil P, Carter BD (2000) Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells. J Biol Chem 275:7558-7565.
Hall SM (1986) The effect of inhibiting Schwann cell mitosis on the re-innervation of acellular autografts in the peripheral nervous system of the mouse. Neuropathol Appl Neurobiol 12:401-414.
Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD (2006) Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219-232.
Meeker RB, Williams KS (2015) The p75 neurotrophin receptor: at the crossroad of neural repair and death. Neural Regen Res 10:721-725.
Provenzano MJ, Minner SA, Zander K, Clark JJ, Kane CJ, Green SH, Hansen MR (2011) p75(NTR) expression and nuclear localization of p75(NTR) intracellular domain in spiral ganglion Schwann cells following deafness correlate with cell proliferation. Mol Cell Neurosci 47:306-315.
Taniuchi M, Clark HB, Johnson EM, Jr. (1986) Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc Natl Acad Sci U S A 83:4094-4098.
Teng KK, Felice S, Kim T, Hempstead BL (2010) Understanding proneurotrophin actions: Recent advances and challenges. Dev Neurobiol 70:350-359.
Yue WY, Clark JJ, Fernando A, Domann F, Hansen MR (2011) Contribution of persistent C-Jun N-terminal kinase activity to the survival of human vestibular schwannoma cells by suppression of accumulation of mitochondrial superoxides. Neuro Oncol 13:961-973.

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Persistent Oxidative Stress in Vestibular Schwannomas After Stereotactic Radiation Therapy

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Objective: Stereotactic radiation therapy is increasingly used to treat vestibular schwannomas (VSs) primarily and to treat tumor remnants following microsurgery. Little data are available regarding the effects of radiation on VS cells. Tyrosine nitrosylation is a marker of oxidative stress following radiation in malignant tumors. It is not known how long irradiated tissue remains under oxidative stress, and if such modifications occur in benign neoplasms such as VSs treated with significantly lower doses of radiation. We immunostained sections from previously radiated VSs with an antibody that recognizes nitrosylated tyrosine residues to assess for ongoing oxidative stress.
Study Design: Immunohistochemical analysis.
Methods: Four VSs, which recurred after excision, were treated with stereotactic radiation therapy. Ultimately each tumor required salvage reresection for regrowth. Histologic sections of each tumor before and after radiation were immunolabeled with a monoclonal antibody specific to nitrotyrosine and compared. Two VSs that underwent reresection of a growing tumor remnant without previous radiation therapy served as additional controls.
Results: Irradiated tumors enlarged in volume by 3.16 to 8.62 mL following radiation. Preradiation sections demonstrated little to no nitrotyrosine immunostaining. Three of four of irradiated VSs demonstrated increased nitrotyrosine immunostaining in the postradiation sections compared with preradiation tumor sections. Nonirradiated VSs did not label with the antinitrotyrosine antibody.
Conclusions: VSs exhibit oxidative stress up to 7 years after radiotherapy, yet these VSs continued to enlarge. Thus, VSs that grow following radiation appear to possess mechanisms for cell survival and proliferation despite radiation-induced oxidative stress. **Key Words:** 3-Nitrotyrosine—Acoustic neuroma—Oxidative stress—Radiation—Reactive oxygen species—Vestibular schwannoma.

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Ionizing-radiation (IR) has been increasingly used in an effort to arrest tumor growth in select patients with vestibular schwannomas (VSs). Most commonly, IR is delivered in the form of stereotactic radiosurgery (SRS) or fractionated stereotactic radiotherapy (FRT). Outcomes of IR in the treatment of VSs have been widely reported and it is generally accepted that SRS and FRT yield high tumor control rates, relatively low associated morbidity and good quality of life outcomes (1,2). Despite the increasing use of IR in the treatment of VSs, the effects of IR on the VS cells themselves are poorly understood. Compared with malignant neoplasms, VS cells in culture possess radioresistant characteristics, consistent with their low proliferative capacity (3–6). Further, many studies that have examined the histopathological features of irradiated VSs fail to find evidence of significant radiation damage to the tumor cells (5,7). Such observations raise the possibility that the consequences of IR on VS growth involve indirect effects such as decreased tumor vascularity.

IR-induced cell damage involves multiple mechanisms and processes. Beyond direct damage to DNA molecules, IR exposure leads to increased production of reactive nitrosative and oxidative free radical species (RONS) (8,9). Furthermore, ROS levels continue to rise beyond those that occur within milliseconds initial radiation exposure leading to cellular oxidative stress (10–12). Radiation-induced oxidative stress may also spread from targeted cells to bystander neighboring cells (13). Such highly reactive oxidizing free-radicals cause cellular damage by oxidizing and nitrosylating cellular macromolecules including nucleic acids, proteins, and lipids (9). One of the byproducts of the oxidative and nitrosative stress, 3-nitrotyrosine (3-NT), has been used as a surrogate marker for radiation-induced oxidative cellular injury (14–16). In this study, we used 3-NT

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immunostaining to evaluate the presence of radiation-induced oxidative cellular stress in VSs before and after radiation in a rare series of four VSs which were initially resected, then irradiated for recurrent growth and ultimately underwent salvage reresection for persistent growth after IR.

METHODS

All procedures for obtaining patient information and VS samples were approved by the Institutional Review Board. Patient demographics and case histories were obtained by retrospective chart review. 3D tumor volumes were calculated based on the axial postcontrast T1 magnetic resonance imaging (MRI) thin section images using Vitrea software [version 6.6.2, Vital images, Toshiba Medical Systems, Minnetonka, MN]. In all cases, the tumor was manually outlined on the axial postcontrast images on all sections followed by computerized compilation of summated tumor volume and generation of a volumetric model. Segmented volumetric data was re-reviewed postanalysis to ensure measurement accuracy. Tumor volumes were calculated from the MRI at the time of presentation, before radiation and before reresection. For controls, we selected two patients with VSs who underwent subtotal resection of a VS with subsequent regrowth and reresection of the VS without any IR therapy.

Samples from each specimen were fixed in 10% neutral buffered formalin and paraffin embedded. Five-micron-thick sections of formalin fixed paraffin embedded tissue were stained with hematoxylin and eosin (H&E) and were also used for immunohistochemical assays.

3-Nitrotyrosine Immunohistochemistry

Paraffin sections were deparaffinized with serial xylene washes and rehydrated with serial levels of ethanol. Slides were retrieved in citrate buffer (pH 6.0) using a Decloaking Chamber (Biocare, Concord, CA). Endogenous peroxidase was quenched with 3% hydrogen peroxide for 8 minutes followed by incubation in 10% goat serum to block nonspecific binding. Anti-3-nitrotyrosine rabbit polyclonal antibody (Millipore; #06284, Billerica, MA, 1:2,000) was applied for 1 hour in Dako buffer (Carpinteria, CA) at room temperature. Dako Rabbit EnVision HRP System reagent was applied for 30 minutes and then slides were developed with Dako diaminobenzidine (DAB) plus for 5 minutes followed by DAB Enhancer for 3 minutes. Slides were counterstained with hematoxylin. Negative control slides were stained using the same procedure, omitting the primary antibody. 3-Nitrotyrosine immunohistochemical staining was scored by a pathologist (A.W.) blinded to the patient information and treatment conditions according to the following system: (−) = no staining, (+) = diffuse background staining of stroma, (++) = strong, specific cytoplasmic staining in cells. 3-NT immunohistochemical staining and scoring were repeated two times. Images were prepared for publication using Adobe PhotoShop and Illustrator software (Adobe, San Jose, CA).

RESULTS

Table 1 provides demographic and tumor characteristics of the patients in this study. Four patients (A–D) had a subtotal resection of a VS with subsequent growth identified on serial imaging. Each patient received a single dose of LinAcSRS to treat the growing tumor remnant (Table 1 lists treatment specifications for each tumor). In each of these four cases, tumor growth continued following SRS prompting repeat tumor resection. Two patients (E, F) underwent subtotal resection of a VS with subsequent growth of the tumor remnant. In these patients, a second tumor resection was performed without intervening IR and thus these patients serve as a control of previously operated, unirradiated tumors. Figure 1 presents the MRI findings for the four patients (A–D) that received IR while Figure 2 presents the MRI finding for the two patients (E, F) that did not receive IR.

Immunohistochemical staining for 3-NT was performed on sections from both the initial resection and from the subsequent resection of the recurrent tumor. Three of the four irradiated tumors (A–C) demonstrated a dramatic increase in 3-NT immunostaining compared with their initial tumor counterparts resected before radiation (Fig. 3). There was little to no 3-NT immunostaining in any of the sections from the initial tumor specimens (Fig. 3). Likewise, there was little to no 3-NT immunostaining in sections from recurrent tumors that had not received previous radiation (E, F) (Fig. 3). In contrast to specimens from patients A–C, sections from the irradiated tumor from patient D failed reveal

TABLE 1. *Patient demographic information and pertinent treatment-related data*

Radiated Patients	Marginal Radiation Dose (gy)	Maximum Radiation Dose (gy)	Prescribed Isodose	Initial tumor Volume (mL)	Interval to Radiation (mo)	Preradiation Tumor Volume (mL)	Interval to Salvage (mo)	Presalvage Tumor Volume (mL)
A	12	17.1 gy	70%	42.23	38	14.13	7	26.95
B	12.5	19 gy	65%	MRI not available	89	2.17	48	6.46
C	12	17.9 gy	67%	0.96	79	1.30	82	4.46
D	12	17.9 gy	67%	22.02	65	5.48	12	7.03

Nonradiated Patients	Gender	Age at Presentation	Initial Tumor Volume (mL)	Interval to Regrowth (mo)	Tumor Volume Prior to 2nd Resection (mL)
E	M	64	15.41	79	9.72
F	F	46	5.67	35	2.25

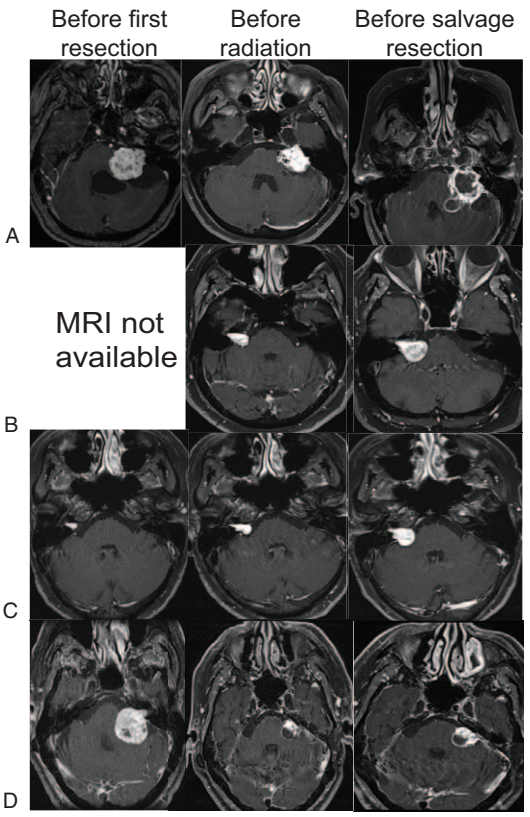

FIG. 1. Representative T1 postgadolinium magnetic resonance imaging results from four patients (*A–D*) who underwent a second resection of a vestibular schwannoma following radiation of a growing tumor remnant.

significant 3-NT immunostaining. Additional sections from each tumor were stained with H&E to examine overall pathological characteristics. Two of the four tumors demonstrated histopathological changes consistent with previous radiation including vessel hyalinization and luminal narrowing and fibrinoid necrosis (Fig. 4).

A pathologist blinded to the specimen treatment scored the 3-NT immunostaining and the results are presented in Table 2. On this scale only ++ is considered a true-positive whereas + likely represents nonspecific background staining. Three of the four tumors (A–C) that had previously been irradiated demonstrated a significant increase in 3-NT immunostaining (++) whereas all tumors that had not been previously irradiated (both initial and recurrent tumors) and one irradiated tumor (patient D) were scored as having no (−) or only background (+) staining.

DISCUSSION

Accumulating evidence suggests that SRS and FRT are safe and effective treatments for appropriately selected VSs (1,2); accordingly, increasing numbers of VSs are being treated with SRS/FRT. While the prolific evaluation of clinical outcomes following IR for VSs continues, there is a paucity of research on the radiobiology of VSs. This is likely due, at least in part, to the low numbers of tumors that have been resected following IR and the challenges of growing large numbers of primary VS cells in culture (17,18). Here, we had a unique opportunity to analyze IR-induced cellular damage in a series of four paired VS specimens that progressed or recurred after microsurgical excision, were treated with IR, progressed again and ultimately required salvage reresection. The decision of what constituted tumor progression and whether or not to resect the tumors after radiation treatment was a complex decision process that involved surgeons, radiation-oncologists, and the patient. Given the retrospective nature of the study it was not possible to determine the specific criteria and decision processes that led to surgery in these cases. It is important to note that different surgeons and services were involved in these cases so the decision-making process was not necessarily uniform. Further, we were able to analyze paired specimens from two other patients that underwent an initial tumor resection followed by re-resection of a recurrent tumor but without receiving any intervening IR. These later specimens provide an excellent control for potential effects of surgery on the histopathological findings.

The data provide evidence for ongoing oxidative stress months and years following IR in three of four irradiated VSs, evidenced by robust 3-NT immunostaining up to 7 years following IR. Despite this oxidative stress, there was ongoing tumor growth indicating that the tumor cells continued to proliferate under this stress. The reason(s) for lack of 3-NT immunostaining in patient D following IR are unknown. One possibility is that the portion of the tumor encompassed in the histopathology specimen represents areas of de novo tumor growth and did not include areas that were previously irradiated. VS control rates are uniformly good with IR therapy and some studies have even demonstrated varying degrees of VS regression following IR therapy in a subset of patients

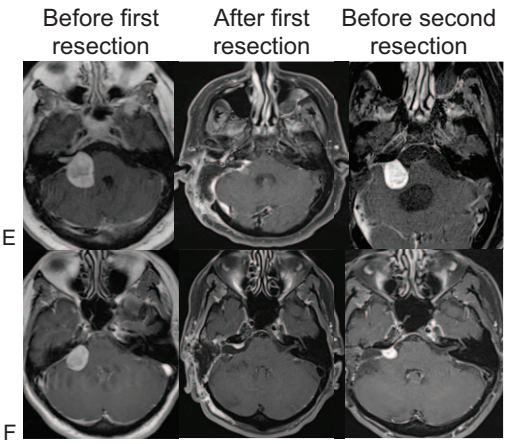

FIG. 2. Representative T1 postgadolinium magnetic resonance imaging results from 2 patients (*E, F*) who underwent a second resection of a vestibular schwannoma after growth of a tumor remnant. These patients did not receive any radiation therapy.

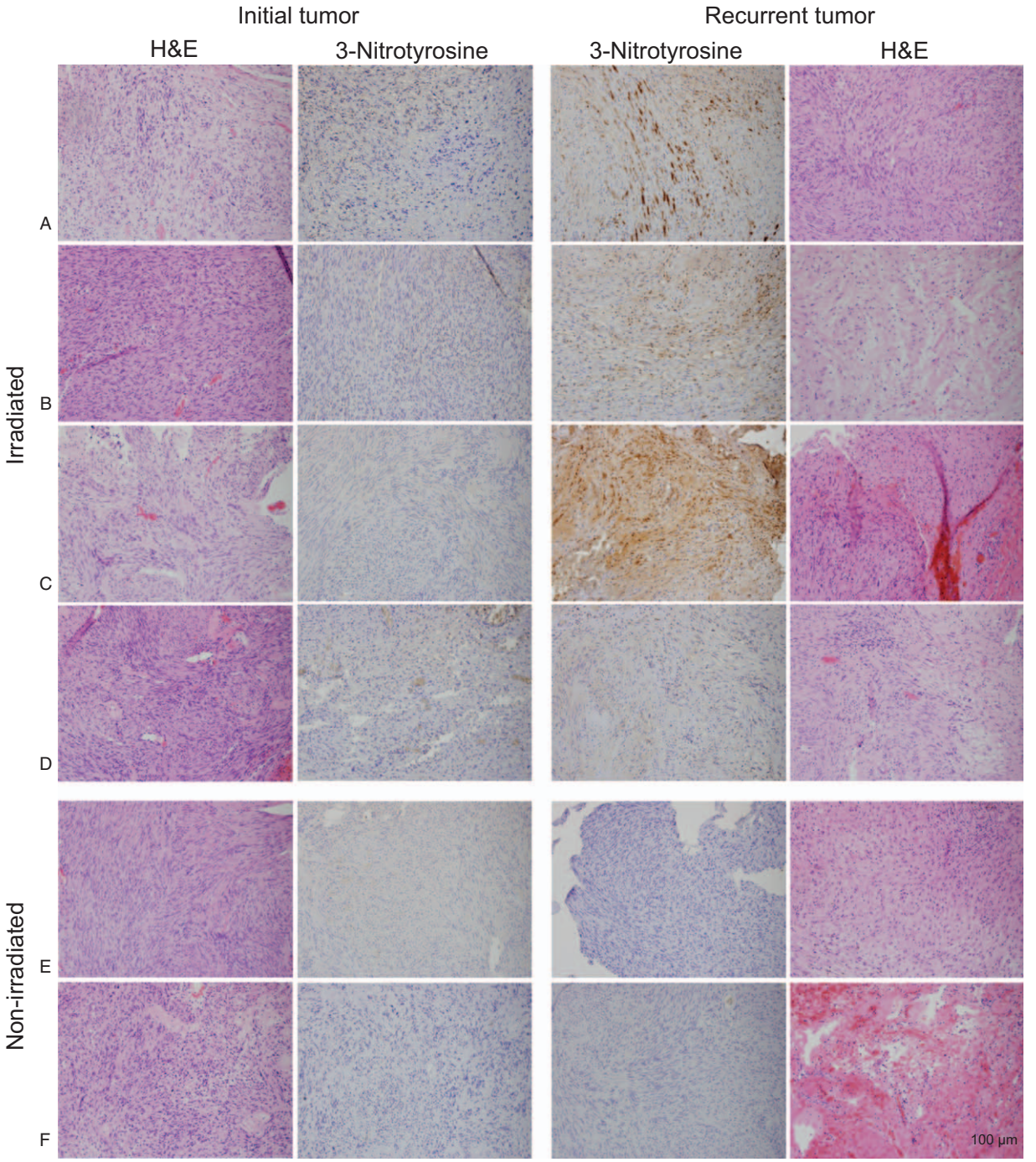

FIG. 3. Radiation increases 3-nitrotyrosine (3-NT) immunostaining in vestibular schwannomas (VSs). Sections of VSs were immunostained with an antibody that detects 3-NT. For four VSs (*A–D*) sections were derived from the initial tumor resection and from the recurrent tumor that had previously been irradiated. For two VSs (E,F) sections were derived from the initial tumor resection and from the recurrent tumor that had not previously been irradiated. Representative hematoxylin and eosin (H&E) stains are shown for each tumor. Scale bar = 100 μm.

(19). However, in contrast to many malignant tumors, rather than inducing tumor regression, IR typically only arrests tumor growth in most responsive VSs (2). These clinical observations are consistent with the fact that IR doses much higher than those currently employed in treatment of VSs (e.g. ≥30 Gy) are required to induce cell cycle arrest and cell death in cultured VS cells (3,6). Amazingly, even after 150 Gy of IR, some cultured VS cells survive and remain viable (6). Others have demonstrated that VSs resected after IR retain viable VS cells

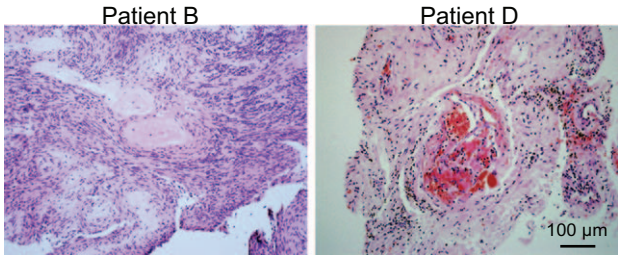

FIG. 4. Histopathological radiation changes to vestibular schwannomas. Hematoxylin and eosin stained sections from the post-irradiated tumors from patients *B* and *D* demonstrating vessel hyalinization and luminal narrowing and fibrinoid necrosis consistent with radiation changes. Scale bar = 100 μm.

with the typical histologic appearance of non-radiated VS cells and a proliferative capacity (4,5,7,20). Consistent with these previous reports, two of the four post-radiation specimens in our study demonstrated no post-radiation changes. Taken together these observations suggest that, compared to malignant neoplasms, VS cells are relatively radioresistant.

There are several potential mechanisms that may contribute to the relative radioresistance in VS cells (4). For instance, radiation-induced cell death depends in part on the proliferation rate of the targeted cells and VS cells have a very low proliferative capacity (18). Indeed, stimulating cultured VS cells to proliferate increases radiosensitivity while inhibiting proliferation protects VS cells from radiation-induced apoptosis (3).

Another mechanism that appears to contribute to the relative radioresistance of VS cells relates to their capacity to mitigate oxidative stress. IR indirectly inflicts molecular and cellular damage through the formation of highly reactive RONS. IR ionizes H_2O molecules into free radicals and induces the action of nitric oxide synthase, mitochondrial oxidase and cytoplasmic NADPH synthase, all of which result in the generation of RONS (11,21,22). Due to their lack of merlin protein expression, VS cells display persistent activation of several intracellular kinase signaling cascades, including phosphatidyl-inositol 3- kinase/Akt/mTORC1, extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK), p21-activated kinase, and c-Jun N-terminal kinase (JNK), among others (23–29). Of these, activation of JNK appears to protect VS cells from cell death by inhibiting accumulation of mitochondrial ROS (23). Indeed, inhibition of JNK increases VS cell radiosensitivity correlated with an increase in mitochondrial oxidative stress following IR (30). Thus, persistent activation of JNK due to a lack of merlin protein expression reduces oxidative stress and decreases sensitivity of VS cells to IR.

RONS result in several molecular modifications including carbohydrate oxidation, lipoprotein oxidation, DNA hydroxylation, and protein oxidation (9,15,16,31). Many of these molecular alterations are detectable and are used as surrogate biomarkers for oxidative cellular stress. One of these markers, 3-NT, is a frequently used marker of cellular oxidative stress (31). 3-NT is the byproduct of the oxidation of tyrosine residues in proteins by peroxynitrite. Using 3-NT, the presence of oxidative cellular stress has been demonstrated acutely in radiated tissues and cellular cultures including lung, cornea, skin, intestine and kidney as well as cultures of hepatocytes, squamous cell carcinoma of the head and neck, and gliomas (15,16,21). However, to our knowledge, no previous studies have determined whether 3-NT persists in the long term in these tissues following IR. Thus, our unique access to paired tumors specimens before and following IR provides the first evidence of persistent 3-NT for several years after exposure to IR. These results indicate ongoing oxidative stress in tissues for months and years following IR.

There is growing evidence that IR results in a chronic oxidative stress. The mechanisms for these chronic oxidative changes following radiation have not been fully elucidated. IR-induced mitochondrial dysfunction likely contributes to chronic overproduction of mitochondrial RONS (22,32–34). 3-NT immunostaining in VSs up to 7 years after IR supports the theory that IR results in long-term chronic oxidative stress; yet, VSs may continue to grow in these conditions.

One aspect of the four irradiated patients in this study that differs from many in the radiotherapy literature is that these tumors had all been observed for tumor growth before receiving IR. Significantly, a recent study of large VSs that underwent subtotal resection found that over 30% of VSs that received IR for growth of tumor remnants demonstrated persistent growth and ultimately required further resection (35). These findings are similar to our patients and highlight the differences in clinical success of IR for VSs when IR use is restricted to tumors that have already been observed and found to have documented growth.

One could argue that the tumors analyzed in this study could be considered ''more aggressive'' or ''less responsive to radiation'' than typical vestibular schwannomas based on the fact that they recurred/progressed after previous surgical removal. However, other vestibular schwannomas that are controlled with radiation seem as, or perhaps even more, likely to demonstrate 3-NT labeling, indicative of ongoing oxidative stress, than tumors that grow after radiation. Ultimately, data from a larger number of vestibular schwannomas will be required to verify chronic oxidative stress following radiation.

TABLE 2. *3-nitrotyrosine immunohistochemistry scoring*

Radiated Patients	First Resection	Second Resection
A	+	++
B	−	++
C	−	++
D	−	+
Nonirradiated patients		
E	+	+
F	−	−

Our findings also highlight the importance of long-term follow-up for VS patients following IR to assess for delayed tumor progression. While studies demonstrating long-term tumor control with microsurgery and IR are emerging (36–38), most of the data in the literature are based on more limited follow-up periods. In two recent systematic reviews of VS outcomes, mean follow-up was reported as ≤5 years in at least 23 studies (1,2). Two of the VSs in this study demonstrated chronic oxidative stress 4 and 7 years after IR and still progressed. The potential for delayed malignant transformation following IR also warrants long-term follow-up. IR induces immediate genetic damage along with a host of long-term indirect cellular carcinogenic effects (22,39). Related to the findings in these VS specimens is the observation that IR-induced mitochondrial dysfunction leads to further genomic instability secondary to chronic overproduction of RONS (32,33). Furthermore, chronic IR-induced oxidative stress appears to promote mutagenesis and tumorigenesis in human thyrocytes (34).

CONCLUSIONS

The data reported here demonstrate chronic oxidative stress in VSs up to 7 years after radiation. Further, VSs may continue to progress in spite of these effects suggesting that VS cells possess mechanisms for cell survival and proliferation despite radiation-induced oxidative stress and highlighting the need for long-term follow-up in patients treated with IR.

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REFERENCES

1. Wolbers JG, Dallenga AH, Mendez Romero A, et al. What intervention is best practice for vestibular schwannomas? A systematic review of controlled studies. *BMJ Open* 2013;22:3.
2. Arthurs BJ, Fairbanks RK, Demakas JJ, et al. A review of treatment modalities for vestibular schwannoma. *Neurosurg Rev* 2011;34:265–77.
3. Hansen MR, Clark JJ, Gantz BJ, et al. Effects of ErbB2 signaling on the response of vestibular schwannoma cells to gamma-irradiation. *Laryngoscope* 2008;118:1023–30.
4. Yeung AH, Sughrue ME, Kane AJ, et al. Radiobiology of vestibular schwannomas: Mechanisms of radioresistance and potential targets for therapeutic sensitization. *Neurosurg Focus* 2009;27:E2.
5. Lee F, Linthicum F Jr, Hung G. Proliferation potential in recurrent acoustic schwannoma following gamma knife radiosurgery versus microsurgery. *Laryngoscope* 2002;112:948–50.
6. Anniko M, Arndt J, Noren G. The human acoustic neurinoma in organ culture. II. Tissue changes after gamma irradiation. *Acta Otolaryngol* 1981;91:223–35.
7. Jacob A, Igarashi S, Platto T, et al. The solid component of radiographically non-growing, post-radiated vestibular schwannoma retains proliferative capacity: Implications for patient counseling. *Ann Otol Rhinol Laryngol* 2015;124:834–40.
8. Biaglow JE, Mitchell JB, Held K. The importance of peroxide and superoxide in the X-ray response. *Int J Radiat Oncol Biol Phys* 1992;22:665–9.
9. Hall E. *Radiobiology for the Radiologist*. Philadelphia, PA: Lippincott Williams & Wilkins; 2000.
10. Du C, Gao Z, Venkatesha VA, et al. Mitochondrial ROS and radiation induced transformation in mouse embryonic fibroblasts. *Cancer Biol Ther* 2009;8:1962–71.
11. Gao Z, Sarsour EH, Kalen AL, et al. Late ROS accumulation and radiosensitivity in SOD1-overexpressing human glioma cells. *Free Radic Biol Med* 2008;45:1501–9.
12. Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Lett* 2012;327:48–60.
13. Hei TK, Zhou H, Chai Y, et al. Radiation induced non-targeted response: Mechanism and potential clinical implications. *Curr Mol Pharmacol* 2011;4:96–105.
14. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol* 1996;271: C1424–37.
15. Tsuji C, Shioya S, Hirota Y, et al. Increased production of nitrotyrosine in lung tissue of rats with radiation-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L719–25.
16. Coleman MC, Olivier AK, Jacobus JA, et al. Superoxide mediates acute liver injury in irradiated mice lacking sirtuin 3. *Antioxid Redox Signal* 2014;20:1423–35.
17. Schularick NM, Clark JJ, Hansen MR. Primary culture of human vestibular schwannomas. *J Vis Exp* 2014;89:e51093.
18. Hansen MR, Roehm PC, Chatterjee P, et al. Constitutive neuregulin-1/ErbB signaling contributes to human vestibular schwannoma proliferation. *Glia* 2006;53:593–600.
19. Slane BG, Goyal U, Grow JL, et al. Radiotherapeutic management of vestibular schwannomas using size- and location-adapted fractionation regimens to maximize the therapeutic ratio. *Pract Radiat Oncol* 2017;7:e233–41.
20. Lee DJ, Westra WH, Staecker H, et al. Clinical and histopathologic features of recurrent vestibular schwannoma (acoustic neuroma) after stereotactic radiosurgery. *Otol Neurotol* 2003;24:650–60.
21. Robbins ME, Zhao W. Chronic oxidative stress and radiation-induced late normal tissue injury: A review. *Int J Radiat Biol* 2004;80:251–9.
22. Szumiel I. Ionizing radiation-induced oxidative stress, epigenetic changes and genomic instability: The pivotal role of mitochondria. *Int J Radiat Biol* 2015;91:1–12.
23. Yue WY, Clark JJ, Fernando A, et al. Contribution of persistent C-Jun N-terminal kinase activity to the survival of human vestibular schwannoma cells by suppression of accumulation of mitochondrial superoxides. *Neurooncology* 2011;13:961–73.
24. James MF, Han S, Polizzano C, et al. NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. *Mol Cell Biol* 2009;29:4250–61.
25. Yi C, Wilker EW, Yaffe MB, et al. Validation of the p21-activated kinases as targets for inhibition in neurofibromatosis type 2. *Cancer Res* 2008;68:7932–7.
26. Flaiz C, Chernoff J, Ammoun S, et al. PAK kinase regulates Rac GTPase and is a potential target in human schwannomas. *Exp Neurol* 2009;218:137–44.
27. Rong R, Tang X, Gutmann DH, et al. Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. *Proc Natl Acad Sci U S A* 2004;101:18200–5.
28. Giovannini M, Bonne NX, Vitte J, et al. mTORC1 inhibition delays growth of neurofibromatosis type 2 schwannoma. *Neurooncology* 2014;16:493–504.
29. Jacob A, Lee TX, Neff BA, et al. Phosphatidylinositol 3-kinase/AKT pathway activation in human vestibular schwannoma. *Otol Neurotol* 2008;29:58–68.
30. Yue WY, Clark JJ, Telisak M, et al. Inhibition of c-Jun N-terminal kinase activity enhances vestibular schwannoma cell sensitivity to gamma irradiation. *Neurosurgery* 2013;73:506–16.
31. Teixeira D, Fernandes R, Prudencio C, et al. 3-Nitrotyrosine quantification methods: Current concepts and future challenges. *Biochimie* 2016;125:1–11.
32. Dayal D, Martin SM, Owens KM, et al. Mitochondrial complex II dysfunction can contribute significantly to genomic instability after exposure to ionizing radiation. *Radiat Res* 2009;172:737–45.
33. Shimura T, Kunugita N. Mitochondrial reactive oxygen species-mediated genomic instability in low-dose irradiated human cells through nuclear retention of cyclin D1. *Cell Cycle* 2016;15:1410–4.

34. Ameziane-El-Hassani R, Talbot M, de Souza Dos Santos MC, et al. NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. *Proc Natl Acad Sci U S A* 2015;112:5051–6.
35. Monfared A, Corrales E, Theodosopoulos P, et al. Facial nerve outcome and tumor control rate as a function of degree of resection in treatment of large acoustic neuromas: Preliminary report of the acoustic neuroma subtotal resection study. *Neurosurgery* 2015;79:194–203.
36. Frischer JM, Gruber E, Schoffmann V, et al. Long-term outcome after Gamma Knife radiosurgery for acoustic neuroma of all Koos grades: A single-center study. *J Neurosurg* 2018;2:1–10.
37. Hasegawa T, Kida Y, Kobayashi T, et al. Long-term outcomes in patients with vestibular schwannomas treated using gamma knife surgery: 10-year follow up. *J Neurosurg* 2013;119 (suppl): 10–6.
38. Hasegawa T, Kida Y, Kato T, et al. Long-term safety and efficacy of stereotactic radiosurgery for vestibular schwannomas: Evaluation of 440 patients more than 10 years after treatment with Gamma Knife surgery. *J Neurosurg* 2013;118:557–65.
39. Kadhim M, Salomaa S, Wright E, et al. Non-targeted effects of ionising radiation—implications for low dose risk. *Mutat Res* 2013;752:84–98.