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.

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, p75NTR, 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 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, 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 p75NTR 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<sup>NTR</sup>, 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 \( p75^{\text{NTR}} \) 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. \( p75^{\text{NTR}} \) 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 \( Nf2 \) (P0Sch\( \Delta \)39-121) demonstrate elevated \( p75^{\text{NTR}} \) levels compared to nerves from wild-type mice. Finally, replacement of merlin into human vestibular schwannoma (VS) cultures reduces \( p75^{\text{NTR}} \) expression. Taken together, these data demonstrate that merlin suppresses \( p75^{\text{NTR}} \) expression in SCs and VSs (appendix, Ahmad, et al. Neurobiol Dis, 2015).

Increased \( p75^{\text{NTR}} \) 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 \( p75^{\text{NTR}} \) expression (appendix, Ahmad, et al. Neurobiol Dis, 2015), suggesting that the phosphorylation status of merlin determines its ability to suppress \( p75^{\text{NTR}} \) expression.

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

To examine the influence of \( p75^{\text{NTR}} \) cleavage on VS cell proliferation, we transduced human VS cultures with adenoviral vectors expressing the intracellular domain (ICD) of \( p75^{\text{NTR}} \) with or without nuclear export or localization signals. Our preliminary results did not find any significant differences in VS

![Figure 1](attachment:image.png)

Figure 1. Overexpression of \( p75^{\text{NTR}} \) intracellular domain (ICD) does not increase VS cell proliferation. Human VS cultures were transduced with adenoviruses that express the ICD \( p75^{\text{NTR}} \) 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.
cell proliferation (as determined by EdU uptake) in cultures that over-express the p75NTR ICD isoforms (Fig. 1).

**Task 3-** Determine the cytoprotective effects of p75NTR signaling in VS cells treated with JNK or mTOR inhibitors.

Activation of p75NTR 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 p75NTR to activate NF-κB (appendix, Ahmad, et al. *Glia*, 2014). mTOR inhibitors did not induce VS cell death singly or in combination with the p75NTR inhibitory peptide, TAT-Pep5 (data not shown).

**Task 4-** Determine the cytoprotective effects of p75NTR 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 p75NTR 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.

**Task 5-** Define the ability of simultaneous inhibition of p75NTR and mTOR or JNK to suppress human schwannoma xenograft growth.
Although activation of p75<sup>NTR</sup> protects VS cells from cell death to JNK inhibitors, we did not find any additive benefit of inhibiting p75<sup>NTR</sup> 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<sup>NTR</sup> 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.](image)

**Task 6** - Define the ability of simultaneous inhibition of p75<sup>NTR</sup> and mTOR or JNK to suppress schwannoma growth in periostinCre;Nf2<sup>f/f</sup> 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<sup>f/f</sup> mice with AS602801 or control vehicle for 75 days with serial audiometric assessment by measuring auditory brainstem response thresholds. The tumors specimens have been transferred to the Hansen lab at Iowa and EdU proliferation and TUNEL labeling will be performed once the sections have been cut. Tumor volume and ABR threshold data are currently being analyzed by Dr. Yates group and will be available in the next few months.

**Training and professional development** - Nothing to report.

**Dissemination of results to communities of interest** - Two manuscripts have been published (see appendix) and a third has been submitted and is under review.
Plans during the next reporting period to accomplish the goals- Our principal objective for the next reporting period is to wrap up the animal tumor model experiments in Tasks 5 and 6. This includes analyzing cell death in the human xenografts, analyzing cell proliferation and death in tumors from the periostinCre; Nf2yper mice, and determining tumor volume and hearing results in the periostinCre; Nf2yper mice. We are encouraged by effectiveness of AS602801 in suppressing VS xenograft growth and will consider this compound for human trials pending the outcome of Task 6. We are also continuing to explore the cytoprotective responses in VS cells that render them radioresistant.
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 p75NTR 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-
No significant changes in objectives and scope are planned. 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, conference papers, and presentations-

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


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


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

<table>
<thead>
<tr>
<th>Name</th>
<th>Project Role</th>
<th>Nearest person month worked</th>
<th>Contribution to project</th>
<th>Funding support</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marlan R. Hansen</td>
<td>Principal investigator</td>
<td>1</td>
<td>Helped design, execute, and interpret experiments; preparation of data for publication and writing.</td>
<td>NIH, Clinical and departmental funds, endowed professorship</td>
</tr>
<tr>
<td>Frank Canady</td>
<td>Pre-doctoral scholar</td>
<td>10</td>
<td>Design, performance, and interpretation of experiments; preparation of data for publication and writing.</td>
<td>University of Iowa Carver College of Medicine and departmental funds</td>
</tr>
<tr>
<td>Jed Rasmussen</td>
<td>Post-doctoral fellow</td>
<td>5</td>
<td>Designed and performed experiments, cell signaling and protein trafficking, cell culture</td>
<td></td>
</tr>
<tr>
<td>J. Jason Clark</td>
<td>Research Assistant</td>
<td>7</td>
<td>Designed and performed experiments, radiation, maintenance of mouse colonies, cell culture</td>
<td>NIH P30, Departmental funds</td>
</tr>
</tbody>
</table>

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^{ff} mice.
8. Special reporting requirements

Nothing to report
9. Appendices

1- Ahmad, I, Yue, WY, Fernando, A, Clark, JJ, Woodson, EA, Hansen, MR. p75NTR is highly expressed in vestibular schwannomas and promotes cell survival by activating NF-κB. *Glia*, 2014, 62(10):1699-712.*cover

p75NTR 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 p75NTR 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 p75NTR expression, VS cells are resistant to apoptosis due to treatment with proNGF, a high affinity ligand for p75NTR. Furthermore, treatment with proNGF protects VS cells from apoptosis due to c-Jun N-terminal kinase (JNK) inhibition indicating that p75NTR 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.lkB.SerS32/36A virus, which inhibits NF-κB activation. Compared with control virus, Ad.lkB.SerS32/36A significantly increased apoptosis including in VS cells treated with proNGF. Thus, in contrast to non-neoplastic SCs, p75NTR 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.

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).

**p75NTR**

p75NTR is the founding member of the tumor necrosis family (TNF) receptor superfamily and was the first identified nerve growth factor receptor (Bothwell, 1995). p75NTR binds mature neurotrophins with low affinity, while proneurotrophins bind avidly to p75NTR (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 p75NTR (Hempestead et al., 1991).

Activation of p75NTR elicits a variety of responses, including apoptosis or cell survival, depending on the cellular context. In the absence of Trk receptors, p75NTR 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 p75NTR signaling initiates cell death, pro-neurone growth factor (NGF) and pro-brain derived neurotrophic factor (BDNF) induce apoptosis in cells expressing p75NTR (Clewes et al., 2008; Koshimizu et al., 2010; Masoudi et al., 2009; Provenzano et al., 2011). This proapoptotic function of p75NTR 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, p75NTR signaling promotes cell survival. What determines whether p75NTR activation leads to cell death or survival remains unknown. However, p75NTR 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).

**p75NTR and JNK Signaling in SCs**

Following axotomy, SCs upregulate p75NTR expression (Provenzano et al., 2008; Taniuchi et al., 1986). In the absence of reinnervation, denervated SCs ultimately undergo p75NTR-mediated apoptosis (Ferri and Bisby, 1999; Petratos, 2003; Syroid et al., 2000). Consistent with a proapoptotic function of p75NTR 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 VVS arise from cells of the SC lineage, they express p75NTR 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 p75NTR in primary VS specimens and the responses of primary cultures derived from acutely resected human VSs to high affinity p75NTR 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, p75NTR 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.IκBα (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), p75NTR (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% trypsin 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 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 (Hansen et al., 2008; Yue et al., 2011). Only S100-positive cells were scored. Given the variability in basal apoptotic rates for each primary culture, the percent of TUNEL-positive VS cells was scored as a fold change in activity relative to the control condition for each repetition. Each condition was performed in duplicate and repeated on 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 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 p75NTR and #4326317E for human glyceroldehyde 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 p75NTR 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-p75NTR (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 ≥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 p75NTR**

SCs upregulate p75NTR expression following denervation by axotomy and in the absence of reinnervation, ultimately undergo p75NTR-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 p75NTR in VSs with normal nerve. We used real-time RT-PCR to compare the
ratio of p75NTR 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 p75NTR to GAPDH transcript levels in VSs compared with normal vestibular nerve. To confirm increased p75NTR protein expression in VSs compared with normal nerves, we quantified p75NTR 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 p75NTR/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-p75NTR antibodies. p75NTR 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 p75NTR 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 p75NTR 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.
These results suggest that VS cells are resistant to proNGF-mediated apoptosis despite high levels of p75NTR expression.

Sortilin functions a p75NTR 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<sup>NTR</sup>-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 p75NTR 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<sup>NTR</sup> ligands protect VS cells from apoptosis we treated cultures maintained in the presence of SP600125 with proBDNF. 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 b<sub>1</sub>, 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 b-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 p75NTR, but not Erb2, signaling promotes survival of VS cells with suppressed JNK activity.

ProNGF Activates NF-κB Independent of JNK

In neuroblastoma cells, p75NTR 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 p75NTR Signaling

We next sought to determine whether the activation of NF-κB by proNGF contributes to the ability of p75NTR signaling to promote VS cell survival. To inhibit NF-κB function, we transduced VS cultures with an adenoviral vector that expresses...
a mutant isoform of IkBα (Ad5.IkBα(S)). This isoform is not able to be phosphorylated on S32 and S36 and thus blocks NF-κB activation by preventing the dissociation of IkBα 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.IkBα(S) 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)
compared with cultures maintained without viral vectors (Fig. 5B) raising the possibility that adenoviral vectors sensitize VS cell cultures to upstream activators of NF-kB. 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-kB abolishes the ability of proNGF to rescue VS cells indicating that activation of NF-kB is necessary for the prosurvival effect of p75NTR 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 p75NTR 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. HE193 cells). We find that p75NTR expression is elevated in VS cells. This elevated expression represents an increase both in transcript and protein levels. Non-neoplastic SCs likewise increase p75NTR expression following axotomy and this increase in p75NTR contributes to SC apoptosis following loss of axonal contact (Ferri and Bisby, 1999; Petrats, 2003; Provenzano et al., 2008; Syroid et al., 2000; Taniuchi et al., 1986). By contrast, activation of p75NTR fails to induce VS cell apoptosis. Further, p75NTR 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 p75NTR 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; Lallemant 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; Lallemant 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 p75NTR expression in VS tissue compared with normal nerve raises the possibility that merlin likewise regulates p75NTR 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; Flaz 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

![Image](https://example.com/image.png)

**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.
Thus, whether Akt promotes VS cell survival in addition to proliferation requires confirmation with more specific, death (James et al., 2009; Lee et al., 2009; Yue et al., 2011).
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-kB 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 p75NTR Signaling**

The response of cells to p75NTR 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 p75NTR inhibits apoptosis (Descamps et al., 2001; Hondermarck, 2012; Koshimizu et al., 2010; Lu et al., 2005). In these cases, p75NTR 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 p75NTR 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 p75NTR 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 p75NTR-mediated apoptosis (Charalampopoulos et al., 2012; Khursigara et al., 2001).

**p75NTR Mediates an Antiapoptotic Response in VS Cells**

The data presented here suggest that proNGF/p75NTR 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 p75NTR 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, p75NTR has been implicated in glioma cell invasion (Johnston et al., 2007; Wang et al., 2010). In breast carcinoma, p75NTR 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, p75NTR 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 (Fernhough et al., 2005; Fu et al., 2010; Ma and Bisby, 1998; Morton et al., 2012; Pollock et al., 2005). Further, activation of p75NTR by NGF treatment increases NF-κB activity and inhibition of NF-κB activation in SCs transiently delays axonal regeneration and compact remyelination (Fernhough et al., 2005; Fu et al., 2010; Ma and Bisby, 1998; Morton et al., 2012; Pollock et al., 2005). Further, activation of p75NTR 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 p75NTR and activation of JNK remains unknown.

Here we find that p75NTR activates JNK and NF-κB in primary VS cells. Typically, NF-κB activation by p75NTR is
TRAF6 and JNK dependent (Costantini et al., 2005). By contrast, p75NTR activates JNK and promotes apoptosis in keratinocytes but suppresses NF-kB activity (Truzzi et al., 2011). The effect of NF-kB activation by p75NTR on cell survival appears to depend on the cell type. For example, p75NTR activates JNK and NF-kB 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-kB in the RN22 schwannoma cell line and induces apoptosis in cells with suppressed NF-kB activity (Gentry et al., 2000). Our results indicate that basal NF-kB activity in primary VS cells depends on JNK activity as both SP600125 and siRNA mediated JNK knock-down reduced apoptosis in cells with suppressed NF-kB activity (Gentry et al., 2000). Our results indicate that basal NF-kB activity in primary VS cells depends on JNK activity as both SP600125 and siRNA mediated JNK knock-down reduced apoptosis in cells with suppressed NF-kB activity (Gentry et al., 2000).

NF-kB activation promotes survival of cells treated with many different classes of chemotherapeutics or ionizing radiation and inhibition of NF-kB potently enhances chemotherapeutic and radiotoxicity (Wang et al., 1996, 1999). JNK inhibitors represent one class of chemotherapeutics that reduce cell survival and anti-apoptosis (Yue et al., 2011). The ability of p75NTR to activate NF-kB independent of JNK appears to provide a cytoprotective effect in VS cells treated with JNK inhibitors. Parallel targeting of the p75NTR/NF-kB 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 p75NTR function in VS cells is that it would represent a fairly specific target since inhibition of p75NTR signaling supports the survival of non-neoplastic SCs.

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References

Ahmad et al.: p75NTR Signaling in Vestibular Schwannomas


James MF, Han S, Poliizzato C, Plotkin SR, Manning BD, Stemmer-Rachamimov AO, Gussela 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.


Merlin status regulates p75NTR expression and apoptotic signaling in Schwann cells following nerve injury

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After nerve injury, Schwann cells (SCs) dedifferentiate, proliferate, and support axon regrowth. If axons fail to re-
generate, denervated SCs eventually undergo apoptosis due, in part, to increased expression of the low-affinity
neurotrophin receptor, p75NTR. 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 de-
generation in a deafness model, temporally correlated with increased p75NTR expression. p75NTR levels are ele-
vated 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 p75NTR expression in primary human schwannoma cul-
tures which otherwise lack functional Merlin. Despite elevated levels of p75NTR, 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 con-
firm that SCs lacking functional Merlin are less sensitive to p75NTR-mediated cell death. Taken together these re-
results point to a model whereby loss of axonal contact following nerve injury results in Merlin phosphoryla-
tion leading to increased p75NTR expression. Further, they demonstrate that merlin facilitates p75NTR-mediated apo-
pptosis in SCs helping to explain how neoplastic SCs that lack functional Merlin survive long-term in the absence of
axonal contact.

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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 prolifer-
ation, and provide support for eventual axonal regrowth (Chen et al.,
2007). They then dedifferentiate and remyelinate regenrated 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 recep-
tor, p75NTR, which promotes SC apoptosis (Taniuchi et al., 1986; Ferri
and Bisby, 1999).

p75NTR promotes SC apoptosis following denervation

p75NTR 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,
p75NTR often interacts with other co-receptors, including sortilin or
Nogo, to mediate cell death (Bandtlow and Dechant, 2004; Barker,
2004). Although p75NTR 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 bind-
ing, p75NTR undergoes intramembrane cleavage by γ-secretase to gen-
erate 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 p75NTR-mediated apoptosis (Kenchappa et al., 2006). Recent data indicate that schwannomas cells express high levels of p75NTR yet, in contrast to non-neoplastic SCs, are resistant to p75NTR-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 p75NTR-mediated SCs responses to loss of axonal contact. We find that merlin suppresses p75NTR expression in a phosphorylation dependent fashion and that merlin facilitates SC cell death in response to p75NTR ligands.

Materials and methods

Mice strains

P0SchΔ(39–121) and NF2E7f mice were obtained from Riken BioResource Center (Tsukuba, Japan) (Giovannini et al., 1999, 2000) and RosaCRE-ERT2 and FVB mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The RosaCRE-ERT2 mice contain a tamoxifen-inducible Cre recombinase system. We crossed the NF2E7f mice with RosaCRE-ERT2 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 p75NTR 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-merlinSS18A-GFP, and Ad5-merlinSS18D-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:NF2E7f 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-p75NTR 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 p75NTR 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-p75NTR and anti-phosphomerlin antibodies to determine the spatial distribution of SCs that express p75NTR 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 p75NTR 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 SPS 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 P0SchΔ39–121 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 p75NTR 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 p75NTR expression in Schwann cells

Denervated SCs with phosphorylated merlin and VS cells that lack functional merlin express high levels of p75NTR raising the possibility that merlin status regulates p75NTR expression in SCs. To test this possibility, we performed sciatric 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-p75NTR antibodies (Fig. 3A). The blots were probed with anti-β-actin antibodies to verify protein loading. Densitometry was performed to quantify the level of p75NTR expression relative to β-actin (Fig. 3B). As expected, axotomy led to a significant increase in p75NTR levels. Consistent with the notion that merlin regulates p75NTR expression, p75NTR levels were elevated in uncut nerves from P0SchΔ39–121 mice compared to WT mice. Further, comparison of p75NTR levels following axotomy demonstrates that p75NTR 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 p75NTR expression, even in SCs that remain in contact with axons.

Merlin status regulates p75NTR expression in SCs

To further explore the relationship between p75NTR expression levels and merlin status, we cultured sciatric nerve SCs derived from a transgenic mouse line with floxed Nf2 and a tamoxifen (Tx)-inducible Cre (RosaCre: Nf2fl/loxP) (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. Sciatric nerve cultures were prepared from these mice. Treatment of cultures with Tx (500 nM) reduced merlin and significantly elevated p75NTR expression levels consistent with the notion that merlin suppresses p75NTR expression in SCs (Fig. 4A, B). The fact that elevated p75NTR expression in SCs following axotomy correlates with merlin phosphorylation raises the possibility that merlin phosphorylation hinders its ability to suppress p75NTR 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-p75NTR antibodies followed by anti-merlin and then anti-β-actin antibodies. Replacement of merlin reduced p75NTR expression in primary VS cultures (Fig. 4C). Further, p75NTR expression was suppressed in cultures transduced with S518A merlin isoform whereas transduction with the S518D isoform resulted in increase in p75NTR expression. Taken together these results indicate that S518 phosphorylation of merlin reduces its ability to suppress p75NTR 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 p75NTR expression. A. Protein lysates from cut and uncut sciatic nerves were collected 7, 21, and 180 days following unilateral axotomy in wild-type and P0Sch39-121 mice. Blots were probed with anti-p75NTR antibody and then stripped and reprobed with anti-β-actin antibodies. B. Average p75NTR/ 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.

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 P0Sch39-121 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 P0Sch39-121 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 P0Sch39-121 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 P0Sch39-121 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 p75NTR-induced Schwann cell apoptosis

SCs from P0SchΔ39-121 mice that lack functional merlin express high levels of p75NTR yet are resistant to apoptosis before and following axotomy (Fig. 6). This observation raises the possibility that functional merlin is necessary for p75NTR-induced SC apoptosis. To test this possibility we cultured SCs from RosaCre::NiF2 mice were treated with (Tx+) or without (Tx−) tamoxifen. Protein lysates were probed with anti-p75NTR and merlin antibodies. The blots were stripped and reprobed with anti-β-actin antibodies. Average p75NTR/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-p75NTR and merlin antibodies. The blots were stripped and reprobed with anti-β-actin antibodies.

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 p75NTR expression (Taniuchi et al., 1986, 1988); the
mechanisms leading to this elevated expression have remained unknown. Eventually in the absence of reinnervation, denervated SCs undergo p75NTR-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 implicates the tumor suppressor merlin as a key mediator of p75NTR 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; Altham 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 (axonotomy) 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 p75NTR 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 p75NTR expression in SCs. Nerve lysates from P0schΔ39-121 mice prior to injury and following axotomy revealed an increase in p75NTR levels in

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.
comparison to wild-type mice. Further, suppression of merlin expression in cultured SCs increased p75NTR expression while replacement of wild-type merlin reduced p75NTR expression in primary VS cells. Taken together these data confirm that merlin suppresses p75NTR levels in SCs in vitro and in vivo and are consistent with the observation of elevated p75NTR levels in neoplastic VS cells (Ahmad et al., 2014). While p75NTR levels were elevated in nerve lysates from P0SchΔ39-121 mice compared to wild-type mice prior to injury, there was a significant increase in p75NTR levels in nerves from P0SchΔ39-121 following axotomy indicating that other factors, in addition to merlin status, contribute to the increase in p75NTR expression following nerve injury.

Following nerve injury, merlin becomes phosphorylated which is temporally correlated with an increase in p75NTR expression. To determine whether merlin phosphorylation promotes p75NTR expression we used a merlin replacement strategy in primary VS cells. Together these data confirm that merlin suppresses p75NTR levels in SCs in vitro and in vivo and are consistent with the observation of elevated p75NTR levels in neoplastic VS cells (Ahmad et al., 2014). While p75NTR levels were elevated in nerve lysates from P0SchΔ39-121 mice compared to wild-type mice prior to injury, there was a significant increase in p75NTR levels in nerves from P0SchΔ39-121 following axotomy indicating that other factors, in addition to merlin status, contribute to the increase in p75NTR expression following nerve injury.

Following nerve injury, merlin becomes phosphorylated which is temporally correlated with an increase in p75NTR expression. To determine whether merlin phosphorylation promotes p75NTR 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 p75NTR expression. By contrast, the S518D mutation functions as a phospho-mimetic and failed to suppress p75NTR expression. Taken together, these data suggest that merlin inactivation by phosphorylation facilitates increased p75NTR expression in SCs following nerve injury.

Merlin is necessary for p75NTR-induced SC apoptosis

Activation of p75NTR 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 p75NTR and resist apoptosis in response to the p75NTR 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 p75NTR. These observations raise the possibility that merlin facilitates p75NTR apoptotic signaling in SCs. To test this possibility we suppressed merlin expression in cultured SCs following axotomy.
Thus, p75NTR represents a potential therapeutic target that would specifically target neoplastic schwannoma cells, but not their non-neoplastic counterparts.

Although p75NTR signaling is often associated with apoptosis, activation of p75NTR 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 carboxy-terminal fragment of p75NTR (Verbeke et al., 2013). Other malignant cell lines (e.g. colorectal) retain sensitivity to p75NTR-mediated apoptosis (Yang et al., 2014). What determines whether p75NTR activation leads to cell death or survival remains unknown; however, p75NTR activation of the nuclear transcription factor NF-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 p75NTR-mediated apoptosis in SCs depends, at least in part, on functional Merlin. Whether sensitivity to p75NTR-mediated apoptosis is likewise Merlin-dependent in these other cell types remains to be determined.

Beyond failing to induce apoptosis, p75NTR 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 p75NTR represents a mechanism whereby VSs are resistant to chemotherapeutics that target kinase signaling (Karaannis et al., 2012; 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 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, Rap, Pak1/2, extracellular regulated kinase/mitogen activated protein kinase (ERK/MAPK), phosphatidylinositol 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 CRL4DCAF1 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 P0SChä39–121 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, immunolabeling and ultrastructural studies identified myelination abnormalities in sciatric nerves from P0SChä39–121 mice but did not reveal significant SC hyperplasia within the peripheral nerve (Denisenko et al., 2008). Rather, SC hyperplasia in P0SChä39–121 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 P0SChä39–121 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 p75NTR expression and apoptotic signaling in peripheral nerve SCs. The ability to decrease p75NTR 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 p75NTR 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


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