The Effect of Protein Nitration on Cell Survival Pathways in Schwann Cells

by
Oliver W. Graumann

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented June 2, 2021
Commencement June 2021

AN ABSTRACT OF THE THESIS OF

Oliver W. Graumann for the degree of Honors Baccalaureate of Science in Microbiology presented on June 2, 2021. Title: The Effect of Protein Nitration on Cell Survival Pathways in Schwann Cells.

Abstract approved:_____

Maria C. Franco

Neurofibromatosis type 2 (NF2) is a genetic disorder caused by the inactivation of the merlin tumor suppressor gene. NF2 patients develop bilateral vestibular schwannomas (VS) and other nervous system tumors with no effective drug treatment option. In pathological conditions, including NF2, production of the oxidant peroxynitrite leads to protein tyrosine nitration. While tyrosine nitration is seen in multiple tumor types, its role in tumorigenesis is unknown, although we have discovered that prevention of tyrosine nitration selectively decreased merlin deficient (MD) Schwann cell survival. This research is the first focused effort to determine the signaling pathways regulated by tyrosine nitration in pathological conditions. Immunoprecipitations followed by mass spectrometry analysis identified proteins endogenously nitrated in VS from NF2 patients, and in human MD-Schwann cells. Then we performed phosphorylation arrays for receptor tyrosine kinases (RTKs), and phospho-kinases, to characterize the relationship between nitrated proteins and survival pathway activation. While RTKs were unaffected, the phospho-MAPK arrays demonstrated a role of nitration in supporting survival pathway activation in MD-Schwann cells, including the PI3K/Akt pathway, and the MEK/ERK pathway. The identification of specific nitrated proteins that promote schwannoma growth could provide novel targets for the treatment of NF2 and possibly other nervous system tumors as well.

Key Words: Nitration, Cell Signaling, Neurofibromatosis 2, Tumor Survival

Corresponding e-mail address: graumano@oregonstate.edu

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APPROVED:

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Maria C. Franco, Mentor, representing Biochemistry and Biophysics

_____

Alvaro Estevez, Committee Member, representing Biochemistry and Biophysics

_____

Maret Traber, Committee Member, representing Biochemistry and Biophysics

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Oliver W. Graumann, Author

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Background

Neurofibromatosis Type 2 (NF2)

Merlin is a tumor suppressor protein coded by the *NF2* gene (1). Mutations to the *NF2* gene result in merlin losing its tumor suppressor function (1). This loss of function is the underlying cause of neurofibromatosis type 2 (NF2), a genetic disease characterized by the development of distinctive bilateral vestibular schwannomas on the vestibulocochlear nerve, along with other nervous system tumors (1). Vestibular schwannomas can result in hearing loss, and progressive nervous system death that can ultimately result in mortality (2).

This disease is inherited in an autosomal dominant manner, affecting one in thirty thousand (3). Tumor resection surgery remains the most common treatment for NF2 patients, as therapeutic options are limited (2). Given that the defective *NF2* alleles will be widely distributed in somatic cells, tumors can recur post-surgery, and patients can develop schwannomas, meningiomas, and ependymomas apart from the characteristic vestibular schwannomas (2). These require additional resection surgeries, causing further brain and peripheral nerve damage. The results of these resection surgeries can be facial palsy, deafness, blindness, or seizures (2).

Efforts to increase treatment options primarily focus on repurposing oncology drugs for NF2 patients (4). Therapies such as Bevacizumab (a monoclonal antibody that inhibits angiogenesis) were successfully repurposed to treat NF2 (4), with clinical trials starting for others such as Selumetinib (MEK inhibitor) (5). Longer-term treatments with

these therapeutics are not advised due to dramatic side effects, ranging from blistering rashes to severe heart problems (4).

These issues highlight the urgent need for long term, non-invasive treatment options for patients suffering from NF2. Our lab discovered nitrated proteins represent a novel target for therapeutic development, as they play a role in tumor cell signaling (6).

Oxidative stress

The toll of evolving an aerobic metabolism is the synthesis of reactive oxygen and nitrogen species such as superoxide and nitric oxide (13). These species, if not kept under strict control, potentially oxidize macromolecules such as proteins, DNA, and lipids (13). The production of oxidants in both tumor cells and tumor microenvironments can play a role in the different stages of tumor development, such as causing the mutations needed for initiation of tumorigenesis. Furthermore, these oxidants can affect signaling pathways within the tumor cells themselves, or their microenvironment (24). This potential for extended regulation of the signaling pathways and cellular processes raises questions regarding the role of reactive species in tumor progression.

For example, peroxynitrite ($ONOO^-$), a reactive nitrogen species, is the product of the diffusion-limited reaction between the secondary messenger nitric oxide (·NO), and superoxide ($\cdot O_2^-$) (Fig. 1) (15). Under normal conditions, ·NO and $\cdot O_2^-$ do not react due to the cellular mechanisms controlling their concentrations (13). ·NO diffuses freely through tissues after its production via nitric oxide synthase (NOS) and is subsequently converted to nitrate after reacting with oxyhemoglobin in red blood cells (16). Intracellular superoxide is controlled via superoxide dismutases (SOD) that are present in high concentration as

scavenging enzymes (13). In this way, peroxynitrite formation can be based on either abnormally high NOS activity, or abnormally low SOD activity.

This thesis focuses on the role of peroxynitrite in the development of NF2 schwannomas. Peroxynitrite production occurs in many pathological conditions, often linked to inflammation, and has previously been implicated in diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's, diabetes, and heart disease (14).

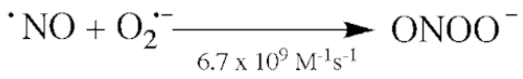


Figure 1: Reaction to form peroxynitrite.

Regarding NF2, we observed that the absence of merlin expression in human Schwann cells (MD-Schwann cells) results in increased neural nitric oxide synthase (nNOS) relative to wild type (WT) human Schwann cells, along with decreased expression of manganese superoxide dismutase (MnSOD) (16). This implies merlin deficiency corresponds to heightened peroxynitrite production in human Schwann cells. Additionally, scavenging peroxynitrite and preventing tyrosine nitration results in decreased survival in MD-Schwann cells, while not affecting WT-Schwann cells (Fig. 3) (16). This provides evidence to support peroxynitrite production as a component of MD-Schwann cell function, as opposed to just being a side effect of merlin deficiency.

Tyrosine nitration

In biology, tyrosine residues within proteins are often subject to phosphorylation, turning them into important players in the activation and inhibition of proteins. Many

survival pathways involve the phosphorylation of tyrosine residues (13). For example, the phosphorylation of JNK, a kinase involved in inflammatory responses and cell apoptosis, is common on specific sites such as tyrosine 185 (7).

The radical products of peroxynitrite decomposition can react with tyrosine residues to form nitrotyrosine by the incorporation of a nitro group ($^{\cdot}NO_2$) in the aromatic ring of the tyrosine (Fig. 2) (18). Production of peroxynitrite and subsequent tyrosine nitration are detected primarily in conditions linked to inflammation, such as those found in tumors and their microenvironment, but it is low or absent in normal tissues (13). Thus, in pathological conditions tyrosine nitration can compete with phosphorylation. However, phosphorylation is a reversible process, whereas nitration is considered irreversible (19). In the context of a proliferative pathway, this means that a protein activated via tyrosine nitration could result in an activated pathway that cannot be turned off until the protein is degraded.

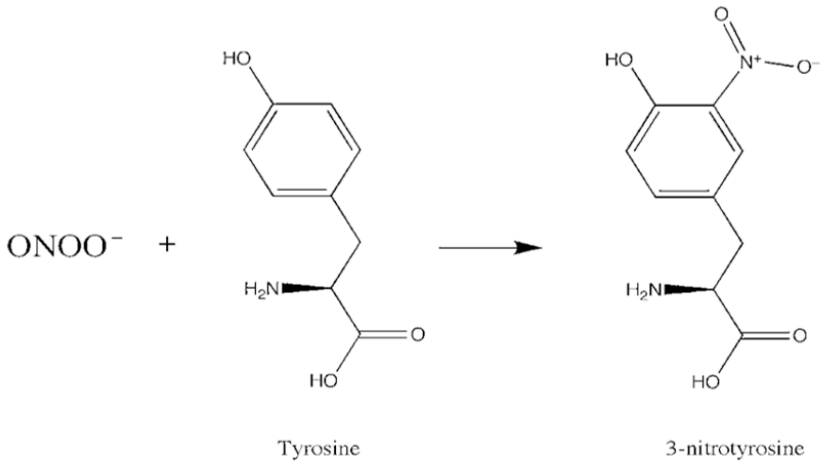


Figure 2: The reaction to form 3-nitrotyrosine from tyrosine by peroxynitrite decomposition products.

A protein with a nitrated tyrosine residue can fold differently from the native protein or incur different protein-protein interactions based on the change in charge (13). For example, tyrosine nitration can induce a gain of function, a new function that the unmodified protein cannot perform of compensate for (6; 20). Additionally, these changes in protein function may alter the role a protein plays in signaling pathways (21).

This is consistent with the findings that nitrotyrosine can serve as a biomarker in a variety of diseases, including cancer, chronic inflammation, and NF2 (13). Despite these findings, the specific functional roles of protein tyrosine nitration are just beginning to be uncovered. One example is the impact on a variety of structural proteins, including tubulin, which often contain higher proportions of tyrosine residues (13). Nitration of these tyrosine residues inhibit the ability for a larger structure to assemble, impacting cellular delivery mechanisms (13). Another example is the nitration of the MnSOD, which is inactivated by nitration (22), as observed in the cerebrospinal fluid of patients with neurodegenerative disorders such as ALS (23).

We found that tyrosine nitration is important for the survival of MD-Schwann cells (Fig. 3), suggesting that one or more nitrated proteins possess a vital function (16). Given that these nitrated proteins are only modified in schwannoma cells, but not in normal Schwann cells, they could be exceptional targets for the development of new pharmacological strategies for the treatment of NF2. Targeting tyrosine nitrated proteins would allow for treatment that avoids negatively impacting healthy cells, and therefore would likely avoid many of the severe long-term side effects present in the drug options currently available for NF2 patients.

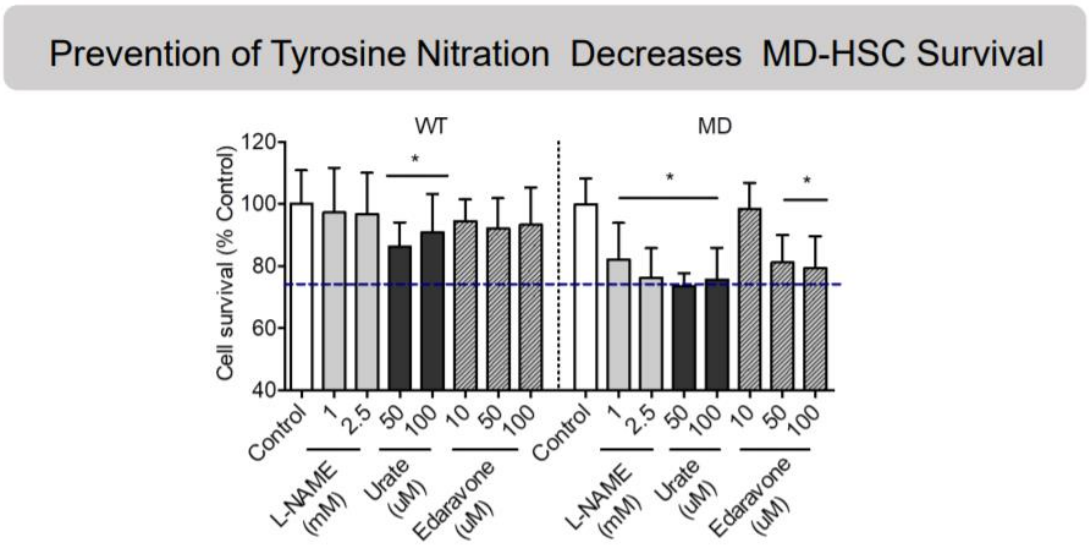


*Figure 3: Prevention of tyrosine nitration decreases human MD-Schwann cell (MD-HSC) survival. Cell survival was evaluated using crystal violet assays. Both wild type (WT) and merlin deficient (MD) human Schwann cells were treated for 96 hours in either urate (50 or 100 µM) or edaravone (50 or 100 µM). Data is reported as a percent survival relative to untreated WT (Left) or MD (Right) human Schwann cells. * p< 0.05 by One-Way ANOVA and Bonferroni post-test.*

Cell Signaling

The shift in cell behavior witnessed in tumorigenesis is the result of a series of cell signaling alterations (18). For a tumor to form, pathways involved in proliferation and survival must be improperly upregulated, and simultaneously, pathways involved in cell apoptosis and anti-proliferation must be improperly downregulated. This requires an alteration of phosphorylation events, the attachment of a phosphoryl (PO_3-) group onto amino acid residues on a protein, often taking place on serine, threonine, and tyrosine residues (18).

Pathways deregulated in cancer include mitogen-activated protein kinases (MAPKs), which serve as intermediates between environmental receptors, cell apoptosis, proliferation, and inflammatory regulation (7). Of all MAPKs, three are of particular interest: extracellular-regulated kinase (ERK), MAPK14 (p38), and c-Jun N-terminal

kinase (JNK). An increase in ERK activity best associated for its roles in cell proliferation and differentiation, although it possesses an anti-apoptotic role, which depends on certain kinetics of its activation (8). However, in response to peroxynitrite, increased ERK phosphorylation is associated with a pro-apoptotic role in neuronal tissue (9). When activated, JNKs appears pro-apoptotic in response to peroxynitrite (10). However, JNKs function ranges from cell cycle regulation, as seen in its apoptotic role, to activation of laminar shear stress in blood vessels (11). Intracellular activation of p38 associates with cell cycle arrest and apoptosis as a response to oxidative stress (12). Another pathway turned on in response to oxidative stress is the PI3K/Akt pathway, which prevents apoptosis (12).

Furthermore, receptor tyrosine kinases (RTKs) located in the membrane precede many of these survival pathways, including the previously mentioned MAPK pathway (13). Certain RTKs act to detect environmental oxidants and initiate a response via the MAPK pathway (40).

Tyrosine Nitration and Cell Signaling

Tyrosine nitration can be specific, occurring in certain proteins, and on certain tyrosine residues within those proteins (13). Knowing this, nitrated proteins could potentially regulate cell signaling, given the ability of tyrosine nitration to modify protein structure, and protein-protein interactions. When exposed to high concentrations of peroxynitrite, such as those observed in conditions of oxidative stress linked to induction of cell death by necrosis, proteins can undergo a high level of tyrosine nitration, in which a high proportion of the protein population within a cell may get oxidized. However, under

pathological conditions, where peroxynitrite is produced at lower levels, specific proteins undergo nitration, such as Heat Shock Protein 90 (Hsp90) (6). The degree of nitration has signaling implications. A protein population that undergoes a low level of nitration may play a role in regulating signaling pathways if nitration results in a gain-of-function, or if the nitration directly activates the protein.

In some cases, peroxynitrite-induced tyrosine nitration impacts signaling pathways dependent on tyrosine phosphorylation. A notable example is the downregulation of T-cell proliferation, suggesting a role for peroxynitrite in immune function (25). Currently, both endogenous peroxynitrite production and therapeutic treatment are known to impact MAPK pathways (such as ERK, JNK, and p38), the PI3K-Akt pathway, NFkB, and other pathways relevant to cell survival and apoptosis (14). However, the connection between these signaling effects and tyrosine nitration is not well understood. A more comprehensive analysis would connect the different observed nitrated proteins to their signaling effects on survival pathways. This thesis represents the first analysis connecting tyrosine nitration to large scale survival signaling effects in NF2 Schwannoma cells.

Materials and Methods

Urate and Edaravone

We used urate and edaravone as a treatment to prevent tyrosine nitration. In general, urate can scavenge radicals, including peroxynitrite derived radicals (16). However, the main method for urate to prevent tyrosine nitration is due to the intermediate production of a tyrosyl radical during the mechanism in which peroxynitrite reacts with tyrosine to produce nitrotyrosine (41). Urate can effectively scavenge the radical from this tyrosyl radical. Cells were treated for 48 hours, which represents a timespan required for many of the cells' proteins to turnover in these nitration-preventing conditions, resulting in a 50% decrease in total tyrosine nitration (16).

Edaravone is also known to scavenge radicals, including peroxynitrite, and is currently being used as a drug to treat ALS (29). We are interested in how its effects compare to urate, as it is possible part of its efficacy is on the basis of this peroxynitrite scavenging ability.

Cell Culture Model

Human WT-Schwann cells were purchased from ScienCell Research Laboratories (catalog number 1700). These cells were used to generate Human merlin deficient (MD) Schwann cells in house as previously described by our collaborator Dr. Fernández-Valle (26) using lentiviral particles to knock down the *NF2* gene. These cells (WT and MD) were cultured on 10 mL Corning CellBIND plates (catalog number 3296) in ScienCell Schwann

cell media (catalog number 1701) and were split 1:3 upon reaching ~90% confluency using 0.25% trypsin.

Mouse WT-Schwann cells were generated in house as previously described (17) from mouse sciatic nerves. These cells were used to generate mouse MD-Schwann cells as previously described (27) where the *NF2* exon was removed via transduction. The MD cells were grown in an N2 supplemented DMEM/F12 media (ScienCell, catalog number 09411) with 1% Penicillin/Streptomycin on Corning CellBIND plates (catalog number 3296). Mouse MD-Schwann cells were split 1:3 upon reaching ~80% confluency using 0.25% trypsin.

Western Blot Analysis

Following 48-hour incubation with urate (100 µM), edaravone (100 µM), or absence of treatment (untreated control), Western blots were performed as described previously (28). Before lysis, cells were washed three times with 10 mL DPBS with 1 mM NaF and 1 mM sodium orthovanadate. Cell lysis and harvesting was performed using 2 steps of 300 µL RnD Systems Lysis Buffer 17 (Catalog number 895943) with 1x phosphoatase inhibitor cocktail, 1x protease inhibitor cocktail,1 mM phenylmethylsulfonyl flouride, 1mM NaF, and 1mM sodium orthovanadate. Samples were cooled for 30 minutes and stored at -80 °C. Thirty µg protein extract was loaded into a 12% gel to be used for SDS-PAGE. Protein was transferred onto a Millipore Sigma background membrane (catalog number IPFL07810), blocked with an Odyssey Blocking Buffer (Li-Cor Biosciences, catalog number 927-40000), and incubated in primary antibodies (listed below), then secondary fluorescent anti-mouse (Cell Signaling, catalog number 7076) or

anti-rabbit (Cell Signaling, catalog number 7074) antibodies at a 1:20000 dilution. Western blots were quantified via a densitometry analysis on the Odyssey System (Li-Cor Biosciences), and bands corresponding to the protein of interest were normalized relative to a previously decided control protein (actin, tubulin, or GAPDH).

Primary Antibodies

JNK 1:1,000 (Cell Signaling, catalog number 2315). Phospho-JNK 1:1,000 (Cell Signaling, catalog number 9261). ERK 1:1,000 (Cell Signaling, catalog number 9102). Phospho-ERK 1:1000 (T202/Y204, Cell Signaling, 9106). Akt 1:1,000 (Cell Signaling, catalog number 9272). Phospho-Akt 1:1,000 (Ser 473, Cell Signaling, catalog number 4051). p38 1:1,000 (Santa Cruz, catalog number sc-7972). Phospho-p38 1:1,000 (T180/Y182, Cell Signaling, catalog number 9216). Actin 1:15,000 (Millipore Sigma, catalog number MAB1501RMI). Tubulin 1:15,000 (Millipore Sigma, catalog number SAB4500087). GAPDH 1:1,000 (Cell Signaling, catalog number 2118).

Phosphorylation Arrays

Following 48-hour incubation with urate (100 µM), edaravone (100 µM), or in the absence of treatment (untreated control), cells were prepared as instructed for R&D's Proteome Profiler Arrays (42).

Arrays were performed using R&D System kits for human phospho-RTKs (catalog number ARY001B), human phospho-kinases (catalog number ARY003B), human MAPKs catalog number ARY002B), mouse phospho-RTKs (catalog number ARY014).

Mass Spectrometry Analysis

Mass spectrometry to identify nitrated proteins was performed as described previously (6). Identification was performed for MD-Schwann cells and NF2 derived vestibular schwannomas (VS).

mRNA extraction and transcriptomics analysis

Extraction was performed with a TRIzol Plus RNA Purification Kit (Thermo Fisher, catalog number 12183555) as instructed. Purity and concentration were confirmed by NanoDrop before analysis. The mRNA will be sent to the Center for Genome Research and Biocomputing (CGRB) and obtained results will undergo computational analysis in collaboration with Dr. David Hendrix. The pathways revealed to be active will be compared to current results.

Statistical Analysis

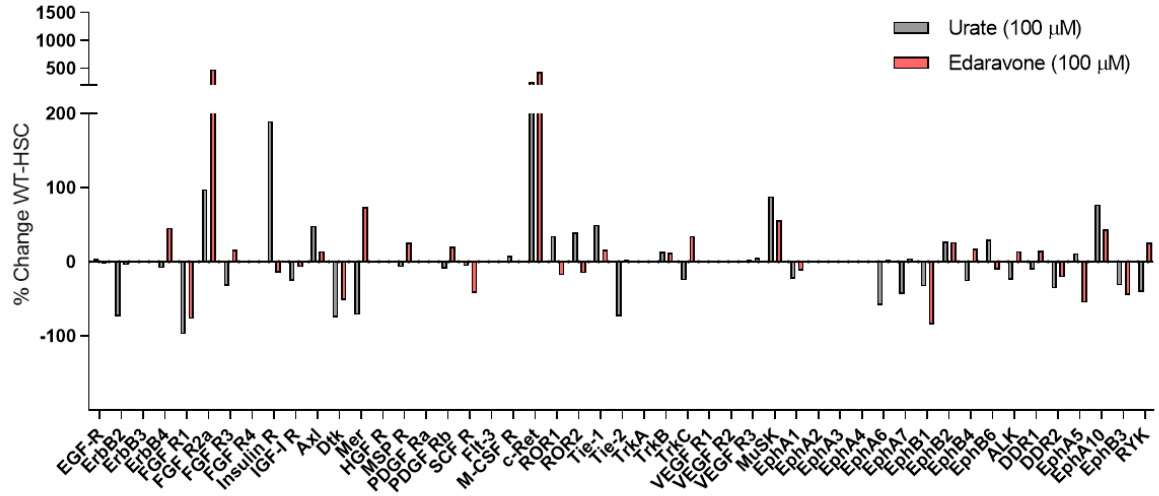
A one-way ANOVA test followed by Bonferroni post-test was performed for the Akt, ERK, and p38 Western blots. Analysis was performed using Prism software (GraphPad Software Inc.).

Results

Focused Signaling Pathway Analysis

Phosphorylation arrays from R&D Systems were used with MD-Schwann cells treated with urate (100 µM), edaravone (100 µM), or in the absence of treatment (untreated control) for a 48-hour period. The arrays provide information about the relative amounts of phosphorylated protein between treatment groups via infrared fluorescence read on the Odyssey System (Li-Cor Biosciences), using either secondary antibodies or streptavidin bound to an infrared fluorochrome.

RTK arrays



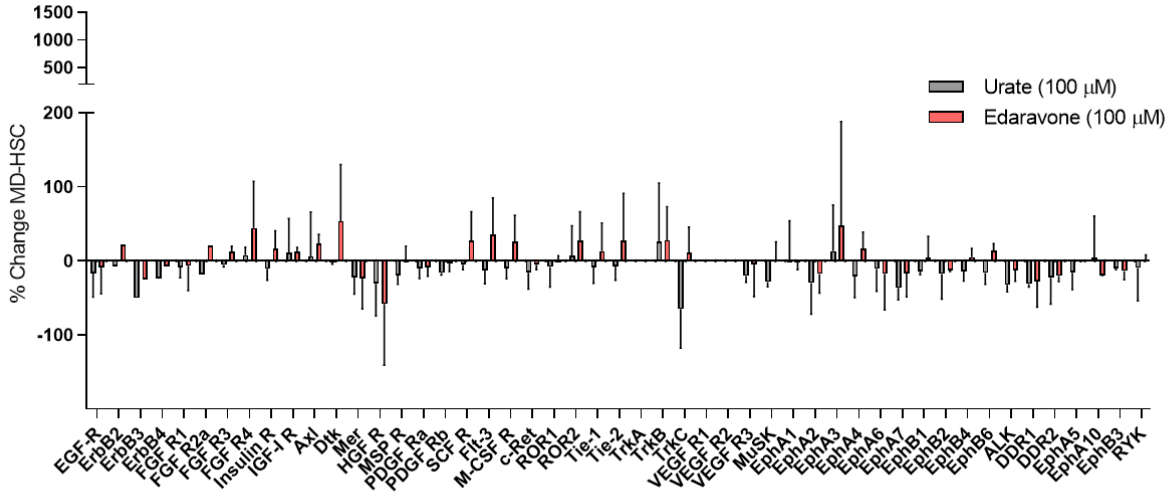
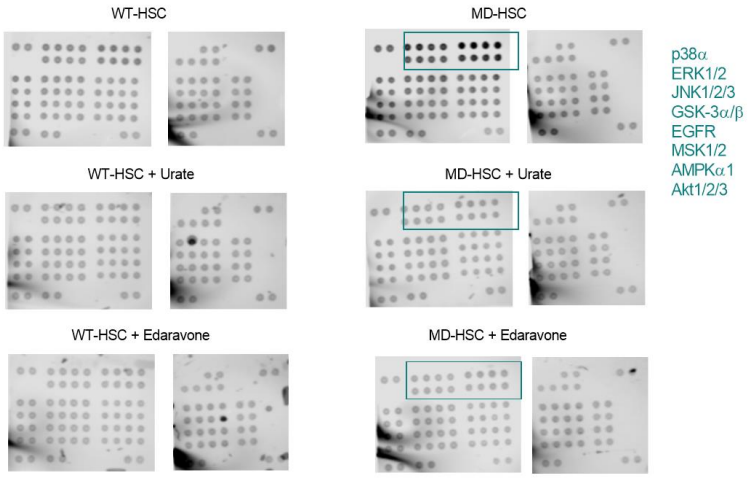
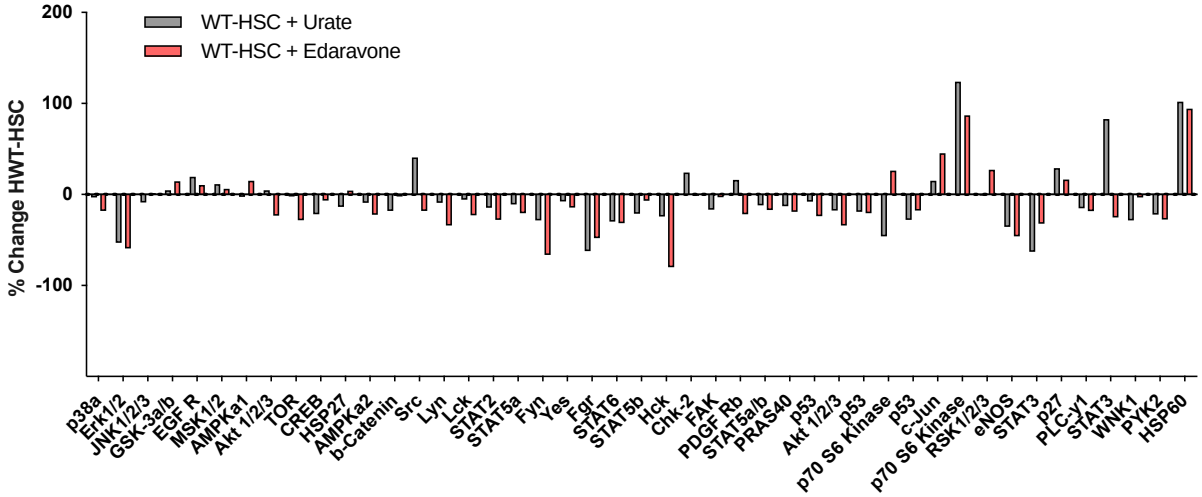


Figure 4: RTK phosphorylation level with urate and edaravone treatments. RTK phosphorylation changes were quantified from RTK arrays and graphed for wild type (WT) (Top) and merlin deficient (MD) (Bottom) human Schwann cells treated for 48 hours with urate (100 µM) or edaravone (100mM). Values represent a percent change relative to an uncreated WT (Top) or untreated MD (Bottom) human Schwann cell plate.

The arrays shown in Figure 4 evaluated the phosphorylation levels of tyrosine kinase receptors such as Axl, which can stimulate cell proliferation and survival (38), or EGFR, a protein known to interact with merlin (39). After 48-hour treatment with urate and edaravone, the phosphorylation levels of RTKs in MD-Schwann cells was not conclusively modified (n=2). In contrast, WT-Schwann cells showed an increase in the phosphorylation of RTK receptors associated with cell proliferation in Schwann cells, such as c-Ret and FGFR2 (n=1) (43). However, these results need to be further confirmed. The results obtained for the MD-Schwann cells suggest that tyrosine nitration may regulate signaling pathways unrelated or downstream of RTKs.

Phospho-Kinase arrays





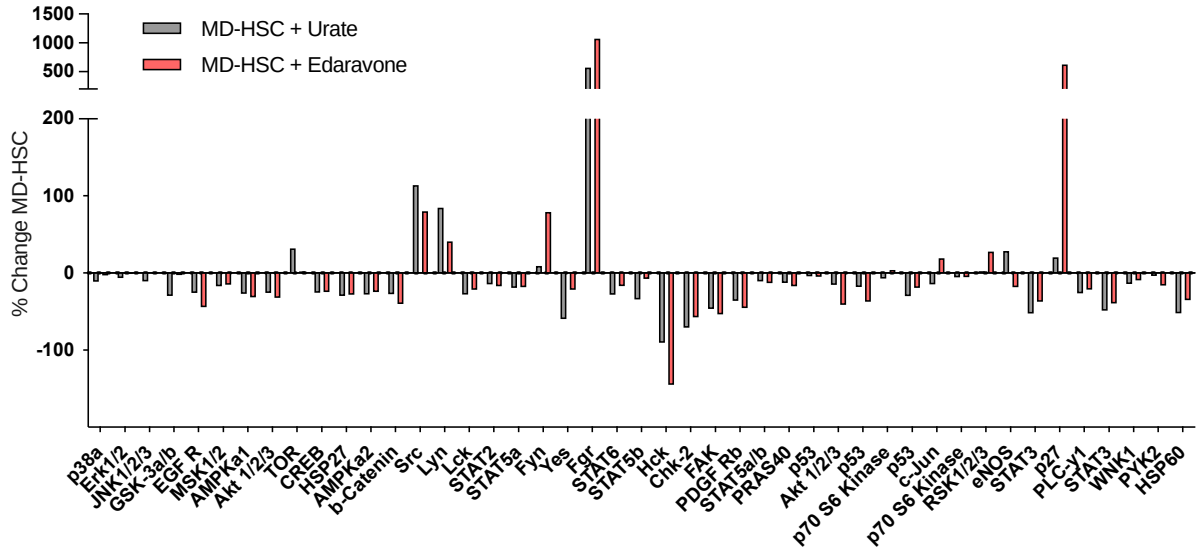
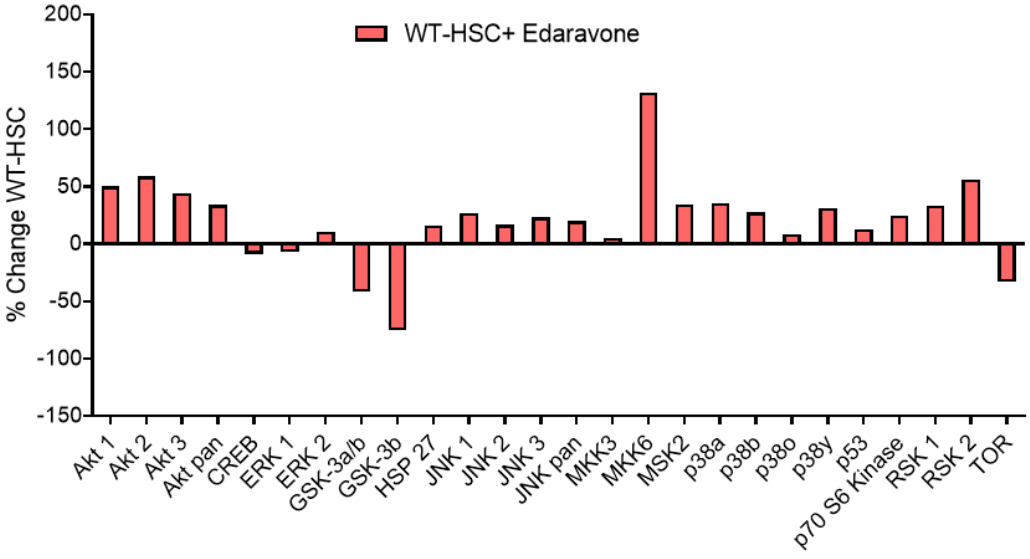


Figure 5: Phospho-kinase level with urate and edaravone treatments Kinase phosphorylation changes were quantified from phospho-kinase arrays (Top) and graphed for wild type (WT) and merlin deficient (MD) human Schwann cells treated for 48 hours with urate (100mM) or edaravone (100mM). Values represent a percent change relative to untreated WT human Schwann cells (Middle) or MD (Bottom) human Schwann cells.

Next, we performed phospho-kinase arrays (n=2). The arrays showed a decrease in the phosphorylation levels of effector kinases involved in pro-survival pathways such as Akt, JNK, and ERK in MD-Schwann cells when treated with urate or edaravone (n=2) (Fig. 5). In contrast milder changes were observed in kinase phosphorylation levels for those kinases dysregulated in NF2 when WT cells were treated with urate or edaravone (n=2). These results need to be further confirmed.

<u>Phospho-MAPK arrays</u>



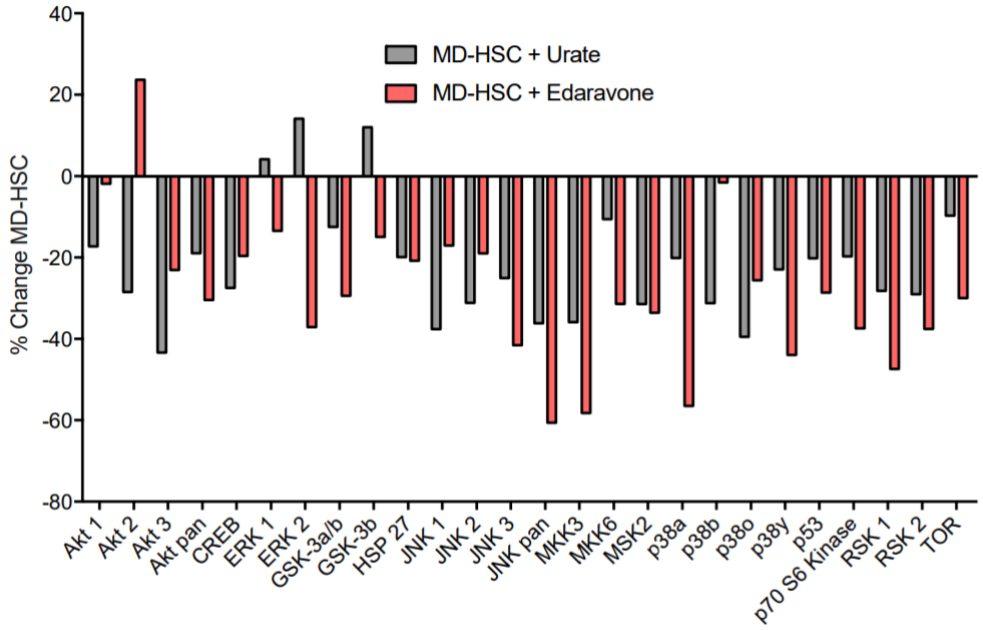


Figure 6: Phospho-MAPK levels with urate and edaravone treatments. MAPK phosphorylation changes were quantified from phospho-MAPK arrays and graphed for wild type (WT) and merlin deficient (MD) human Schwann cells treated for 48 hours with urate (100mM) or edaravone (100mM). Values represent a percent change relative to untreated WT (Top) or MD (Bottom) human Schwann cells.

Similar results were obtained using phospho-MAPK arrays (Fig. 6, n=2). These results were consistent with the phospho-kinase arrays in showing decreased pro-survival kinase phosphorylation in MD-Schwann cells after treatment with urate or edaravone, with limited effect on WT- Schwann cells after edaravone treatment.

The arrays identified JNK, p38, Akt, and ERK1/2 as proteins involved in important cell signaling pathways associated with endogenously nitrated proteins. To confirm these results, we performed Western blots for all of these proteins.

Western Blots

Western blots quantifying phosphorylated and total protein were performed in triplicate for p38, ERK1/2, and Akt.

p38

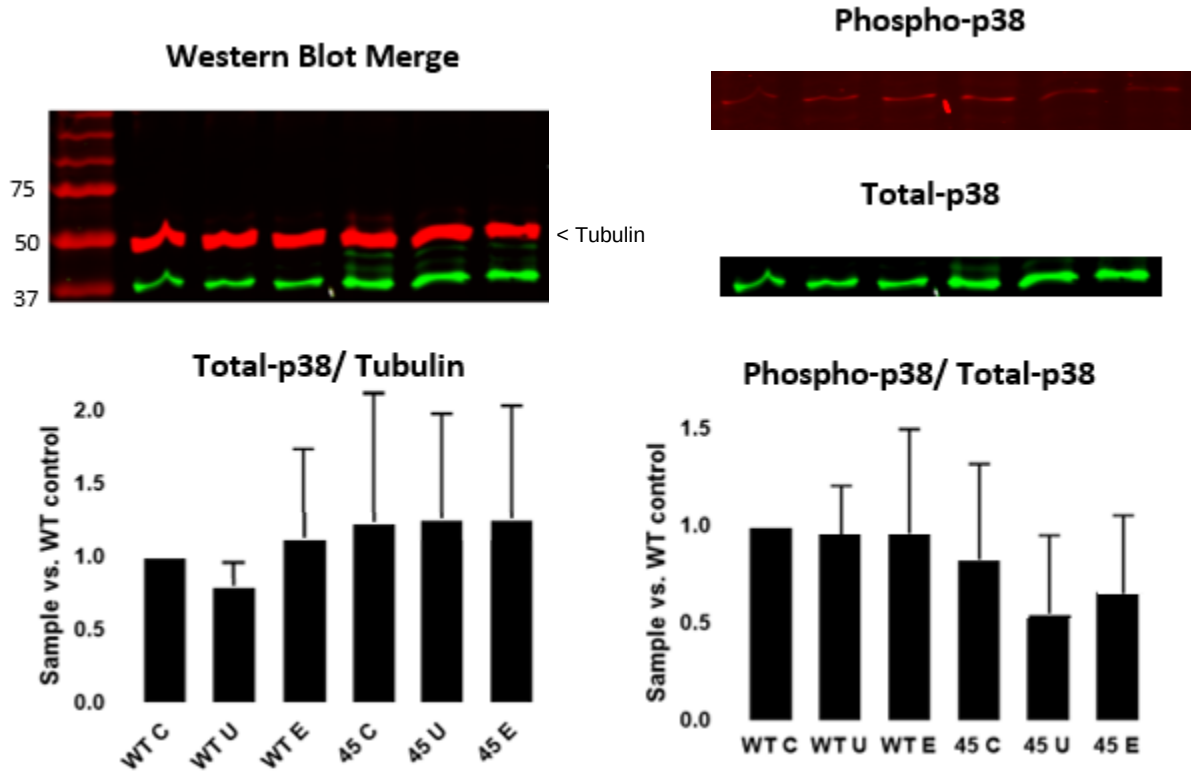


Figure 7: Phosphorylation state and level of p38 with urate and edaravone treatments. P38 phosphorylation state and level was examined in wild type (WT) and merlin deficient (MD) human Schwann cells through infrared Western blot. Antibodies were used for total p38 (green), phosphorylated p38 (red), and tubulin as control (red). Control cultures were compared to cultures with 48 hours urate (100 µM) or edaravone (100 µM). Below, a densitometry analysis was performed comparing the ratio of total p38 to tubulin control (Left) and the ratio of phospho-p38 to total p38 (Right). (n = 3, mean ±3SD)

No significant changes were seen in total p38, or phosphorylation levels of p38 between WT and MD Schwann cells. For both WT and MD-Schwann cells, treatment with urate or edaravone did not result in significant changes (Fig. 7).

ERK 1/2

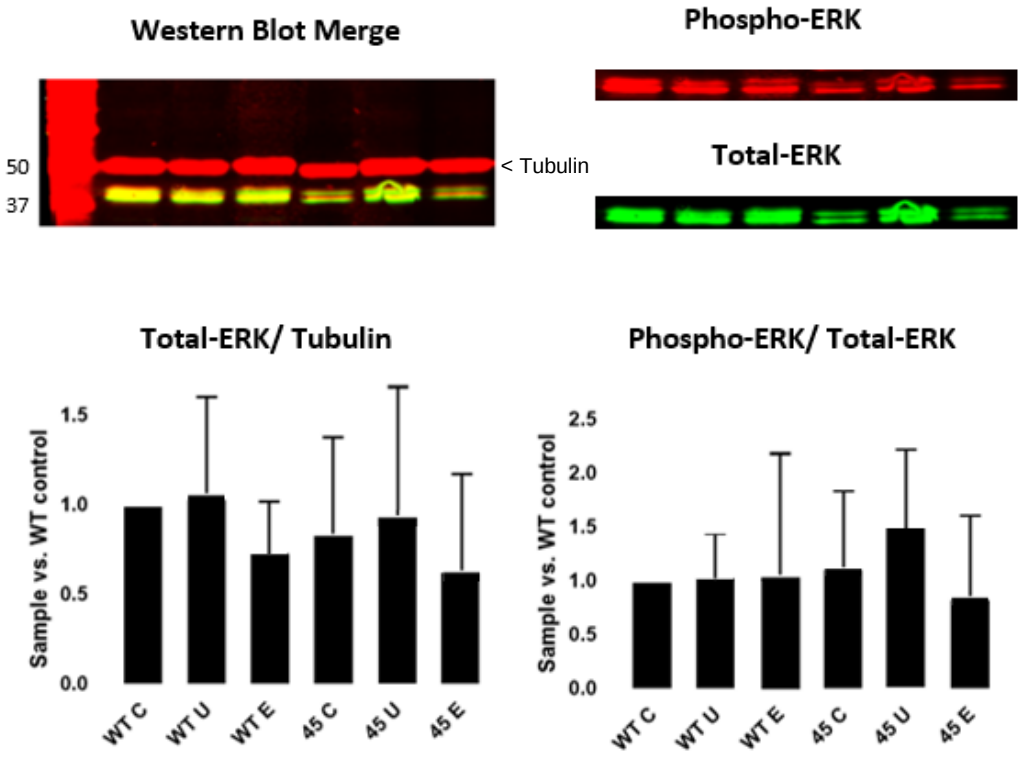
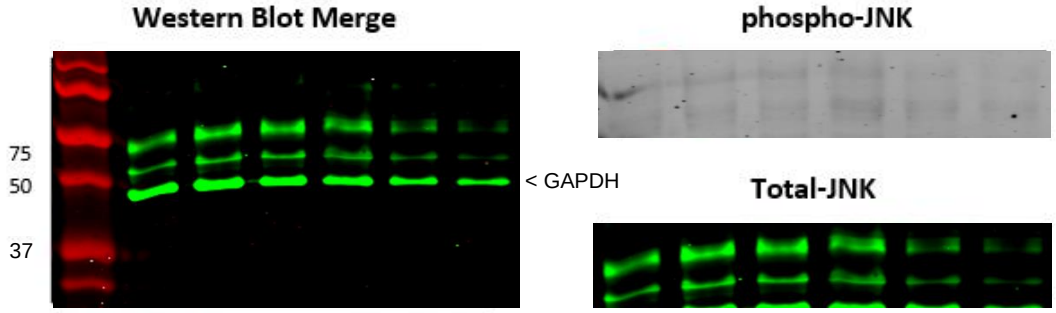
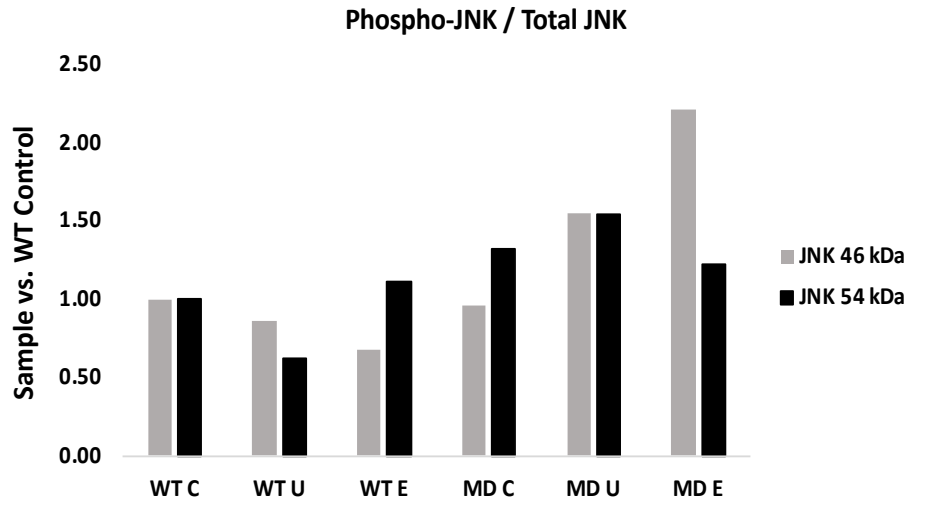


Figure 8: Phosphorylation state and level of ERK with urate and edaravone treatments. ERK phosphorylation state and level was examined in wild type (WT) and merlin deficient (MD) human Schwann cells through infrared Western blot. Antibodies were used for total ERK (green), phosphorylated ERK (red), and tubulin as control (red). Control cultures were compared to cultures with 48 hours urate (100 µM) or edaravone (100 µM) Below, a densitometry analysis was performed comparing the ratio of total ERK to tubulin control (Left) and the ratio of phospho-ERK to total ERK (Right). (n = 3, mean ±3SD)

No significant changes were seen in total ERK 1/2, or phosphorylation of ERK 1/2 between WT and MD Schwann cells. Treatment with urate and edaravone did not have an effect on total ERK ½ (Fig. 8).

JNK





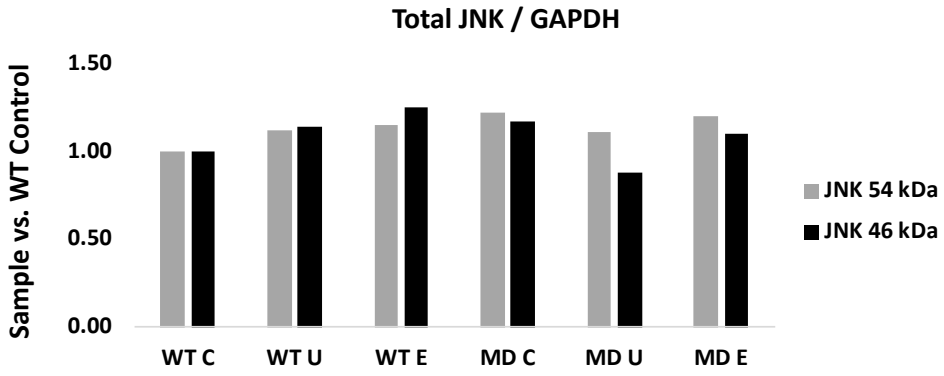


Figure 9: Phosphorylation state and level of JNK with urate and edaravone treatments. JNK phosphorylation state and level was examined in wild type (WT) and merlin deficient (MD) human Schwann cells through infrared Western blot. Antibodies were used for total JNK (green), and phosphorylated JNK (red). The

bands corresponding to Phospho-JNK are shown in grayscale (red channel). Control, untreated cultures were compared to cultures after 48 hours urate (100 µM) or edaravone (100 µM) treatment. Below, a densitometry analysis was performed comparing the ratio of phospho-JNK 46 kDa to total-JNK 46 kDa and the ratio of phospho-JNK 54 kDa to total-JNK 54 kDa, and both total JNK 54 kDa and Total JNK 46 kDa to GAPDH levels (n = 2).

The data for JNK has currently been done in duplicate, rather than triplicate. On this basis, there is inconclusive evidence of the effects of urate and edaravone on total JNK, or phosphorylated JNK of either isoform (Fig. 9).

Akt

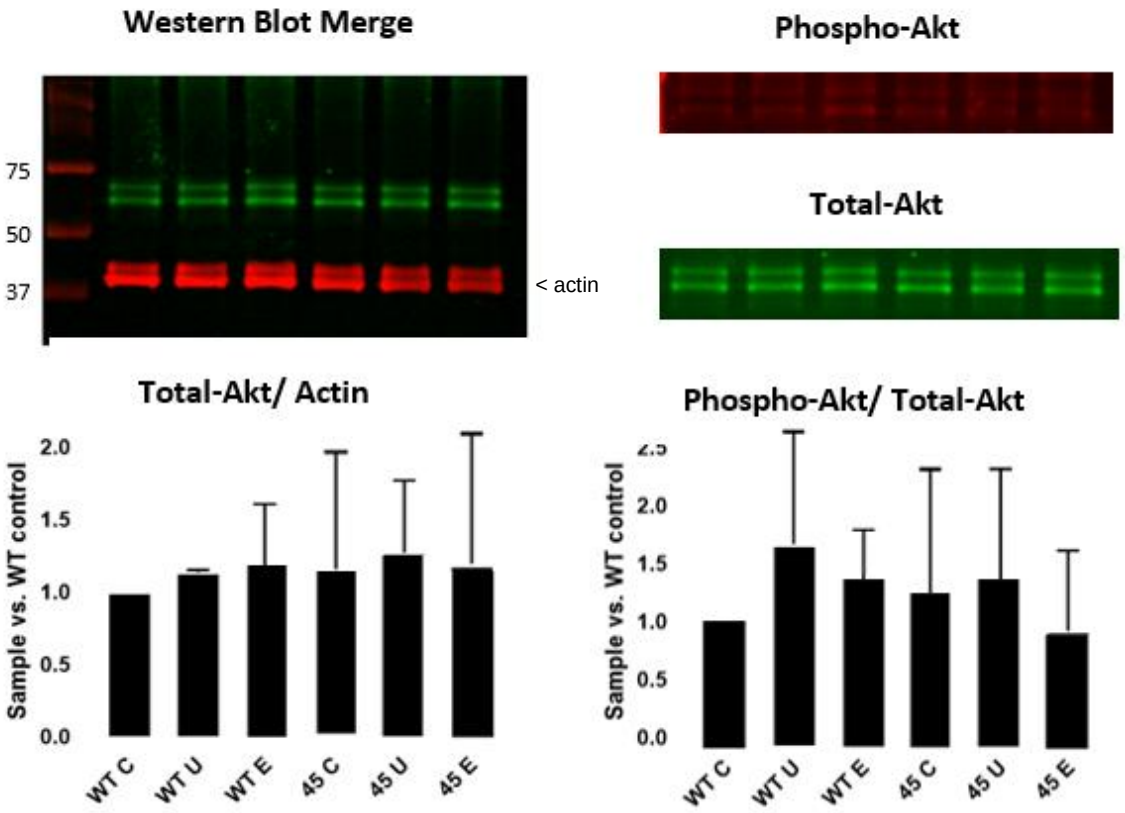


Figure 10: Phosphorylation state and level of Akt with urate and edaravone treatments. Akt phosphorylation state and level was examined in wild type (WT) and merlin deficient (MD) human Schwann cells through infrared Western blot. Antibodies were used for total Akt (green), phosphorylated Akt-Ser473 (red), and actin as control (red). Control cultures were compared to cultures with 48 hours urate (100 µM) or

edaravone (100 μM). Below, a densitometry analysis was performed comparing the ratio of total Akt to actin control (Left) and the ratio of phospho-Akt to total Akt (Right). (n = 3, mean ± SD)

No significant changes were seen in total Akt, or phosphorylation of Akt on Ser 473 between WT and MD Schwann cells. Treatment with urate and edaravone did not have an effect on total Akt (Fig. 10).

The results from the Western blots were inconsistent with the array results. This opened the door to the transcriptome analysis to help find which results reflect reality.

Unbiased Transcriptome and Pathway Analysis

Cells were grown at low passage, and had RNA extracted to be sent to the CGRB. The results will be sent back within the following month for further analysis.

Identification of Endogenously Nitrated Proteins in NF2 Schwannomas and Human MD-Schwann Cells

Nitrated proteins are potential pharmaceutical targets, so we aimed to identify the proteins that are endogenously nitrated in NF2 schwannomas and MD-Schwann cells. To this end, we performed immunoprecipitation followed by mass spectrometry analysis and identified ~40 proteins endogenously nitrated in vestibular schwannomas from NF2 patients (Table 1), and MD-Schwann cells (Table 2). Of these, a subset of nitrated proteins that participate in the survival pathways that are dysregulated in NF2 were identified, as discussed previously. There proteins were not found nitrated in WT-Schwann cells, making them fit as potential targets for further investigation (Fig. 11).

Nitrated proteins in VS from NF2 patients

Function	Protein	Peptides (CI 95%)
Cytoskeleton	Vimentin	41
	β-actin	17
	β-tubulin	13
Structural (nucleus)	Histone H4	4
	Histone H2A	4
	Histone H2B	3
	Histone H3	1
Chaperones/ Protein folding	Hsp90	4
	Hsp70	3
	14.3.3	7
	Peptidyl-prolyl cis-trans isomerase A	1
Translation	Elongation factor 1-α 1	4
Metabolism (cytosol)	GAPDH	4
	L-lactate dehydrogenase	3
	Phosphoglycerate kinase	2
	Alpha enolase	1
	Pyruvate kinase	1
Metabolism (mitochondria)	ATP/ADP translocase	3
	ATP synthase subunit alpha	1
Antioxidant defense	Peroxiredoxin 1	1
	Peroxiredoxin 2	2
	Peroxiredoxin 6	1
Signaling	Galectin 1	2
	Ras-related protein Rab	2
	Moesin	2
	Ezrin	1
	Radixin	1
	Rho-related GTP-binding protein RhoC	1
	Transforming protein RhoA	1
	S100 protein A8	1
	S100 protein A9	1

Table 1: Endogenously nitrated proteins in merlin deficient Schwann cells and patient vestibular Schwannomas. Immunoprecipitations with a polyclonal anti-nitrotyrosine antibody was followed by mass spectrometry analysis and Western blot analysis was performed to identify proteins endogenously nitrated in patient vestibular Schwannomas (VS). This work was performed by Dr. Franco in collaboration with this thesis.

Nitrated proteins in MD-HSC

Function	Protein	Peptides (CI 95%)
Cytoskeleton	Vimentin	20
	β-actin	28
	β-tubulin	8
	cofilin-1	1
	cofilin-2	1
Structural (nucleus)	Histone H4	10
	Histone H2A	6
	Histone H2B	11
	Histone H3	3
Chaperones/ Protein folding	Hsp90	2
	Hsp70	6
	14.3.3	1
	SERPINH1(endoplasmic reticulum)	8
	Peptidyl-prolyl cis-trans isomerase B	3
	Stress-70 (mortalin) (mitochondrial)	2
	Hsp60 (mitochondrial)	2
Translation	Elongation factor 2	2
	Eukaryotic initiation factor-4A	2
	Eukaryotic initiation factor-5A	1
Metabolism (cytosol)	Pyruvate kinase	3
Metabolism (mitochondria)	ATP/ADP translocase	1
	ATP synthase subunit alpha	5
Antioxidant defense	Peroxiredoxin 1	1
	Peroxiredoxin 2	1
Signaling	Galectin 1	2
	Ras-related protein Rab	2

*Underlined proteins are also found in WT-Schwann Cells

Table 2: Endogenously nitrated proteins in merlin deficient Schwann cells and patient vestibular Schwannomas. Immunoprecipitations with a polyclonal anti-nitrotyrosine antibody was followed by mass spectrometry analysis and Western blot analysis was performed to identify proteins endogenously nitrated in merlin deficient (MD) human Schwann cells. This was performed by Dr. Franco in collaboration with this thesis.

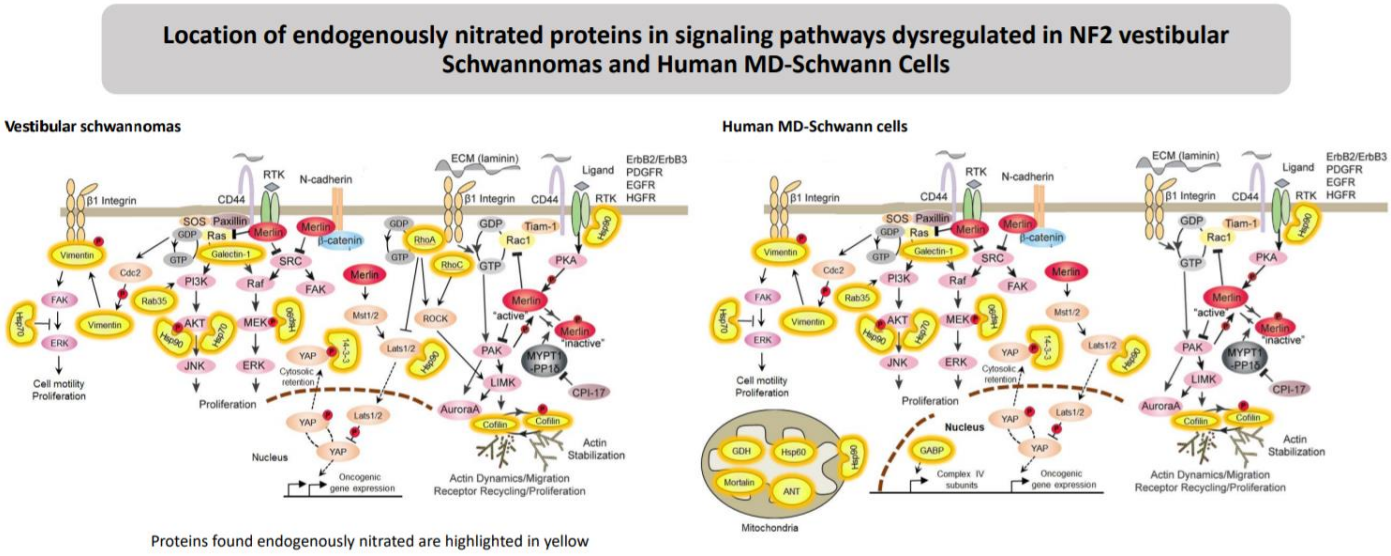


Figure 11: Depiction of protein pathways between Vestibular Schwannomma cells (Left), as well as human MD-Schwann cells (Right). Produced based on described findings on endogenously nitrated proteins (Table 1,2)

Discussion

The integration of the information provided by the identification of signaling pathways regulated by nitrated proteins, together with the determination of the proteins that are endogenously nitrated in vestibular schwannomas from NF2 patients allowed us to create a list of proteins that participate in signaling pathways known to be dysregulated in NF2 (Fig. 11). Between vestibular Schwannomas from NF2 patients, Merlin deficient (MD) human Schwann cells, and wild type (WT) human Schwann cells, both Hsp90 and Galectin-1 appeared to be endogenously nitrated in pathological conditions only. Hsp90 is a heat shock protein that chaperones a variety of other proteins, including those involved in survival pathways (Fig. 11) (6). Hsp90 makes up 1-2% of the total cytosolic protein content (6). Previous research from our group showed that while nitrated Hsp90 can result in motor neuron apoptosis, nitration of Y-33 on Hsp90 can result in metabolic reprogramming via mitochondrial downregulation in tumor cells (16). We previously identified Hsp90 as nitrated in MD-Schwann cell survival (16). When nitrated, we identified Hsp90 as a regulator of a metabolic shift away from mitochondrial ATP production, due to its association with decreased mitochondrial performance (16). Additionally, in motor neurons, Hsp90 nitration results in cell death via activation of the Fas death pathway (19). In both cases, nitration can act as a key cell regulator in pathogenic contexts, suggesting that it could play a role in supporting the metabolic reprogramming in MD-Schwann cells, and supporting cell survival.

We are the first to describe galectin-1 in the context of nitration. Galectin-1 is associated with multiple survival pathways, including the PI3K/Akt pathway, and the

MEK/ERK pathway. Galectin-1 primarily supports Ras signaling, which results in increased downstream MAPK activity (30). In pathology, galectin-1 supports malignant behavior in tumors, and is associated with poorer prognosis in patient prognosis in glioblastoma multiforme outcome (31). Furthermore, galectin-1 is implicated in the formation of malignant peripheral sheath tumors in NF1 (32). In both cases, galectin-1 activity was associated with upregulation of the PI3K/Akt pathway (31, 32). While NF2 is not associated with malignant tumors, this draws attention to nitrated galectin-1 as a target for future pharmaceuticals, suggesting a critical role for survival pathway dysregulation in the proliferative success of NF2-associated vestibular schwannomas.

Upstream of these proteins, as well as merlin itself, are the RTKs, which are important proteins that interpret and integrate external signals. We did not observe differences in RTK phosphorylation levels that would be associated with decreased survival and proliferation. This suggests that nitrated proteins regulate signaling pathways downstream of RTKs, but at a level upstream of MAPK phosphorylation, in agreement with the role of galectin-1 and Hsp90 in these pathways.

Kinases such as ERK, JNK, Akt, and p38 all showed a phosphorylation increase in the arrays. While activation of ERK and Akt support cell proliferation, activation of JNK and p38 could lead to induction of apoptosis. In addition, ERK and JNK possess competing functions in the context of oxidant-induced stress, with the balance of both being an important decider in cell survival (33). However, in NF2, persistently phosphorylated JNK specifically supports survival through suppressing reactive oxygen species buildup (37). Aside from JNK activity, the activation of Akt and ERK both contribute to proliferation in vestibular schwannoma cells (37). However, inhibiting either

pathway independently does not result in cell death (37). In the phospho-MAPK arrays, prevention of tyrosine nitration in MD-Schwann cells was associated with the downregulation of these pro-survival pathways (Akt, ERK, JNK). This supports a role for nitrated proteins in the regulation of crucial pro-proliferative signaling pathways in NF2.

Furthermore, our phospho-MAPK arrays showed the downregulation of p38 associated with prevention of tyrosine nitration. This does not fit the narrative given p38 role in apoptotic signaling, especially in the context of peroxynitrite stimulation (8). Additionally, while p38 is activated in an ERK dependent matter, independent activation due to peroxynitrite exposure has also been observed (34). Therefore, it remains unclear whether the activation of both ERK and p38 is multifactorial.

The PI3K/Akt pathway is associated with both galectin-1 and rab35, and both proteins associated with pathogenic conditions in the results for endogenously nitrated proteins. Therefore, activation of the PI3K/Akt pathway could prove promising, especially considering this pathway represents a pro-survival and anti-apoptotic signal playing an important role in NF2 schwannomas (37). The downregulation of this pathway was shown to decrease cell survival in the face of oxidative stress, suggesting the pathway could play a protective role when a cell is exposed to oxidants (35). This pathway could therefore be acting to counteract the upregulation of pro-apoptotic pathways such as p38.

Arrays provide information on the increase in the quantity of phosphorylated protein, but not the proportion of total protein phosphorylated. If the expression of a protein increased substantially, it may falsely, appear to increase in activation on the arrays. This is why the supplementation of this method with Western blots as well as the transcriptome and pathway analysis are necessary. The Western blots did not point to

the same conclusions that the arrays did. The lack of consistency between Western blots, along with the lack of consistency with any of the array results, suggests that the issue lies in methodology. A potential culprit is the difference in protein conformity, with arrays detecting folded, native proteins, and the Western blots detecting denatured proteins.

Future Directions

While the RNA for the transcriptome analysis has been sent out, the results are pending, and likely represent the main objective before further action. Once the results are obtained from the CGRB, computational analysis will be performed in collaboration with Dr. David Hendrix to determine which pathways are active. Integration with current results will determine further pathways of interest. A comparison analysis between the transcriptomic data, array data, and Western blot data will follow.

It is warranted to repeat the Western blots for all of the four observed proteins, with a priority for JNK to increase the number of biological replicates.

Endogenously nitrated proteins seen in these results will be investigated as potential pharmaceutical targets. Further characterization of the role of nitrated proteins in cell survival will either result in narrowing down the selection of potential targets, or motion towards a future therapeutic option. Both galectin-1 with its link to cell survival signaling (30), and Hsp90 with its link to cell metabolism (16), show promise as nitrated proteins to further investigate as pharmaceutical targets.

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