Supplemental information

Table S1. KEGG pathway of differentially expressed genes from gene expression profiling of sciatic nerves from 1-month-old OGT-SCKO and littermate control mice.

Gene set analysis for upregulated genes in OGT-SCKO			Gene set analysis for downregulated genes in OGT-SCKO		
KEGG PathwayName	**#Gene**	**adjP**	**KEGG PathwayName**	**#Gene**	**adjP**
MAPK signaling pathway	26	1.93e-11	Metabolism of xenobiotics by cytochrome P450	5	0.0002
Cytokine-cytokine receptor interaction	24	6.78e-11	Peroxisome	5	0.0002
Graft-versus-host disease	12	1.42e-09	Metabolic pathways	15	0.0003
Focal adhesion	20	1.81e-09	Arachidonic acid metabolism	4	0.0019
Pathways in cancer	25	1.84e-09	Amino sugar and nucleotide sugar metabolism	3	0.0026
Viral myocarditis	14	1.93e-09	Fat digestion and absorption	3	0.0026
Type I diabetes mellitus	12	1.93e-09	Linoleic acid metabolism	3	0.0026
Cell adhesion molecules (CAMs)	17	3.18e-09	Neuroactive ligand-receptor interaction	5	0.0118
Antigen processing and presentation	13	3.32e-09	Drug metabolism - cytochrome P450	3	0.0118
Allograft rejection	11	5.03e-09	Pentose and glucuronate interconversions	2	0.0151

A total of 1099 differentially expressed genes (887 upregulated and 212 downregulated) (2-fold change, $p<0.05$) were identified in the mutant nerve. Note the over-representation of MAPK signaling and inflammation pathways in the upregulated gene subset and of metabolic pathways in the downregulated gene group.

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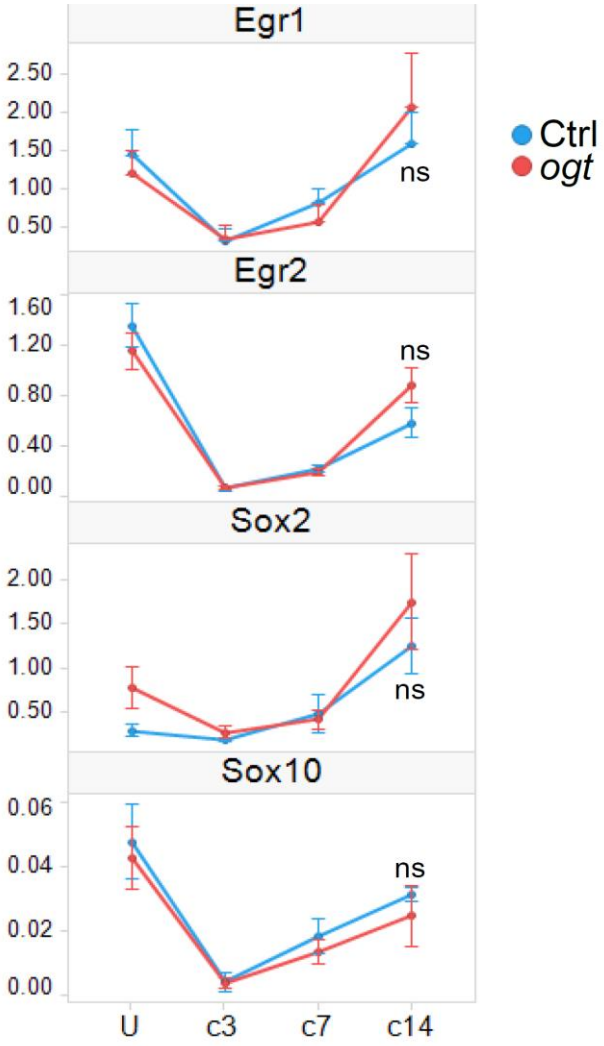
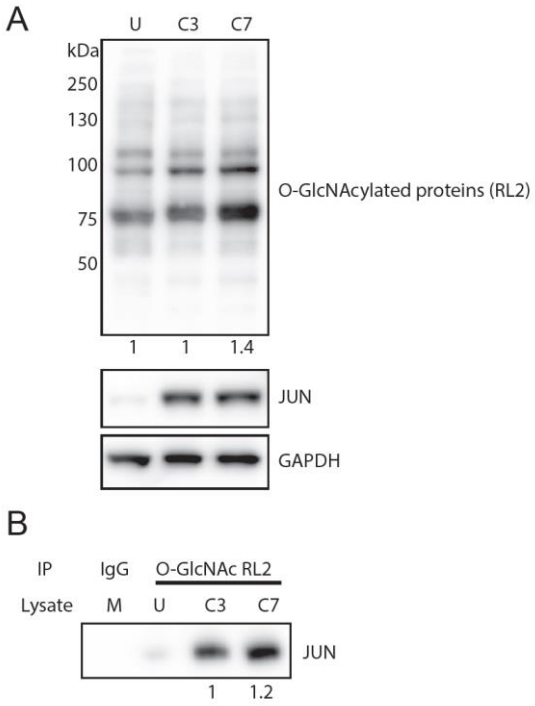
Fig. S1. Expression of key SC developmental genes in OGT-SCKO nerves after injury.



Quantitative RT-PCR analysis of control (Ctrl) vs. OGT-SCKO (ogt) sciatic nerves at indicated times after nerve injury. Note expression of *Egr1*, *Egr2*, *Sox2*, and *Sox10* were not significantly different after nerve injury. U (uninjured), C3 (3 days post injury), C7 (7 days post injury), C14 (14 days post injury). Y-axis: mRNA expression (Scaled per gene, normalized to *Gapdh*). ** ($p<0.01$), *** ($p<0.001$). N=3~5 nerves per genotype per injury time point.

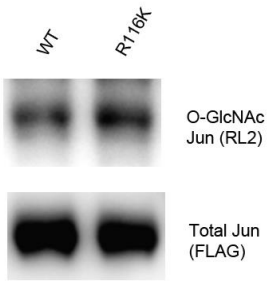
Fig. S2. JUN is O-GlcNAcylated *in vivo*



A. Western blot analysis shows significant amount of total O-GlcNAcylation in rat sciatic nerves, either uninjured (U), post-injury day 3 (C3), or day 7 (C7). Note higher (~40%) total O-GlcNAcylation at C7 compared to C3. Note JUN expression is minimal in uninjured nerves (U), and dramatically induced in post-injury nerves (C3 and C7). Total O-GlcNAcylated proteins (normalized by GAPDH) is significantly increased (~40%) at C7 compared to C3.

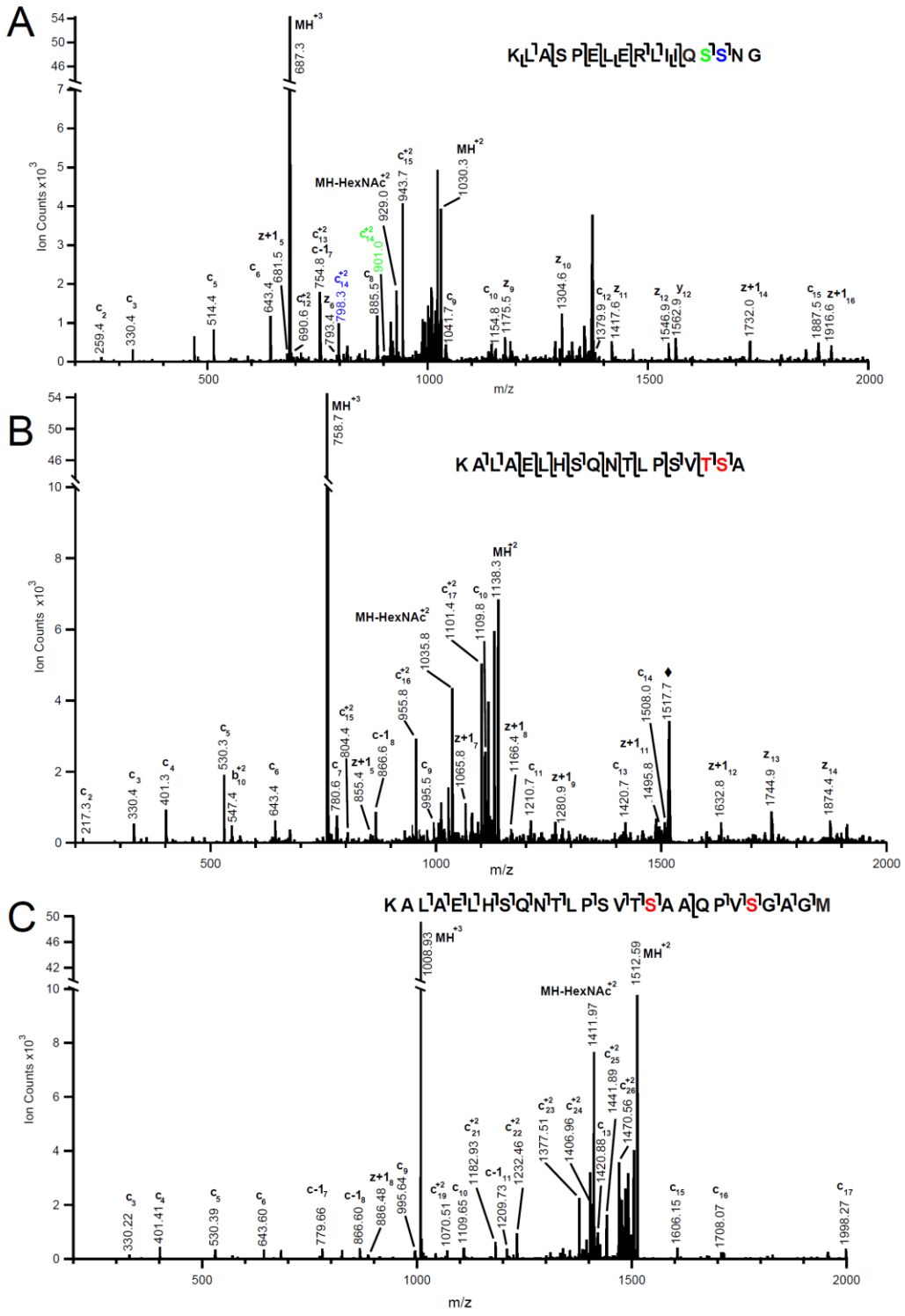
B. Rat sciatic nerve lysates from (A) were used to enrich O-GlcNAcylated proteins via immunoprecipitation using anti-GlcNAc RL2 antibody. Immunoblot analysis of these immunoprecipitated proteins detects JUN in the injured nerve O-GlcNAcylated protein pool. JUN O-GlcNAcylation was approximately 20% higher at C7 vs. C3 (measured by comparing JUN IP sample and JUN RL2 signal at each time point). IgG (control mouse IgG). M (mixed, equal volume of U, C3 and C7 lysates were combined). N=3 nerves for uninjured and 1 nerve each for C3 and C7. A representative western blot of three independent experiments is shown. 1% lysate input in (A).

Fig. S3. O-GlcNAcylation of JUN R116K is similar to that of JUN wildtype.



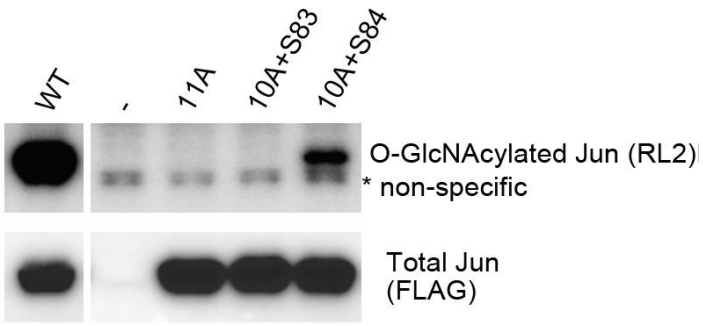
Flag-tagged versions of JUN wildtype (WT) and R116K mutant were expressed in HEK 293T cells expressing exogenous OGT and treated with Thiamet-G. Cell lysates were analyzed by immunoblot for O-GlcNAcylation (anti-O-GlcNAc RL2) and total JUN (anti-FLAG). Note comparable O-GlcNAcylation between wild type and mutant JUN, indicating that the R116K mutation does not interfere with JUN O-GlcNAcylation.

Fig. S4. JUN is O-GlcNAcylated at Ser84, Thr131, Ser132, Ser138.



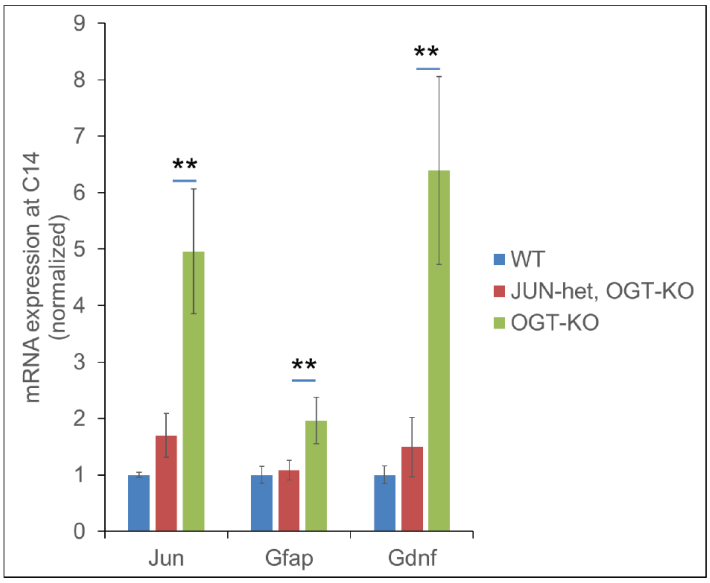
A-C. Mass spectrometry analysis of JUN O-GlcNAcylation. MS profile of S83 or S84 O-GlcNAc (A), T131 and S132 O-GlcNAc (B), T131 or S132 and S138 O-GlcNAc (C). A. Blue and green 'S' are ambiguous site assignments for GlcNAc modification. B. O-GlcNAc sites are marked in red. ♦ is a coeluting +2 precursor. C. Red 'S' are O-GlcNAc modified.

Fig. S5. JUN S84, but not S83, is O-GlcNAcylated.



Flag-tagged versions of JUN wildtype (WT) and Ala substitution mutants were expressed in HEK 293T cells expressing exogenous OGT and treated with Thiamet-G. Cell lysates were analyzed by immunoblot for O-GlcNAcylation (anti-O-GlcNAc RL2) and total JUN (anti-FLAG). Note lack of O-GlcNAcylation in JUN 11A mutant (substitutions at S29, S48, S83, S84, T131, S132, S138, S199, T234, T242, and S246). The S84 revertant of 11A, which is similar to above but with S84A reverted back to Ser (10A+S84), but not S83 revertant of 11A, which is similar to above but with S83A reverted back to Ser (10A+S83) undergoes O-GlcNAcylation. This result indicates that JUN residue S84 can be O-GlcNAcylated whereas S83 is not a site for O-GlcNAcylation.

Fig. S6. Decreased JUN gene dosage normalizes the expression of JUN and its target genes in OGT-SCKO.



qRT-PCR analysis of 14 d post-injury nerves showed that expression of JUN and indicated JUN target genes was significantly lower in JUN-het/OGT-SCKO mice vs. OGT-SCKO mice, and was reduced to levels comparable to that observed in wildtype mice. N=4~5 nerves per genotype. **($p<0.01$).

Dataset S1. Annotated microarray gene expression profiles of sciatic nerves from wild type and OGT-SCKO mice at 1 month of age.

Dataset S2. List of genes with ± 2 fold-change in expression level (OGT-SCKO / wild type) at the cut-off $p<0.05$.

Supplemental Materials and Methods

Nerve Injury

Mice and rats were anesthetized by intraperitoneal injection of 2-2-2 tribromoethanol at a dose of 500 mg/kg. The sciatic nerve was exposed at the sciatic notch and a reproducible crush injury was created by using #5 jeweler's forceps for 30 sec. The site of injury was marked with a single 10-0 nylon epineural suture. Nerve lesions were produced on the right side and the contralateral nerve was left intact and served as the uninjured control. At the appropriate time, nerves were harvested and processed for histology.

Nerve histology and morphometry

Sciatic nerves were dissected and placed in 3% glutaraldehyde overnight. After washing with phosphate buffer, nerves were post-fixed in 1% osmium tetraoxide in phosphate buffer overnight at 4°C. Specimens were then dehydrated in graded alcohols and embedded in 100% epoxy (Araldite 502). For light microscopy, semi-thin plastic embedded sections (300 – 600 nm thick) were prepared and stained with toluidine blue. For electron microscopy, ultrathin sections (40-50 nm) were prepared, stained with uranyl acetate and lead citrate, and imaged with a JEOL (Akishima) 1200 electron microscope. All nerves underwent qualitative assessment of neural architecture followed by detailed histomorphometric analysis carried out as previously described (1). Morphometry was performed using ImageJ.

Immunostaining and fluorescent microscopy

Sciatic nerves were dissected and fixed in 4% paraformaldehyde for 1 h at 4°C. The fixed nerves were cryopreserved in 30% sucrose and embedded in Tissue-Tek OCT Compound (Sakura Finetek) prior to preparing 6 µm sections. For analysis of teased nerve fibers, mouse sciatic nerves were dissected and fixed in 4% paraformaldehyde in PBS for 30 min 4°C. The nerves were washed three times in PBS for 5 min, de-sheathed, and nerve bundles were dissected with fine needles in PBS on Fisherbrand Superfrost/Plus microscope slides. Slides were air-dried for at least 2 h at room temperature and stored at -20°C. For immunostaining, frozen sections were washed and blocked in 5% fish skin gelatin in PBS with 0.2% Triton for 1 h at room temperature. Sections were then incubated with primary antibody diluted in blocking buffer overnight at 4°C. Secondary antibody incubation was performed at room temperature for 1 h also in blocking buffer. The immunostained sections were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories) for microscopic visualization. Images were captured using an upright microscope equipped for epifluorescence microscopy (Nikon 80i; CoolSnapES camera) and were processed using MetaMorph. All quantifications were performed with the observer blinded to genotype. ImageJ (National Institutes of Health, Bethesda, MD) was used for all quantitative manipulation and analysis of images.

RNA Preparation and qRT-PCR

Total RNA was isolated after homogenization (for sciatic nerves) in Qiazol lysis reagent (QIAGEN) using a miRNeasy Minikit (QIAGEN) according to the manufacturer's protocol. RNA concentration was quantified using an ND-1000 spectrophotometer (Nanodrop Technologies). mRNA was reverse transcribed from 100 ng of total RNA using qScript cDNA SuperMix (Quanta Biosciences). mRNA qRT-PCR was performed using a SYBR green-based detection system on a 7900 HT Sequence Detector instrument (Applied Biosystems) as described previously (2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize samples and obtain relative expression values that were used to calculate percent changes. Primer sequences: The sequences of the qRT-PCR primers used are as follows (5'-3'; mouse unless specified):

Shh: F, AAAGCTGACCCCTTTAGCCTA; R, TTCGGAGTTTCTTGTGATCTTCC.
Ngfr: F, GTACCCAGTACAGTGGCGGA; R, CTGTTCCACCTCTTGAAAGCAAT.
Ccnd1: F, GCGTACCCTGACACCAATCTC; R, CTCCTCTTCGCACTTCTGCTC.
Gap43: F, ACCACTGATAACTCCCCGTCC; R, GCTTCGTCTACAGCGTCTTTCTC.
Gdnf: F, CGTCATCAAACTGGTCAGGA; R, CCGGTAAGAGGCTTCTCG.
Gfap: F, CGGAGACGCATCACCTCTG; R, AGGGAGTGGAGGAGTCATTCG.
Jun: F, CCTTCTACGACGATGCCCTC; R, GGTTCAAGGTCATGCTCTGTTT.
Fos: F, CGGGTTTCAACGCCGACTA; R, TTGGCACTAGAGACGGACAGA.
Artn: F, TCTTCCACTGCACCAGCGGGCCGTGCGT; R, TGCTCAGCAGAGCCAGAGCGGCCAGGGT.
Sox10: F, TCAAGAAGGAACAGCAGGAC; R, CTTTCGTTCAGCAACCTCCAG.
Sox2: F, GCGGAGTGGAAACTTTTGTCC; R, CGGGAAGCGTGTACTTATCCTT.

Egr2: F, CATGGGCAAATTCTCCATTGA; R, TTGCAAGATGCCCGCAC.
Egr1: F, CACCTGACCACAGAGTCC; R, CCATCGCCTTCTCATTATTC.
Gapdh: F, TGCCCCCATGTTTGTGATG; R, TGTGGTCATGAGCCCTTCC.

Microarray and Computational analysis
Total RNA samples were prepared by isolating and pooling RNA from at least 3 different 1-month-old OGT-SCKO and Ctrl mice. Triplicates were prepared entirely independently from three separate pools of at least three animals each. Total RNA quality was then determined by Agilent 2100 bioanalyzer (Agilent Technologies) according to manufacturer's recommendations. RNA transcripts were amplified by T7 linear amplification messageAmp TotalPrep amplification kit (ABI-Ambion) using 400 ng of each total RNA sample. The amplified RNA samples (aRNA) were then cleaned with RNA columns and quantified on a spectrophotometer, and RNA quality was determined by Agilent 2100 bioanalyzer (Agilent Technologies). 1500 ng of each aRNA were hybridized onto Illumina Mouse WG-6 v2.0 Expression Beadchips according to manufacturer's recommendations. Arrays were scanned on an Illumina BeadArray Reader. Images were quantitated by Illumina Beadscan, v3 and the resulting data was imported into Illumina GenomeStudio software, where on-slide spot replicates were averaged and individual spot probe was reported. This signal intensity data was background subtracted and quantile normalized in Illumina Genome Studio. Probes having detection p-value>0.05 in all samples were removed and a two-class unpaired SAM analysis was performed. Differentially expressed genes with at least 2.0 fold differential regulation between OGT-SCKO and Ctrl nerves at a false discovery rate (FDR) of 0.5% were selected for further analysis. Gene enrichment analysis was performed using WebGestalt (3).

Cell culture, transfection, and luciferase reporter assay
HEK293T cells were routinely cultured in DMEM (Invitrogen) containing 10% FBS (Hyclone). Lipofectamine 2000 (Invitrogen) was used for transfections following the manufacturer's protocol. Luciferase reporter assays were performed using Dual-Glo® Luciferase Assay System (Promega) according to manufacturer's protocols. AP-1 driven firefly luciferase units were normalized by thymidine kinase (TK) driven renilla luciferase units. The following plasmids were used: 3xAP1pGL3 reporter (3xAP-1 binding sites in pGL3-basic; a gift from Alexander Dent (Addgene plasmid # 40342), pRL-TK (Promega), shOGT (Sigma, TRCN0000110398), Flag-JunWT, 4A, 4D-Myc (FLAG-mouse JUN-Myc in pcDNA3, a gift from Axel Behrens (Addgene).

Immunoprecipitation and western blotting
Sciatic nerves (N=1 nerve per sample, minimum 3 mice per genotype tested) were isolated, de-sheathed in PBS, and immediately frozen in liquid nitrogen. Nerve lysates were prepared by homogenizing the tissue in a lysis buffer containing 150 mM sodium chloride, 20 mM Tris-Cl (pH 7.4), 1% Triton X-100, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail (Sigma). The lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C and quantified using the MicroBCA Protein Assay kit (Pierce). For analysis of JUN O-GlcNAcylation *in vivo*, O-GlcNAcylated proteins in rat sciatic nerve lysates (N=3 nerves pool for uninjured, 1 nerve for C3 and C7 injured) were enriched by immunoprecipitation using anti-O-GlcNAc RL2 antibody. For analysis of JUN O-GlcNAcylation in cell cultures, HEK 293T cells were transfected with expression plasmids encoding FLAG-tagged JUN variants (mouse). If indicated, the cells were also transfected with human OGT expression vector was also transfected, and/or treated with 1 µM Thiamet-G at the time of transfection. Two-day post-transfection, cells lysates were prepared using a lysis buffer (150 mM sodium chloride, 20 mM Tris-Cl (pH 7.4), 1% NP-40, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, complete protease inhibitor cocktail (Sigma), and 10 µM Thiamet-G), and FLAG-JUN was immunoprecipitated using FLAG-agarose beads for 1 hour at 4°C. For Western blotting, proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes were blocked in 5% milk in 0.05% PBS-Tween and incubated overnight with the appropriate primary antibody. Following incubation with secondary antibodies conjugated to HRP (GE Healthcare), membranes were developed with Western Chemiluminescent HRP Substrate (ECL) (EMD Millipore Immobilon). Densitometry of the blots was performed using ImageJ.

Antibodies
The following antibodies were used at the indicated dilution. For western blot: O-GlcNAc RL2 (Santa Cruz, sc-59624 HRP, 1:1,000), JUN, phospho-JUN S63 (Cell Signaling, 1:1000), phospho-JUN S73 (Cell Signaling, 1:1000), phospho-JUN T91 (Cell Signaling, 1:1000), Phospho-JUN T93 (Cell Signaling, 1:1000), p-JNK, (Cell

Signaling, 1:1000), JNK (Cell Signaling, 1:1000), p-ERK (Cell Signaling, 1:1000), ERK (Cell Signaling, 1:1000), p-p38 (Cell Signaling, 1:1000), GAPDH (Cell signaling, 1:1000), β-actin (Sigma, 1:1000), FLAG M2 (Sigma, 1:1000), β-tubulin E7 (Developmental Studies Hybridoma Bank, 1:1000). For immunostaining: MBP (Millipore, 1:1000), NF (Aves, 1:1000), JUN (Cell Signaling, 1:1000), NGFR (Millipore, 1:1000).

Mass Spectrometry

JUN-R116K protein bands were excised from an SDS-PAGE gel and subjected to in-gel digestion. The gel bands were reduced with 10 mM dithiothreitol (Sigma-Aldrich) at 56°C for 1 hour, followed by alkylation with 55 mM iodoacetamide (Sigma) at room temperature in the dark for 45 minutes. The samples were incubated overnight with Lys-N (ThermoFisher Scientific) at 37°C followed by further proteolysis with 50mM CNBr (Sigma-Aldrich) in 25% TFA for an additional 6 hours. The resulting peptides were extracted using 50% acetonitrile and 5% formic acid, dried, resuspended in 20ul of 0.1% formic acid, and desalted using C_{18} ZipTips (Millipore). Peptides were analyzed by on-line LC-MS/MS using an Orbitrap Velos (Thermo Scientific, San Jose, CA) equipped with a nano-Acquity UPLC (Waters, Milford, MA). Peptides were fractionated on a 15 cm x 75 µm ID 3 µm C18 EASY-Spray column using a linear gradient from 2-35% solvent B over 60 min. Survey mass measurements were performed using the Orbitrap, scanning from m/z 350-2000. Two injections were run, one selecting the 6 most abundant multiply charged ions for ETD analysis and one selecting the 3 most abundant multiply charged ions for ETD and HCD analysis. Supplemental activation was enabled. The ETD fragments were measured in the linear trap, whereas HCD fragments were measured in the Orbitrap. Peak lists were generated from the raw data using in-house software, PAVA (4). The MS/MS data were searched against the human SwissProt database (downloaded 12.01.2015) including the tagged cJUN-R116K sequence and a randomized concatenated database using Protein Prospector (v5.20.0) Cleavage specificity was set as Lys-N, allowing for 3 missed cleavages and non-specific cleavage at the C-termini. Carbamidomethylation of Cys was set as a constant modification. All searches allowed for a maximum of three of the following variable modifications: acetylation of protein N-termini, oxidation of Met, cyclization of N-terminal Gln, deamidation of Asn and Glu, conversion of C-terminal Met to Hse or Hsl, and HexNAc modification of Ser, Thr, and Asn. HCD searches included HexNAc as a neutral loss. The required mass accuracy was 20 ppm for precursor ions, 0.8 Da for ETD fragments, and 30 ppm for HCD fragments. HexNAc containing peptides were manually verified.

Supplemental References

1. Kim S, et al. (2016) Schwann Cell O-GlcNAc Glycosylation Is Required for Myelin Maintenance and Axon Integrity. *J Neurosci Off J Soc Neurosci* 36(37):9633–9646.

2. Viader A, et al. (2013) Aberrant Schwann Cell Lipid Metabolism Linked to Mitochondrial Deficits Leads to Axon Degeneration and Neuropathy. *Neuron* 77(5):886–898.

3. Wang J, Duncan D, Shi Z, Zhang B (2013) WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res* 41(W1):W77–W83.

4. Guan S, Price JC, Prusiner SB, Ghaemmaghami S, Burlingame AL (2011) A data processing pipeline for mammalian proteome dynamics studies using stable isotope metabolic labeling. *Mol Cell Proteomics MCP* 10(12):M111.010728.