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Supplemental Information

Schwann-Cell-Specific Deletion of Phosphatidylinositol 4-Kinase Alpha Causes Aberrant Myelination

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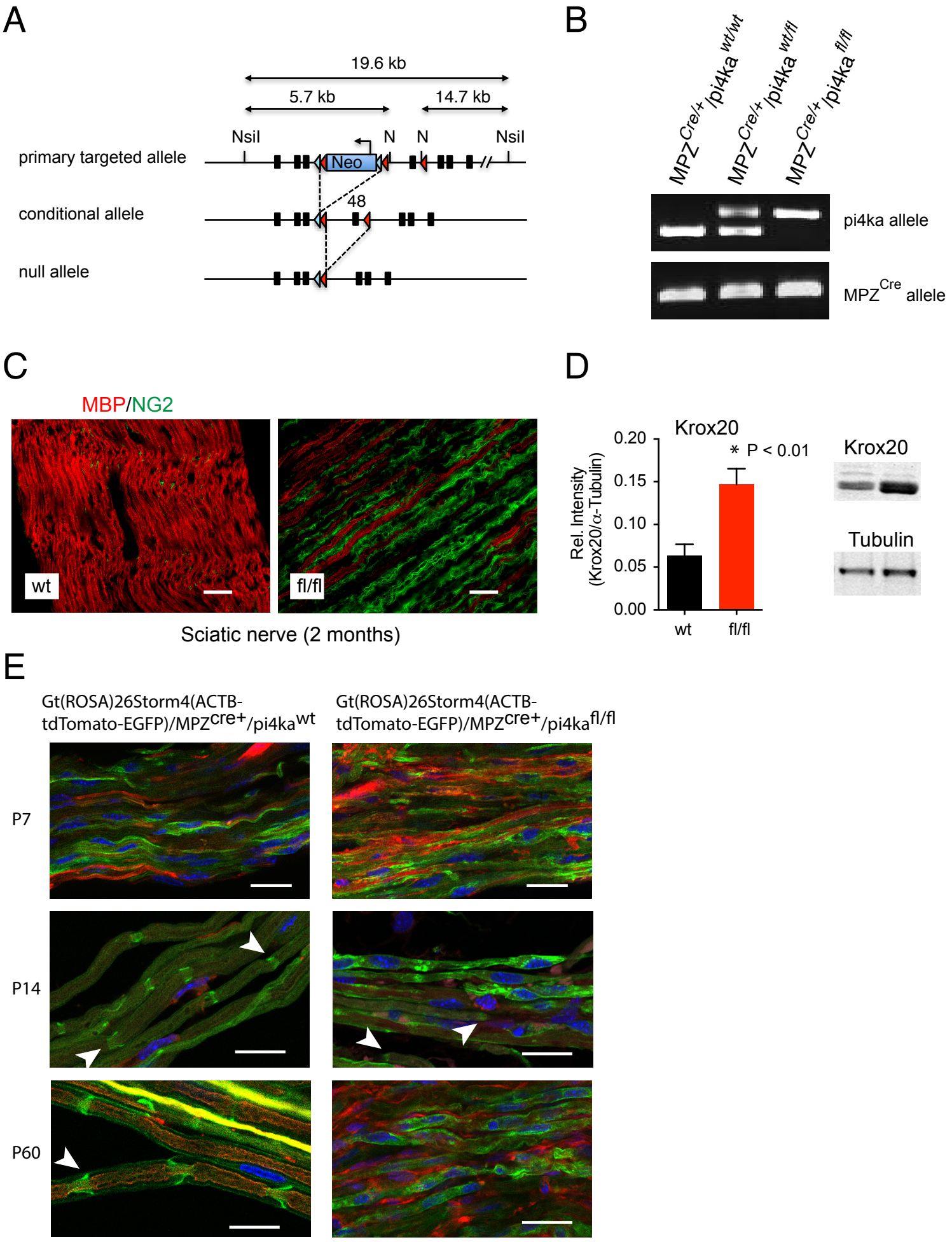

Figure S1 related to Figure 1

Legend to Figure S1 complementing Figure 1. (*A*) Cartoon showing the design of the conditional allele of pi4ka. The conditional allele was generated from the primary target allele where the NsiI restriction sites (N) and the neomicin selection cassette (NEO) are shown. The NEO casette was eliminated by Flp–mediated deletion. The null allele is obtained by the action of the Cre-recombinase that removes exon 48 that is critical in the catalytic domain. The final product is a truncated PI4KA with no catalytic activity. To obtain Schwann cell-specific deletion, these pi4Ka mice were crossed with mice with the Cre recombinase driven by the Mpz promoter (MPZCre). Mice were then classified as "controls" ($MPZ^{Cre/+}$/$pi4ka^{fl/wt}$ and $MPZ^{Cre/+}$/$pi4ka^{wt/wt}$) or "mutants" ($MPZ^{Cre/+}$/$pi4ka^{fl/fl}$). (*B*) PCR analysis showing the presence of the indicated alleles. (*C*) The oligodendrocyte marker, NG2 is highly expressed in the nerves obtained from mutant animals, when compared to the controls (bars show 20 μm). (*D*) Krox20, a transcription factor that regulates the expression of MPZ and MBP and is also expressed in oligodendrocytes, shows significantly higher expression in the mutant nerves (Means ± SEM, n =3, * denotes $P < 0.01$ by Student's t-test). (*E*) Sciatic nerves isolated from Gt(ROSA)26Storm4(ACTB-tdTomato-EGFP)/MPZ^{cre+}/$pi4ka^{wt/wt}$ and their $pi4ka^{fl/fl}$ counterpart littermates were teased and analyzed by confocal microscopy at the indicated ages. Cre recombination in these reporter mice causes switching plasma membrane targeted red fluorescence to Green. There is no obvious difference between the nerves at age P7, but myelinating Schwann cells are already recognizable at P14 with the appearance of Schmidt–Lanterman I(SLIs, white arrowheads) in control nerves. SLI structures are barely recognizable at this age (P14) in the mutant nerves. At two months of age, myelinating Schwann cells are clearly recognizable in wild type nerves whereas they are disorganized in the mutant nerves. There is little difference between the nerves from different ages in the mutant animals.

Supplemental Experimental Procedures (supplementing Experimental Procedures):

Materials - The PI4KA inhibitor, A1 (GSK2736460A), was synthesized by (Glaxo Smith-Kline) (Bojjireddy et al., 2014). Inhibitors were dissolved in DMSO and added freshly to the medium (final DMSO 0.1%) to treat the mouse Schwann cells. A1 was used at a concentration of 100 nM for 24 h. The following antibodies were used mouse anti-S100 (Millipore MAB079-1) mouse anti MBP (Biolegend 808401) rabbit anti-Mpz (polyclonal from MBL Life Science #PD046, Nagoya, Aichi, Japan) anti Krox20 (Egr-2, Santa Cruz Biotechnologies, Santa Cruz, CA) rabbit anti NG2 (Millipore AB5320). Anti pAkt-Ser374, Thr308, pan Akt and S6K were from Cell Signaling (Boston, MA). Anti-tubulin was from Sigma (St. Louis, MO). The polyclonal PI4KA antibody was kindly provided by Dr. Pietro De Camilli. The antibodies for Drp2 and Periaxin were produced in rabbits and were kindly provided by Dr. Peter Brophy (The University of Edingburgh, Scotland, UK). Fluorescent secondary antibodies were from Invitrogen (Thermofisher Scientific). The plasmids used are described as follows: For PI4P and PI(4,5)P2 analysis we used the PI4P-specific lipid sensor P4M of the SidM protein of Legionella pneumophila (Hammond et al., 2014) and the PLCδ_1-PH domain (Várnai and Balla, 1998), respectively. LifeAct was created by cloning the 5'-accatgggcgtggccgacctgatcaagaagttcgagag-catctccaaggaggaggggat-3' nucleotide sequence which codes the lifeact protein between the EcoRI and BamH1 sites of either the mRFP-N1 or eGFP-N1 expression plasmids.

Preparation of MEF cells - Embryos were removed and briefly washed with 70% ethanol in PBS and placed in ice-cold PBS. After removing the heads and the red tissues, the remaining tissue was minced and digested with Trypsin/EDTA (0.05%) for 15 minutes at 37C. The digested tissue was filtered through a nylon mesh and the MEF cells were plated on 10 cm culture dishes using DMEM high glucose complemented with Penicillin-Streptomycin 1% and Serum 10%. MEFs were immortalized by using the serial passages method. Briefly, cells were taken through 20-25th passage, using a 3-day transfer schedule. In order to keep the same level of confluency and since the growth rate decreased from passage 5th to 15th, the cells were transferred to 6 cm dishes during that stage of the immortalization process. Once the MEFs recovered their growth rate (passage 20th to 25th) they were transferred back to 10 cm dishes, expanded, and properly stored in liquid nitrogen. When PI4KA was to be inactivated, immortalized MEF cells were treated with (Z)-4-hydroxytamoxifen (4-OHT) (final concentration, 1 uM) and cultured for additional 2 weeks without Tamoxifen before transfection and microscopy analysis.

Immunohistochemistry and Histology – Sciatic nerves of 2 months old mice were fixed in Bouin's solution and embedded in paraffin. Longitudinal and cross sections (7 μm thick) were cut on microtome. Following deparaffinization, tissue was processed for immunohistochemistry. Briefly, tissue was blocked for non-specific binding in 1% bovine serum albumin (BSA) in 0.01 M phosphate buffered saline pH 7.4 (PBS). Primary antibodies were diluted in 1% BSA in PBS and applied to sections overnight at 4˚C. Secondary antibody (donkey anti-mouse Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 555) was applied for two hours at room temperature at 1:500 dilution in 1% BSA in PBS. Sections were mounted in Mowiol (Sigma) and examined under an inverted confocal microscope (Zeiss LSM 710). Collagen was visualized by Masson's trichrome staining. These sections were then dehydrated, cleared in xylene mounted in DPX (Sigma) and examined under Zeiss Axiovert microscope equipped with camera. Where stated, sciatic nerves were teased after removing the epineurium using extra-fine forceps and the nerve fibers were spread out on a Superfrost®Plus/Colorfrost®Plus slide. Subsequently, the specimen was immunostained with the correspondent antibodies and/or phalloidin Alexa Fluor 647 (1:100-200). To study apoptosis, the Click-iT™ TUNEL Alexa Fluor™ 488 kit (ThermoFisher Scientific) was used on teased nerves following the manufacturer's instructions. Slides were mounted with ProLong™ Gold Antifade Mountant with DAPI to visualize cells nuclei. Cells were examined in a Zeiss 710 scanning confocal microscope. The brightness levels were adjusted in Adobe Photoshop CS4 but the same setting and adjustments were used for images obtained from wild-type and mutant nerves.

Electron microscopy - After anesthetizing 2 months old mice with 2% isoflurane in 100% oxygen, transcardial perfusion fixation was performed on a downdraft table equipped with a chemical waste collection system. Phosphate buffer was perfused briefly (<5min) through the left ventricle, followed immediately by a fixative solution consisting of 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer. Excised sciatic nerve samples were post-fixed for 72 hours in the same fixative at 4°C. In preparation for resin embedding, nerve samples were equilibrated in 0.1M sodium cacodylate buffer, intensified in 2% osmium tetroxide (OsO_4), dehydrated through a graded ethanol series and equilibrated to 100% propylene oxide. Next, an Embed-812 resin (Electron Microscopy Sciences, Hatfield, PA.) infiltration series, using propylene oxide as the solvent, up to 100% resin was completed. The epoxy resin was polymerized for 24 hours in a vacuum oven set at 60° C. Ultra-thin sections (80nm) were prepared on a Reichert-Jung Ultracut-E ultramicrotome using a Diatome diamond knife (Electron Microscopy Sciences, Hatfield, PA.). The ultra-thin sections were transferred to 300 mesh cooper grids (Electron Microscopy Sciences, Hatfield, PA.) and were

post-stained with uranyl acetate and lead citrate. Imaging was performed on a JEOL 1400 transmission electron microscope operating at 80kV.

Sciatic motor nerve conduction velocity (MNCV) - Mice (P60) were anesthetized with 2% isoflurane in 100% oxygen, and the fur covering the hind limbs was shaved and depilated with a commercially available hair-removal cream. The sensing electrode was placed at the position where the gastrocnemius muscle has its maximum diameter, while the reference electrode was placed just distal to it. To target the sciatic nerve accurately, a small incision was made, and distal and proximal stimulation of the nerve were performed using a monopolar disposable 30 G needle electrode (Natus, Cat# S53153). Stimuli were delivered by a Grass model S48G stimulator and were monitored with a Coulbourn Instruments model V75-04 amplifier with 100x gain and bandpass filtered between 150 Hz and 1 kHz. Stimulus and response voltage data were digitized using a National Instruments USB-6212 acquisition card and Labview Software. Analysis was carried out using custom software implemented in Matlab. Conduction time was determined as the interval from stimulus onset to the time where the tangent to the initial deflection of the muscle recording intersected the abscissa. Measurements were taken at points both distal and proximal to the spinal cord, and the conduction velocity was determined as the distance between the two recording sites divided by the difference in conduction times.

Western blotting - Sciatic nerves were carefully dissected, pulling the perineurium and epineurium away from the endoneurium along the whole length of the nerve (Verheijen et al., 2003). Cells and sciatic nerves were lysed in different ice-cold lysis buffers: (M-PER; Thermo Scientific) and (T-PER; Thermo Scientific), respectively. To avoid protein degradation, both buffers were previously supplemented with protease inhibitors (Complete tablets; Roche). Protein concentration was determined by using the bicinchoninic acid (BCA) assay (Thermo Scientific) with BSA as a standard. High molecular weight proteins (>200 kDa) were resolved with NuPAGE 3-8% Tris-Acetate gels (Invitrogen), and middle molecular weight proteins (30-100 kDa) with NuPAGE 4-12% Tris-Glycine gels (Invitrogen). After performing electro-transference onto nitrocellulose membranes (Invitrogen), blots were blocked in Odyssey® blocking buffer (PBS) for one hour. Subsequently, blots were incubated overnight at 4°C in the same buffer supplemented with antibodies against Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), pS6 ribosomal protein, α-Tubulin, Krox-20, and PI4KA. After washing with TBS-T, blots were incubated for 1 h at room temperature within the proper Odyssey® secondary antibody diluted in TBS-T. Finally, blot membranes were scanned and quantitated using the Odyssey® instrument and software.

Lipidomic analysis – Sciatic nerves (~10 mm in length) were dissected, measured and weighed. After turning the perineurium inside out nerves (together with the perineurium) were homogenized and processed for lipid extraction and analysis of lipid content. Likewise, Schwann cells cultured on 10 cm diameter plates and treated with 100 nM of A1 or DMSO for 24h were processed for lipid extraction and analysis of lipid content. Samples were extracted by a mixture of Chloroform: Methanol: 1N HCl (2:4:1.6) (in ml v/v) followed by phase separation by adding 2 ml of Chloroform and 2 ml 1 N HCl. After removing the lower phase, the upper phase was re-extracted with 1 ml Chloroform and the lower phase was combined with the lower phase of the first extraction. The combined chloroform phase was washed once with 0.5 ml 1M KCl and then with 0.5 ml H_2O before drying under nitrogen. The samples were than subjected to analysis by mass spectrometry performed by the Kansas Lipidomics Research Center. Analysis was performed in two separate experiments and duplicate or triplicate samples were analyzed. For analysis of PIPs, Schwann cells were cultured on 6 cm culture dishes to ~80% confluence and after washing twice with PBS (w/o Ca^{2+} and Mg^{2+}) cells were terminated by the addition of ice-cold TCA (0.5M, final). After 30 min on ice, cells were scraped into 2 ml microfuge tubes and span at 20,000 x g for 3 min. The pellet was vortexed with 1 ml TCA (5%, w/v) and 10 mM EDTA, recentrifuged, (this was repeated one more time) and frozen on dry ice and kept at -80 °C. An internal standard of $PI(4,5)P_2$ (37:4) was added to the TCA pellets followed by 670 µL of ice cold chloroform:methanol:12.1 M HCl, (10/20/1, v/v/v). Samples were vigorously vortexed for 2-5 min and let sit on ice for 10 min before the addition of 650 µL of ice cold chloroform and vortexing for additional 5 min. At this point 300 µL of 1 M HCl was added for phase separation and samples were vortexed for 2 min, followed by centrifugation at 10,000 x g for 5 min at 4 °C. The lower phase was then collected into a 2 mL fresh tube and 990 µL of "lower phase" (generated from a mixture of chloroform:methanol:1.74 M HCl (86/14/1, v/v/v) was added to the remaining upper phase for re-extraction. After vortexing for 2 min, and centrifugation, the lower phase was combined with the previously collected lower phase. The combined lower phases were evaporated to dryness, flushed with argon and stored at -80 ˚C until further analysis. Samples are kept at 4 °C throughout these extraction steps. Derivatization and MS analysis of the samples were done by ATK Analytics and Discovery as described in (Traynor-Kaplan et al., 2017). Averages calculated from three separate cell culture samples from one experiments are shown.

Supplemental Refrences

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