PMP22 Regulates Cholesterol Trafficking and ABCA1-Mediated Cholesterol Efflux

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The absence of functional peripheral myelin protein 22 (PMP22) is associated with shortened lifespan in rodents and severe peripheral nerve myelin abnormalities in several species including humans. Schwann cells and nerves from PMP22 knock-out (KO) mice show deranged cholesterol distribution and aberrant lipid raft morphology, supporting an unrecognized role for PMP22 in cellular lipid metabolism. To examine the mechanisms underlying these abnormalities, we studied Schwann cells and nerves from male and female PMP22 KO mice. Whole-cell current-clamp recordings in cultured Schwann cells revealed increased membrane capacitance and decreased membrane resistance in the absence of PMP22, which was consistent with a reduction in membrane cholesterol. Nerves from PMP22-deficient mice contained abnormal lipid droplets, with both mRNA and protein levels of apolipoprotein E (apoE) and ATP-binding cassette transporter A1 (ABCA1) being highly upregulated. Despite the upregulation of ABCA1 and apoE, the absence of PMP22 resulted in reduced localization of the transporter to the cell membrane and diminished secretion of apoE. The absence of PMP22 also impaired ABCA1-mediated cholesterol efflux capacity. In nerves from ABCA1 KO mice, the expression of PMP22 was significantly elevated and the subcellular processing of the overproduced protein was aberrant. In wild-type samples, double immunolabeling identified overlapping distribution of PMP22 and ABCA1 at the Schwann cell plasma membrane and the two proteins were coimmunoprecipitated from Schwann cell and nerve lysates. Together, these results reveal a novel role for PMP22 in regulating lipid metabolism and cholesterol trafficking through functional interaction with the cholesterol efflux regulatory protein ABCA1.

Key words: apoE; ATP-binding cassette transporter 1; cholesterol efflux; hereditary neuropathy; peripheral myelin protein 22; Schwann cell

Significance Statement

Understanding the subcellular events that underlie abnormal myelin formation in hereditary neuropathies is critical for advancing therapy development. Peripheral myelin protein 22 (PMP22) is an essential peripheral myelin protein because its genetic abnormalities account for ~80% of hereditary neuropathies. Here, we demonstrate that in the absence of PMP22, the cellular and electrophysiological properties of the Schwann cells' plasma membrane are altered and cholesterol trafficking and lipid homeostasis are perturbed. The molecular mechanisms for these abnormalities involve a functional interplay among PMP22, cholesterol, apolipoprotein E, and the major cholesterol-efflux transporter protein ATP-binding cassette transporter A1 (ABCA1). These findings establish a critical role for PMP22 in the maintenance of cholesterol homeostasis in Schwann cells.

Introduction

Cholesterol, one of the main lipids in myelin, is essential for glial membrane expansion, as severe myelin defects in both the CNS

and PNS occur after genetic ablation of squalene synthase, an enzyme in the cholesterol synthetic pathway (Saher et al., 2005, 2009). Cellular lipid content is controlled by the sterol-regulatory

Received Nov. 14, 2018; revised April 22, 2019; accepted April 26, 2019.

Author contributions: Y.Z., H.T., H.K., D.R.B., S.F., and L.N. designed research; Y.Z., J.R.M., M.L., and H.B. performed research; Y.Z., J.R.M., H.T., M.L., H.K., and S.F. analyzed data; Y.Z. wrote the first draft of the paper; Y.Z., H.T., H.K., D.R.B., H.B., G.E.L., S.L., S.F., and L.N. edited the paper; G.E.L. contributed unpublished reagents/analytic tools; L.N. wrote the paper.

This work was supported by the Facial Pain Research Foundation and the University of Florida College of Medicine (L.N.). Studies at Oregon Health & Sciences University were partially supported by the National Institutes of Health (Grant HL57986 to S.F.). We thank Angela Corona for collection of the ABCA1-deficient mouse nerves, Joseph J.

Lebowitz for advice in fluorescent image quantification, and the NIH Shared Instrumentation Grant program (Grant 1S100D020026) for the scanning confocal images.

We dedicate this article to the memory of Professor Eric M. Shooter, who was instrumental in the establishment of this mouse model of PMP22 deficiency.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.2942-18.2019>

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element (SRE) binding proteins (SREBP) 2 and 1c (Shimano et al., 1996) and by the liver X-receptor (LXR) (Keller et al., 1993; Venkateswaran et al., 2000) pathways. The SRE is a sensing system that monitors membrane cholesterol deficiency and regulates de novo lipogenesis of cholesterol (through SREBP-2), and triglycerides and phospholipids (through SREBP-1c) (Horton et al., 2002). Conversely, the LXR system senses cholesterol excesses and modulates the cholesterol efflux machinery through LXR/LXR or LXR/RXR dimerization and activation, including the expression of ATP-binding cassette transporter 1 (ABCA1) and the acceptor protein apolipoprotein E (apoE) (Joseph and Tontonoz, 2003). In Tangier disease, due to loss-of-function mutations in the gene for ABCA1, and as characterized by intracellular lipid accumulation in Schwann cells, ~50% of the patients develop peripheral neuropathy (Hobbs and Rader, 1999; Zyss et al., 2012).

Peripheral neuropathies comprise a heterogeneous group of disorders with >100 distinct types. Approximately 80% of hereditary neuropathies are linked with abnormal expression of the *peripheral myelin protein 22* (PMP22) gene (Ekins et al., 2015), which causes disease by three distinct mechanisms, including overexpression (gene duplication), gene deletion or truncation (haploinsufficiency), and point mutations (Li et al., 2013; van Paassen et al., 2014). Heterozygous deletion of the PMP22 gene is responsible for hereditary neuropathy with liability to pressure palsies (HNPP), an autosomal-dominant, compression-induced neuropathy (Chance et al., 1994). Homozygous HNPP, a rare condition, presents as an early-onset severe neuropathy with pronounced motor and sensory deficits (Saporta et al., 2011). Various rodent models confirm the requirement for PMP22 in peripheral nerve myelination and provide insights on the role of the protein in glial biology.

In the PNS, Schwann cells synthesize PMP22, the culprit protein in the majority of hereditary neuropathies. PMP22 is a hydrophobic integral membrane glycoprotein with demonstrated roles in cell differentiation and membrane expansion (Suter and Snipes, 1995). Whereas PMP22 is broadly expressed in the body, affected individuals and animals with PMP22 mutations develop demyelinating or dysmyelinating peripheral neuropathy with varying severities. In the adult mature nerve, PMP22 is localized to the compact portion of myelin, where it potentially serves a structural role (Suter and Snipes, 1995). Binding partners of PMP22 include the major protein constituent of PNS myelin, protein zero (P0), and the $\alpha 6 \beta 4$ integrin complex (D'Urso et al., 1999; Amici et al., 2006). Based on the identification of a highly conserved cholesterol recognition CRAC motif in the fourth transmembrane domain of PMP22 (Gould et al., 2005; Sedzik et al., 2013) and a conserved palmitoylation site at C85 (Zoltewicz et al., 2012), a potential interaction with lipids is also likely.

How altered expression of PMP22 causes the distinct forms of hereditary neuropathies and how the pathologies relate to the biological function of the protein in Schwann cells remains a puzzle. Given the vital role of lipids in myelin biology, the recognized lipid abnormalities in PMP22-mutant neuropathic Trembler mice and the potential connection between cholesterol and PMP22 (Heape et al., 1986; Juguelin et al., 1986; Lee et al., 2014), we investigated whether PMP22 directly affects lipid metabolism. For our studies, we used systemic PMP22 knock-out (KO) mice, which develop early-onset severe neuropathy with altered cholesterol distribution and elevated apoE production in affected peripheral nerves (Amici et al., 2006; Lee et al., 2014). Here, we demonstrate that PMP22 is critical for cholesterol trafficking and ABCA1-dependent cholesterol efflux in Schwann cells, implicating

aberrant cholesterol metabolism in the pathogenesis of dysmyelinating and demyelinating neuropathies.

Materials and Methods

Ethics statement. All experiments with animals were performed after approval by the University of Florida Animal Care and Use Committee. Breeding colonies of PMP22 KO mice (Amici et al., 2006) were housed at the University of Florida under specific-pathogen-free conditions. WT and PMP22 KO littermates generated from heterozygous breeders on 129S1/SvImJ background were used for tissue collection and cell culture. Genotyping was performed on genomic DNA isolated from tail biopsies and determined by PCR (Amici et al., 2006). Before each tissue collection, animals were fasted for 16 h and weighed. After euthanasia, blood was cleared by transcardial perfusion with cold PBS. ABCA1 KO (Abca1^{tm1jdm}) mice (Aiello et al., 2003) were purchased from The Jackson Laboratory.

Cell culture studies. Mouse Schwann cells were isolated from sciatic nerves of genotyped postnatal day 5 (P5) to P8 littermates as described previously (Fortun et al., 2006) with modifications. In brief, dissected nerves were maintained in DMEM supplemented with Ham's F12 (Thermo Fisher Scientific), 10% FBS, and gentamicin (20 μ g/ml; Invitrogen) for 2–4 d. Nerves were then digested with 0.03% collagenase, 1.25 units/ml dispase, and 0.01% hyaluronidase type 1 (Worthington). After trituration, the cell suspension was preplated for 30 min to allow the adhesion of fibroblasts and unattached cells were moved onto poly-L-lysine-coated dishes. After treatment with 5 μ M cytosine arabinoside (Sigma-Aldrich) for 2–4 d, Schwann cells were maintained in DMEM-F12 with 10% FBS, 20 μ g/ml bovine pituitary extract (Biomedical Technologies), and 5 μ M forskolin (Calbiochem). Mouse nerve fibroblasts (MNFs) represented the cells that adhered to the tissue culture plate during the first 30 min after plating. MNFs were expanded in DMEM medium supplemented with gentamicin and 10% FCS (Hyclone). Rat Schwann cells were cultured from the sciatic nerves of neonatal pups, as described previously (Notterpek et al., 1999).

shRNA and WT-PMP22 plasmid transfection. Before transfection, rat Schwann cells were plated on poly-L-lysine-coated plates and maintained until reaching 70% confluence. Transient transfections with WT-PMP22-Myc plasmids (Johnson et al., 2005) and scrambled or pooled rat PMP22 shRNA plasmids containing GFP reporters (Applied Biological Materials) were performed using lipofectamine 3000 (Invitrogen) in Opti-MEM medium (Thermo Fisher Scientific) (Rao et al., 2015). The sequences (from 5'-3') of PMP22 shRNA are as follows: 40 GCGGT GCTAGTGTGCTCTTCGTCTCCAC; 161 ACTCCTCATCTGTGAG CGAATGGCTTCAG; 313 CTTGCTGGTCTGTGTGTGATGAGTGC AGC; and 438 CCTCCTTAGTGGCATCATCTACGTGATCC.

Twenty-four hours later, cells with WT-PMP22 transfection were lysed for coimmunoprecipitation (co-IP). Cells transfected with scrambled or PMP22 shRNA were directly processed for cholesterol efflux assay or switched to DMEM with 10% lipoprotein-deficient serum (LPDS) medium for another 48 h before immunostaining.

Whole-cell current-clamp recording. The membrane properties of WT and PMP22 KO Schwann cells were determined via whole-cell current-clamp recording. The WT or PMP22 KO Schwann cells were continuously perfused with artificial CSF (aCSF) containing the following additives (in mM): 126 NaCl, 2.5 KCl, 2 CaCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 MgSO₄, and 10 dextrose equilibrated with 95% O₂/5% CO₂, pH adjusted to 7.4. Patch electrodes were fabricated from borosilicate glass (1.5 mm outer diameter; World Precision Instruments) with the P-2000 puller (Sutter Instruments). The tip resistance was in the range of 3–5 M Ω . The electrodes were filled with a pipette solution containing the following (in mM): 120 potassium-gluconate, 20 KCl, 2 MgCl₂, 10 HEPES, 0.1 EGTA, 2 ATP, and 0.25 GTP, pH adjusted to 7.25 with KOH. The electrophysiology data were acquired using the ClampEx 10 software package (Molecular Devices). The data were analyzed offline using pClamp 10.

RT-PCR. Freshly frozen sciatic nerves and rat Schwann cells were processed for RNA extraction using an RNeasy Lipid Tissue Mini Kit or an RNeasy mini kit (Qiagen). Nerves from three to four animals were combined for lyses as one-sample. Total RNA (1 μ g) was synthesized to cDNA in 20 μ l of reaction mixture using an iScript cDNA synthesis kit

Table 1. Primary antibodies used in the present study

Species	Antigen	Source and catalog # (RRID)	Dilution	
			WB	IF
Rabbit	ABCA1	Novus Biologicals, NB400-105 (RRID: AB_10000630)	1:2000	N/A
Rabbit	ABCA1	Thermo Fisher Scientific, PA1-16789 (RRID: AB_2288917)	1:2000	1:150
Mouse	ABCA1	Novus Biologicals, NB100-2068 (RRID: AB_10002789)	N/A	1:100
Rabbit	ACC	Abcam, ab45174 (RRID: AB_867475)	1:2000	N/A
Goat	ApoE	Millipore, AB947 (RRID: AB_2258475)	1:3000	N/A
Rabbit	ApoE	Abcam, ab20874 (RRID: AB_449883)	N/A	1:300
Rabbit	CD11b	Abcam, ab133357 (RRID: AB_2650514)	1:2000	N/A
Rat	CD11b	Thermo Fisher Scientific, MA5-16528 (RRID: AB_2538033)	N/A	1:300
Mouse	c-Myc	Santa Cruz Biotechnology, sc-40 (RRID: AB_627268)	1:300	N/A
Mouse	Dystrophin	Sigma-Aldrich, D8043 (RRID: AB_259241)	1:2000	N/A
Rabbit	GAPDH	Santa Cruz Biotechnology, FL-335 (RRID: AB_10167668)	1:5000	N/A
Mouse	G515	BD Biosciences, 610960 (RRID: AB_398273)	N/A	1:200
Rabbit	Laminin	Sigma-Aldrich, L9393 (RRID: AB_477163)	1:20,000	N/A
Rabbit	LAMP1	Abcam, ab24170 (RRID: AB_775978)	1:2000	N/A
Rabbit	LC3	Cell Signaling Technology, 27755 (RRID: AB_915950)	1:2000	N/A
Chicken	LDLR	Abcam, ab14056 (RRID: AB_2135124)	1:5000	N/A
Mouse	MAG	Millipore, MAB1567 (RRID: AB_2137847)	1:2000	N/A
Mouse	P0	Archelos et al., 1993	1:300	N/A
Rabbit	P62	Abcam, ab91526 (RRID: AB_2050336)	1:1000	N/A
Rabbit	p75	Abcam, ab8874 (RRID: AB_306827)	1:20,000	1:800
Mouse	PHH3	Millipore, 05-806 (RRID: AB_310016)	1:2000	N/A
Rabbit	PMP22	Pareek et al., 1997	1:3000	N/A
Mouse	PMP22	Santa Cruz Biotechnology, sc-515280	1:500	N/A
Mouse (IgM)	PMP22	NeoMarkers, 20–14	N/A	1:50
Mouse	Rab8	BD Biosciences, 610844 (RRID: AB_398163)	1:1000	N/A
Rat	Thy-1	Abcam, ab3105 (RRID: AB_2287350)	N/A	1:500
Rabbit	Vinculin	Abcam, ab129002 (RRID: AB_11144129)	1:10,000	N/A
Rabbit	β -actin	Abcam, ab75186 (RRID: AB_1280759)	1:10,000	N/A
Rabbit	β -tubulin	Proteintech, 10094 (RRID: AB_2210695)	1:1000	N/A

The isotype of all antibodies is IgG except when noted otherwise under "Species."

WB, Western blot; IF, immunofluorescence staining; N/A, not applicable.

(Bio-Rad). Real-time PCR was performed using TaqMan reagents and probes in microfluidic array cards (Applied Biosystems). Briefly, 100 ng of cDNA in a 100 μ l reaction mixture was loaded into the reservoir of the microfluidic array cards and analyzed using QuantStudio software (Thermo Fisher Scientific). RPL32 mRNA was used as an internal control and the relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Western blotting. Total nerve protein lysates were prepared by homogenization in modified radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, 150 mM NaCl, pH: 8.0, 1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with complete protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. Cultured mouse Schwann cells and mouse nerve fibroblasts were lysed in RIPA buffer with protease and phosphatase inhibitors. Protein concentrations were determined using a BCA (Pierce) assay kit and samples were resolved on polyacrylamide gels and transferred to nitrocellulose or PVDF membranes. After immunoblotting with primary antibodies and HRP-conjugated secondary antibodies (Cell Signaling Technology), blots were reacted with ECL reagent (Bio-Rad) and visualized via X-ray film or the ChemiDoc MP System (Bio-Rad). Primary antibodies used are shown in Table 1.

For PMP22 detection in lysates of cultured MNFs, we prepared detergent-insoluble protein fractions (Fortun et al., 2006). For endoglycosidase (endo H) or N-glycosidase digestions, 2 μ g of sciatic nerve lysates and 20 μ g of transfected cell lysates were treated with endo H or N-glycosidase (New England Biolabs) for 16 h (Pareek et al., 1997). For native gel electrophoresis, sciatic nerves from 6-week-old WT and PMP22 KO mice were minced under liquid nitrogen and lysed in Tris-buffered saline, pH 7.6, with protease inhibitors (Corona et al., 2016). After centrifugation at 7000 rpm for 10 min, supernatants were separated on 4–15% mini-protein TGX precast gels (Bio-Rad) under native conditions at 90 V for 3 h. A high-molecular-weight calibration kit (GE Healthcare) was used to estimate protein migration. For semiquantitative analyses, densitometry was performed using ImageJ software and

relative protein levels were determined after correction for a constitutive marker such as GAPDH, actin, or tubulin.

Co-IP assays. Rat Schwann cells and sciatic nerves were lysed in IP buffer (0.15 M NaCl, 0.05 M Tris, 5 mM EDTA, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche) and 500 μ M phenylmethylsulfonyl fluoride (Sigma-Aldrich) (Amici et al., 2006). Lysate viscosity was reduced with benzonase nuclease (Novagen). After centrifugation, protein pellets were resolubilized in IP buffer containing 1% (rat Schwann cells) or 2% (sciatic nerves) SDS and added back to the supernatant. The concentration of SDS in the final protein lysates was 0.25%. The samples were then precleared with nonspecific rabbit serum and protein A-Sepharose (preblocked with 5% BSA at room temperature; Sigma-Aldrich) for 30 min each, followed by incubation with rabbit anti-ABCA1 IgG antibody (Invitrogen) overnight at 4°C. The antigen–antibody complexes were captured by the addition of protein A-Sepharose. Immunoprecipitates were eluted with 3% SDS and 2% β -mercaptoethanol-containing gel sample buffer and boiling for 10 min at 95°C. Western blotting for PMP22, c-Myc, ABCA1, and dystrophin detections were performed as above.

Morphological studies. Sciatic nerves were collected from genotyped mice after 16 h of fasting. For lipid staining, longitudinal sciatic nerve sections were incubated with 0.5% Oil Red O solution (O0625; Sigma-Aldrich) at 60°C for 10 min followed by a brief immersion in Meyer's hematoxylin (MHS16; Sigma-Aldrich). Images were digitally captured from an Olympus BX43 upright microscope outfitted with an Olympus DP80 camera and exported to Adobe Photoshop CS3 for figure preparation.

Ultrastructural studies. Ultrastructural examination was conducted on nerves from WT and PMP22 KO mice after fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h at 4°C. Nerves were transferred to 0.1 M phosphate buffer and processed for electron microscopy, as described previously (Amici et al., 2006).

Fluorescent dye staining and immunolabeling. For filipin–cholesterol labeling, mouse Schwann cells were fixed with 4% paraformaldehyde for 10 min. After rinsing in PBS, samples were incubated with 50 $\mu\text{g}/\text{ml}$ filipin III (F4767; Sigma-Aldrich) in PBS for 1 h in the dark. For filipin costaining with GS15, after 4% PFA fixation, cells were incubated with filipin (50 $\mu\text{g}/\text{ml}$) for 1 h before adding GS15 antibody. For CD11b and apoE costaining, sciatic nerve sections (8 μm thickness) were fixed with 4% PFA for 1 h and permeabilized with ice-cold acetone for 2 min. Thy-1 and p75 costaining on mouse nerve fibroblasts was completed after fixation with 4% PFA for 10 min and permeabilization with ice-cold methanol for 5 min. For PMP22 or intracellular ABCA1 and PMP22 co-detection cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature; for cell surface staining, the permeabilization step was omitted. For ABCA1 and PMP22 costaining, sciatic nerve sections were permeabilized with ice-cold methanol for 5 min. For ABCA1 staining on teased fibers and sectioned nerves, samples were fixed for 15–30 min with 4% PFA and permeabilized with 0.1% Triton X-100 for 15 min. After blocking in 15% normal goat serum for 1 h and incubating with primary antibodies overnight at 4°C, bound primary antibodies were detected with appropriate fluorescent-dye conjugated secondary antibodies (Invitrogen). Hoechst dye (1:1000; Invitrogen) was applied to label nuclei, when indicated. Primary antibodies used are listed in Table 1. Coverslips were mounted on glass slides using Prolong Gold antifade (Invitrogen) and images were acquired on a Nikon A1RMPsi laser-scanning confocal microscope. For CD11b-positive cell counting, nerve sections were observed under a Nikon Eclipse E800 microscope and five random areas were counted in a full-view field at 40 $\times$ magnification.

Images were analyzed using Nikon NIS Elements Software version 4.40 or 4.51. All colocalization studies were performed on background-reduced images after 2D deconvolution and Pearson correlation coefficient was determined (Adler and Parmryd, 2010; Dunn et al., 2011); Pearson coefficient >0.7 is considered a high-positive correlation (Mukaka, 2012). For colocalization quantification, individual dots in the red and green channels were selected by a binary/mask setup and the numbers of dots were automatically calculated by the Nikon software. Dots in yellow (merged channel) were manually counted on individual images. Colocalization percentage was determined as the number of yellow dots/number of red or green dots. For subcellular filipin intensity quantification, regions of interest (ROIs) of the whole cell were autodetected; ROIs of GS15-positive regions were selected after automatic binary detection by the Nikon software; ROIs of the plasma membrane were manually selected. For cell surface ABCA1 fluorescence intensity quantification, ROIs at the cell surface were automatically selected by the Nikon software.

LDL cholesterol uptake. Mouse Schwann cells were equilibrated for 18 h in DMEM-F12 medium supplemented with 10% lipoprotein-deficient FBS (Sigma-Aldrich) (Loregger et al., 2017). Cells were then treated with 10 $\mu\text{g}/\text{ml}$ BODIPY-LDL (L3483; Invitrogen) for 3, 8, or 24 h. After rinsing, cells were fixed, mounted, and imaged under a Nikon eclipse E800 microscopy system. Fluorescent intensity was determined via Nikon's NIS Elements software after autodetecting ROIs.

ApoE secretion and cholesterol efflux assays with pharmacological treatment paradigms. For apoE secretion assays, the conditioned medium was collected and concentrated using Ultra-4 centrifugal filter units (Amicon). The levels of secreted apoE and laminin were determined by Western blotting. At the time of media collection, cells from respective culture plates were lysed and processed for Western blotting with anti-ABCA1, LDLR, and apoE antibodies, as above.

Cholesterol efflux assays were conducted with ^3H -cholesterol, as described previously (Dove et al., 2005). Briefly, mouse Schwann cells were plated at a density of 5×10^4 cells per well onto poly-L-lysine-coated 96-well plates and allowed to grow for 2 d. Rat Schwann cells were plated at a density of 5×10^4 cells per well onto poly-L-lysine-coated 24-well culture plates and allowed to adhere overnight. Scrambled or PMP22 shRNA transfection was performed as described above. Cells were treated with DMEM or DMEM/F12 containing 1% FBS, 1% penicillin/streptomycin (pen/strep), 1% Sandoz (58–03; Sigma-Aldrich), 100 $\mu\text{g}/\text{ml}$ acLDL prepared in-house, and 6 $\mu\text{Ci}/\text{ml}$ ^3H -Cholesterol (NET139001MC; PerkinElmer) for 48 h. Cells were then washed twice

each with DMEM, 1% BSA, 25 mM HEPES, and then with DMEM/HEPES without BSA. After the washes, cells were maintained in the efflux medium overnight. For mouse Schwann cells, the efflux medium contained DMEM/F12, 1% Sandoz, 1% pen/strep, and 1% LPDS; the rat Schwann cell efflux medium contained DMEM, 1% Sandoz, 1% pen/strep, and no LPDS. Percentage efflux was calculated as the ratio of counts per minute in the efflux medium over the total combined counts per minute of the medium plus cells.

BODIPY–cholesterol efflux assays were performed as described previously (Sankaranarayanan et al., 2011) with minor modifications. Schwann cells, seeded in 48-well plates (1×10^5 cells per well) coated with poly-L-lysine and 5 $\mu\text{g}/\text{ml}$ laminin, were incubated with sonicated labeling medium containing 0.017 mM BODIPY-cholesterol (Avanti Polar Lipids), 0.067 mM cholesterol (Sigma-Aldrich), and 3.33 mM methyl- β -cyclodextrin (Sigma-Aldrich) in DMEM-F12 for 3 h at 37°C. After 2 washes with DMEM-F12 (HEPES), cells were equilibrated in DMEM-F12, 0.2% BSA for 1 h at 37°C. After two rinses in FluoroBrite DMEM (Thermo Fisher Scientific), samples were maintained in FluoroBrite DMEM supplemented with 15 mM HEPES, 2 mM L-glutamine, 0.2% BSA with or without pharmacological agents, and 20 $\mu\text{g}/\text{ml}$ lipid-free human apoA-I (Calbiochem) for 18 h. To increase ABCA1 activity, 0.1 mM 8-bromo-cAMP (8-br-cAMP; Sigma-Aldrich) was applied (Haidar et al., 2002); ABCA1 activity was inhibited with 2 μM cyclosporine A (CsA) (Sigma-Aldrich) (Le Goff et al., 2004). After the indicated incubation periods, conditioned media were collected and filtered via 96-well 0.45 μm filter plates (VWR Scientific) by centrifuging at 2000 rcf for 10 min. Intracellular BODIPY–cholesterol was extracted with 1% cholic acid by shaking for 3 h. Fluorescence intensities in the collected medium and in the corresponding cell lysates were determined by a BioTek Gen5 reader (excitation 485 nm, emission 516 nm). Total cholesterol efflux was calculated by dividing the fluorescence intensity of the medium by the sum of fluorescence intensities of the medium plus the cell lysates. Background efflux in the absence of apoA-I acceptor was subtracted from total cholesterol efflux value (Shrestha et al., 2016).

Experimental design and statistical analyses. For all experiments, $n = 3$ –5 independent cultures or tissues from $n = 3$ –6 genotyped male and female mice were used. For quantitative assays, all raw data were exported to GraphPad Prism software for statistical analyses. Statistical significance was determined based on p -values obtained from an unpaired two-tailed Student's t test unless specified otherwise. $p < 0.05$ was considered to be statistically significant. Graphs were plotted representing the mean $\pm$ SEM.

Results

Absence of PMP22 alters subcellular cholesterol distribution and the membrane properties of Schwann cells

Previous studies identified perturbed lipid rafts and intracellular cholesterol retention in Schwann cells from PMP22 KO mice (Lee et al., 2014). To define the subcellular localization of cholesterol in the absence of PMP22, we performed high-resolution imaging of filipin–cholesterol with the Golgi marker GS15 (Fig. 1A). In Schwann cells from PMP22 KO mice, we detected prominent filipin–cholesterol signal in the perinuclear area, with reduced fluorescence intensity at the plasma membrane, compared with WT. Quantification of data from three independent experiments revealed a $\sim 44\%$ increase ($t = 3.1$, $p = 0.0027$, $R^2 = 0.15$, unpaired Student's t test) in Golgi-associated filipin–cholesterol and a concomitant $\sim 32\%$ reduction ($t = 6.9$, $p < 0.001$, $R^2 = 0.46$, unpaired Student's t test) at the plasma membrane in PMP22-deficient cells (Fig. 1A,B). We confirmed the influence of PMP22 on cholesterol trafficking by shRNA knock-down in normal rat Schwann cells. Similar to the PMP22 KO cells, in cells expressing PMP22 shRNA-GFP, PMP22 immunoreactivity was reduced and cholesterol was prominent in the perinuclear region (Fig. 1C, top, arrows). Cells transfected with the scrambled shRNA-GFP and nontransfected cells displayed

Figure 1. Intracellular cholesterol retention and perturbed membrane electrical properties in PMP22-deficient Schwann cells. **A, B**, Representative images of WT and PMP22 KO mouse Schwann cells (**A**) and quantification of filipin intensity (**B**) in the Golgi and at the plasma membrane (PM) after costaining with anti-GS15 antibody ($n = 80$ cells each genotype). “Rest” indicates the rest of a cell minus the Golgi and PM. **C**, Representative images of PMP22 (red) and cholesterol (blue) localization after scrambled or PMP22 shRNA-GFP plasmid (green) transfection in rat Schwann cells. Arrows point to transfected cells. **D**, Images depicting the location of patch pipettes relative to the cells subjected to current-clamp recordings. **E–G**, Current-clamp recordings of WT (gray bars) and PMP22 KO (black bars) mouse Schwann cells showing the membrane capacitance (**E**), membrane resistance (**F**), and time constant (**G**) for each experimental group ($n = 10$ cells per group from three independent replicates). For all experiments, values represent the mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$, two-tailed Student’s t test.

prominent, plasma-membrane-associated filipin–cholesterol (Fig. 1C, bottom).

Lipids, and cholesterol in particular, are known to affect the electrical properties of biological membranes (Stephens and Shinitzky, 1977; Tillman and Cascio, 2003; Starke-Peterkovic et al., 2006), which is of fundamental importance for myelin regulation of electrical properties of the neuronal membrane, and thus conduction. Therefore, we examined the functional consequences of the observed membrane cholesterol deficiency in PMP22 KO Schwann cells by whole-cell current-clamp recordings (Fig. 1D–G). As shown in Figure 1 E–G, we found significantly increased membrane capacitance (WT 16.7 ± 1.6 pF vs PMP22 KO 28.9 ± 3.9 pF, $t = 4.1$, $p = 0.0006$, $R^2 = 0.49$, two-tailed Student's t tests) and decreased membrane resistance (WT 1710.2 ± 304.2 m Ω vs PMP22 KO 206.9 ± 44.4 m Ω , $t = 5.8$, $p < 0.0001$, $R^2 = 0.65$, two-tailed Student's t tests) in cells from PMP22 KO mice. The increased membrane capacitance was unlikely the consequence of a larger cell volume because Schwann cells from PMP22 KO mice display shortened axial length and collapsed lamellipodia (Lee et al., 2014). There was no change in the time constant (WT 481.9 ± 161.4 μ s vs PMP22 KO 607.0 ± 142.3 μ s, $t = 0.7$, $p = 0.5$, $R^2 = 0.026$, two-tailed Student's t tests), probably due to the concomitant increased capacitance and decreased resistance. It is plausible that the reduction in membrane-associated cholesterol in the absence of PMP22 alters the biophysical properties of the Schwann cell membrane and has functional consequences that could be a key factor in impaired nerve conduction in PMP22-deficient animals and patients (Hong et al., 2003). These data serve as the first evidence of an important role for PMP22 in maintaining the electrical properties of the Schwann cell membrane.

Lipid accumulation and altered expression of lipid-metabolism-related genes in nerves from PMP22 KO mice

To determine the extent of cholesterol abnormalities in samples from PMP22 KO mice, we determined the expression of SREBP- and LXR-regulated genes (Fig. 2A). In nerves from 6-week-old mice, RT-PCR analyses revealed a 48.6% decrease in fatty acid synthase (*FAS*) ($t = 7.4$, $p = 0.018$, two-tailed one-sample t test), a 72.2% decrease in low-density lipoprotein receptor (*LDLR*) ($t = 10.8$, $p = 0.0084$, two-tailed one-sample t test), and a 64.0% decrease in HMG-Co-A reductase (*HMG-CoA R*) ($t = 6.8$, $p = 0.02$, two-tailed one-sample t test) expression in the absence of PMP22 compared with WT. Within the same samples, we detected a 3.2-fold increase in *APOE* ($t = 5.0$, $p = 0.037$, two-tailed one-sample t test) and a 9.2-fold increase in *ABCA1* ($t = 5.3$, $p = 0.033$, two-tailed one-sample t test) (Fig. 2A). Consistent with these results, we identified highly upregulated apoE (7.8-fold, $t = 3.3$, $p = 0.016$, $R^2 = 0.65$, unpaired Student's t test) and ABCA1 (9.8-fold, $t = 7.3$, $p = 0.0003$, $R^2 = 0.9$, unpaired Student's t test) protein levels in affected nerves (Fig. 2B,C), whereas LDLR remained stable ($t = 0.61$, $p = 0.56$, $R^2 = 0.036$, unpaired Student's t test) (Fig. 2D,E).

To investigate lipid distribution in the nerves, we stained tissue sections with Oil Red O (Fig. 2F), a fat-soluble diazot dye that labels neutral lipids and cholesteryl esters in red (Mehlem et al., 2013). As shown in the representative images, compared with WT, PMP22 KO nerves accumulated neutral lipids in the endoneurial and perineurial regions (Fig. 2F, arrows). By ultrastructural examination, we identified lipid droplets (arrow) and vacuoles (V) within affected Schwann cells (Fig. 2G). Evaluation of 68 different Schwann cell profiles on images from nerve sections from three different PMP22 KO mice, 54 cells (79%) con-

tained lipid droplets. These lipid droplets were unlikely derived from myelin fragmentation and/or dysfunctional autophagy because we observed only moderate activation of autophagy-related protein in PMP22 KO nerves, including LAMP1 (2.5-fold, $t = 9.3$, $p < 0.0001$, $R^2 = 0.93$), p62 (1.6-fold, $t = 4.8$, $p = 0.0031$, $R^2 = 0.79$), and LC3 II/I (2.2-fold, $t = 6.9$, $p = 0.0023$, $R^2 = 0.92$) (Fig. 2H,I).

Because nerve fibroblasts and macrophages are potential sources for the elevated apolipoproteins and lipids (Stoll and Müller, 1986; Saada et al., 1995), we isolated Thy1-positive MNFs and analyzed the abundance of CD11b-reactive macrophages (Fig. 3). Although both ABCA1 and apoE appeared elevated in whole-protein lysates of cultured PMP22 KO nerve fibroblasts, this increase was not significant (ABCA1: WT $100 \pm 2.2\%$ vs PMP22 KO: $145.1 \pm 24.6\%$, $t = 1.8$, $p = 0.12$, $R^2 = 0.36$; apoE: WT $100 \pm 2.4\%$ vs PMP22 KO: $127.2 \pm 15.6\%$, $t = 1.7$, $p = 0.13$, $R^2 = 0.33$; unpaired Student's t test) (Fig. 3A,B). The expression of PMP22 in cultured WT MNFs was low but detectable in detergent-insoluble membrane fractions (Fig. 3C). We monitored the number of Schwann cells in the fibroblast cultures by immunostaining for p75, which identified very few glial cells (Fig. 3D). Next, we assessed the potential contribution of CD11b-positive macrophages to the elevated apoE in PMP22 KO nerves (Fig. 3E–I). We identified an ~ 2.0 -fold increase in the occurrence of CD11b-positive macrophages (WT 5.8 ± 1.3 vs PMP22 KO 11.7 ± 1.7 ; $t = 2.8$, $p = 0.03$, $R^2 = 0.57$, unpaired Student's t test), along with an ~ 2.1 -fold increase of CD11b protein level in affected samples ($t = 2.8$, $p = 0.02$, $R^2 = 0.47$, unpaired Student's t test) (Fig. 3E–G). Double immunolabeling with anti-apoE and CD11b antibodies revealed that macrophages were reactive for apoE (Fig. 3H) and contributed to the elevated nerve apoE. However, macrophages were not prominent in the perineurial region (Fig. 3I), where we observed pronounced accumulation of lipid droplets (Fig. 2F). These results indicate that, compared with microphages and fibroblasts, Schwann cells are the predominantly affected cell type in PMP22 KO nerves that contribute to the observed dyslipidemia.

ABCA1 deficiency causes elevated expression and compromised processing of PMP22

Given the pronounced increase of ABCA1 in nerves from PMP22-deficient animals (Fig. 2), we examined samples from ABCA1 KO mice, which model Tangier disease (Aiello et al., 2003). By Western blot analyses, we studied the levels of myelin-associated glycoprotein (MAG), P0, and p75 in total sciatic nerve lysates from heterozygous and homozygous ABCA1-deficient mice (Fig. 4A,B). As shown on the blots and semiquantitative analyses, the levels of these key myelin proteins remained steady across the three genotypes (MAG: $F = 0.76$, $p = 0.50$, $R^2 = 0.16$; P0: $F = 0.06$, $p = 0.94$, $R^2 = 0.016$; p75: $F = 0.55$, $p = 0.60$, $R^2 = 0.14$; one-way ANOVA followed by the Tukey's post test). Schwann cell dedifferentiation and hyperproliferation have been observed in neuropathies (Niemann et al., 1999); therefore, we also determined the levels of phospho-histone H3 (PHH3), a mitotic marker. In nerves from heterozygous and homozygous ABCA1-deficient mice, PHH3 levels remained below detection, whereas hypertrophic phenotype was evident in PMP22 KO samples (Fig. 4C). In contrast to MAG, P0, and p75, nerves from 10-month-old ABCA1 KO mice contained ~ 1.53 -fold higher levels of PMP22 relative to WT, with an intermediate 1.37-fold increase in the heterozygous (HET) samples ($F = 7.2$, $p = 0.013$, $R^2 = 0.62$; one-way ANOVA followed by the Tukey's post test) (Fig. 4D,E). To determine the subcellular processing of the ele-

Figure 2. Lipid accumulation and altered expression of lipid-metabolism-related genes in peripheral nerves from PMP22 KO mice. **A**, In the absence of PMP22, genes involved with cholesterol efflux (regulated by LXR/RXR) are increased, whereas genes linked to lipid synthesis (SREBP-1c and SREBP-2 pathways) are reduced ($n = 3$ independent experiments). RPL32 served as the internal control. **B–E**, Immunoblot detection (**B**, **C**) and quantification (**D**, **E**) of ABCA1 and apoE and LDLR in nerves from WT and PMP22 KO mice. **F**, Oil Red O staining detects accumulated neutral lipids in the perineurium and endoneurium (arrows) on longitudinal sections of PMP22 KO nerves. Nuclei (purple) are stained with hematoxylin. **G**, Representative images from P18 sciatic nerves showing vacuoles (V) and lipid droplets (arrow) in PMP22 KO Schwann cells. Boxed areas are magnified on the right of the panels. **H**, **I**, Immunoblot detection (**H**) and quantification (**I**) of autophagy markers LAMP1, p62, and LC3 in nerves from WT and PMP22 KO mice. Actin and GAPDH are shown as protein-loading controls. Values represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n.s., non-significant, one-sample t test or two-tailed Student's t test. **B–I**, $n = 3–6$ mice each genotype.

Figure 3. Nerve macrophages and fibroblasts make a limited contribution to lipid abnormalities in PMP22 KO nerves. **A, B**, Immunoblot detection (**A**) and quantification (**B**) of ABCA1 and apoE in cultured nerve fibroblasts from WT and PMP22 KO mice. **C**, Representative immunoblot showing the expression of PMP22 in WT MNFs. **D**, Characterization of nerve fibroblast cultures by immunostaining for Thy-1 and the Schwann cell marker P75. **E**, Number of CD11b-positive cells in WT and PMP22 KO nerve sections after immunostaining and counting. **F, G**, Immunoblot detection (**F**) and quantification (**G**) of CD11b in whole-nerve lysates from WT and PMP22 KO mice. Actin or GAPDH is shown as the protein-loading control. **H**, Co-staining for CD11b and apoE showing macrophages positive for apoE. **I**, CD11b-positive macrophages are detected in the endoneurium of PMP22 KO nerves. Nuclei are stained with Hoechst dye (blue). Data were collected from three to four independent culture preparations (**A–D**) or from four animals per genotype (**E–I**). Values represent the mean $\pm$ SEM. * $p < 0.05$, two-tailed Student's *t* test.

lated PMP22 in the absence of ABCA1, we treated sciatic nerve lysates with endo H or N-glycosidase F (Pareek et al., 1993, 1997). Quantification of data from independent experiments identified a decrease in the endo H-resistant PMP22, with a corresponding increase in the endo H-sensitive fraction (endo H resistant: WT $81.9 \pm 4.2\%$ vs ABCA1 HET $62.7 \pm 7.8\%$ vs ABCA1 KO $52.2 \pm 1.8\%$; $F = 8.3$, $p = 0.019$, $R^2 = 0.73$; one-way ANOVA followed by the Tukey's post test) (Fig. 4F, G). The elevated levels of endo H-sensitive PMP22 in the absence of ABCA1 indicates slowed intracellular processing of the newly synthesized protein through the secretory pathway (Pareek et al., 1997). The overexpression and aberrant processing of PMP22 in the absence of ABCA1 (Fig. 4D–G), together with the increase in ABCA1 in nerves from PMP22 KO mice (Fig. 2), suggest a functional relationship between these two integral membrane proteins. Because nerves from patients with Tangier disease are known to contain tomacula and accumulate lipids (Hobbs and Rader, 1999; Cai et al., 2006), we stained samples from ABCA1 KO mice with Oil Red O (Fig. 4H). As expected, nerve fibers of ABCA1 KO mice contained elevated levels of neutral lipids compared with WT (Fig. 4H). Therefore, the absence of PMP22 or ABCA1 in Schwann cells results in similar pathologies, including accumulation of neutral lipids and formation of tomacula.

ABCA1 and PMP22 are codistributed within nerves and Schwann cells

To further examine the connection between PMP22 and ABCA1, we analyzed the localization of the two proteins on single-plane

confocal images of detergent-permeabilized, double-immunolabeled Schwann cells isolated from WT mice. As shown on the representative images (Fig. 5A, B), there was a pronounced association between ABCA1-like (red) and PMP22-like (green) signals, indicating intracellular colocalization. Calculation of a Pearson correlation coefficient of 0.75 ± 0.01 further supported significant colocalization between the two proteins (Dunn et al., 2011). To assess the degree of codistribution of PMP22 and ABCA1 at the plasma membrane, we performed cell surface immunolabeling. Single-plane confocal images and subsequent quantification of co-detection (yellow spots) determined a partial colocalization of the two proteins on the cell surface of a given WT mouse Schwann cell (Fig. 5C), with $33.5 \pm 5.3\%$ of surface ABCA1 and $40.5 \pm 4.7\%$ of surface PMP22 colocalized. We next costained for ABCA1 and PMP22 in longitudinal sciatic nerve sections and observed colocalization within Schwann cells (arrow) and at the outer cell membrane (arrowheads). Based on the results from the double-immunolabeling experiments, we investigated a potential association between PMP22 and ABCA1 by co-IP in normal samples (Fig. 5E–G). Both ABCA1 and PMP22, or ABCA1 and PMP22-Myc, were identified within the total (T) protein homogenates and, upon IP with an anti-ABCA1 antibody, PMP22, or c-Myc, were detected in the pulldown fraction (Fig. 5E–G). As a control of specificity, PMP22 was absent in the anti-ABCA1 antibody pulldown from PMP22 KO nerves, whereas dystrophin, a known ABCA1-binding partner (Albrecht et al., 2008), was detected (Fig. 5H). Collectively, these data indicate that in normal nerves, a substantial fraction of PMP22 and

Figure 4. Elevated expression and altered processing of PMP22 in sciatic nerves from ABCA1-deficient mice. **A, B**, Immunoblot detection (**A**) and quantification (**B**) of myelin proteins MAG, P0, and P75 in nerve samples from control (WT), heterozygous (HET), and homozygous (KO) ABCA1-deficient mice. **C**, Immunoblot detection of PHH3 from WT, ABCA1 HET, and ABCA1 KO nerves. Nerve lysate from 6-week-old PMP22 KO mice is shown as a positive control. **D, E**, Immunoblot detection (**D**) and quantification (**E**) of PMP22 in nerves from WT, ABCA1 HET, and ABCA1 KO mice. Actin is shown as a protein-loading control. **F, G**, Immunoblot detection (**F**) and quantification (**G**) of PMP22 after incubation in buffer without enzyme (C), with endo H (H), or with N-glycosidase (N). A decrease in the endo H resistant (R) PMP22 and a corresponding increase in endo H sensitive (S, arrow) PMP22 is detected in the absence of ABCA1 (**G**). **H**, Oil Red O staining detects accumulated neutral lipids in the perineurium and endoneurium (arrows) on longitudinal sections of ABCA1 KO nerves. Results are from four to five mice per genotype. Values represent the mean $\pm$ SEM. * $p < 0.05$, one-way ANOVA followed by the Tukey's post test.

ABCA1 proteins coexpress within Schwann cells and are codistributed at the plasma membrane.

ABCA1-dependent cholesterol efflux is compromised in the absence of PMP22

Given the observed relationship between PMP22 and ABCA1 (Figs. 2, 4, 5) and the aberrant distribution of cholesterol and lipids in the absence of PMP22 (Figs. 1, 2), we tested cholesterol secretion by cells from WT and PMP22 KO mice (Fig. 6). Because apoE contributes to cellular cholesterol efflux and is co-secreted with cholesterol *in vitro* (Shanmugaratnam et al., 1997; Mahley et al., 2006), we analyzed apoE levels in Schwann-cell-conditioned media (Fig. 6A,B). Glial cells from PMP22 KO mice secreted $\sim 29.1\%$ less apoE compared with WT ($t = 2.5$, $p = 0.047$, $R^2 = 0.51$, unpaired Student's t test), whereas the abundance of laminin in the media was similar among the samples ($t = 0.42$, $p = 0.69$, $R^2 = 0.03$, unpaired Student's t test). Immunoblot analyses on total protein lysates of the same cells detected elevated apoE (~ 2.2 -fold, $t = 3.4$, $p = 0.029$, $R^2 = 0.74$, unpaired Student's t test) and ABCA1 (~ 1.5 -fold, $t = 6.6$, $p = 0.0027$, $R^2 = 0.92$, unpaired Student's t test) in the absence of PMP22 (Fig.

6C,D). Conversely, LDLR expression was similar between the normal and mutant cells ($t = 0.0007$, $p = 1.0$, $R^2 = 7.1 \times 10^{-8}$, unpaired Student's t test). Consistent with the unaltered expression of LDLR, cholesterol uptake of BODIPY-LDL through LDLR-mediated endocytosis (Loregger et al., 2017) was comparable between WT and PMP22 KO Schwann cells (Fig. 6E,F).

As a direct measure of cholesterol efflux from the Schwann cells, we loaded cells with ^3H -cholesterol (Dove et al., 2005) and studied the efflux over 16 h (Fig. 6G). Quantification from independent cultures identified a significant impairment in cholesterol efflux from PMP22 KO Schwann cells compared with WT (WT $17.4 \pm 0.8\%$ vs PMP22, KO $7.9 \pm 1.8\%$; $t = 4.9$, $p = 0.0081$, $R^2 = 0.86$, unpaired Student's t test). To further substantiate the influence of PMP22 on ABCA1 function, we studied ABCA1-mediated cholesterol efflux in the presence of lipid-free human apoA-I in normal rat Schwann cells when PMP22 was suppressed (Fig. 6H). Rat Schwann cells transfected with a PMP22 shRNA secreted $\sim 44\%$ less ABCA1-dependent cholesterol compared with scrambled control ($t = 3.2$, $p = 0.018$, $R^2 = 0.63$, unpaired Student's t test) (Fig. 6H). In these transfection experiments, the level of PMP22 knock-down was $46 \pm 4\%$ compared with scram-

Figure 5. ABCA1 and PMP22 are codistributed within Schwann cells. **A, B**, ABCA1 and PMP22 costaining in cultured WT mouse Schwann cells after Triton X-100 permeabilization. Control for staining specificity with secondary antibody alone is shown in **A**. Arrows point to the cell magnified in **B**. **B**, Representative high-magnification image after Z-stack (step size = 0.15 μ m). Single XY plane in the middle, and XZ and YZ plane on the side display the colocalization of the two proteins (yellow) ($n = 150$ cells). **C**, Cell surface ABCA1 and PMP22 costaining in WT mouse Schwann cells revealing colocalization at the plasma membrane (yellow on merge) ($n = 100$ cells). **D**, ABCA1 and PMP22 costaining in longitudinal sections of WT mouse sciatic nerves (arrowheads). Arrow points to Schwann cell body, with coexpression of the two proteins near the nucleus (blue). Boxed areas are magnified in the upright corner. **E, F**, Coimmunoprecipitation of ABCA1 and PMP22 (**E**) or ABCA1 and PMP22-Myc (**F**) from WT rat Schwann cells or cells transfected with PMP22-Myc, respectively. **G, H**, Coimmunoprecipitation of ABCA1 and PMP22 from WT (**G**) and PMP22 KO (**H**) mouse sciatic nerve (SN) homogenates. Dystrophin, a known ABCA1-binding partner, was precipitated as a positive control. Total protein lysate (T), preclear (PC), and IP are included for each experiment ($n = 3$ –5 independent experiments).

bled. We also performed ABCA1-dependent BODIPY–cholesterol efflux assays with or without pharmacological modulation of ABCA1 (Fig. 6I–K). Under basal conditions, cholesterol efflux to apoA-I was $\sim 27.3\%$ lower in PMP22 KO mouse Schwann cells compared with WT (WT $4.2 \pm 0.4\%$ vs PMP22 KO $3.0 \pm 0.2\%$; $t = 2.4$, $p = 0.04$, $R^2 = 0.36$, unpaired Student's t test). Upregulation of the expression and activity of ABCA1 with 8-bromo-cAMP (Haider et al., 2002) significantly increased cholesterol efflux from both cell types, but to a lower extent from PMP22 KO cells (WT $6.0 \pm 0.5\%$ vs PMP22 KO $3.8 \pm 0.2\%$; $t = 4.7$, $p = 0.0012$, $R^2 = 0.71$, unpaired Student's t test) (Fig. 6I–K). The immunosuppressant drug CsA blocks the endocytosis of membrane-associated ABCA1 and thus inhibits its activity (Le Goff et al., 2004). As expected, 18-h-long treatment with CsA increased the levels and blocked the activity of ABCA1 in WT cells; however, it had no effect on PMP22 KO cells (WT $2.4 \pm 0.4\%$ vs PMP22 KO $2.6 \pm 0.2\%$; $t = 0.56$, $p = 0.59$, $R^2 = 0.03$, unpaired Student's t test) (Fig. 6I–K). The atypical response of PMP22 KO cells to 8-bromo-cAMP and CsA indicates a reduction in functional ABCA1 at the plasma membrane.

ABCA1 expression is reduced at the plasma membrane when PMP22 is absent

To determine the levels of ABCA1 in the Schwann cell plasma membrane, we performed cell surface immunolabeling on nonpermeabilized cells (Fig. 7A, B). Confocal imaging and quantification detected an $\sim 25\%$ decrease in ABCA1-like immunoreactivity on the glial cell surface in the absence of PMP22 ($t = 3.5$,

$p = 0.0006$, $R^2 = 0.07$, unpaired Student's t test), which likely contributed to the decreased cholesterol efflux (Fig. 6G–I, J). To confirm the influence of PMP22 on ABCA1 localization, we inhibited PMP22 in normal rat Schwann cells. As shown on the images, cells expressing PMP22 shRNA-GFP displayed reduced surface ABCA1-like fluorescence (Fig. 7C, arrow) compared with nontransfected cells (Fig. 7C, asterisk). Furthermore, ABCA1 was reduced at the Schwann cell membrane in PMP22 KO mouse nerves and this phenotype was evident on both nerve cross-sections (Fig. 7D) and teased fibers (Fig. 7E).

Because Rab8 is known to facilitate cholesterol efflux and regulate the cell surface expression of ABCA1 (Linder et al., 2009), we tested Rab8 levels in WT and PMP22 KO nerves (Fig. 7F, G). Rab8 levels were increased (~ 1.5 -fold, $t = 2.7$, $p = 0.034$, $R^2 = 0.56$, unpaired Student's t test) in sciatic nerves from PMP22 KO mice, likely as a compensatory mechanism to facilitate the presence and activity of ABCA1 at the cell surface. To further substantiate the relevance of the aberrant cholesterol efflux by PMP22-deficient Schwann cells, we examined the extent of apoE lipidation within whole sciatic nerve lysates using native gel electrophoresis (Corona et al., 2016). In agreement with the retention of cholesterol and ABCA1 within Schwann cells in the absence of PMP22 (Figs. 1, 2, 6, 7A–E), lipidated apoE in particles of the size of high-density lipoproteins (>8 nm or 140 kDa) was more abundant in PMP22 KO nerves relative to WT (Fig. 7H). Therefore, results from both *in vitro* and *in vivo* studies indicate that PMP22 is critical for lipid homeostasis in peripheral glial cells.

Figure 6. Decreased apoE secretion and cholesterol efflux in the absence of PMP22. **A, B**, Immunoblot detection (**A**) and quantification (**B**) of secreted apoE and laminin in the conditioned media of cultured mouse Schwann cells after 24 h. **C, D**, Immunoblot detection (**C**) and quantification (**D**) of ABCA1, apoE, and LDLR in whole WT and PMP22 KO Schwann cell lysates. Actin or Ponceau staining are shown as protein-loading controls. **E, F**, Visualization (**E**) and quantification (**F**) of BODIPY-LDL uptake by WT and PMP22 KO mouse Schwann cells after 3, 8, and 24 h incubation times. **G**, $[^3\text{H}]$ -cholesterol efflux to medium without apolipoprotein induction from WT and PMP22 KO mouse Schwann cells. **H**, $[^3\text{H}]$ -cholesterol efflux to apoA-I in normal rat Schwann cells transfected with scrambled or PMP22 shRNA. **I**, BODIPY-cholesterol efflux to apoA-I in WT and PMP22 KO mouse Schwann cells after treatments with 8-Br-cAMP (cAMP) or CsA of ABCA1. **J, K**, Quantification (**J**) and immunoblot detection (**K**) of ABCA1 protein expression after treatments with vehicle (DMSO), 8-Br-cAMP (cAMP), or CsA. Results are representative of three to five independent experiments. Values represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$, two-tailed Student's t test.

Discussion

We sought to elucidate the role of PMP22 in Schwann cell biology and to better understand the subcellular events involved in the pathobiology of PMP22-linked hereditary neuropathies. Utilizing a previously characterized PMP22 KO mouse line (Amici et al., 2006), we discovered the critical requirement for PMP22 in maintaining the biophysical properties of the Schwann cell membrane and in cholesterol homeostasis. At the plasma membrane, the presence of PMP22 affects Schwann cell membrane resistance and capacitance, two key elements in myelin biology. Furthermore, by partnering with cholesterol, apoE, and ABCA1, PMP22 is involved in intracellular cholesterol trafficking and cholesterol efflux, which are central for the

maintenance of PNS myelin integrity. Cholesterol not only serves as an essential building block of myelin, but it also affects the intracellular transport of the major PNS myelin protein P0 (Saher et al., 2009). Further studies will be required to determine whether PMP22 directly interacts with cholesterol; however, elegant *in vitro* studies have demonstrated the formation of compressed and cylindrically wrapped protein-lipid vesicles when purified PMP22 is reconstituted (Mittendorf et al., 2017). Therefore, our cellular and molecular studies described here, along with previously published work (Mittendorf et al., 2017), support the notion that altered expression of PMP22 causes peripheral myelin defects, in part through altering the organization and/or integrity of the glial membrane.

Figure 7. Reduced ABCA1 expression at the plasma membrane in the absence of PMP22. **A, B**, Representative images of ABCA1 immunoreactivity on the surface of WT and PMP22 KO mouse Schwann cells (**A**) and quantification of fluorescence intensity (**B**) ($n = 90$ cells per genotype). **C**, Representative images of ABCA1 immunoreactivity on the surface of normal rat Schwann cells after PMP22 shRNA-GFP transfection. Arrow points to a transfected cell as indicated by GFP expression. Asterisk marks a nontransfected cell. **D, E**, Representative images of ABCA1 localization in WT and PMP22 KO nerve cross-sections (**D**) and teased nerve fibers (**E**). Arrowheads point to plasma-membrane-associated ABCA1 in WT nerves, and asterisks mark Schwann cell bodies. **F, G**, Immunoblot detection (**F**) and quantification (**G**) of Rab8 in lysates from WT and PMP22 KO sciatic nerves. **H**, Native immunoblot detection of lipidated (arrowheads) and poorly lipidated (arrows) apoE in whole sciatic nerve lysates. GAPDH or actin is shown as the protein-loading control. In **A–C**, Results are representative of three to five independent experiments; in **D–H**, results are from three to four animals per genotype. Values represent the mean $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$, two-tailed Student's t test.

In the CNS, loss of ABCA1, a key regulator of cholesterol metabolism, is associated with functional and structural deficits (Karasinska et al., 2009). To date, the role of ABCA1 in peripheral nerve biology has not been investigated in detail, even though almost half of the patients with Tangier disease develop peripheral neuropathy (Hobbs and Rader, 1999). Here, we demonstrate a role for ABCA1 in Schwann cells and a functional linkage between ABCA1 and PMP22. In normal cells, PMP22 and ABCA1 are co-detected in subcellular compartments and at the plasma membrane. Our results from PMP22 KO cells indicate that loss of PMP22 results in compromised ABCA1-mediated cholesterol efflux with consequent accumulation of intracellular cholesterol, apoE, and ABCA1. The coordinated upregulation of ABCA1 and apoE in PMP22-deficient Schwann cells, along with the increase in PMP22 in ABCA1 KO nerves, support a functional relationship between these three proteins in cholesterol metabolism.

The nature of cholesterol dictates its essential role in myelin, the insulator of nerve axons. In addition to being a structural myelin component, cholesterol inhibits the activity of phospholipid-dependent Na^+ and K^+ channels and thus increases membrane resistance (Tobias et al., 1962; Papahadjopoulos et al., 1973). As a constituent of lipid rafts through interactions with sphingolipids, cholesterol reduces membrane fluidity and permeability (Barenholz, 2002). Cholesterol is rate-limiting for myelin membrane growth because the lack of cholesterol synthesis results in profound abnormalities in both the CNS and PNS (Saher et al., 2011). The mistrafficking of cholesterol can also lead to

severe dysmyelination, such as seen in Niemann-Pick disease type C (NPC) (Qiao et al., 2018). NPC is characterized by the sequestration of cholesterol in lysosomes due to NPC gene mutations and associated hypoplasia in cerebellar white matter (Palmeri et al., 1994) and severe PNS myelin abnormalities, including vacuolization in the Schwann cell cytoplasm (Bagel et al., 2013). Therefore, aberrant expression of distinct genes involved with cholesterol metabolism leads to myelin defects, supporting the essential role for cholesterol in the glial membrane.

Myelin proteins with known cholesterol interaction include proteolipid protein (PLP) in the CNS and P0 in the PNS (Saher et al., 2009; Werner et al., 2013). Both of these glial proteins contain a cholesterol recognition amino acid consensus (CRAC) sequence, which is known to mediate the interaction of cholesterol with membrane proteins (Fantini and Barrantes, 2013). In oligodendrocytes, PLP, a tetraspan glycoprotein (Snipes et al., 1992) maintains cholesterol levels in the secretory pathway for myelin biogenesis (Werner et al., 2013). Our findings on the intracellular, Golgi-associated retention of cholesterol in PMP22 KO samples support a similar role for PMP22 in cholesterol secretion. In the PNS, the exit of P0 from the ER is dependent on cholesterol, which was discovered in cells with ablated squalene synthase (Saher et al., 2009). In the same model system, however, PMP22 was not retained in the ER, suggesting an independent mechanism for the transport of PMP22 and P0. PMP22 contains an evolutionary conserved CRAC motif in its fourth transmembrane domain (Gould et al., 2005; Sedzik et al., 2013) and mutations in this

region have been linked to severe neuropathies (Ionasescu et al., 1997; Ikegami et al., 1998; Parman et al., 1999; Ohnishi et al., 2000; Maki et al., 2011). Therefore, given the intracellular retention of cholesterol in the absence of PMP22 shown here, it is plausible that, similar to PLP, PMP22 is key for the cholesterol trafficking from trans-Golgi to the plasma membrane. This hypothesis is supported by reduced levels of cholesterol secretion by PMP22 KO Schwann cells (Fig. 6).

A key role for PMP22 in lipid metabolism is reinforced by the observed induction of ABCA1 and apoE in PMP22 KO cells, likely through LXR activation. This hypothesis is supported by reports on the activation of LXR in various demyelinating conditions (Cermenati et al., 2013; Norrmén et al., 2014; Meffre et al., 2015), although in our samples, we did not detect an increase in LXR expression (data not shown). *APOE*, one of the genes regulated by LXR, has been linked to peripheral nerve injury and repair and is significantly upregulated in the absence of PMP22 (Ignatius et al., 1986; Lee et al., 2014). In agreement with our findings (Fig. 2A), the downregulation of SREBP pathways have been observed previously in other PMP22-linked neuropathic animal models (Vigo et al., 2005; Fledrich et al., 2012). The coordinated upregulation of ABCA1 and apoE and the associated decrease in some SREBP-1c and SREBP-2 dependent genes likely reflect counterregulatory maneuvers in cells reacting to abnormal cholesterol accumulation in PMP22 KO peripheral nerves (Lee et al., 2014). The identification of upstream and downstream signaling pathways mediating the observed phenotype will require further studies and may involve PI3K/AKT/mTOR (Goebbels et al., 2012).

ABCA1 is present in lipid rafts, where it interacts with apolipoproteins, free cholesterol, and sphingomyelin (Oram and Heinecke, 2005; Klappe et al., 2009; Phillips, 2018). Similarly, PMP22 is predicted to bind cholesterol through the cholesterol recognition (CRAC and CARC) motifs (Gould et al., 2005; Sedzik et al., 2013). Therefore, whereas it is currently unknown whether PMP22 and ABCA1 directly interact, it is conceivable that cholesterol acts as an intermediate in the association between these two proteins within lipid rafts. Our study shows that PMP22 and ABCA1 are codistributed in the plasma membrane and the absence of PMP22 impairs both ABCA1 trafficking and cholesterol efflux. This discovery is supported by the detection of ABCA1 in the Schwann cell membrane in association with dystrophin (Albrecht et al., 2008), a component of the dystrophin–glycoprotein complex. This dystrophin–glycoprotein complex binds laminin (Yamada et al., 1994), an extracellular matrix molecule that is complexed with PMP22 (Amici et al., 2006). In addition, ABCA1 directly associates with flotillin-1 (Bared et al., 2004), a constituent of lipid rafts whose distribution is perturbed in the nerves of PMP22 KO mice (Lee et al., 2014). Finally, the recognition of tomacula in ABCA1 KO nerves (Fig. 4H) and in a patient with Tangier disease (Cai et al., 2006) is another commonality between PMP22- and ABCA1-linked neuropathies (Lupski et al., 1993). Together, our findings support a synergistic functional connection between PMP22 and ABCA1 in cholesterol homeostasis in Schwann cells.

Better understanding of lipid metabolism in peripheral nerve biology has wide implications for neuropathies and provides critical information for the development of therapies. It was recently shown that defective cholesterol clearance limits remyelination in the aged CNS (Cantuti-Castelvetri et al., 2018) and could similarly play a critical role in the repair of demyelinated nerves in neuropathies. Indeed, an apoE mimetic peptide has been shown to benefit postcrush peripheral nerve repair (Li et al., 2010), likely

through improving cholesterol and lipid reutilization during regeneration. In the cardiovascular field, ABCA1 has been considered as a therapeutic target (Oram, 2002; Kuntz et al., 2015) and such an approach could benefit patients with PMP22-linked peripheral nerve disorders such as CMT1A and HNPP. In related experiments, dietary cholesterol was shown to repair CNS myelin lesions, including in Pelizaeus–Merzbacher disease due to PLP overexpression (Saher et al., 2012; Berghoff et al., 2017). Considering the co-secretion of phospholipids and cholesterol through ABCA1-mediated efflux (Oram and Heinecke, 2005), it is conceivable that PMP22 misexpression disturbs phospholipid homeostasis, which could explain the effectiveness of phospholipid therapy in CMT1A rodents (Fledrich et al., 2018). Therefore, our findings provide significant new knowledge to advance our understanding of the connection between peripheral nerve biology and lipid metabolism and how this connection can be exploited for the development of therapies.

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