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Supplementary Information for

Dysregulation of myelin synthesis and actomyosin function underlies aberrant myelin in CMT4B1 neuropathy

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Extended Material and Methods

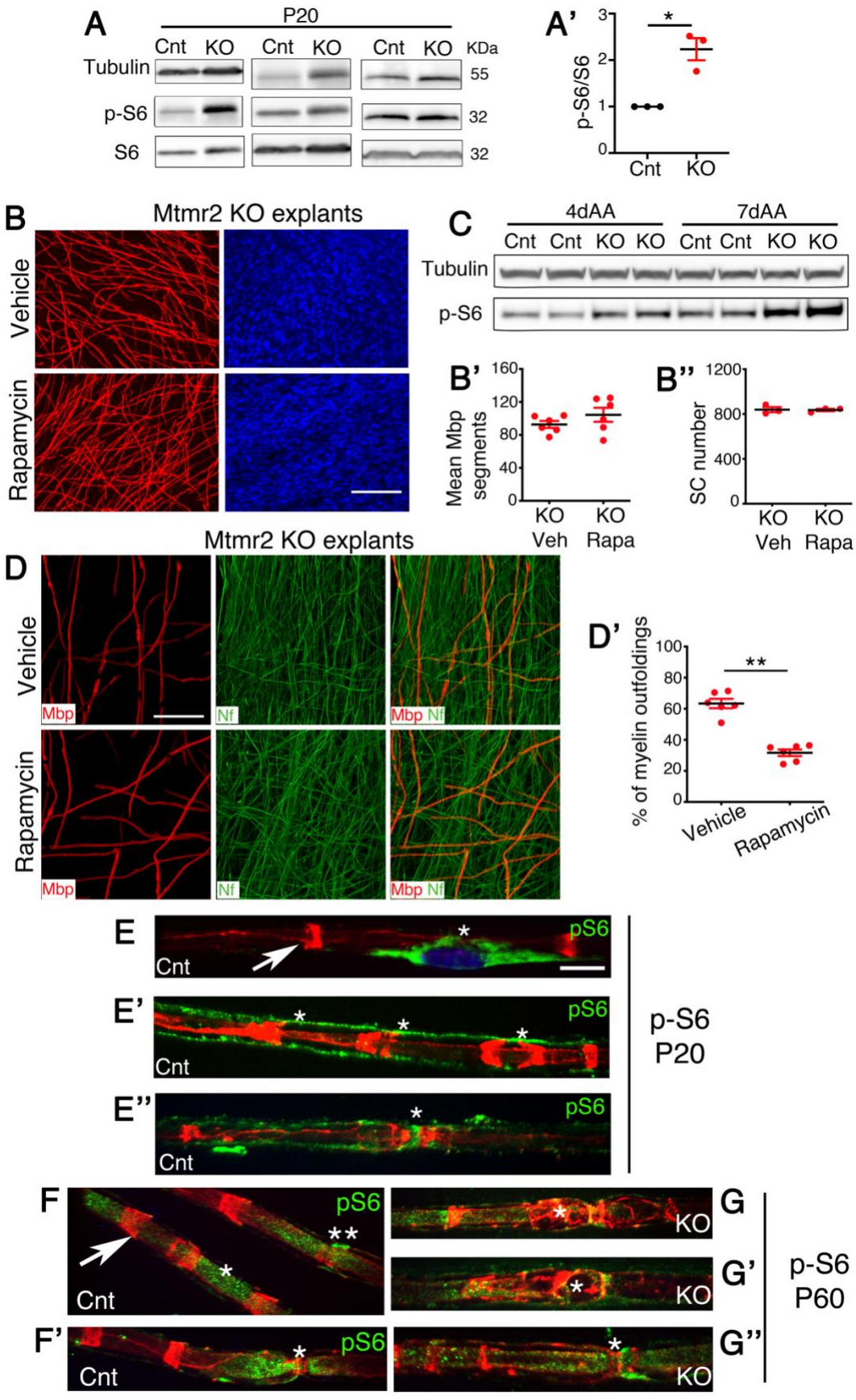
A
P20
Cnt KO Cnt KO Cnt KO KDa
Tubulin
p-S6
S6
55
32
32
A'
p-S6/S6
Cnt
KO
B
Mtmr2 KO explants
Vehicle
Rapamycin
C
4dAA
7dAA
Cnt Cnt KO KO Cnt Cnt KO KO
Tubulin
p-S6
B'
Mean Mbp segments
KO Veh
KO Rapa
B''
SC number
KO Veh
KO Rapa
D
Mtmr2 KO explants
Vehicle
Rapamycin
Mbp
Nf
Mbp Nf
D'
% of myelin outfoldings
Vehicle
Rapamycin
E
E'
E''
pS6
Cnt
p-S6
P20
F
F'
G
G'
G''
KO
p-S6
P60

Fig. S1. Loss of Mtmr2 results in enhanced mTORC1 signaling.

(A) Increased S6 phosphorylation in *Mtmr2* KO sciatic nerve lysates at P20, and quantified in A', n=3 pools of nerve lysates per genotype in three independent experiments, one sample t-test, t=5.183, df=2, P=0.0353.

(B-B'') Rapamycin treatment does not interfere with myelination in *Mtmr2* KO Schwann cell/DRG neuron co-culture explants as assessed by measuring the number of Mbp-positive fibers (B') and Schwann cell nuclei (B''). Mbp-positive segments: n=6 DRG/coverslips vehicle-treated and n=6 DRG/coverslips rapamycin-treated, two-tailed Mann-Whitney U test, P=0.3095. Schwann cell nuclei, n=3 DRG/coverslips vehicle-treated and n=3 rapamycin-treated, two-tailed Mann-Whitney U test, P=0.999. Representative of three independent experiments.

(C) Increased S6 phosphorylation in *Mtmr2* KO Schwann cell/DRG neuron co-culture explants as compared to control following 4 and 7 days of ascorbic acid treatment in differentiating conditions.

(D, D') Confocal microscope representative images of *Mtmr2* KO Schwann cell/DRG neuron co-culture explants vehicle (ethanol)-treated and rapamycin-treated where myelin outfoldings are rescued, as quantified in (D') by measuring the percentage of Mbp-positive fibers carrying myelin outfoldings on the total number of Mbp-positive fibers. n=6 DRG/coverslips vehicle-treated, 252 total fibers analyzed and n=6 DRG/coverslips rapamycin-treated, 250 total fibers analyzed, two-tailed Mann-Whitney U test, P=0.0022. Representative of three independent experiments.

(E) Teased fibers analysis shows enrichment of p-S6 in the Schwann cell perinuclear region (asterisk) of control sciatic nerves at P20. The arrow indicates Schmidt-Lanterman incisures (SLI).

(E') Asterisks along the internode indicate p-S6 localization at abaxonal regions of control myelinated fibers at P20.

(E'') The asterisk shows paranodal localization of p-S6 in control fibers at P20.

(F) The arrow indicates Schmidt-Lanterman incisures (SLI) and the asterisk marks abaxonal p-S6 staining also surrounding SLI (double asterisk).

(F') The asterisk marks paranodal localization of p-S6 in control and *Mtmr2* KO fibers at P60 (G'').

(G, G') Examples of aberrant myelin structures indicated by asterisks and co-localization with pS6 staining in yellow.

“Cnt” is control; “KO” is *Mtmr2* KO. Green= p-S6; Red=phalloidin; Blue=DAPI in E’.

Mbp=Myelin basic protein; Nf= neurofilament. Bar is 45 µm in B and 112 µm in D.

Bar in E is 4.7µm for E, F; in E' 4.88 µm; in E'' 4.201 µm; in F’ 6.1 µm; in G 5.64 µm; in G’ is 6.71 µm, and in G’’ is 8.75 µm.

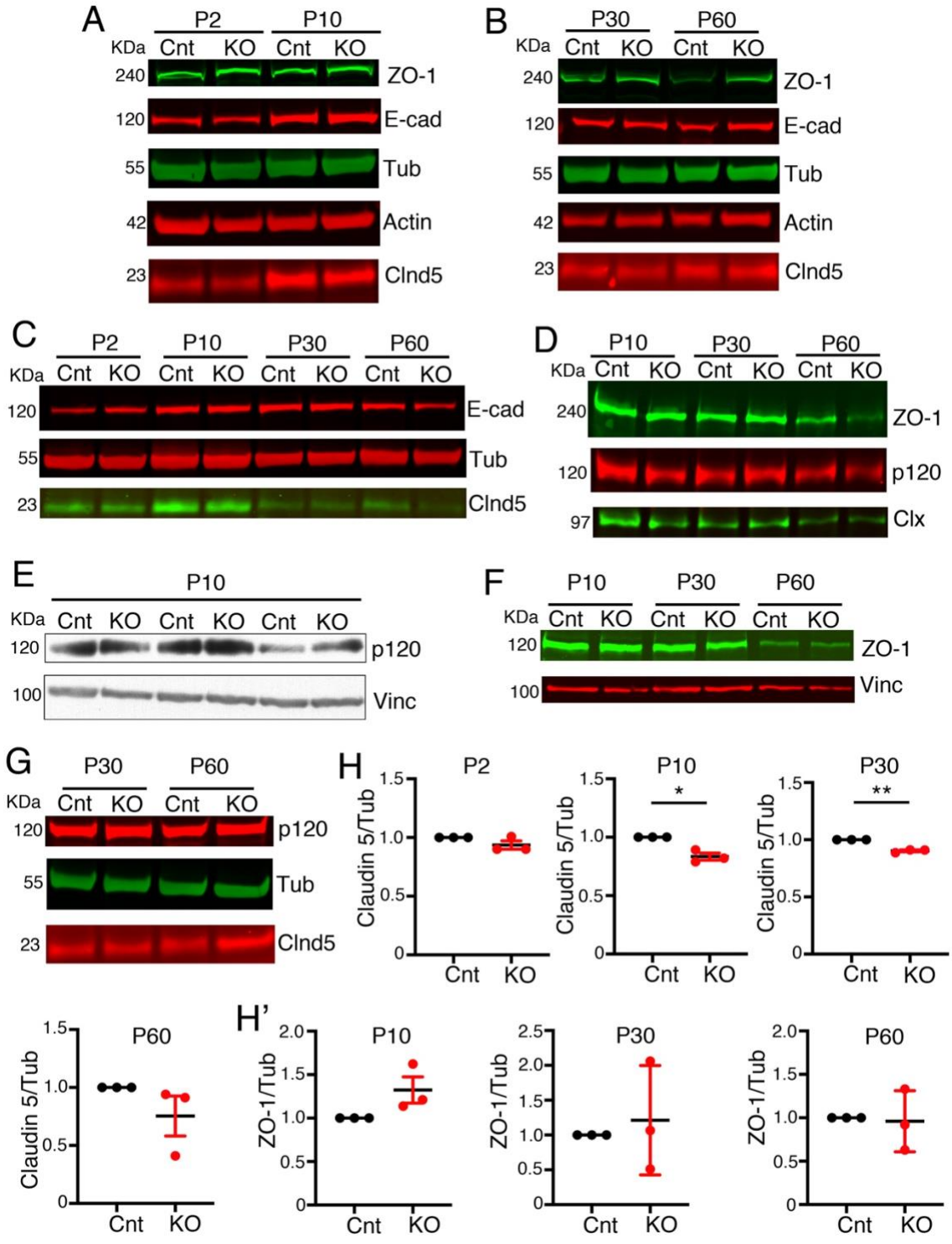

Fig. S2. Replicates of western blot analyses to detect expression of tight and adherens junction markers in sciatic nerves (shown in Figure 3).

(A-G) Technical and biological replicates of western blot analyses to detect expression of ZO-1, E-cadherin, p120 catenin, and claudin 5 in sciatic nerve lysates from control and *Mtmr2* KO mice at different time points of postnatal development.

(H) Quantification of claudin 5 expression at P2, P10, P30, and P60. P10: n=3 independent experiments, one sample t-test, P=0.0314; t=5.509; df=2. P30: n=3 independent experiments, one sample t-test, P=0.0048; t=14.37; df=2.

(H') Quantification of ZO-1 expression at P10, P30, and P60.

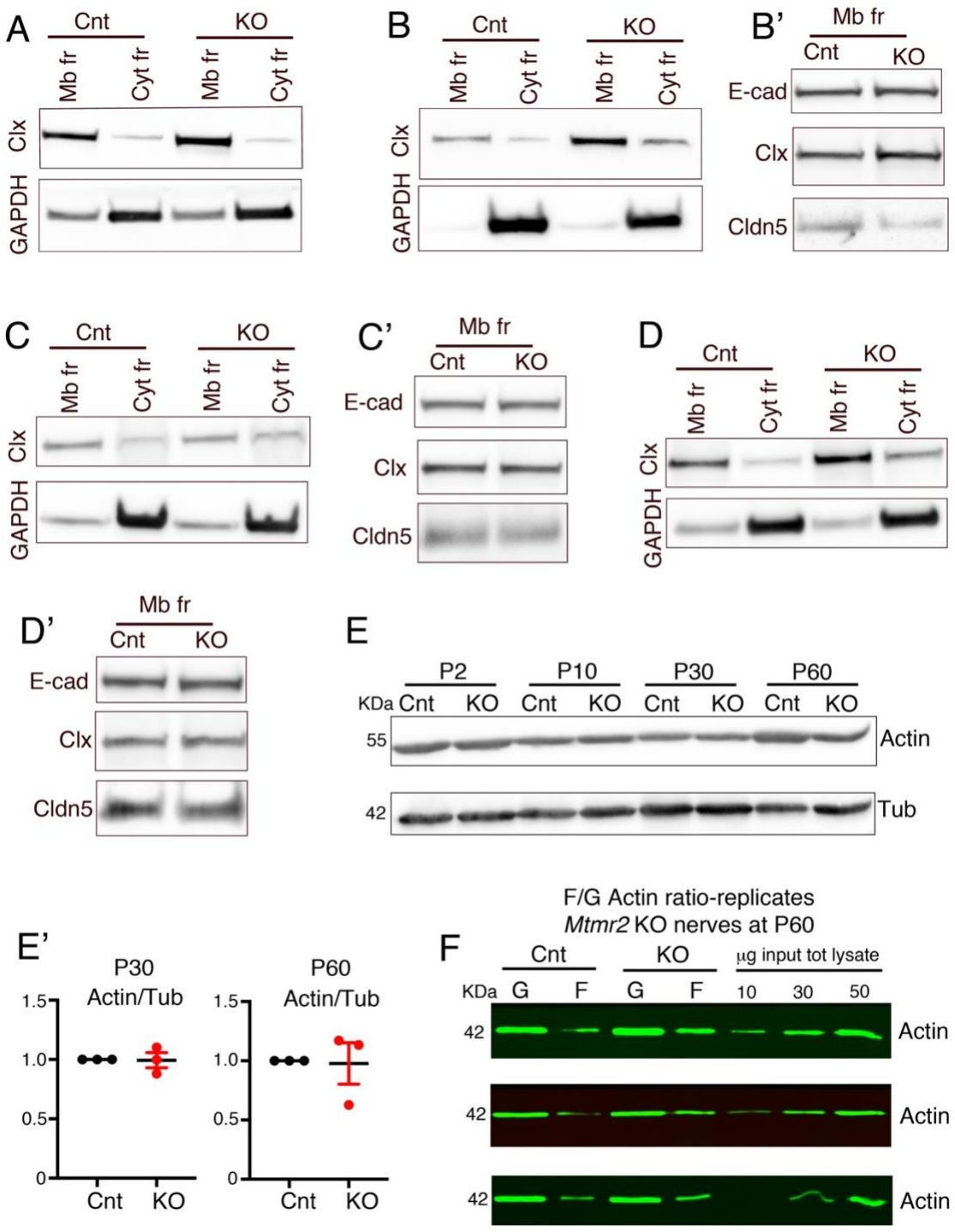

Fig. S3. Technical and biological replicates of western blot analysis shown in Figure 3.

(A) Membrane (Mb)-calnexin enriched, and cytosolic (Cyt) fraction (fr)-GAPDH enriched from the fractionation experiment of sciatic nerves at P30 shown in Figure 3A'.

(B, C, D) Technical and biological replicates of fractionation experiments of sciatic nerves at P30 showing membrane fraction-calnexin enriched, and cytosolic fraction-GAPDH enriched, along with the corresponding western blot analysis performed on membrane fraction lysates only (B', C', D') to assess reduced expression levels of claudin 5 in mutant nerves (quantified in Figure 3A').

(E) Technical and biological replicate of western blot analysis to detect total actin expression levels in *Mtmr2* KO nerves, and quantification in E' of n=3 independent experiments as shown in Figure 3F and Suppl. Fig. 2A, B.

(F) Technical and biological replicate of Figure 3G, G'. The first row is the same experiment shown in Figure 3G, here with input lysates.

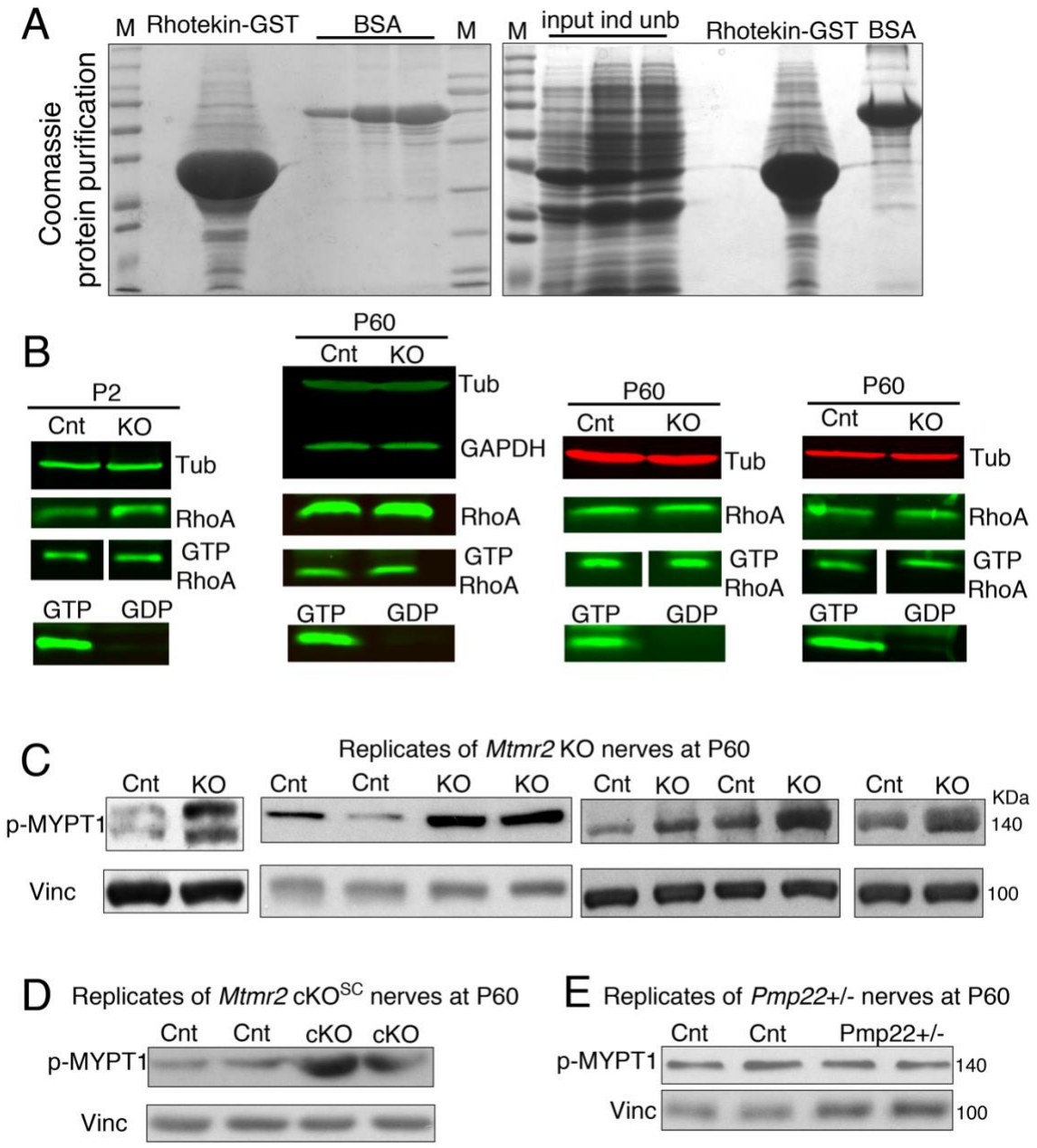

Fig. S4. Technical and biological replicates of western blot analysis shown in Figure 4.

(A) Examples of Rhotekin-GST purified from bacterial lysates, M is marker, input is bacterial lysates, “ind” is bacterial lysates after IPTG induction, and “unb” is the unbound fraction after GST-sepharose beads binding. BSA is bovin serum albumin used as a standard.

(B) Technical and biological replicates of pull down experiments as shown and quantified in Figure 4 A, A’.

(C) Technical and biological replicates of western blot analyses of Figure 4C, C'. p-MYPT1 is detected with two bands or one band depending on the homogenization procedure, manually using a plastic pestel or with the Precellys Cryolys Evolution System (Bertin Instruments), respectively.
(D, E) Technical and biological replicates of western blot analyses of Figure 4D-E.

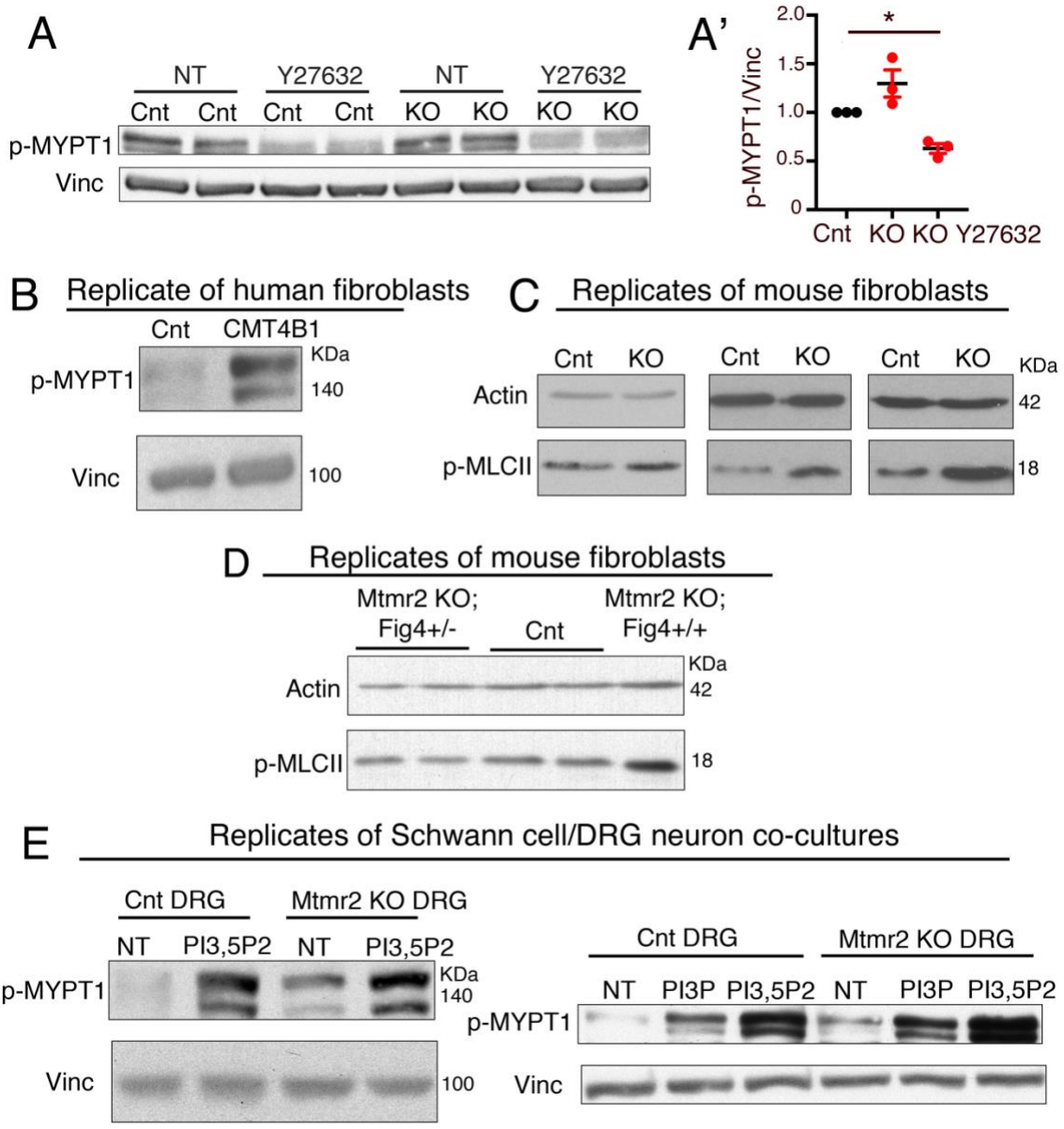

Fig. S5. Technical and biological replicates of western blot analysis shown in Figures 5, 6 and S7.

(A, A') Technical and biological replicates of western blot analysis shown in Figure 5A, with quantification in (A'), n=3 DRG/coverslips pools per condition per genotype, one sample t-test, P= 0.0178, t=7.386, df=2.

(B) Technical and biological replicate of western blot analysis shown in Fig. S7B.

(C, D) Technical and biological replicates of western blot analysis shown in Fig. S7C, C'.

(E) Technical and biological replicates of western blot analysis shown in Figure 6D, D'.

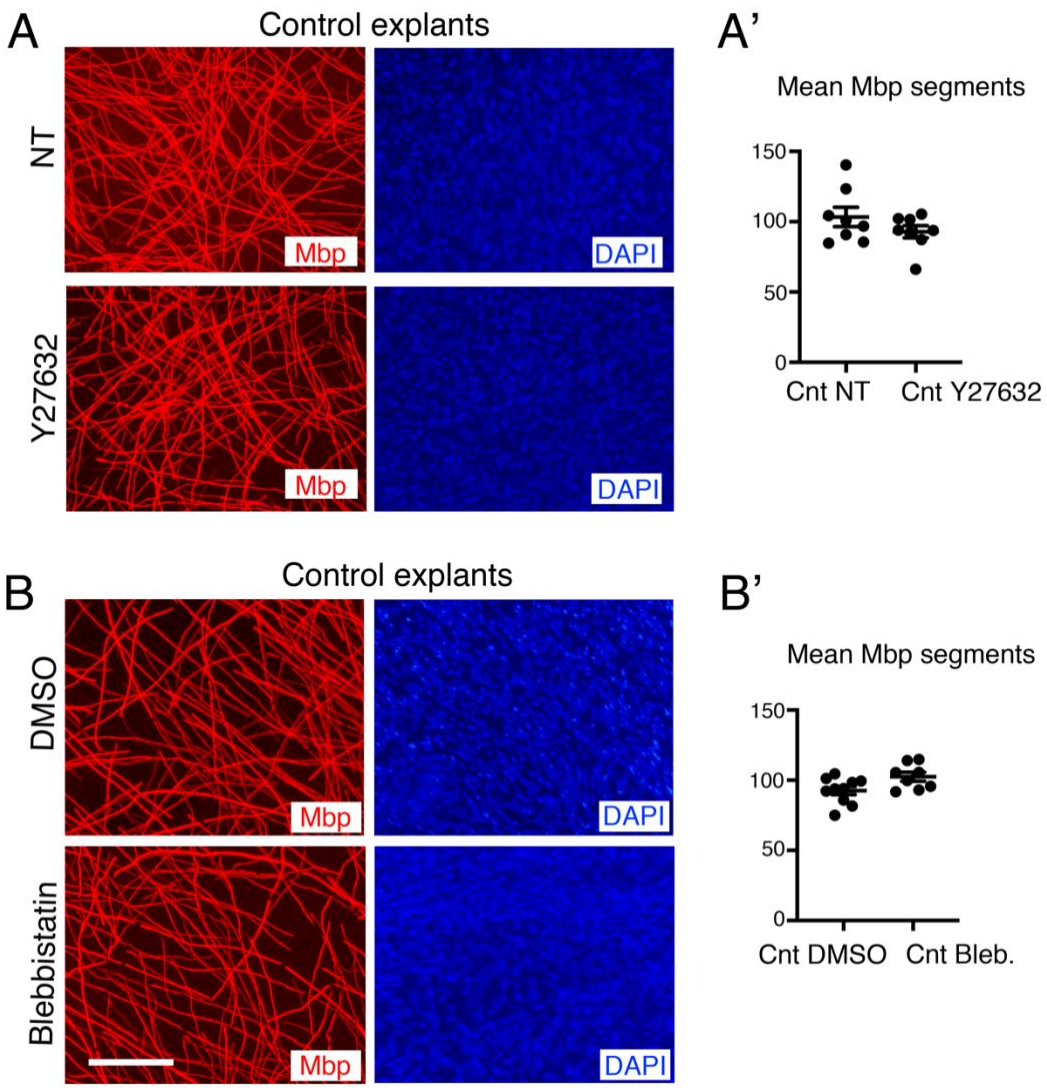

Fig. S6. Y27632 and blebbistatin treatment of control cultures.

(A) Representative fluorescence microscope images of control cultures NT and treated using Y27632. (A') Quantification of Mbp-positive segments: n=8 DRG/coverslips NT and n=8 DRG/coverslips Y27632-treated, two-tailed Mann-Whitney test, P=0.6454. Representative of three independent experiments.

(B) Representative fluorescence microscope images of control cultures DMSO-treated and treated using blebbistatin. (B') Quantification of Mbp-positive segments: n=10 DRG/coverslips DMSO-

treated and n=8 DRG/coverslips blebbistatin-treated, two-tailed Mann-Whitney test, P=0.07. Representative of two independent experiments.

Cnt is control cultures. Bar in B is 108 µm for Mbp and DAPI.

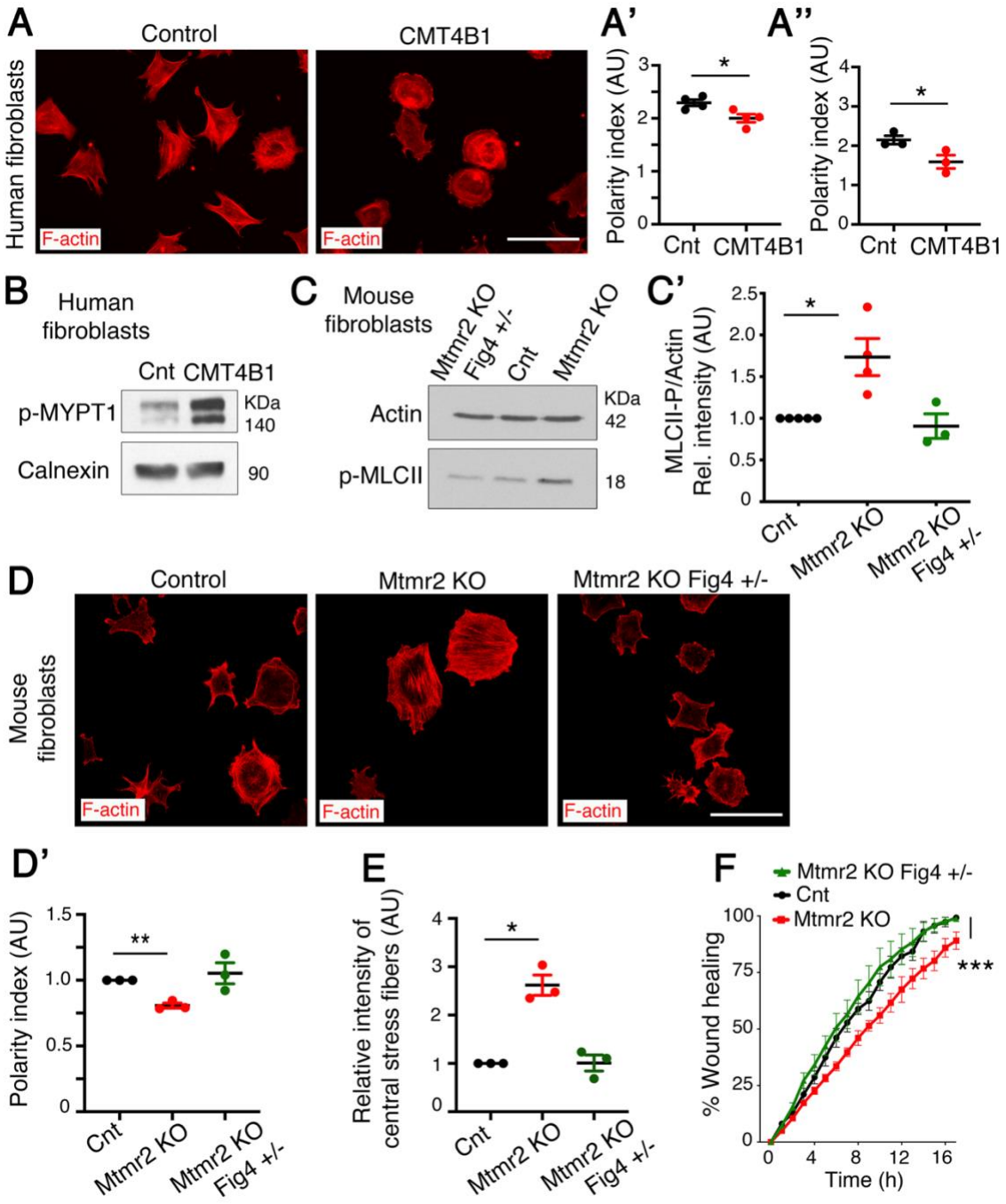

Fig. S7. Lipid-mediated increase of RhoA-myosin II pathway enhances cortical cytoskeleton contractility in *MTMR2/Mtmr2* KO cells.

(A) Representative images showing cell morphology of CMT4B1 patients and control fibroblasts plated for 1 hour on fibronectin and stained with phalloidin labelling F-actin.

(A') Quantification of the polarity index of CMT4B1 patient and control cells shows that patient cells have a reduced polarity index, suggesting a rounder cell shape, with n=4 coverslips (for a total of

166 cells analyzed) and n=4 CMT4B1 coverslips (for a total of 158 cells analyzed), two-tailed Mann-Whitney U test, P=0.0226.

(A'') Quantification of the polarity index of a different set of CMT4B1 and control lines, which supports results shown in A, with n=3 coverslips (for a total of 151 cells analyzed) and n=3 CMT4B1 coverslips (for a total of 113 cells analyzed), two-tailed Mann-Whitney U test, P=0.049.

(B) Western blot analysis shows that phosphorylation levels of MYPT1 are increased in CMT4B1 patient fibroblasts plated on fibronectin for 2 hours, representative of two independent experiments using n=2 controls and n=2 CMT4B1 primary lines (SI Appendix, Fig. S5B).

(C) Western blot analysis shows that phosphorylation levels of MLCII are increased in *Mtmr2* KO fibroblasts as compared to both wild-type and *Mtmr2*-/-; *Fig4*+/- cells, and quantified in C': n=5 wild-type, n=4 *Mtmr2* KO, and n=3 *Mtmr2*-/-; *Fig4*+/-, five independent experiments analyzed (SI Appendix, Fig. S5C, D). One sample t-test, wild-type and *Mtmr2* KO, P=0.0458, t=3.299, df=3; wild-type and *Mtmr2*-/-; *Fig4*+/-, P=0.5882, t=0.6391, df=2 .

(D) Representative confocal microscope images showing the cell morphology of *Mtmr2* KO, wild-type and *Mtmr2*-/-; *Fig4*+/- primary fibroblasts plated for 2 hours on fibronectin and stained with phalloidin labelling F-actin.

(D') Quantification of the polarity index shows that *Mtmr2* KO cells have a reduced polarity index as compared to both wild-type and *Mtmr2*-/-; *Fig4*+/-, suggesting a rounder cell shape, n=3 primary lines per genotype in three independent experiments, one-sample t-test, P=0.0099, t=9.99, df=2. Each "n" is the mean of the different coverslips analyzed in a single experiment, with at least 100 cells per line, per experiment.

(E) Quantification of the central stress fibers relative intensity labelled using phalloidin (F-actin), n=3 primary lines per genotype in three independent experiments, one-sample t-test, P=0.0165, t=7.69, df=2. Each "n" is the mean of the different coverslips analyzed in a single experiment, with at least 100 cells per line, per experiment.

(F) Wound healing assay of n=2 primary lines per genotype analyzed, n=4 wells per genotype, nonparametric one-way ANOVA, followed by Tukey's multiple comparisons test, P<0.001,

representative of three independent experiments. Wild type and *Mtmr2* KO, P<0.001; *Mtmr2* KO and *Mtmr2*-/-; *Fig4*+/-, P<0.001.

Bar is 119 µm in A and 114 µm in D.

Extended Materials and Methods

Schwann cell/DRG neuron co-cultures

Myelin-forming Schwann cell/DRG neuron co-culture organotypic explants have been established from *Mtmr2* KO at E13.5. Neurobasal (NB) medium supplemented with 2.5S NGF (Nerve Growth Factor) at 50 ng/ml final concentration and B27 (Thermo Fisher) was used for 5-7 days prior to induce myelination. For myelination, C-media supplemented with ascorbic acid for additional 15 days (50 µg/ml, SIGMA) was used.

To perform immunohistochemistry, co-culture explants were fixed for 15 min in 4% paraformaldehyde, permeabilized for 5 min in ice-cold methanol at -20°C, blocked for 20 min with 10% NGS, 1% BSA, incubated with primary antibodies for 1 hour at room temperature in PBS 1x (anti-Mbp) and overnight in 5%BSA, 1% NGS, 0.6% Triton in PBS1x at 4°C (anti-NF-M). After washing, the coverslips were incubated with the secondary antibody for 30 min, washed and mounted.

Co-culture explants were treated with the following compounds every other day starting from two days of ascorbic acid treatment to avoid interference with Schwann cell proliferation: compound 19 (Selleckchem, #S8456) 2.5 µM or 10 µM; apilimod (MedChem Express, #HY-14644) 300 nM; blebbistatin (SIGMA, #B0560) 1µM; Y27632 (Merck Millipore, #688000) 10µM; rapamycin (Merck Millipore, #553210) 25 nM.

Primary fibroblast cultures

To monitor morphological changes during adhesion, fibroblasts were plated on 12 mm coverslips coated with fibronectin 2,5ug/ml (Merck Millipore, #FC010) for 1 hour at 37°C. Then, cells were fixed for 3 min with paraformaldehyde 4% and F-actin was revealed by TRITC-conjugated phalloidin. Actin cytoskeleton was observed with fluorescence (human fibroblasts) or confocal (mouse fibroblasts) microscopes (Leica), and images were analyzed with ImageJ software.

The Polarity index was calculated as the ratio between the cell length and the cell width.

To analyze the proportion of the central stress fibers in each cell, the ratio of central F-actin integrated intensity over the total F-actin integrated intensity was calculated.

To perform the wound healing assay, mouse fibroblasts were seeded on plates coated with fibronectin (2.5 µg/ml) and cultured for one day, allowing cells to adhere and spread on the substrate completely. Then, the confluent monolayer of fibroblasts was scratched using a sterile p10 pipet tip. Cells were washed, supplied with complete medium, and imaged every 10 min for 16 h with a Zeiss Axiovert S100 TV2 microscope equipped with 10x lens. Quantification of the wound areas was performed with the ImageJ software using the polygon selection mode. Images at time zero were captured to record the initial area of the wounds and the migration of cells toward the wounds was expressed as percentage of wound closure.

Antibodies

The following antibodies were used in this study: rat anti-LAMP1 (Developmental Studies Hybridoma Bank, DSHB #1D4B-s); chicken anti-NF-M (Covance, #PCK-593P); hybridoma rat anti-MBP (kindly provided by Dr V. Lee); rabbit anti-phosphorylated-S6 (Ser235/236, Cell Signalling, #4858 and #2211); mouse anti-ß Tubulin (SIGMA, #T4026); rabbit anti ZO-1 (Thermofisher, #40-2200); mouse anti-Claudin 5 (Thermofisher, #35-2500); mouse anti-E-Cadherin (Becton Dickinson, #610181); rabbit anti-Calnexin (SIGMA, #C4731); rabbit anti-Actin (SIGMA, #A2066); mouse anti-RhoA (Santa Cruz, #sc418); rabbit anti-phosphorylated MLCII (Ser19, Cell Signalling, #3671); rabbit anti-phosphorylated MYPT1 (Thr853, Cell Signalling, #4563S); mouse anti-Vinculin (Millipore, #05-386).

For immunofluorescence, secondary antibodies included fluorescein (FITC)-conjugated and rhodamine (TRITC)-conjugated donkey anti-mouse or rabbit IgG (Jackson ImmunoResearch), and Alexa (488), #A11034.

For western blotting, secondary antibodies included horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-mouse immunoglobulins (Dako), and IRDye 800- and 680-conjugated goat anti-mouse, goat anti-rabbit and goat anti-rat IgG (Li-Cor Biosciences).

Western blot and pull down analyses

Protein lysates from mouse sciatic nerves for western blot analysis were prepared by homogenizing the tissue using a plastic pestel in a 1.5ml tube or for most antigens the Precellys Cryolys Evolution System (Bertin Instruments) with a lysis buffer containing 2% SDS, 25 mM Tris buffer pH 8.0, 95 mM NaCl, 10 mM EDTA, complete protease inhibitors (Roche), 1mM β-Glycerophosphate (SIGMA), and Phosphatase inhibitor Cocktail 1 and 2 (SIGMA). For the detection of phosphorylated antigens, samples were lysed with a buffer containing 1%TX-100, 50 mM Tris buffer pH 8.0, 150 mM NaCl, complete protease inhibitors (Roche), 1mM β-Glycerophosphate (SIGMA), Phosphatase inhibitor Cocktail 1 and 2 (SIGMA), and 10mM NaF and 1 mM Na_3VO_4. Protein quantification was performed using BCA assay (Pierce, Thermo Scientific).

For western blotting, SDS-PAGE gels were transferred to PVDF membranes (Millipore) or to nitrocellulose (Millipore) at 4°C in 20% methanol blotting buffer. Filters were blocked in 5% dry milk in PBS-0.1% Tween 20 overnight at 4°C and immunoblotted with primary antibodies diluted in 3% dry milk in PBS-0.1% Tween. For phosphorylated antigens, an additional blocking was performed for 30 min at RT in 3% bovine serum albumin (BSA) (Sigma-Aldrich), 0.5% gelatin, 0.1% Tween, 1 mM EDTA pH 8.0, 0.15 M NaCl, 10 mM Tris buffer pH 7.5, followed by incubation with primary antibodies diluted in the same blocking solution. Secondary antibodies, either horseradish peroxidase-conjugated (Dako) or IRDye 800- and 680-conjugated (Li-Cor Biosciences), were used and immunoblots revealed by using either ECL/ECL-prime developing systems and films for chemiluminescent detection (Amersham) or by Odyssey CLx Infrared Imaging System (Li-Cor Biosciences).

RBD-Rhotekin recombinant proteins were purified directly from bacterial extract on glutathione-Sepharose 4 Fast Flow beads. Protein lysates for pull-down experiments were prepared from 54 sciatic nerves at P2 and 4-6 sciatic nerves at P60 per genotype using 0.5%TX-100, 20 mM Tris buffer pH 7.5, 150 mM NaCl, and protease/phosphatase inhibitors as before. Equal amounts of protein lysates were incubated for 1 hr at 4°C with immobilized the GST-RBD-Rhotekin protein. In addition, as controls, control protein lysates were also incubated with 2 µl of 10mM GTPγs or with 2 µl GDPs 10mM for 15 minutes at 37°C. After blocking the reaction, lysates were then incubated

with conjugated sepharose beads and processed as the control and *Mtmr2* KO nerve sample lysates.

Immunofluorescence on teased fibers

Teased fibers were dried on a TESPA treated glass slide, rehydrated with PBS, permeabilized with 0.2% Triton X- 100 in PBS at room temperature for 5 minutes, and then blocked at room temperature for 2 h in 5% fish skin gelatin containing 0.5% Triton X-100 in PBS. Slides were incubated overnight at 4°C with primary antibodies.

At least 3 different slides were prepared per sciatic nerve per genotype and localization of ZO-1 was expressed as the percentage of SLI positive for ZO-1 staining on the total number of SLI morphologically recognized by phalloidin (Phalloidin-TRITC, #R145, Lifetech). At least three slides per nerve/animal were examined and the average was calculated. Final results were expressed as ± SEM with n=5 animals per genotype.

Morphological analysis

To perform morphometric analysis, digitalized images of fiber cross sections were obtained from corresponding levels of sciatic nerves with a 100x objective and Leica DFC300F digital camera (Milan, Italy). Five images per animal were analyzed using the Leica QWin software (Leica Microsystem) and the g-ratio calculated as the ratio between the mean diameter of an axon (without myelin) and the mean diameter of the same axon including the myelin sheath.

Neurophysiology

Mice were anesthetized with trichloroethanol, 0.02 ml/g of body weight, and placed under a heating lamp to avoid hypothermia. The sciatic nerve motor conduction velocity was obtained with steel monopolar needle electrodes: a pair of stimulating electrodes was inserted subcutaneously near the nerve at the ankle; and a second pair of electrodes was placed at the sciatic notch, to obtain two distinct sites of stimulation, proximal and distal, along the nerve.