CMTM6 expressed on the adaxonal Schwann cell surface restricts axonal diameters in peripheral nerves

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The velocity of nerve conduction is moderately enhanced by larger axonal diameters and potently sped up by myelination of axons. Myelination thus allows rapid impulse propagation with reduced axonal diameters; however, no myelin-dependent mechanism has been reported that restricts radial growth of axons. By label-free proteomics, STED-microscopy and cryo-immuno electron-microscopy we here identify CMTM6 (chemokine-like factor-like MARVEL-transmembrane domain-containing family member-6) as a myelin protein specifically localized to the Schwann cell membrane exposed to the axon. We find that disruption of Cmtm6-expression in Schwann cells causes a substantial increase of axonal diameters but does not impair myelin biogenesis, radial sorting or integrity of axons. Increased axonal diameters correlate with accelerated sensory nerve conduction and sensory responses and perturbed motor performance. These data show that Schwann cells utilize CMTM6 to restrict the radial growth of axons, which optimizes nerve function.
B y facilitating salutary propagation of nerve impulses \(^1,^2\), myelination of axons accelerates nerve conduction 20–100-fold \(^3\). In the peripheral nervous system (PNS), salutary conduction requires the close cellular association between myelinated axons and myelinating Schwann cells \(^4,^5\). Indeed, the development of functional axon/myelin-units involves complex signaling between neurons/axons, Schwann cells and the extracellular matrix \(^6,^7\). A critical morphogenetic prerequisite for myelination is the radial sorting of axons out of bundles \(^8\). At this stage, axons that have a threshold diameter of 1 µm are sorted out by immature Schwann cells for subsequent individual myelination. It is thought that receptor tyrosine kinases (erbB2, erbB3) on Schwann cells sense and integrate the abundance of neuregulin-1 type-III on the axonal surface as a measure of its diameter \(^8\). Several additional signaling molecules steer peripheral myelination, including the G-protein-coupled receptor ADGRG6/GPR126, neurotrophic factors, integrin β1, and Schwann cell-derived LGH interacting with axonal ADAM23 \(^9,^10\). Thus, multiple extrinsic factors regulate the formation of myelin by Schwann cells. Less is known about signaling from Schwann cells to axons. Multiple extrinsic factors regulate the formation of myelin by Schwann cells and axons, we biochemically purified a light-weight membrane fraction from sciatic nerves of wild-type mice (Supplementary Data 1) including known markers of axolemma and adaxonal myelin (ATP1A1, MAG, NFASC). By STED microscopy on sciatic nerve teased fiber preparations, CMTM6 localizes to Schmidt–Lanterman-incisures (SLI) and adaxonal myelin (Fig. 1c, d and Supplementary Fig. 3a), in which it displays periodic distribution with an autocorrelation peak visible at 200 nm (Fig. 1e). Cryo-immuno electron microscopy confirmed adaxonal localization of CMTM6 (Fig. 1g). By immunoblot-analysis of sciatic nerves, CMTM6 displayed developmentally increasing abundance (Fig. 1f) similar to myelin markers (MPZ, MAG, CNP). When subjecting teased fiber preparations of Cmtm6\(^{flox/−}\)mice (Supplementary Fig. 4a) to X-Gal histochemistry (Fig. 1h), the labeling pattern indicated gene expression in Schwann cells. We then used Dhh\(^{Cre}\)-driver \(^25\) for recombination of an engineered Cmtm6\(^{lox/−}\)-mice (Supplementary Fig. 4) to delete CMTM6-expression specifically in Schwann cells. Indeed, qRT-PCR (Supplementary Fig. 4c), immunoblotting (Fig. 1i) and immunolabeling of teased fiber preparations (Supplementary Fig. 3b) confirmed the absence of CMTM6-expression from sciatic nerves in Cmtm6\(^{lox/−}\;Dhh^{Cre}\)-mice (also termed conditional knockout, cKO, in the following). Together, these data indicate that CMTM6 is a Schwann cell protein localized to the adaxonal myelin membrane.

Results

Proteome analysis of an axogliasome-enriched nerve fraction. We hypothesized that CMTM6 mediates important functional interactions between Schwann cells and axons, we assessed the consequences of its deletion in Cmtm6\(^{−}\)-KO mice. Strikingly, Cmtm6\(^{−}\)-KO mice displayed strongly increased axonal diameters in phrenic nerves (Fig. 2a, b), dorsal roots (Fig. 2d, e), sciatic nerves (Supplementary Fig. 5a, b) and ventral tail nerves (Supplementary Fig. 5f). Axonal loss was not a feature (Fig. 2c, f and Supplementary Fig. 5c, e, g), at least up to an age of 2 months. This indicates a role for CMTM6 in restricting the radial growth of axonal diameters. Importantly, the ratio between myelin sheath thickness and axonal diameter in Cmtm6\(^{−}\)-KO mice was appropriate as indicated by normal g-ratios in all assessed nerves and ages (Supplementary Fig. 6).

Nerve conduction velocity and behavioral performance. Considering that nerve conduction velocity (NCV) in myelinated fibers is roughly linear proportional to axonal diameter and myelin sheath thickness \(^3,^17,^18\), we assessed NCV in peripheral nerves of Cmtm6\(^{−}\)-KO mice. Indeed, sensory nerve action potentials (SNAP) were enlarged, and sensory nerve conduction velocity (SNCV) was accelerated in Cmtm6\(^{−}\)-KO mice (Fig. 3a, b). Internode length, nodal and paranodal dimensions may also affect NCV \(^16,^19\) but were unaltered in Cmtm6\(^{−}\)-KO mice (Supplementary Fig. 7), implying that the increased NCV is owing to the increased axonal diameters and myelin thickness.

We then tested whether CMTM6-deficiency alters behavioral performance. Indeed, Cmtm6\(^{−}\)-KO mice displayed accelerated sensory reaction on the hot plate (Fig. 3c) and increased slipping frequency when walking over a regular grid (Fig. 3d). We also assessed breathing of Cmtm6\(^{−}\)-KO mice considering the increased axonal diameters in the phrenic nerve (Fig. 3a, b), which controls respiration. Wild-type C57BL/6N mice display breathing pauses of >750 ms/s termed spontaneous apnea \(^26\). Notably, these breathing pauses were absent in Cmtm6\(^{−}\)-KO mice (Fig. 3e). Together, among the identified proteins, CMTM6 (chemokine-like factor-like MARVEL-transmembrane domain-containing family member-6) constitutes an average relative protein amount of 50 PPM (10 %CV) in the AE in the AE according to quantitative proteome analysis (Supplementary Data 1). We selected CMTM6 for further analysis because immunoblot validated its enrichment in the AE compared to nerve lysate (Fig. 1b) similar to markers of axolemma and adaxonal myelin (ATP1A1, MAG, NFASC). Any variation of axonal diameters thus has a fln (NCV) along myelinated cellular matrix \(^5,^6\). A critical morphogenetic prerequisite for development of functional axon/myelin-units involves complex signaling between neurons/axons, Schwann cells and the extracellular matrix \(^3,^6\). A critical morphogenetic prerequisite for myelination is the radial sorting of axons out of bundles \(^7\). At this stage, axons that have a threshold diameter of 1 µm are sorted out by immature Schwann cells for subsequent individual myelination. It is thought that receptor tyrosine kinases (erbB2, erbB3) on Schwann cells sense and integrate the abundance of neuregulin-1 type-III on the axonal surface as a measure of its diameter \(^8\). Several additional signaling molecules steer peripheral myelination, including the G-protein-coupled receptor ADGRG6/GPR126, neurotrophic factors, integrin β1, and Schwann cell-derived LGH interacting with axonal ADAM23 \(^9,^10\). Thus, multiple extrinsic factors regulate the formation of myelin by Schwann cells. Less is known about signaling from Schwann cells to axons. Multiple extrinsic factors regulate the formation of myelin by Schwann cells and axons, we biochemically purified a light-weight membrane fraction from sciatic nerves of wild-type mice (Supplementary Data 1) including known markers of axolemma and adaxonal myelin (ATP1A1, MAG, NFASC). By STED microscopy on sciatic nerve teased fiber preparations, CMTM6 localizes to Schmidt–Lanterman-incisures (SLI) and adaxonal myelin (Fig. 1c, d and Supplementary Fig. 3a), in which it displays periodic distribution with an autocorrelation peak visible at 200 nm (Fig. 1e). Cryo-immuno electron microscopy confirmed adaxonal localization of CMTM6 (Fig. 1g). By immunoblot-analysis of sciatic nerves, CMTM6 displayed developmentally increasing abundance (Fig. 1f) similar to myelin markers (MPZ, MAG, CNP). When subjecting teased fiber preparations of Cmtm6\(^{flox/−}\)mice (Supplementary Fig. 4a) to X-Gal histochemistry (Fig. 1h), the labeling pattern indicated gene expression in Schwann cells. We then used Dhh\(^{Cre}\)-driver \(^25\) for recombination of an engineered Cmtm6\(^{lox/−}\)-mice (Supplementary Fig. 4) to delete CMTM6-expression specifically in Schwann cells. Indeed, qRT-PCR (Supplementary Fig. 4c), immunoblotting (Fig. 1i) and immunolabeling of teased fiber preparations (Supplementary Fig. 3b) confirmed the absence of CMTM6-expression from sciatic nerves in Cmtm6\(^{lox/−}\;Dhh^{Cre}\)-mice (also termed conditional knockout, cKO, in the following). Together, these data indicate that CMTM6 is a Schwann cell protein localized to the adaxonal myelin membrane.

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Increased axonal diameters are a plausible explanation for increased NCV and altered behavioral performance of Cmtm6-cKo mice.

Radial axonal growth is frequently associated with altered density and phosphorylation of neurofilaments. However, by electron microscopy (Supplementary Fig. 8a, b) and immunoblotting (Supplementary Fig. 8c–f), Cmtm6-cKo mice displayed unaltered neurofilament density and phosphorylation, implying that the increase of axonal diameters reflects genuine radial growth.
Fig. 2 Diameters of myelinated axons are abnormally increased when CMTM6 is lacking from Schwann cells. a Electron micrographs of cross-sectioned phrenic nerves reveal increased axonal diameters in Cmtm6-cKO compared to control mice at 2 months (2 mo). Scale bar, 5 μm. b Genotype-dependent assessment of myelinated axons on semi-thin sections confirms shift towards larger axonal diameters in phrenic nerves of Cmtm6-cKO compared to control mice. Data are presented as frequency distribution with 0.5 μm bin width; n = 1293 axons from n = 5 control mice and n = 1172 axons from n = 5 Cmtm6-cKO mice; Mean axonal diameter (control+/−Cmtm6-cKO) = 2.87 μm ± 0.84 μm; P = 2.2e−16 by two-sided Kolmogorov-Smirnow test of frequency distributions. For sciatic nerves and tail nerves, Supplementary Fig. 5 g-ratios Supplementary Fig. 6 g nodal parameters Supplementary Fig. 7: neurofilament density Supplementary Fig. 8; tamoxifen-induced deletion in adult mice Fig. 4. c Genotype-dependent quantification on semi-thin sections shows unchanged number of myelinated axons in the phrenic nerves of Cmtm6-cKO-mice. n = 5 mice per genotype; P = 0.1929 by two-tailed Student’s t-test. d Representative electron micrographs of cross-sectioned dorsal roots show increased axonal diameters in Cmtm6-cKO compared to control mice at 2 mo. Scale bar 5 μm. e Genotype-dependent assessment of the diameters of myelinated axons on semi-thin sections confirms the shift towards larger axonal diameters in dorsal roots of Cmtm6-cKO-mice. Data are presented as frequency distribution with 0.5 μm bin width, n = 10387 axons from n = 5 control mice and n = 9298 axons from n = 5 Cmtm6-cKO mice; mean axonal diameter (control+/−Cmtm6-cKO) = 2.47 μm ± 0.27 μm; P = 2.2e−16 by two-sided Kolmogorov-Smirnow test of frequency distributions. f Genotype-dependent quantification on semi-thin sections shows unchanged numbers of myelinated axons in the dorsal root of Cmtm6-cKO-mice. n = 5 mice per genotype; P = 0.3894 by two-tailed Student’s t-test. Data in c and f presented as mean ± SD. n.s. = non-significant P > 0.05; ***P < 0.001. Source data see Source Data file.

Fig. 3 Electrophysiological properties and behavioral performance of mice lacking CMTM6 from Schwann cells. a, b Electrophysiological measurement reveals a larger sensory nerve action potential (SNAP) and accelerated sensory nerve conduction velocity (SNCV) in the tails of Cmtm6-cKO compared to control mice at P75. n = 15 control and n = 12 Cmtm6-cKO mice; a P = 0.0061 and b P = 0.0386 by two-tailed Student’s t-test. c The latency of retracting a hindlimb upon a heat stimulus was reduced in Cmtm6-cKO compared to control mice. n = 16 control and n = 19 Cmtm6-cKO mice; P = 0.0089 by two-tailed Student’s t-test. d Compared to control mice, Cmtm6-cKO-mice showed an increased number of fore- and hindlimb slips while traveling a distance of 2 m on a regular grid. n = 12 mice per genotype; P = 0.0111 by two-tailed Student’s t-test. e Cmtm6-cKO-mice did not show breathing pauses longer than 750 ms. n = 6 mice per genotype; P = 0.0152 by two-sided Mann-Whitney rank-sum test. Data presented as mean ± SD; *P < 0.05; **P < 0.01. Source data see Source Data file.
Increased axonal diameters in tamoxifen-inducible Cmtm6-cKO. The results presented thus far were gained with mice, in which DhhCre-driven recombination of the Cmtm6-gene occurred early in Schwann cell-development, i.e., before the onset of myelination. We thus utilized PlpCreERT2-mice28 for tamoxifen-induced recombination in myelinating cells of young adult mice. Indeed, Cmtm6lox/lox;PlpCreERT2-mice injected with tamoxifen at the age of 2 months (termed Cmtm6-iKO in the following) displayed increased axonal diameters both 2 and 6 months after tamoxifen injection (PTI) (Fig. 4). CMTM6 thus continues to restrict the growth of axonal diameters after developmental myelination is largely completed in young adult mice.

Assessment of Remak bundles. Peripheral axons below 1 µm in diameter are ensheathed by non-myelinating Schwann cells in Remak bundles7. Developmentally, axons reaching a diameter above this 1 µm threshold are radially sorted out of bundles to be myelinated by individual Schwann cells. Considering that mutations in numerous genes impair radial sorting7, we assessed Remak bundle-associated axons in Cmtm6-cKO sciatic nerves. Surprisingly, the diameters of these non-myelinated axons were also significantly increased at P9 and, more strikingly, at 2 months (Supplementary Fig. 9a-d). Yet, the number of axons per Remak bundle was unaltered (Supplementary Fig. 9e, f). Importantly, Remak bundles did not comprise axons above the 1 µm threshold diameter (Supplementary Fig. 9g, h), and the frequency of developmentally sorted, promyelinated axons were unchanged (Supplementary Fig. 10c). We note that the diameters of myelinated axons were not yet significantly increased at the early developmental time point of P9 (Supplementary Fig. 10a, b); indeed, significance was reached by 1 month of age (Supplementary Fig. 10e, f). Together, CMTM6 restricts radial growth of...
both non-myelinated and myelinated axons without interfering with radial sorting or myelin biogenesis.

Expression of CMTM6 can affect the presence of programmed death ligand-1 (PD-L1/CD274) at the surface of cells in a tumor environment at least in vitro, a possible mechanism to regulate anti-tumor immunity. To test whether CMTM6 regulates CD274 in non-tumorigenic Schwann cells in vivo, we assessed Cmtm6-cKO mice. However, immunoblotting of homogenized peripheral nerves (Supplementary Fig. 11a–c) and immunohistochemistry of cross-sectioned sciatic nerves (Supplementary Fig. 11d) showed unaltered abundance and preferentially abaxonal localization of CD274. *Vice versa,* the abundance of CMTM6 was also unchanged in C274-Ko sciatic nerves by immunoblot (Supplementary Fig. 11e).

**Increased axonal diameters in Cmtm6-cKO;Mag-KO mice.** It was previously reported that mice lacking myelin-associated glycoprotein (MAG) have mildly reduced diameters of peripheral axons and our assessment confirmed this finding (Supplementary Fig. 12). Considering that the two adaxonal Schwann cell molecules that increase (MAG) or restrict (CMTM6) axonal diameters may functionally interact, we assessed their abundance and localization in the absence of the respective other. Abundance and localization of MAG is clearly independent of CMTM6 (Fig. 1i and Supplementary Fig. 3b). *Vice versa,* however, in Mag-Ko mice CMTM6 displays a mainly perinuclear localization in Schwann cells according to immunolabeling of teased fiber preparations (Fig. 5a, b), and its abundance was strongly reduced by immunoblotting (Fig. 5c). Thus, MAG facilitates normal abundance and localization of CMTM6 in Schwann cells.

Considering this interaction between MAG and CMTM6, we finally assessed axonal diameters in the absence of both proteins. Strikingly, axonal diameters were increased in Cmtm6-cKO;Mag-Ko double-knockout mice like in Cmtm6-cKO single-knockout mice (Fig. 5d, e). This indicates that loss-of-CMTM6-function overrides loss-of-MAG-function regarding axonal diameters (scheme in Fig. 6). Taken together, Schwann cells regulate the diameters of peripheral axons via adaxonal surface proteins including MAG and CMTM6.

**Discussion**

The velocity of impulse propagation along fibers in the nervous system depends on myelination, axonal diameters, nodal features, and internode length. In non-myelinated species, only a few vital reflexes are mediated by giant-diameter axons, probably owing to constraints by space and energy consumption. Indeed, NCV along non-myelinated fibers increases approximately proportional to the square root of the interior axonal diameter. Compared to non-myelinated fibers, variation of the diameters of myelinated axons affects NCV much more potently when considering the roughly linear proportionality. Strikingly, myelin facilitates saltatory conduction, thereby accelerating NCV 20–100-fold compared to unmyelinated axons of the same diameter. Theoretically, this would allow more fast-propagating axons with a reduction of required space and metabolic demand. It may thus appear counter-intuitive that so far no myelin-dependent mechanism was identified that restricts axonal diameters. Indeed, the expression of MAG in Schwann cells further increases the diameters of myelinated peripheral axons. Here, we used label-free proteome analysis to assess the constituents of a biochemical fraction from peripheral nerves termed axogloseosome-enriched fraction. Utilizing STED microscopy and cryo-immuno electron microscopy, we identified CMTM6 as a constituent of the adaxonal Schwann cell membrane. To our knowledge, CMTM6 is the first myelin protein found to actually restrict axonal diameters. Importantly, deficiency of CMTM6 does not impair the radial sorting of axons, myelin biogenesis, axonal integrity, internode length, or nodal/paranodal parameters. This implies that the function of CMTM6 in Schwann cells is specifically the restriction of axonal diameters.

**CMTM6 is a member of the chemokine-like factor-like MARVEL-transmembrane domain-containing (CMTM)-family of proteins.** Structurally, CMTM-proteins are predicted to comprise four transmembrane domains, short N- and C-terminal stretches protruding into the cytoplasm and two extracellular loop domains. Its name notwithstanding, CMTM6 does not comprise an apparent chemokine-like domain. Expression of CMTM6 was reported to limit anti-tumor immunity via regulating abundance and localization of programmed death ligand-1 (PD-L1/CD274) at the surface of cancer cells, at least in vitro. However, we did not find evidence of CMTM6 influencing CD274 in Schwann cells, at least in non-tumorigenic peripheral nerves in vivo. It will be interesting to elucidate in future work whether CMTM6 in Schwann cells affects the abundance or localization of other proteins or possibly lipids.

Our finding that Cmtm6-cKO nerves display normal g-ratios at multiple time points of sciatic nerve development implies that axonal diameters and myelin thickness effectively increase at the same time. We can thus not formally rule out that CMTM6 in Schwann cells may function in cis to increase myelin thickness, which, by an unknown indirect mechanism may secondarily enhance radial axonal growth. However, considering the adaxonal localization of CMTM6 and MAG it is likely that Schwann cells regulate axonal diameters directly in trans via a CMTM6-dependent mechanism. In turn, appropriate myelin sheath thickness in Cmtm6-cKO nerves is expected to emerge secondary to axonal diameter increase via neuregulin-1 signaling to erbB-receptors. We did not find evidence of altered neurofilament phosphorylation or density in Cmtm6-cKO axons, suggesting that other axonal molecules execute Schwann cell-dependent regulation of radial axonal growth. For example, axonal diameters are increased in mice lacking the cytoskeletal regulator a-adducin (ADD1), probably by regulating the diameters of axonal submembrane actin/spectrin rings. Though speculative, Schwann cells may restrict secondary axonal growth via axonal actin/spectrin rings. Circumstantially, CMTM6 in the adaxonal Schwann cell membrane displays periodic distribution similar to axonal β2-spectrin.

Given that the number of myelinated axons, internode length and nodal features are unaltered in Cmtm6-cKO nerves, the observed increase in SNCV is expected to be due to the shift toward increased axonal diameters. Notably, as measured, NCV is mainly determined by the fibers with the largest diameters. Yet, the normal g-ratios in Cmtm6 cKO nerves indicate that coinciding with the increased axonal diameters, myelin sheath thickness is also appropriately increased. Indeed, thicker myelin sheaths increase the transverse resistance, and thus reduce the transverse capacitance of a fiber, by itself correlating with increased NCV. The effects of axonal diameters and myelin thickness on NCV can thus not be experimentally uncoupled in the present model. Conversely, in theoretical calculations and simulation studies usually one parameter is theoretically altered while assuming all other relevant parameters as fixed, ultimately limiting direct correlation with experimental data and biological complexity. Finally, we cannot formally rule out the existence of yet unidentified additional factors that may affect NCV in Cmtm6-cKO nerves despite careful morphological and quantitative assessment.

It is of note that increased axonal diameters in Cmtm6-cKO mice were observed in four different peripheral nerves, implying that the effect is not limited to a particular nerve type. Yet, among...
the assessed nerves the shift was most considerable for the phrenic nerve, a vital nerve that controls breathing. Indeed, Cmtm6-cKO mice displayed anomalous breathing, i.e., the lack of normal breathing pauses. Interestingly, phrenic nerves display a bimodal frequency distribution of axonal diameters in control mice, which was altered toward unimodal, roughly normal distribution in Cmtm6-cKO mice. We cannot exclude that the effect of CMTM6-deficiency may be somewhat more pronounced for particular axonal sub-populations, e.g., for sensory versus motor fibers. However, it is not straightforward to conclude from bimodal versus unimodal distributions if a different susceptibility of sensory or motor axons exists when considering that bimodal distribution of axonal diameters are observed both in mixed (e.g., phrenic) and non-mixed nerves (e.g., ventral root, a motor nerve) while other mixed nerves do not display bimodal distribution (e.g., sciatic nerve, tail nerve). In the phrenic nerve, the frequency distributions of axonal diameters, as well as the total number of fibers, vary markedly among species. For example, phrenic fibers display bimodal distribution of diameters in cats and dogs, but unimodal distribution in rabbits. When comparing young and adult rats, the number of myelinated fibers is roughly similar; however, the unimodal distribution of phrenic fibers in young rats shifts to bimodal distribution in adult rats. Together, Cmtm6-cKO mice display a shift toward larger axonal diameters in multiple types of peripheral nerves and at multiple developmental stages; yet, it is difficult to interpret from the distribution patterns whether particular axonal sub-populations are more susceptible than others.

Expression of MAG and CMTM6 affect axonal dimensions toward enhancing and restricting radial growth, respectively, suggesting functional interplay of both adaxonal myelin proteins in optimizing nerve function. However, the functional relevance of the expression of MAG and CMTM6 is more complex. For example, MAG-deficiency causes early postnatal loss of
motoneurons\(^4\^-1\) and of peripheral axons\(^8\)\(^\text{,}\) whereas Cmtm6\(^\text{-}\)cKO mice did not display altered numbers of myelinated axons, at least up to an age of 2 months. Moreover, CMTM6-deficiency does not impair abundance or localization of MAG; vice versa MAG-deficiency leads to reduced abundance of CMTM6 in peripheral nerves coinciding with perinuclear accumulation and probably accelerated turnover in Schwann cells. Yet, axonal diameters are increased when both proteins are lacking, similar to deficiency of only CMTM6. This implies that CMTM6 may affect axonal diameters also if at low abundance and retained in intracellular compartments. Future work will show whether experimentally overexpressing CMTM6 restricts axonal diameters beyond its effect at wild-type levels. We hypothesize that besides MAG and CMTM6 additional, unidentified molecules are involved in the extrinsic regulation of secondary radial axonal growth by Schwann cells. If such molecules exist and are proteins, they are probably comprised in the axoglialome-enriched fraction. Further exploitation of the proteomic dataset may thus lead to their identification and enable characterizing their functional interplay with MAG and CMTM6.

In conclusion, CMTM6 emerges as a key player in the interactions between Schwann cells and axons. We speculate that counteracting the function of CMTM6 may restore the reduced axonal diameters and slowed NCV\(^1\^-4\) in rodent models of Charcot–Marie–Tooth spectrum disorders. If confirmed in future preclinical assessment, this may allow developing a rational therapy concept toward functional improvement for neuropathy patients.

**Methods**

**Mouse models.** Mice lacking expression of MAG (Mag\(^\text{null}\) mice; also termed Mag Ko) or CD274 (Cd274\(^\text{null}\) mice; also termed Cd274-Ko) were previously described\(^14\^-15\). Genotypes were determined by genomic PCR. Primers to determine the Mag allele were sense 5\'TTGCCGCGGCA ATGGGCTGAC, sense 5\'ACGGCA GGGA ATGGAGACAC and antisense 5\'ACCCGGCCGTCGGTTTGAT, amplifying a 600 bp band for the Mag\(^\text{null}\) allele and a 300 bp band for the WT allele. Primers to determine the Cldn4 allele were sense 5\'AGAACGGGAG CTGGACC TGTTTTGGAT, amplifying a 600 bp band for the Cldn4\(^\text{null}\) allele and a 300 band for the WT allele.

Experimental homozygous mutant mice and control littermates were the progeny of heterozygous mice. Frozen mouse sperm comprising the Cntn1\(^\text{null}/\text{null}\)/Cldn4\(^\text{null}/\text{null}\) allele (also termed Cntn1\(^\text{null}/\text{null}\)/Cldn4\(^\text{null}/\text{null}\)) was obtained from the European Mouse Mutant Archive (EMMA, Neuherberg/Munich, Germany) and used for in vitro fertilization, yielding mice harboring the Cntn1\(^\text{null}/\text{null}\)/Cldn4\(^\text{null}/\text{null}\) allele. Upon interbreeding with mice expressing FLIP recombinase (129S4/SvJaeSor-Plp\(^\text{CreERT}\)\(^\text{2}\) mice; termed control) mice and induced conditional knockout mice (Cmtm6\(^\text{cKo}\)) was by genomic PCR using sense primer (5\'-GCTGCTGTTT CTCATTGCTG; P1 in scheme in Supplementary Fig. 4a) in combination with antisense primers (5\'-ACGGCA ACCGGTAAT; P2 in scheme in Supplementary Fig. 4a) to determine the Cmtm6\(^\text{null}\) allele and a 300 bp band for the WT allele.

**Supplementary methods.** The diameters of peripheral axons are markedly increased (arrows) when Schwann cells lack CMTM6, indicating that Schwann cells regulate the diameters of peripheral axons via adaxonal surface proteins including MAG and CMTM6. Scheme created by Maria A. Eichel with Biorender.com.

**Fig. 6 Model of the role of adaxonal myelin proteins CMTM6 and MAG in regulating the diameters of peripheral axons.** a, b Compared to wild-type mice (a), the diameters of peripheral axons (light brown) are decreased in the absence of myelin-associated glycoprotein (MAG) (b) (see ref.\(^14\) and Supplementary Fig. 12), which is specifically expressed by Schwann cells (green). This implies that MAG-dependent signaling by Schwann cells increases axonal diameters\(^14\). c The diameters of peripheral axons are markedly increased (arrows) when Schwann cells lack CMTM6, indicating that Schwann cells utilize CMTM6 to restrict axonal diameters. d Diameters of peripheral axons are increased (arrows) when Schwann cells lack both MAG and CMTM6, indicating that loss-of-CMTM6-function overrides loss-of-MAG-function regarding axonal diameters. Schwann cells thus regulate the diameters of peripheral axons via adaxonal surface proteins including MAG and CMTM6. Scheme created by Maria A. Eichel with Biorender.com.
of Experimental Medicine. All animal experiments were performed in accordance with the German animal protection law (TierSchG) and approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) under license 33.19-42502-04-16/2128.

Biochemical purification of an axoglialosome-enriched fraction. To purify a light-weight membrane fraction enriched for the plasma membrane of peripheral axons and the adaxonal Schwann cell membrane, we adapted protocols originally established for the brain12–14. Briefly, for each biological replicate, we pooled the sciatic nerves from five mice. One day after postnatal day 75 (P75) rats were killed, the sciatic nerves were dissected from ten adult C57BL/6N mice at postnatal day 75 (P75) in centrifugation tubes containing 1.25 M sucrose supplemented with complete protease inhibitor tablets (Roche Diagnostics GmbH, Mannheim, Germany) for homogenization using Polytron PT3000 (Kinemetica, Eschbach, Germany). The nerve lysate was carefully overlaid with first 1 M then 0.9 M sucrose (see scheme in Supplement Figure 1) and centrifuged at 25,000 × g for 40 min at 4 °C. The supernatant was then centrifuged using a Beckman TLX rotor (Beckman Coulter, Krefeld, Germany) at 100,000×g for 1 h. After a Pasteur pipette, we then collected the myelin-enriched fraction at the 0.29 M/I M interface and the axoglialosome-enriched fraction (AEF) at the 1 M/1.25 M interface. Two subsequent washing steps and osmotic shocks were performed on both fractions. The interface fraction, which contains the plasma membrane mass, 1 ml i-cold dH2O was added and vortexed. Then, the tubes were filled up with i-cold dH2O up to 0.5 cm below the tube and centrifuged at 15 min at 100,000×g. After centrifugation, the supernatant was discarded carefully and the remaining fluid was removed using paper tissue. This washing step was repeated once. The AEF fraction was then loaded onto a 50 µl 10× TBS (with protease inhibitor (Roche)) centrifuge tube carried out using an ultracentrifuge with a rotor (OptimaTM TLX rotor (Beckman Coulter, Krefeld, Germany) at 100,000×g for 16 h at 4 °C. For each biological replicate, we pooled the sciatic nerves of 8-week-old male mice of the indicated genotypes. Frozen sciatic nerves were homogenized in Trizol (Life Technologies, ThermoFischer Scientific, Carlsbad, California, USA) and RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Integrity of the RNA was determined with the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, California, USA). RNA integrity was considered as a minimum RNA integrity of 9.0. cDNA was synthesized using random nonamer primers and SuperScript III RNA Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). A pipetting robot (Eppendorf) was used for pipetting the RNA into the 384-well optical plates (96) × 12 columns. The cDNA was purified with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) and RNA was analyzed in relative quantities to the mean of the standards Rpi13 and Ube2l3, which did not differ between genotypes. Statistical analysis was performed in GraphPad Prism 6.0. Primers were specific for Ctnm3 (forward 5′-GAGGACACCAA CGTACAGCAGT, reverse 5′-GGAGCAC CGTACAGCATAGT), Ctnm4 (forward 5′-GGAGGATCGCCCC CAGATACACCT, reverse 5′-ACCTCCGGTGAC CAAGTGACG), Ctnm5 (forward 5′-GATACGTGAA AGAAGCTCATCG, reverse 5′-AATGAGTGGGA...
GACAAAAATGA, Ctnmt7, forward 5′-TGCGCTCCAT AGTGATAGCC, reverse 5′-GCTGCTAGGC AGAGAAGC, Ctnmt8, forward 5′-CAGACGAGAGA AGGCTGAC, reverse 5′-TCCCTTCAATGGGAGGAC, and Ube2B1, forward 5′-AAGCAGAAATG AGGCTGAC, reverse 5′-TTCCTTCAATGGGAGGAC, and Ube2B1) were used. For quantitative
analysis of sciatric nerve teased fibers, images were obtained randomly at 10× or ×40 magnification.

**STED nanoscopy.** Sciatic nerves were fixed in 4% PFA for 45 min at 4 °C, per-
meabilized in ice-cold methanol for 20 min and rehydrated in PBS. Free-floating partially
 teased fibers were blocked for 45 min in PBS containing 1% BSA. Both primary and secondary
antibody incubations were performed for 1–2 h at RT or overnight at 4 °C in PBS supplemented
with 0.05% Triton X-100. After each step, samples were washed three times for 10 min in PBS with
0.05% Triton X-100. Finally, samples were completely teased on a coverslip and embedded in a
mounting media containing 9% [w/v] Mowiol (Sigma Aldrich, Darmstadt, Germany) supplemented
with DABCO (Sigma Aldrich, Darmstadt, Germany). For preparing cross sections, immunolabeled
nerves were teased on a coverslip and embedded in melamine resin following a controlled temperature
regime (24 h at RT, 24 h at 40 °C, and 48 h at 60 °C). Samples were washed again in PBS 3 × 2 min, secondary
48 mg of the resin catalyst p-toluenesulfonic acid monohydrate (Sigma Aldrich, Darmstadt,
Germany) in 0.576 ml distilled water and then adding 1344 g of 2,4,6-
Tris[bis(methoxymethyl)amino]-1,3,5-triazine (TCI, Tokyo, Japan)29. After complete
polymerization, sections of 200–300 nm thickness were cut using an ultra-
microtome (U MT, Leica Microsystems, Wetzlar, Germany). Sections were mounted directly on to glass
coverslips and embedded in mounting media. Primary antibodies were specific for CMTM6 (OríGene, Cat# TA232204, 1:100) and
betalin-spectrin (BD Biosciences, San Jose, United States, Cat# 612563, 1:200). Secondary antibodies (sheep anti-mouse, Dianova, Hamburg, Germany, Cat# 515-003-000
and 515-003-145) and donkey anti-rabbit (Dianova, Hamburg, Germany, Cat# 111-005-003) were labeled with STAR580
(zeiss, Göttingen, Germany, Cat# ST580-0002) and STAR635P (Abberior, Göttingen, Germany, Cat# ST635P-002) and used at 1:100 dilution.
Imaging was performed on a two-color Abberior STED 775 QUAD scanning microscope (Abberior Instruments GmbH, Göttingen, Germany) equipped with an Olympus
vertical stand and an Olympus Planapo ×100/1.4 Oil lens. Excitation was achieved with 488, 561, and 640 nm pulsed lasers, while for depletion pulsed lasers at 775 and 595 nm are available. Images were processed and visualized using the ImSpector software package (Max-Planck Innovation; version 14.1-16.1)
and ImageJ (imagej.nih.gov/ij; version 1.50b). Smoothing was performed using a 1-
pixel low-pass Gaussian filter within the software ImSpector. Brightness and contrast
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(SNAP) reached a plateau. SNAP measurements were averaged over 20 stimuli. The sensory nerve conduction velocities (nNCV) were calculated from sensory action potential latency measurements over the 50 mm distance.

**Behavioral analysis.** All phenotypical analyses were performed by the same investigator blinded toward genotypes using 8–10-week-old mice. To assess motor capabilities (Fig. 3d), mice were placed on a metal grid (1 cm grid size) and allowed to run a distance of 2 m while being videotaped. The number of fore- and hindlimb slips were assessed on a slow-motion video. Assessment was performed once per mouse, at a time, once per mouse, and without prior habituation to the grid. To assess sensory reaction (Fig. 3e), mice were placed on a hot plate (Leica HI 1220, Nussloch, Germany) which was heated to constant 52 °C and surrounded by a clear acrylic cage (open top). A timer was started once mice were placed on the hot plate and the time until mice respond with either licking or retracting one of their hindlimbs was stopped and measured as retraction latency. Afterward mice were immediately removed from the hot plate, placed back in the home cage. Assessment was performed bluntly in the same mouse at a time, once per mouse, and without prior habituation to the hot plate62. All behavioral experiments and analyses were performed blinded to the genotype.

**Plethysmography.** Breathing was analyzed by unrestrained whole-body-plethysmography (Fig. 3e)63. Mice were placed in a plethysmograph chamber and could habituate for at least 12 min. Breathing cycles from a subsequent period of 3 min were analyzed. Since mice were allowed to explore the chamber freely, some pressure changes were induced from.sources. During analysis, we did not discriminate between respiratory cycles associated to different types of behavior. The chamber was used in the flow-through configuration with a negative bias flow of 150 ml/min introduced by a CO2/O2 sensor (ADInstruments, Sydney, Australia)64. Pressure difference between the recording chamber and a reference chamber were captured by a DP103-12 pressure transducer (Validyne Engineering, Northridge, CA, United States) and passed through a sine wave carrier demodulator (CD-15, Validyne Engineering) for digitization (100 Hz sampling rate) with an analog-digital interface (PowerLab/4s) and LabChart-software (ADInstruments; version 8.1.13). The signal was smoothed (averaging 50 samples) and offline band pass filtered (3–20 Hz) to remove noise and movement artifacts. The peak detection module of LabChart was used to identify peaks of inspiratory flow. The respiratory rate was calculated as the reciprocal of the averaged peak-to-peak interval using Excel (Microsoft). Intervals longer than 750 ms were considered as breathing pauses, and the number per minute was calculated. Plethysmography and analyses were done blinded to the genotype.

**Statistics and reproducibility.** Statistical analyses were mainly performed in GraphPad Prism (GraphPad Software, Inc., San Diego, United States). Data are mainly shown as mean ± SD (error bars). 11a) and the distribution was assumed to be normal but was not formally tested, except for plethysmography data. Sample sizes were not predetermined but are as commonly used in the field. Exact sample size/number of mice is mostly shown in the figures and indicated in the figure legends. Outlier tests were performed on all data except axonal diameter values using GraphPad (https://www.graphpad.com/quickcalcs/Grubbs1.cfm). Only for nerve conduction velocity measurement one outlier was found in the control group; no further outliers were identified. For comparing two groups, unpaired two-tailed Student’s t-test was applied. For qRT-PCR (Supplementary Fig. 3c), two-way analysis of variance (ANOVA) with Sidak post hoc correction (Fig. 3). The p-value was calculated as the reciprocal of the averaged peak-to-peak interval using Excel (Microsoft). Intervals longer than 750 ms were considered as breathing pauses, and the number per minute was calculated. Plethysmography and analyses were done blinded to the genotype.

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Author contributions

M.A.E. and H.B.W. designed experiments. U.D. and S.T. performed proteome analysis. E. Der and H.B.W. performed and analyzed STED microscopy. K.P. and T.K. performed and analyzed electrophysiology. S.H. performed and analyzed phlethysmography. M.A.E., V.-I.G., T.B., K.-A.L., C.M., R.B.J., and K.K. performed all other experiments. M.A.E., W.M., K.-A. N., and H.B.W. analyzed and interpreted data. H.B.W. initiated and directed the project and obtained funding. M.A.E. and H.B.W. wrote the paper with assistance and proof-reading by all authors.

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Competing interests

The authors declare no competing interests.

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