Mtmr13/Sbf2-deficient mice: an animal model for CMT4B2

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Charcot-Marie-Tooth (CMT) disease denotes a large group of genetically heterogeneous hereditary motor and sensory neuropathies and ranks among the most common inherited neurological disorders. Mutations in the Myotubularin-Related Protein-2 (MTMR2) or MTMR13/Set-Binding Factor-2 (SBF2) genes are associated with the autosomal recessive disease subtypes CMT4B1 or CMT4B2. Both forms of CMT share similar features including a demyelinating neuropathy associated with reduced nerve conduction velocity (NCV) and focally folded myelin. Consistent with a common disease mechanism, the homodimeric MTMR2 acts as a phosphoinositide D3-phosphatase with phosphatidylinositol (PtdIns) 3-phosphate and PtdIns 3,5-bisphosphate as substrates while MTMR13/SBF2 is catalytically inactive but can form a tetrameric complex with MTMR2, resulting in a strong increase of the enzymatic activity of complexed MTMR2. To prove that MTMR13/SBF2 is the disease-causing gene in CMT4B2 and to provide a suitable animal model, we have generated Mtmr13/Sbf2-deficient mice. These animals reproduced myelin outfoldings and infoldings in motor and sensory peripheral nerves as the pathological hallmarks of CMT4B2, concomitant with decreased motor performance. The number and complexity of myelin misfoldings increased with age, associated with axonal degeneration, and decreased compound motor action potential amplitude. Prolonged F-wave latency indicated a mild NCV impairment. Loss of Mtmr13/Sbf2 did not affect the levels of its binding partner Mtmr2 and the Mtmr2-binding Dlg1/Sap97 in peripheral nerves. Mice deficient in Mtmr13/Sbf2 together with known Mtmr2-deficient animals will be of major value to unravel the disease mechanism in CMT4B and to elucidate the critical functions of protein complexes that are involved in phosphoinositide-controlled processes in peripheral nerves.

INTRODUCTION

Charcot-Marie-Tooth (CMT) disease, also called hereditary motor and sensory neuropathies (HMSN), comprise a genetically heterogeneous group of inherited disorders affecting myelinated axons in the peripheral nervous system (1–3) with a prevalence of approximately 1:2500 (4). The disease is characterized by progressive distally accentuated muscle weakness and atrophy. Based on clinical, electrophysiological, and histological data, CMT has been subdivided into demyelinating and axonal forms. Demyelinating neuropathies are diagnosed by reduced nerve conduction velocity (NCV). Axonal loss and muscle atrophy are also observed most likely as secondary effects due to the tight interaction and communication between myelinating Schwann cells, axons and muscle cells (5). Axonal forms of CMT are characterized by a reduction of the compound muscle action potential (CMAP) amplitude due to a loss of myelinated axons (6). Dissection of the cellular functions of the gene products altered in CMT as well as the generation of detailed pathophysiological models are of crucial importance to understand the underlying common as well as distinct disease mechanisms which may affect Schwann cells, axons or both (2).
The subtype CMT4B is a severe demyelinating autosomal recessive inherited neuropathy with onset in early childhood. Histological analysis of human nerve biopsies revealed demyelination and focally folded myelin as the particular pathological hallmark (7). The disease was mapped to chromosome 11q22 and 11p15 indicating that mutations in two different genes lead to indistinguishable pathologies (8–10). Further mapping refinements and molecular cloning lead to the identification of Myotubularin-Related-Protein-2 (MTMR2) and MTMR13/ Set-Binding-Factor-2 (SBF2) as the disease causing genes of CMT4B1 and CMT4B2 (11–13). Mtmr2-deficient mice have been generated and mimic the pathology of CMT4B1 closely (14,15).

MTMR2 and MTMR13/SBF2 belong to the family of myotubularin-related proteins (16). This family consists of 14 members in human and orthologues have been found throughout the eukaryotic kingdom but not in bacteria. Myotubularin, the founding member of the family, has been originally identified as the disease-causing gene in X-linked myotubular myopathy (17). The domain signature of MTMRs is defined by the presence of a phospoinositide (P1)-binding PH-GRAM (pleckstrin homology–glucosyltransferases, Rab-like GTPase activators and myotubularins)-homology domain, a phosphatase domain, and a coiled coil motif. The phosphatase domain was initially described as a potential protein tyrosine/dual specific phosphatase, but the PIs (PtdIns)-3-phosphate (P) and PtdIns-3,5-P2 (with myotubularin phosphatase activity specific for the D3 position of the inositol ring) have emerged as the main substrates (16,18). The membrane-associated PtdIns-3-P and PtdIns-3,5-P2 regulate trafficking in the endosomal–lysosomal pathway suggesting that the disease mechanisms in MTMR-based diseases are likely to be related to these processes. Interestingly, disturbances of regulatory mechanisms involving PIs seem to become a recurrent theme in CMT since mutations in FIG4, a D5 phosphatase using PtdIns-3,5-P2 as substrate, have been recently described to cause CMT4J (19).

Six members of the myotubularin family, including MTMR13/ SBF2, contain inactivating substituations in the phosphatase domain. Catalytically inactive MTMRs can interact with active MTMRs as modulators of the phosphatase activity, substrate traps or they influence the subcellular localization. Specifically and in addition to the common motifs, MTMR13/ SBF2 contains a classical Pths-3,4,5-P3-binding PH domain (20), a DENN (differentially expressed in normal versus neoplastic) domain and a stretch of 500 amino acids with no homologies to other proteins (Fig. 1A). MTMR13/ SBF2 exists in cells independently as a homodimer, as well as in complex with a homodimer of MTMR2 (20–22). The latter association dramatically increases the enzymatic activity of the binding partner MTMR2 (20).

Here, we have generated Mtmr13/Shb2-deficient and Mtmr2// Mtmr13/Shb2 double-deficient mice to dissect the role of this pair of myotubularins in health and disease in vivo.

RESULTS

Gene-trapped disruption of Mtmr13/Shb2

We have used mouse embryonic stem cells carrying a gene trap insertion in the Mtmr13/Shb2 locus (XH212; Bayogenomics Gene Trap Resource) for the generation of an Mtmr13/Shb2-deficient mutant mouse line using established procedures (15). The insertion site of the gene trap cassette was mapped 1267 bp downstream of exon 14 of Mtmr13/Shb2 (Fig. 1A). Based on this information, primers I, II and III were designed to discriminate between different alleles and for genotyping (Fig. 1B). Genotyping PCR for homozygous (−/−), or heterozygous (+/−) Mtmr13/Shb2 mutant mice, or wt (+/+). (C) Western blot analysis of sciatic nerve lysates of Mtmr13/Shb2-deficient (−/−) and wt (+/+ control mice. A rabbit polyclonal antibody was used to detect the 210 kDa Mtmr13/ Shb2 protein. Purified CBP-tagged Mtmr13/Shb2 protein from a baculovirus expression system served as positive control (20). Bands below 210 kDa represent degradation products of Mtmr13/Shb2.
ratios. Upon visual inspection, the behavioral phenotype of both Mtmr13/Sbf2-deficient and Mtmr2//Mtmr13/Sbf2-double deficient mice appeared normal compared to control littermates. Starting at the age of 2 months, however, both mutant lines showed an unusual but very mild hind limb clamping upon tail suspension (data not shown). Double-heterozygous Mtmr2//Mtmr13/Sbf2 mutant animals appeared indistinguishable from their wt littermates up to 15 months of age (latest time point examined).

Expression analysis of Mtmr2, Mtmr13/Sbf2 andDlg1/Sap97 in sciatic nerves of mutant animals

In a first step, we analysed whether alterations in Mtmr2 or Mtmr13/Sbf2 expression alter the protein levels of its respective binding partner in the sciatic nerve of mutant animals. Western blot analysis of sciatic nerve lysates from 12-month-old animals revealed that Mtmr2 levels were unchanged in Mtmr13//Shy2-deficient mice (Fig. 2A). Vice versa, Mtmr13/Sbf2 levels remained unaltered in Mtmr2-deficient mice (Fig. 2B). Bolino et al. (14) and Bolis et al. (23) have reported an interaction of Mtmr2 with Sap97. They detected also reduced expression of Sap97 in the sciatic nerves of their strain of Mtmr2-deficient mice. Here, we confirmed these findings in our strain of Mtmr2-mutant mice (15). We continued to test whether loss of Mtmr13/Sbf2 would also reduce the levels of Sap97 by reasoning that loss of the Mtmr2 interaction partner Mtmr13/Sbf2 might affect indirectly the interaction between Mtmr2 and Sap97 within a putative larger complex. However, the levels of Sap97 were not significantly different to wt in Mtmr13//Sbf2-deficient mice (Fig. 2C). Consistent with these findings, we found a comparable reduction of Sap97 in Mtmr2//Mtmr13//Sbf2-double deficient mice as in Mtmr2-single mutants (Fig. 2D). We conclude that the interaction of Mtmr13//Sbf2 with Mtmr2 and the interaction between Mtmr2 and Sap97 are unlikely to be intimately connected. This conclusion was also supported by co-immunoprecipitation experiments revealing no apparent differences in the interaction of Mtmr2 with Sap97 between wt and Mtmr13/Sbf2-deficient sciatic nerves (data not shown).

Behavioral analysis

Visual examination of both Mtmr13/Shy2-deficient and Mtmr2//Mtmr13//Shy2-double-deficient mice revealed no obvious signs of tremor or major functional disability, similar to what we had observed in the Mtmr2-deficient model of CMT4B1 (15). Therefore, we performed a rotarod test to assess whether a behavioral difference related to motor function was detectable using this assay. At the age of 4 months, Mtmr13/Shy2-deficient mice were not distinguishable from wt mice (Fig. 3A). At 12 months of age, however, Mtmr13/Shy2 mutant animals showed a significantly reduced performance compared to their wt control littermates (Fig. 3B) and similar results were obtained with Mtmr2//Mtmr13//Shy2-double-deficient mice (Fig. 3C). No significant difference between the two mutant strains was observed. Based on the results at this point, we decided not to perform further detailed studies on Mtmr2//Mtmr13//Shy2-double deficient mice. Qualitatively, we did not observe major pathology differences compared to the single Mtmr13/Shy2 mutants (data not shown).

Electrophysiology of peripheral nerves of Mtmr13//Shy2-deficient mice

Motor nerve conduction of sciatic nerves was studied in Mtmr13//Shy2-deficient mice at the age of 4 and 12 months (Fig. 4). In 4-month-old animals, the CMAP amplitude was not significantly different in Mtmr13//Shy2-deficient mice compared to wt (18.0 ± 3.7 versus 14.0 ± 3.6 mV). At 12 months of age, however, Mtmr13//Shy2-deficient mice showed a significant reduction of the CMAP (7.3 ± 1.1 versus 16.3 ± 2.2 mV in wt mice) and a slightly prolonged F-wave latency of 5.1 ± 0.2 ms (4.4 ± 0.1 ms in wt). Such a very mildly prolonged F-wave latency was already present in 4-month-old Mtmr13//Shy2-deficient animals (4.7 ± 0.2 versus 4.2 ± 0.2 ms in wt). A tendency towards NCV slowing was not significant at both ages examined.

Progressive myelin abnormalities in peripheral nerves of Mtmr13//Shy2-deficient mice

Focally folded myelin is the histological hallmark of both CMT4B1 and CMT4B2. Thus, we focused our analysis on the presence, time course and distribution of focal dysmyelination. At 4 months of age, Mtmr13//Shy2-deficient mice showed numerous sciatic nerve fibres with focally folded myelin (Fig. 5). Abnormalities included both infoldings and outfoldings of the entire myelin sheath which particularly affected large calibre fibres but also smaller, thinly myelinated fibres. Non-myelinated fibres appeared normal. At higher magnification, sciatic nerve cross sections of mutant mice were littered with various degrees of dysmyelination ranging from focal budding of the myelin sheath to multiple or combined infoldings and outfoldings. Abnormal myelin structures were first but rather sporadically observed in the sciatic nerves of Mtmr13//Shy2-deficient mice already at the age of 3 weeks (Fig. 6A and B). Irregular myelin folds were easily detectable although of low complexity. Quantitative analysis of these pathological structures revealed a significant increase in numbers compared to wt animals (Fig. 7A). Next, since CMT is usually associated with a clinically progressive time course, we followed the qualitative and quantitative progression of the pathology over time. Thus, we examined sciatic nerves at the age of 4 and 15 months (Fig. 6C–F; Fig. 7A). We found a progressive increase in both, the complexity (age-dependent tendency to double and multiple infoldings and outfoldings) as well as the number of misfolded myelin sheaths in Mtmr13//Shy2-deficient animals suggesting that the disease process is continuous throughout the timeframe analysed. Particularly in older mice, some signs of axonal damage were recognized although we did not observe an obvious major loss of myelinated axons. Next we reasoned, considering the suggested molecular function of myotubulins, that the misfoldings of myelin observed in Mtmr13//Shy2-deficient mice might be due to altered vesicular trafficking and myelin-membrane turnover. This could potentially lead to generally altered myelin sheath thickness as we have observed in myelin
mutants with multi-folded myelin extensions in the central nervous system (24). However, using computer-aided morphometry, we did not detect significant alterations in myelin thickness and axon diameter in Mtmr13/Sbf2-deficient sciatic nerves (Fig. 7B and C), consistent with our identical previous findings in Mtrm2-deficient mice (15). In agreement with these data, we did not observe Schwann cell onion bulb formation as the classical indicators of demyelination and remyelination.

Figure 2. Western blot analysis of the relative Mtmr13/Sbf2, Mtmr2 and Dlg1/Sap97 levels in sciatic nerve lysates from 12-month-old wt, Mtmr2-deficient (Mtmr2−/−), Mtmr13/Sbf2-deficient (Sbf2−/−) and Mtmr2/Mtmr13/Sbf2-double deficient (MTMR2−/− Sbf2−/−) mice. Each pool contains the sciatic nerves from two or three animals. Protein levels of Mtmr13/Sbf2, Mtmr2 and Sap97 were quantified by normalizing the relative protein levels to beta-actin, illustrated in a bar chart. (A) The relative protein level of Mtmr2 does not differ significantly between wt and Mtmr13/Sbf2-deficient (Sbf2−/−) sciatic nerves. (B) The Mtmr13/Sbf2 expression level shows no difference in Mtmr2-deficient (Mtmr2−/−) compared to wt control lysates of sciatic nerves. The Sap97 protein levels in Mtmr2-deficient (Mtmr2−/−) lysates are significantly reduced (P < 0.05, Student’s t-test). (C) The relative protein level of Sap97 protein level is not altered in the lysates of Mtmr13/Sbf2-deficient (Sbf2−/−) mice compared to wt control lysates. (D) The relative protein levels of Mtmr13/Sbf2, Sap97 and Mtmr2 were compared in wt and Mtmr2/Mtmr13/Sbf2-double deficient (MTMR2−/− Sbf2−/−) sciatic nerves. As expected, no expression of Mtmr13/Sbf2 or Mtmr2 protein was found. The Sap97 protein is significantly reduced compared to wt (P < 0.05; Student’s t-test) in the lysates of the double mutant mice. Error bars indicate the SEM.
Motor and sensory nerves are affected in Mtmr13/Sbf2-deficient mice

CMT4B2 is classified as a motor and sensory neuropathy. Thus, we analysed whether these mixed symptoms were reflected in pathological aberrations in both motor and sensory nerves. We chose to examine the ventral roots containing exclusively axons derived from motor neurons and dorsal roots for sensory axons. In both locations, the pathological hallmarks of myelin misfoldings were barely detectable at the age of 4 months (Fig. 8A and B), in contrast to the more distally (with respect to the neuronal cell bodies) located sciatic nerve (Fig. 6D) which contains both motor and sensory axons. These findings indicate that the pathology is more severe in distal compared to proximal parts of PNS nerves. At the age of 15 months, myelin misfoldings were prominently visible in ventral and dorsal roots suggesting that both motor and sensory nerves become affected in a progressive manner over time (Fig. 8C and D).

Complex structures of misfolded myelin in Mtmr13/Sbf2-deficient mice

In order to get some detailed insights into the fine structure of aberrant Schwann cell-axon units in our mutant mice, we performed ultrastructural analysis using electron microscopy. Figure 9 shows a collection of pictures to provide a sampling overview of the different aberrant structures that we have observed. The myelin misfoldings invariably originated from compacted myelin and showed an identical number of myelin lamellae in both myelin misfoldings and the myelin sheath they originated from (Fig. 9D, quantitative data not shown). Within myelin misfoldings, we observed normal compaction and periodicity of the myelin sheath. In general, the impression of pathological alterations was dominated by myelin outfoldings with strongly variable complexity (Fig. 9A–D). The most common formation consisted of one or multiple outfoldings of different size adjacent to a myelinated large calibre axon, and multiple outfoldings showed a tendency to form groups (Fig. 9C). Aberrant myelin loops were always ensheathed by the plasma membrane of the related Schwann cell (Fig. 9A). Myelin infoldings were also prominent. They usually presented as finger-like inversions of the myelin sheath (Fig. 9E) or circular inclusions within the myelinated nerve fibre (Fig. 9F). Entrapping Schwann cell cytoplasm on their outer surface, they protrude far into the axon and displace the axoplasm (Fig. 9E). The formation of double circles (Fig. 9F and G) is most likely due to the retrograde inversion of a single infolding since, at higher magnifications, we observed axonal material in the gap between the inner and outer infolding. Alternatively, the nesting of two distinct infoldings may have led to the double-circle appearance.

On electrophysiological examination, older Mtmr13/Sbf2-deficient mice showed a reduction of the CMAP indicating axonal loss or damage. Thus, we also carefully looked for axonal pathology. Degeneration of whole Schwann cell-axon units (Fig. 9K and L) were not observed in young mutants, but were sporadically present at 4 months and rather frequent at 15th month. Lateral dislocation of the axon by myelin infoldings and vacuolar alteration of the axoplasm (Fig. 9I and J) was often observed. We occasionally found also myelinated nerve fibres not affected by myelin misfoldings but with a conspicuous widening of the periaxonal space (Fig. 9H).

The amazing complexity of myelin misfoldings and the consequences for the affected Schwann cell-axon units, however,
can be best appreciated on longitudinal sections (Fig. 9M–P). Complex myelin formations are preferentially, although not exclusively, located in the nodal and paranodal regions. At the same time, misfoldings contain axoplasm-like structures suggesting the entrainment of axonal parts during their formation (Fig. 9M and P). Occasionally, misfoldings of the myelin sheath had a larger diameter than the original nerve fibre, or lead to spatial disarrangement of the normal fibre anatomy like affecting the node of Ranvier (Fig. 9N). Relating these structural changes back to the molecular and cellular functions (and misfunctions in disease) of MTMR13/SBF2 will be a major challenge for the future. On a pure morphological level, the pathological observations in Mtmr13/Sbf2−/− mice are very reminiscent of our observations in Mtmr2−/− animals consistent with the biochemical finding suggesting a crucial role for a high-molecular complex containing both MTMR13/SBF2 and MTMR2 in the regulation of membrane trafficking.

**DISCUSSION**

We report the generation and analysis of the first animal model for the human neuropathy CMT4B2 by disruption of Mtmr13/Sbf2 in mice. To our knowledge, this is also the first animal model for a genetically inherited human disease caused by mutations affecting an inactive member of the myotubularin phosphatase family. The aim of this study was three-fold. First, we wanted to provide the formal proof that mutations in Mtmr13/Sbf2 are indeed causative for CMT4B2. Second, we anticipated that the generation of an animal model for this particular form of CMT will contribute critically to the dissection of the underlying disease mechanisms, especially with regard to the development and the progression of the disorder but also by providing appropriate means for further cell biological and biochemical analysis. Finally, recent reports relying on the analysis of appropriate CMT animal models have provided hopeful projections for treatment strategies encouraging the development of further refined animal models (25–28).
Initial analysis of Mtmr13/Shb2-deficient mice as animal models for CMT4B2 revealed behavioural deficits in the rotarod assay not in 4 months but in 12-month-old mice indicating significant motor impairments. This finding is consistent with the general clinical features of CMT4B2 patients that involve motor and sensory disturbances (11,12). Further analysis by light microscopy and ultrastructural analysis of peripheral nerves by electron microscopy showed prominent and complex myelin infoldings and outfoldings reminiscent of CMT4B2 (9). Our mouse model allowed us to follow the development of these pathological features in a temporal fashion by analysing peripheral nerves at different time points. Both the number and complexity of the unusual myelin figures increased gradually over time in a continuous fashion with little inter-animal variability. Some signs of axonal damage were also observed, in particular in older animals. Nevertheless, we did not find obvious evidence for primary demyelination and remyelination as judged from the lack of major Schwann cell onion bulb formations in the mouse mutant nerves up to the age of 15 months (last time point examined) and no decrease in myelin thickness. Consistent with these findings, we observed only minor signs of reduced NCV (prolonged F-wave latency). This appears to be in contrast to the strongly reduced NCV in CMT4B2 patients (9,12). Although the reason for this discrepancy is not yet clear, it should be noted that animal models for the related condition CMT4B1, associated with Mtmr2 deficiency, showed also much milder electrophysiological abnormalities compared to the corresponding human patients (14,15). It remains to be determined whether this feature is due to some specific mechanistic aspects of CMT4B-type neuropathies. Similarly, we did not observe a striking loss of myelinated large calibre axons in Mtmr13/Shb2-deficient mice as has been reported based on qualitative assessments of sural nerve biopsies of CMT4B2 patients (9,12). Demyelination and in particular secondary axonal loss might be more pronounced in humans in these distally accentuated neuropathies due to basic physiological species differences that include much longer nerves and the longer life span of humans compared to mice (5). We found, however, a correlation between the rather poor performance of the mutants in the functional testing on the rotarod and the reduction of the CMAP amplitude. Since we did not observe major axonal loss, we favour the hypothesis that distal conduction block occurs in a significant proportion of fibres, possibly due to axonal constrictions by major myelin infoldings. Perinodal abnormalities may also lead to conduction block in the affected fibres resulting in reduced CMAP amplitudes.

The progressive formation of aberrant myelin outfoldings and infoldings is a robust feature caused by both MTMR2 and MTMR13/SBF2-deficiency in human and mice. This poses the major question how these abnormalities are generated and lead to disease. The first important conceptual point in this context concerns the determination of the cell type(s) which require the correct function of these proteins in a cell-autonomous or potentially non-cell autonomous...
manner. Although it appears likely from the data presented here and other reports that myelinating Schwann cells are malfunctioning in CMT4B1 and CMT4B2, the close interactions between axon and Schwann cells require a careful direct experimental analysis. Using conditional gene targeting in the mouse, Bolis et al. (23) have elegantly shown that loss of MTMR2 is sufficient to cause the typical myelin abnormalities in a Schwann cell-autonomous manner. Complementary experiments will be necessary to answer this question also for MTMR13/SBF2.

Although it is not known in detail which signalling cascades are involved in the molecular pathogenesis of CMT4B1 and CMT4B2, the related processes involve regulation at various levels (20,22,29). MTMR13/ SBF2 appears to act at least (i) as a regulator of the MTMR2 phosphatase activity, (ii) as a protector of the PI substrates, and (iii) as an adaptor regulating the localization of active MTMR2. Membrane PI-binding GRAM-PH and PH domains in MTMR2 as well as MTMR13/ SBF2 play a major role in at least some of these processes, possibly by regulating vesicular and membrane trafficking in Schwann cells since loss of these proteins is associated with uncontrolled folding of myelin and CMT4B. Recently, mutations in the Rho-type GTPase guanine nucleotide exchange factor (GEF) Frabin/FGD4 have been shown to be associated with CMT4H characterized by abundant myelin outfoldings (30,31). In addition to a RhoGEF (DH) domain, Frabin contains two PH domains and a presumably PI-binding FYVE domain. It is tantalizing to speculate that the presence of PI-binding domains in the disease-causing MTMR2, MTMR13/ SBF2 and Frabin/FGD4 genes and the striking correlation with myelin outfoldings might be related to overlapping disease mechanisms. Further, how Dlg1/Sap97 and neurofilament light chain protein as described interaction partners of MTMR2 are involved in the disease process causing CMT4B remains to be determined (14,32).

In conclusion, we have generated an animal model for CMT4B2. In combination with the available Mtmr2−/− mice, the Mtmr13/Sbf2−/− mice provide valuable tools for future mechanistic studies in hereditary neuropathies characterized by myelin outfoldings and infoldings.

**MATERIALS AND METHODS**

**Generation of Mtmr13/Sbf2−/− mice**

The Mtmr13/Sbf2−/− mutant cell line (XH212) was obtained from MMRRRC / BayGenomics ES Cell Clones (http://baygenomics.ucsf.edu/cgi-bin/BaySearch.py). Details for
gene-trap mutagenesis (33) in mouse embryonic stem cells can be found at http://baygenomics.ucsf.edu/protocols/index.html. The precise insertion of the gene-trap vector (pGT1lxI) in the ES cell genome was determined by Inverse PCR and DNA sequencing. The cell line was used for blastocyst injection (Institute of Laboratory Animal Science, University of Zürich) to obtain chimeric mice and these were crossed with C57Bl/6 mice. The offspring resulted in expected mendelian frequencies (wt: 26.1%; heterozygous: 49.2%; homozygous: 24.7%; n = 412).

Figure 9. Electron microscopic analysis of focally folded myelin and axonal degeneration in sciatic nerve of Mtmr13/Shf2-deficient mice. The figure shows a collection of different morphologies at age P21 (A–D), 4 months (E–F, M, O, P), and 15 months (G–L, N). Outfolding of the myelin sheath is the most frequent type of dysmyelination (A–C). Single or multiple redundant myelin loops are visible adjacent to the original myelinated fibre. The Schwann cell membrane surrounds both the outfoldings and the Schwann cell-axon unit they arise from. Simple outfoldings and the original myelin sheath share the same periodicity and number of lamellae (D). Infoldings of the myelin sheath may severely affect the axonal shape by leading to constriction of the axonal cytoplasm (arrow in E) or by forming extensions giving the cross-sectioned fibre a target-like appearance (F and G). The double circles in F and G likely reflect the retrograde inversion of infolded myelin loops. Note that the interspaces between the inner and outer infolding and the original myelin sheath show the structure of axonal cytoplasm (insert in F). Some fibres exhibit both infoldings and outfoldings (G). Apart from abnormalities of the myelin sheath, some fibres show disintegration or even degradation of the entire Schwann cell-axon unit. We observed widening of the periaxonal space (white arrow in H), vacuolar disruption of the inner myelin layers (I), compression and lateralization of the axon by massive infoldings (J) and various stages of axonal degeneration (K and L). The white arrow in L points to residual myelin. Longitudinal sections (M–P) revealed preferential location of myelin abnormalities in the nodal and paranodal segments (white arrow in M). Note that the aberrant myelin loops ensheath axonal processes and lead to massive disruption of the normal architecture of the node, which here is forced off the cutting plane (white arrow in N). Infoldings and outfoldings also occur in the internodal segment of the myelinated fibre (white arrowhead in O) or near the Schmidt-Lantermann incisures (arrows and white arrowhead in P). Scale bars for D: 1 μm, L–P: 5 μm.
Genotyping PCR
Tail genomic DNA was used for the genotype analysis. Primer pairs I and III were used to characterize the wt allele. Primer pairs II and III were used for the detection of the mutated allele. I: 5’-TCA GCA GCC AGG GAA CGG AGA C -3’. II: 5’-GGA GCA GAC AAG CCC GTC AOG-3’. III: 5’-AAG AA GGA AGA AAG ACA GTC CG-3’.

Protein expression analysis on mutant and wt sciatic nerves
Sciatic nerve tissue was dissected, frozen in liquid nitrogen, pulverized with a chilled mortar and pestle and dissolved in SDS gel sample buffer (34). Proteins were electrophoresed on 10% SDS-polyacrylamide gels, transferred onto a Polyvinylidene fluoride (PVDF) membrane (Millipore), and immunobotted with antibodies against Mtmr13/Sbf2 [1:500; rabbit polyclonal anti-peptide antibodies raised against the peptide CKNKLRAASAGDWES (20)], Mtmr2 [1:500; rabbit polyclonal anti-peptide antibodies raised against the peptide CSTSHSNSVHTKSAS (20)],Dlg1/Sap97 (BD Transduction Laboratories; 1:1000) and beta-actin (Sigma; 1:5000). After incubation with goat anti-rabbit horseradish peroxidase-(Jackson Laboratories; 1:10000) or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Promega; 1:1000) or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Promega; 1:10000), immunoreactive bands were visualized by West (Jackson Laboratories; 1:5000) or CDP-Star (Roche). Blots were quantified using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized to the beta-actin signal.

Rotarod test
Mice were placed on a rotarod apparatus (TSE Systems, Germany) and the time spent on the rotating rod, rotating from 4 to 40 rpm, was measured. Four trials per day, every 2 h, on four consecutive days were performed with a maximal trial length of 10 min.

Neurophysiology
Motor nerve conduction of sciatic nerves was investigated in anesthesized animals as described previously (35). CMAP and F-wave were recorded with needle electrodes in the foot muscles after supramaximal stimulation of the tibial nerve at the ankle and stimulation of the sciatic nerve at the sciatic notch. Statistical analysis was performed using Student’s t-test.

Electron microscopy
Animals were anesthetized and sequentially perfused with 0.25 mg/ml heparin in 0.1 M PBS and a fixative solution containing 4% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Sciatic nerves were dissected, postfixed for 24 h at 4°C, osmicated for 2 h in 2% osmium tetroxide, washed in distilled water several times, dehydrated in ascending acetone and embedded in Spurr’s medium (36). Ninety nanometer ultrathin sections cut on an Ultracut E microtome (Reichert-Jung, Heidelberg, Germany) were mounted on copper grids and contrasted with uranyl acetate and lead citrate. Digital photographs were taken on a Philips EM 208 S transmission electron microscope using the iTEM® software (Olympus Soft Imaging Solutions, Muenster, Germany).

Morphometric analysis and quantification of focally folded myelin
Sciatic nerves from intracardially perfused animals were osmicated and embedded in Spurr’s medium as described above. Semithin sections (0.5 µm) were stained with alkaline toluidine blue under standardized conditions to obtain identical results with regard to contrast and colour intensity. Semithin sections were visualized in a Leica DMRX microscope at 400-fold magnification, and a series of partially overlapping images covering the cross-sectional area was captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). In order to obtain a composite image of the entire cross section, single images were pasted together using a Gaussian filter. Image processing and morphometric data collection were performed using the analySIS® software (Olympus Soft Imaging Solutions, Muenster, Germany). A macro was designed to meet the specific requirements of morphometric analysis. To discriminate axons and myelin sheaths, we defined a grey-scale threshold level for axon and myelin profiles. For each nerve fibre, both axon and myelin area were measured, and the diameter of axons and myelinated fibres were mathematically deduced from a circle of an equivalent area. Paranodes, Schwann cell nuclei, nerve fibres with focally folded myelin, and unmyelinated fibres were manually excluded from morphometry. We analysed two sciatic cross sections per animal (mutant and wt), and sections were taken from different levels 1.5 mM apart. For quantification of dysmyelinated nerve fibres, we determined the total number of fibres on each cross section, and the proportion of myelinated fibres showing focally folded myelin was calculated.

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