Genetic interaction between MTMR2 and FIG4 phospholipid phosphatases involved in Charcot-Marie-Tooth neuropathies.

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Abstract

We previously reported that autosomal recessive demyelinating Charcot-Marie-Tooth (CMT) type 4B1 neuropathy with myelin outfoldings is caused by loss of MTMR2 (Myotubularin-related 2) in humans, and we created a faithful mouse model of the disease. MTMR2 dephosphorylates both PtdIns3P and PtdIns(3,5)P2, thereby regulating membrane trafficking. However, the function of MTMR2 and the role of the MTMR2 phospholipid phosphatase activity in vivo in the nerve still remain to be assessed. Mutations in FIG4 are associated with CMT4J neuropathy characterized by both axonal and myelin damage in peripheral nerve. Loss of Fig4 function in the plt (pale tremor) mouse produces spongiform degeneration of the brain and peripheral neuropathy. Since Fig4 has a role in generation of PtdIns(3,5)P2 and MTMR2 catalyzes its dephosphorylation, these two phosphatases might be expected to have opposite effects in the control of PtdIns(3,5)P2 homeostasis and their mutations might have compensatory effects in vivo. To explore the role of the MTMR2 phospholipid phosphatase activity in vivo, we generated and characterized the Mtmr2/Fig4 double null mutant mice. Here we provide strong evidence that Mtmr2 and Fig4 functionally interact in both Schwann cells and neurons, and we reveal for the first time a role of Mtmr2 in neurons in vivo. Our results also suggest that imbalance of PtdIns(3,5)P2 is at the basis of altered longitudinal myelin growth and of myelin outfolding formation. Reduction of Fig4 by null heterozygosity and downregulation of PIKfyve both rescue Mtmr2-null myelin outfoldings in vivo and in vitro.

Introduction

Phosphoinositides (PIs) constitute potent signaling molecules with a specific and restricted distribution at intracellular membranes that is strictly controlled by the concerted action of kinases and phosphatases [1,2]. PIs are key regulators of membrane trafficking as they contribute to assembly of molecular machineries that promote and control membrane dynamics and vesicle movement, tethering and fusion. In the nervous system, both neurons and glia rely on efficient membrane trafficking for many functions, such as axonal transport or myelination.

Charcot-Marie-Tooth (CMT) neuropathies are very heterogeneous disorders from both the clinical and genetic point of view [3-6]. Several CMT genes encode proteins that regulate or are connected with PI metabolism, including FRABIN/FGD4, FIG4, DNM2, RAB7, SIMPLE, LRSAM1, SH3TC2, MTMR2, and MTMR13, supporting the idea that regulation of intracellular trafficking is a key process in peripheral nervous system biology [7] (http://www.molgen.ua.ac.be/CMTMutations/default.cfm).

We first demonstrated that loss of function mutations in the MTMR2 (Myotubularin-related 2) gene cause autosomal recessive demyelinating Charcot-Marie-Tooth type 4B1 (CMT4B1, OMIM #601382) neuropathy with myelin outfoldings [8]. MTMR2 is a phospholipid phosphatase that dephosphorylates both PtdIns3P and PtdIns(3,5)P2 phosphoinositides at the D3 position of the inositol ring, thus generating PtdIns5P [9-16]. We have generated a Mtmr2-null mouse which models the CMT4B1 neuropathy and we reported that loss of Mtmr2 specifically in Schwann cells is both sufficient and necessary to provoke myelin outfoldings [17,18]. We recently identified a potential mechanism using in vivo and in vitro models of CMT4B1 and proposed that Mtmr2 belongs to a molecular machinery that titrates membrane formation during myelination. According to this model, myelin

Genetic Interaction between MTMR2 and FIG4 Phospholipid Phosphatases Involved in Charcot-Marie-Tooth Neuropathies

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Funding: This work was supported by the Italian Telethon (Grant N. GPP10007), Association Française contre les Myopathies (AFM), and ERA-Net for research in Charcot-Marie-Tooth neuropathies from both the clinical and genetic point of view [3-6]. Several CMT genes encode proteins that regulate or are connected with PI metabolism, including FRABIN/FGD4, FIG4, DNM2, RAB7, SIMPLE, LRSAM1, SH3TC2, MTMR2, and MTMR13, supporting the idea that regulation
outfoldings arise as a consequence of the loss of negative control on the amount of membrane produced during myelination [19]. Despite these findings, the function of MTMR2 and the role of the MTMR2 phospholipid phosphatase activity in the nerve still remain to be assessed.

Loss of the FIG4/SAC3 phospholipid phosphatase in human provokes another form of autosomal recessive demyelinating CMT, the CMT type 4J (OMIM #611228) neuropathy [20,21]. FIG4 is a 5-phosphatase involved in the dephosphorylation of PtdIns(3,5)P2, a predicted substrate of MTMR2. Loss of Fig4 in the mouse causes the plt (pal tremor) phenotype, characterized by extensive neuronal vacuolization and degeneration and by a peripheral neuropathy [20,22]. Yeast Fig4 is localized at the vacuolar membrane-the yeast lysosomal compartment- and is required for both the generation and turnover of PtdIns(3,5)P2 [23,24]. In addition to the 5-phosphatase activity, yeast Fig4 appears to activate Fab1, the kinase that produces PtdIns(3,5)P2 from PtdIns3P [23,24]. Deletion of yeast Fig4 reduces rather than increases PtdIns(3,5)P2 leading to defects in vacuole homeostasis and function. A significant decrease of PtdIns(3,5)P2 was found also in plt (fig4-null) fibroblasts, suggesting conserved enzymatic and cellular functions of Fig4 from yeast to mouse [20]. Moreover, the most common human mutation of FIG4 acts by reducing its affinity for the PtdIns(3,5)P2 biosynthetic complex [25].

Since Fig4 has a role in generation of PtdIns(3,5)P2 and MTMR2 catalyzes its dephosphorylation, these two phosphatases might have opposite effects in the control of PtdIns(3,5)P2 homeostasis and their mutations might have compensatory effects in vivo. To explore the role of the MTMR2 phospholipid phosphatase activity in vivo, we took advantage of the Fig4 and Mtmr2-null mice and generated and characterized the Mtmr2/Fig4 double null mutant. Here we provide strong evidence that Mtmr2 and Fig4 functionally interact in both Schwann cells and neurons, and reveal for the first time a role of Mtmr2 in neurons in vivo. We also report that the imbalance of PtdIns(3,5)P2 might be at the basis of myelin outfoldings in the nerve. Reduction of Fig4 by null heterozygosity and downregulation of PIKfyve both rescue Mtmr2-null myelin outfoldings in vivo and in vitro.

### Results

#### Generation of Mtmr2/Fig4-null mice

The generation and characterization of Mtmr2-null and Fig4-null (plt) mice have been reported [17,20]. Mtmr2/Fig4 double null mice and controls were analyzed in the F2 generation. At postnatal day three (+/Fig4−/− mice had reduced body size and dilated pigmentation of the coat similar to the Mtmr2+/−/+Fig4−/− mice in the same litter, and as reported for the plt mouse [20]. Tremor and abnormal gait developed in the second week after birth. Mtmr2+/+Fig4−/− mice show juvenile lethality and die around 1 month of age. The viability of Mtmr2−/−/+Fig4−/− mice was lower than for Mtmr2+/+Fig4−/− littersmates. A reduced number of both Mtmr2+/−/+Fig4−/− and Mtmr2−/−/+Fig4−/− mice were present at P8, compared to the expected Mendelian ratio (Table 1 and Table 2). The longest survival of the double mutant was 20 days. The data indicate that loss of Mtmr2 reduces viability of Mtmr2+/+Fig4−/−. We therefore hypothesized that loss of Mtmr2 might provoke a worsening of the Mtmr2+/+Fig4−/− neurodegeneration.

#### Mtmr2 loss exacerbates Fig4-null neurodegeneration

To explore this possibility, we performed semithin section analysis of DRG ganglia, brain and spinal cord from Mtmr2+/−/+Fig4−/− and Mtmr2−/−/+Fig4−/− mice. DRG ganglia from both Mtmr2+/−/+Fig4−/− and Mtmr2−/−/+Fig4−/− mice at P3 were severely affected, exhibiting neuronal loss and massive vacuolization (Figure 1A−1C). In the cerebellum of both Mtmr2+/−/+Fig4−/− and Mtmr2−/−/+Fig4−/− mice at P8 and at P20 we observed a thickening of the molecular layer as compared to wild-type, and cells with cytoplasmatic vacuoles were present in the granular layer. At P20, a consistent loss of Purkinje and basket cells was observed in both genotypes (Figures S1 and S2). These cerebellar findings have not been previously reported in the plt mouse.

In the cortex and brainstem of Mtmr2−/−/+Fig4−/− mice at P3 we noted more cells with vacuoles and inclusions than in Mtmr2+/−/+Fig4−/− mice, which were never been observed in wild-type animals (Figure 1D−1F). In particular, in the brainstem of Mtmr2−/−/+Fig4−/− mice at P8 the number of neurons carrying pathological abnormalities was significantly increased as compared to Mtmr2+/−/+Fig4−/− mice (Figure 1J−1L). We also analyzed the spinal cord of Mtmr2+/−/+Fig4−/− and Mtmr2−/−/+Fig4−/− mice at P3 and P8 (Figure 2A−2F). Vacuolated cells and cells with inclusions were observed, as previously described for the plt phenotype, which were not present in wild-type spinal cords [20,26]. At P8, we observed a significant decrease in the number

#### Table 1. Number of mice with Mtmr2+/+Fig4−/−, Mtmr2−/− Fig4, and Mtmr2−/−/+Fig4−/− genotypes scored at P8 on a total of 608 mice

| Mtmr2 | Fig4 | Predicted | Observed | P values |
|-------|------|-----------|----------|----------|
| +/-   | −/−  | 38        | 34       | 0.28     |
| +/-   | −/+  | 76        | 56       | 0.007    |
| −/+   | −/−  | 38        | 10       | <0.0001  |

"Predicted" refers to the number of expected mice among 608 mice born, based on Mendelian ratios. "Observed" refers to the mice identified by genotyping at P8. The number of mice decreases with loss of Mtmr2 on a Fig4-null background. P values were calculated for a binominal distribution, using the chi-square test. doi:10.1371/journal.pgen.1002319.0001

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**Author Summary**

Charcot-Marie-Tooth type 4B1 (CMT4B1) and Charcot-Marie-Tooth type 4J (CMT4J) are severe autosomal recessive demyelinating neuropathies with childhood onset. We previously demonstrated that loss of the phospholipid phosphatase MTMR2 causes CMT4B1 with myelin outfoldings in human and mouse and that loss of the phospholipid phosphatase FIG4 causes CMT4J and neurodegeneration in the mouse. MTMR2 has a predicted role in membrane trafficking, which is crucial for myelin membrane biogenesis and homeostasis. However, the biochemical activity of MTMR2 in vivo and the role of MTMR2 in myelination still remain to be assessed. MTMR2 and FIG4 act on the same phospholipid substrate PtdIns(3,5)P2, but with predicted opposite effects. We generated a double Mtmr2/Fig4-null mouse which showed that Mtmr2 and Fig4 interact in neurons and Schwann cells to control phospholipid metabolism. Moreover, Mtmr2-null myelin outfoldings are rescued by Fig4 heterozygosity, suggesting that imbalance of PtdIns(3,5)P2 is at the basis of the excessive myelin growth and hypermyelination.
of motor neurons and cells in Mtmr2−/−Fig4−/− mice as compared to Mtmr2+/+Fig4+/− mice (Figure 2H, 2I). These findings demonstrate that loss of Mtmr2 exacerbates the Mtmr2+/*Fig4*+/− neurodegeneration.

This effect could be the consequence of loss of Mtmr2 in neurons or in other cells, such as in astrocytes. For example, in the plt mouse, a block of autophagy in astrocytes has been reported. In plt mice at 1 week of age, the p62 autophagy marker was increased in GFAP-positive astrocytes from brain regions severely affected at later stages, suggesting that autophagy impairment contributes to the pathogenesis [26]. Elevated p62 co-localized with LAMP2-positive late endosomes/lysosomes (LE/LY) in astrocytes, showing

| Genotype 1              | Genotype 2              | RR (95%CI) | Chi-square | P values |
|------------------------|-------------------------|------------|------------|----------|
| Mtmr2+/+Fig4−/−         | Mtmr2+/−Fig4−/−         | 2.5 (0.92, 6.80) | 2.91       | 0.09     |
| Mtmr2+/+Fig4−/−         | Mtmr2−/−Fig4−/−         | 7 (2.72, 18.03)  | 28.55      | 9×10⁻⁴   |
| Mtmr2+/−Fig4−/−         | Mtmr2−/−Fig4−/−         | 2.8 (1.84, 4.27) | 21.42      | 4×10⁻⁴   |

The relative risk analysis was performed on the 608 animals scored at P8, as also reported in Table 1. DOi:10.1371/journal.pgen.1002319:002

Figure 1. Neurodegeneration in Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mice. Semithin section analysis of DRG ganglia, cortex and brainstem of wild-type (A, D, G) and mutant mice (B, C, E, F, H, I). (B, C) DRG sensory neurons from Fig4-null and Mtmr2/FIG4 double null mice at P3 with massive vacuolization. (F, I) More cells carrying inclusions and vacuoles were observed in Mtmr2−/−Fig4−/− cortex (F) and brainstem (I) at P3 as compared to Mtmr2+/+Fig4−/− mice (E, H). (K, L) Brainstem analysis at P8 showed increased number of pathological neurons in Mtmr2−/−Fig4−/− (L) as compared to Mtmr2+/+Fig4−/− mice (K) and quantification in (J), P = 2.75×10⁻⁶. A total of 10030, 10290, and 9620 neurons were counted and analyzed in wild-type Mtmr2+/+Fig4−/−, and Mtmr2−/−Fig4−/− mice, respectively. All neurons scored were normal in wild-type, whereas 48.3% and 81.1% of neurons displayed pathological features in Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/−, respectively. Arrows indicate inclusions; arrowheads vacuoles. Three mice per genotype were analyzed. Bar is 35 μm in (A–C), 10 μm in (D–L). DOi:10.1371/journal.pgen.1002319:001

Table 2. The relative risk (RR) of lethality for the Mtmr2+/+Fig4−/−, Mtmr2+/−Fig4−/−, and Mtmr2−/−Fig4−/− genotypes.
that the block of autophagy occurred subsequent to the fusion of autophagosomes with LE/LY [26]. To determine whether loss of Mtmr2 in astrocytes might further impair autophagy, we evaluated p62 levels in total brain extracts from Mtmr2+/+Fig4−/− mice as compared to Mtmr2−/−Fig4−/− mice. Increased p62, LAMP1 and GFAP expression levels were confirmed in Mtmr2−/−Fig4−/− double null mice (Figure 3A). This finding indicates that the block of autophagy occurred subsequent to the fusion of autophagosomes with LE/LY [26]. To determine whether loss of Mtmr2 does not further impair the block in the autophagic process in astrocytes of Fig4-null mice.

To further investigate the cell autonomy of the Mtmr2/FIG4 interaction, we established dissociated Schwann cell/DRG neuron co-cultures from Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mice, in which mutant Schwann cells were cocultured with exogenous wild-type rat Schwann cells. Mtmr2−/−Fig4−/− DRG neurons cultured with wild-type Schwann cells were significantly more severely vacuolated (57.8%) as compared to Mtmr2+/+Fig4−/− cultures (37.4%) (Figure 3B–3D'). This finding provides strong evidence that the loss of Mtmr2 in neurons leads to the worsening of the Fig4−/− neurodegeneration.

Like neurons, mouse primary fibroblasts (MFs) from plt mutants display enlargement and vacuolization of the LAMP2-positive LE/LY compartment [20,22]. To provide further evidence for functional interaction between MTMR2 and FIG4, we established MF cultures from Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mice. By LAMP1 staining and confocal microscopy, we observed that the number of fibroblasts carrying enlarged LE/LY was significantly increased in Mtmr2−/−Fig4−/− double mutants as compared to Mtmr2+/+Fig4−/− (Figure 4). This finding indicates that Mtmr2 loss exacerbates Fig4-null vacuolar phenotype by further impairment of the endo/lysosomal trafficking pathway.

Mtmr2 exacerbates Fig4-null hypomyelination in sciatic nerve

The plt mouse phenotype is characterized by a peripheral neuropathy. Loss of large diameter myelinated axons, hypomyelination (reduced myelin thickness), reduced amplitude of compound motor action potential (cMAP) and slowing of the nerve conduction velocity (NCV) have been reported in plt mice and the presence of demyelinating features in CMT4J patient biopsies such as onion bulbs suggested that FIG4 has also a cell autonomous role in Schwann cells [22].

We investigated sciatic nerves from Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mice. At P3 and P8, mutant sciatic nerves showed a normal development. In both genotypes at P8, Schwann cells often contained cytoplasmic inclusions and occasionally

Figure 2. Semithin section analysis of spinal cord from Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mice. At P3 (A–C) and P8 (D–F), cytoplasm inclusion and vacuolization, leading to extensive cell loss were observed in the spinal cord of Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mutant mice. By counting the number of cells in at least 15 sections of spinal cord per genotype (H, I), a significant decrease in the number of motorneurons (H) and of cells (I) (per mm²) was observed at P8 in Mtmr2−/−Fig4−/− spinal cords as compared to Mtmr2+/+Fig4−/− (and wild-type). In Mtmr2+/+Fig4−/− as compared to Mtmr2−/−Fig4−/−, P = 0.04893; wild-type as compared to Mtmr2−/−Fig4−/−, P = 0.02107; in (I), Mtmr2+/+Fig4−/− as compared to Mtmr2−/−Fig4−/−, P = 0.0134245; wild-type as compared to Mtmr2−/−Fig4−/−, P = 0.008836. (G) Electron microscopy of a motorneuron showing cytoplasmic electron-dense inclusion and vacuoles. Arrows indicate inclusions; arrowheads vacuoles. Bar is 10 μm in (A–F) and 1 μm in (G).

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Figure 3. Mtmr2 loss in neurons exacerbates Fig4-null neurodegeneration. (A) Western blot analysis of brain extracts from wild-type, Mtmr2+/+Fig4−/−, and Mtmr2−/−Fig4−/− mice at P20. p62, GFAP, and LAMP1 are increased in Mtmr2+/+Fig4−/− as compared to wild-type as already reported for the plt mouse. No differences were detected between Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/−, suggesting that loss of...
Mtmr2 does not further impair astrocytosis and the block in the autophagic process in astrocytes. (B–G) Dissociated DRG explants from wild-type (B, B’, E), Mtmr2+/+Fig4−/− (C, C’, F), and Mtmr2−/−Fig4−/− (D, D’, G) mice, in which mutant Schwann cells were replaced by exogenous wild-type rat Schwann cells. (D, D’) More Mtmr2−/−Fig4−/− neurons (n = 303 neurons on 523 total neurons) are vacuolated as compared to Mtmr2+/+Fig4−/− neurons (C, C’; n = 236 on 635 total neurons; P = 7.56271 × 10−8, 16 covers per genotype, and quantification in H). (G) Mtmr2−/−Fig4−/− co-cultures produced less myelinated MBP-positive segments after 7 days of ascorbic acid treatment (n = 52 segments on 16 coverslips) as compared to Mtmr2+/+Fig4−/− explants are severely hypomyelinated as compared to wild-type (E) (n = 6560 segments on 16 coverslips). Green is neurofilament (NF-L); red is myelin basic protein (MBP), staining compact myelin. Arrowheads in (C, D, C’, D’) indicate vacuoles within sensory neurons. Bar in (B–G) is 50 μm.

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Figure 4. Enlarged late endosomal compartment in primary fibroblasts from Mtmr2+/+Fig4−/− and Mtmr2−/−Fig4−/− mutant mice. (A–C) The number of fibroblasts carrying enlarged late endosomal/lysosomal compartment (LAMP1 positive, green) is significantly increased in Mtmr2−/−Fig4−/− (B) as compared to (A) Mtmr2+/+Fig4−/−. In red is phalloidin which stains actin filaments. Blue, DAPI. The quantification in (D) was made by counting cells established from three animals per genotype. n = 526 on 17 coverslips for Mtmr2+/+Fig4−/−; n = 583 cells on 20 coverslips for Mtmr2−/−Fig4−/−; P = 0.000079; confocal microscopy analysis. Asterisks mark cells carrying enlarged late endosomal compartments. Bar in (A–C) is 25 μm.

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Phospholipid analysis in mouse fibroblasts

To correlate MTMR2 and FIG4 functional interaction with changes in PI levels, we measured PtdIns3P and PtdIns(3,5)P2 levels from wild-type; Mtmr2+/−Fig4+/+; Mtmr2+/+Fig4−/−; Mtmr2−/−Fig4+/+; and Mtmr2−/−Fig4−/− fibroblasts by metabolic labeling and HPLC analysis (Figure 8B). PtdIns3P levels were similar in all the genotypes analyzed (data not shown). In mammalian cells, PtdIns3P generation and turnover are controlled by multiple redundant pathways, so that ablation of one particular enzyme such as myotubularin does not necessarily result in an imbalance of PtdIns3P, as already reported [9,30,31].

On the other hand, we found that loss of Fig4 in Fig4−/−fibroblasts results in a significant decrease of PtdIns(3,5)P2 as compared to control cells, thus confirming previous findings [20] (Figure 8B). As also suggested by the in vitro mass assay performed on Mtmr2−/−myelin-forming co-cultures (Figure 8A), loss of Mtmr2 in Mtmr2−/−fibroblasts leads to a significant increase in PtdIns(3,5)P2 level, consistently with the 3-phosphatase activity of MTMR2 (Figure 8C). Moreover, PtdIns(3,5)P2 was equally reduced in Mtmr2+/−Fig4+/+ and Mtmr2−/−Fig4−/− cells (Figure 8B), possibly because the PtdIns(3,5)P2 substrate is already severely affected by loss of Fig4, and Mtmr2 acts downstream of Fig4 in the control of this lipid level.

To support the hypothesis that myelin outfoldings in Mtmr2−/−Fig4+/+ co-cultures were rescued by restored PtdIns(3,5)P2 levels, we also measured PtdIns(3,5)P2 in Mtmr2+/−Fig4+/+ and Mtmr2−/−Fig4+/+ fibroblasts. However, PtdIns(3,5)P2 did not differ in Mtmr2−/−Fig4+/+ and Mtmr2−/−Fig4−/− fibroblasts (Figure 8C). Small changes in PtdIns(3,5)P2 levels due to loss of 50% of phosphatase expression may be below the level of detection of this method.

Overall, these findings indicate that Mtmr2 and Fig4 control PtdIns(3,5)P2 with opposite effects. If Fig4 is totally absent and PtdIns(3,5)P2 is low, the absence of Mtmr2 which dephosphorylates PtdIns(3,5)P2 has no influence. On the other hand, when PtdIns(3,5)P2 is high due to loss of Mtmr2, a partial reduction in PIKfyve activity due to heterozygosity of Fig4 might lead to PtdIns(3,5)P2 rebalance and rescue of myelin outfoldings.

We finally tested for interaction between phosphatases using a pull-down assay. GST-MTM2 was not able to pull-down Fig4 enzyme.
from brain or isolated rat Schwann cell lysates, suggesting that the functional interaction between MTMR2 and FIG4 demonstrated here is not mediated by physical interaction between the two proteins (Figure S3C–S3E).

Mammalian MTMR2 converts PtdIns(3,5)P$_2$ and PtdIns3P in yeast

The mutant yeast strain fig4+a displays enlarged vacuoles caused by reduced PtdIns(3,5)P$_2$, which in yeast controls the homeostasis of the vacuole (the lysosomal compartment). To further test MTMR2 function, and further test functional interactions between Mtmr2 and Fig4, we transformed FLAG-MTMR2 in the mutant yeast strain fig4+a. Overexpression of wild-type MTMR2 in fig4+a caused a further enlargement of the vacuolar compartment and defects in vacuole fission whereas the catalytically inactive mutant FLAG-MTMR2C417S did not cause these changes (Figure 9). To determine the substrates and products of mammalian MTMR2 in yeast, we measured phosphorylated phosphoinositide lipid levels from cells expressing FLAG-MTMR2 as compared to the vector alone. To enhance the sensitivity of the assay, we subjected the

Figure 7. A rebalance of PtdIns(3,5)P$_2$ rescues Mtmr2-null myelin outfoldings in vitro. (A, B) Heterozygosity of Fig4 in Mtmr2-null explants rescues myelin outfoldings. (C) The number of MBP positive segments carrying myelin outfoldings on the total of MBP segments on random fields was assessed. Mtmr2+/−/Fig4+/+ n = 383 fibers; Mtmr2+/−/Fig4−/− n = 405 fibers, on at least 5 covers per genotype; P = 1.9754 × 10$^{-2}$. (D) Decrease of PIKfyve expression on lysates from myelin-forming Schwann cells/DRG explants transduced with PIKfyve shRNA lentiviral vector (LV), as compared to a scramble sequence. (E–F) Myelin outfoldings are rescued in Mtmr2-null transduced by PIKfyve shRNA LV as compared to cultures transduced with a scramble sequence (PIKfyve, n = 423 and scramble, n = 484; P = 6 × 10$^{-6}$). (G, H, and J) Myelin outfoldings are rescued in Mtmr2-null treated with 70 nM of YM201636 PIKfyve inhibitor as compared to cultures treated with DMSO (YM201636 n = 913; DMSO, n = 857; P = 6.5 × 10$^{-7}$). Bar is 20 µm in (A, B, E–F, and G, H). Green is neurofilament (NF-L); red is myelin basic protein (MBP), staining compact myelin. Arrowheads indicate myelin outfoldings.
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yeast to hyperosmotic shock. In wild-type yeast, this results in a transient increase in PtdIns(3,5)/P2 levels (green line) and concomitant decrease in PtdIns3P (blue line) (Figure 10, 5 min time point). If MTMR2 acts on PtdIns(3,5)/P2, then there should be a decrease in PtdIns(3,5)/P2 and a corresponding increase in PtdIns3P. Moreover, if MTMR2 acts on PtdIns3P there will be a decrease in that lipid as well. Each of these changes was observed (Figure 10, solid lines and Table S1). These findings demonstrate that MTMR2 and FIG4 coordinately regulate the production of PtdIns(3,5)/P2, and the production of PtdIns3P is generated [16]. Interestingly, another phospholipid phosphatase, FIG4/SAC3, is involved in both dephosphorylation and the production of PtdIns(3,5)/P2 and is mutated in autosomal recessive demyelinating CMT4J neuropathy [20]. Loss of Fig4 in mouse provokes the plt phenotype characterized by massive neurodegeneration and peripheral neuropathy. In Fig4-null fibroblasts a decrease in PtdIns(3,5)/P2 has been demonstrated, suggesting that Fig4 promotes PtdIns(3,5)/P2 production by PIKfyve activation or stabilization [20]. Thus, MTMR2 and FIG4 could have opposite effects in the control of PtdIns(3,5)/P2.

To explore the biological role of MTMR2 phosphatase activity in the nerve in vivo, we generated a Mtmr2/Fig4 double null mutant. Analysis of these mice provides evidence that Mtmr2 and Fig4 functionally interact in neurons, fibroblasts, and Schwann cells. Loss of Mtmr2 reduces the viability and exacerbates the neurodegeneration of Fig4-null mice.

These results also provide the first evidence for a role for MTMR2 in neurons in vivo, consistent with the marked axonal loss in CMT4B1 patients [32]. Although conditional ablation of Mtmr2 in motorneurons in mice did not reveal signs of axonal degeneration or neuronopathy, a cell autonomous role of Mtmr2

Discussion

The MTMR2 3-phosphatase activity toward PtdIns3P and PtdIns(3,5)/P2 has been demonstrated by a number of studies using recombinant MTMR2 in vitro as well as conventional cell lines overexpressing MTMR2 [10–16]. Overexpressed MTMR2 has been co-localized with Rab7 in A431 cells at the level of late endosome/lysosomes, where PtdIns(3,5)/P2 is
in neurons was not excluded since axonopathies are length dependent and not easily reproduced in mice [18]. Interestingly, a role for MTMR2 in neurons in vitro has been recently reported suggesting that Mtmr2 is localized to excitatory synapses of central neurons via direct interaction with the PSD-95 scaffolding protein [33]. Knockdown of Mtmr2 in cultured neurons markedly

Figure 9. MTMR2 and FIG4 interaction in yeast. Expression of human FLAG-MTMR2 in the mutant yeast strain fig4Δ, caused enlarged vacuoles and a defect in vacuole fission. Wild-type and fig4Δ expressing pVT102U (vector), pVT102U-MTMR2 or pVT102U-MTMR2-C417S, were grown to mid-log phase and the vacuole membrane was labeled with SynaptoRed C2. Arrow indicates an example of a bud vacuole that has not properly separated from the mother vacuole. Scale bar, 6 μm.
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Figure 10. MTMR2 3-phosphatase activity in yeast. Mouse MTMR2 has 3-phosphatase activity in vivo and converts PtdIns(3,5)P2 and PtdIns3P to PtdIns5P and phosphatidylinositol, respectively. Expression of MTMR2 in yeast causes a decrease in PtdIns3P and PtdIns(3,5)P2 as well as an increase in PtdIns5P. Mouse MTMR2 cDNA was subcloned into a yeast expression vector, pVT102U (vector) to express MTMR2 from the ADH promoter, pVT102U-MTMR2. Yeast expressing MTMR2 or with vector alone were labeled with 3H-inositol for 18 h. Cells were exposed to 0.9 M NaCl for the times indicated, lipids were extracted, deacylated, and the corresponding glycerol-inositol phosphates were quantitated by high-pressure liquid chromatography, reported as the percent of total [3H]phosphatidylinositol extracted. Two independent experiments are shown.
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reduced excitatory synapse density and function and it was proposed that the MTMR2/PSD95 complex contributes to the maintenance of excitatory synapses by inhibiting excessive endosome formation and destructive endosomal traffic to lysosomes.

Here, we assessed MTMR2 and FIG4 interaction in yeast and found that overexpression of MTMR2 reduces both PtdIns3P and PtdIns(3,5)P2 leading to an increase in vacuole size in the fig4Δ mutant. These findings support the in vivo role of MTMR2 as a 3-phosphatase that acts on both PtdIns3P and PtdIns(3,5)P2.

Fig4 heterozygosity rescues myelin outfoldings due to Mtmr2 deficiency both in vivo and in vitro, thus providing evidence of the Fig4 and Mtmr2 interaction in Schwann cells as well as neurons. Loss of Mtmr2 specifically in Schwann cells provokes myelin outfoldings. The presence of cytoplasmic inclusions in Schwann cells and the reduced NCV in the Fig4-null mouse, and the typical demyelinating features (onion bulbs) of CMT1J patients, all strongly support a Schwann cell autonomous role for Fig4. But how does loss of Fig4 in Schwann cells rescue Mtmr2-null myelin outfoldings? We hypothesized that a 50% reduction of Fig4 might be sufficient to rebalance the PtdIns(3,5)P2 elevation in Mtmr2-null cells (Figure 11), thus reducing myelin outfoldings. MTMR2 loss should lead to an increase of both PtdIns3P and PtdIns(3,5)P2, whereas FIG4 loss reduces PtdIns(3,5)P2 levels. In agreement with this model, we observed that downregulation of PIKfyve expression or inhibition of its activity in Mtmr2-null co-cultures reduced myelin outfoldings, as also observed with Fig4 heterozygosity (Figure 11). Our results therefore suggest that imbalance of PtdIns(3,5)P2 is at the basis of altered longitudinal myelin growth and formation of myelin outfoldings. The observed rescue of myelin outfoldings is likely mediated by restored PtdIns(3,5)P2 rather than PtdIns5P. PtdIns5P may be produced via dephosphorylation of PtdIns(3,5)P2 by MTMRs, and can also be generated, at least in vitro, by PIKfyve acting on phosphatidylinositol [34]. Therefore, Fig4 heterozygosity in Mtmr2-null cells would lead to a further reduction in PtdIns5P rather than restoration, as for PtdIns(3,5)P2.

PtdIns(3,5)P2 is thought to be localized to EE and the limiting membranes of LE/LY, although it cannot be excluded that this lipid might also be generated at other membranes. The lack of specific probes to detect PtdIns(3,5)P2 prevents the definition of other membrane localization [35,36]. Our studies raise the question of how dys-regulation of PtdIns(3,5)P2 leads to aberrant longitudinal myelin growth. Excessive longitudinal myelin growth and myelin outfoldings might arise as a consequence of reduced endocytosis/recycling and degradation or as a consequence of increased exocytosis. One can speculate that increased PtdIns(3,5)P2 due to loss of MTMR2 might favor exocytosis from

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**Figure 11. Model of MTMR2 and FIG4 phosphatase activities in the control of PtdIns3P-PtdIns(3,5)P2 metabolism.** In red, altered levels of Pls due to loss of MTMR2 and/or FIG4 are depicted. In blue, rebalanced levels of PtdIns(3,5)P2 in Mtmr2-null cells upon Fig4 heterozygosity and downregulation of either PIKfyve expression or inhibition of its activity are shown.

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the LE/LY compartment during myelin biogenesis. However, this mechanism, which has been recently suggested to occur in oligodendrocytes [37], accounts for the assembly of myelin components during the active phase of myelination. In myelin outfoldings, myelin thickness is normal, so a more subtle mechanism of regulation would be involved. Increased PtdIns(3,5)P2 might alter autophagy dynamics. However, we did not observe any change on LC3II/I ratio and/or p62 levels in Mtmr2-null nerves or in myelin-forming DRG co-cultures (unpublished results).

Alternatively, MTMR2 may favor endocytosis and counteract ectoeytosis during later stages of myelin biogenesis. The myelin outfoldings may thus arise as a consequence of the loss of negative control on the amount of membrane produced during myelination. Another alternative is that MTMR2 might control endocytosis of specific transmembrane proteins linking Schwann cell plasma membrane to the axonal plasma membrane, which then act as signaling molecules to control longitudinal myelin growth. Note that myelin outfoldings often contain axoplasm and axons branches at paranodal regions thus following myelin membrane outgrowth [17]. Along these lines, enhanced surface localization of putative adhesion molecules due to loss of Mtmr2-mediated endocytosis might result in the loss of control of myelin elongation and thus in myelin outfoldings. Other members of the MTMR family seem to possess similar biological functions. MTMR4 was recently demonstrated to regulate the sorting of endosomal cargos into recycling endosomes [38]. In C. elegans, MTM6 and MTM9 were found to be involved in endocytosis [39] whereas Drosophila Mtm (homologous to catalytically active MTM1, MTMR1, and MTMR2) regulates both actin-based plasma membrane morphogenesis and the endosomal influx toward the endo-lysosomal axis [40]. Whether and how MTMR2 might regulate endocytosis in Schwann cells during postnatal development remains to be assessed.

Materials and Methods

Ethics statement

All experiments involving animals were performed in accordance with Italian national regulations and covered by experimental protocols reviewed by local Institutional Animal Care and Use Committees.

Mice

Mtmr2-null mice were backcrossed for at least 5 generations to strain C57BL/6N.

Fig4+/− heterozygous mice were maintained on the recombinant inbred line CB.plt derived predominantly from strains CAST/Ei and C57BL/6j (25%) [25].

Heterozygous Fig4+/− males were crossed with Mtmr2-null females to obtain Mtmr2+/−−/Fig4+/−−/ double heterozygous mice. Double heterozygotes were crossed to generate Mtmr2+/−−/−/Fig4+/−−/− null mice as well as Mtmr2+/−−/−/−Fig+/−−/− mice for analysis. Genotyping was performed as described [17, 20].

Morphological analysis

Semithin morphological analysis was performed as described previously [41]. For morphometric analysis in brainstem at P8, neuronal damage was evaluated in the facial nucleus at the level of the upper medulla oblongata (Bregma −5.88). For each experimental sample, microscopic images (130 μm×90 μm, nine images per slide, three slides for each brain) were taken with a digital camera and processed by Adobe Photoshop 7.0 software. To be counted, a cell (diameter >20 μm) had to be located in the facial nucleus and

100–150 cells were scored per section. Cells with abnormal cytoplasm vacuolization were scored as pathological. The average percentage of normal and damaged neurons for each sample was considered for each experimental group to represent the neuronal density. Counts were performed in double blind by 2 investigators on slides with a number-code system, and results were analyzed.

The number of motoneurons and of total cells in spinal cord was assessed by performing at least 15 sections for each spinal cord from three animals per genotype as before and by counting the number of cells per area-cell density (mm²).

The proportion of fibers carrying myelin outfoldings in Mtmr2-null nerves as compared to Mtmr2-null mice with Fig4+/−−/− heterozygosity was determined by measuring the number of fibers carrying myelin outfoldings normalized to the total number of axons per section (the entire nerve section was reconstructed).

Ultrasound morphological analysis was conducted as reported previously [41]. For morphological analysis, three to five animals were evaluated at each time point in most cases.

Primary mouse fibroblast (MF) culture

MFs were established at P3 from tails and legs chopped in pieces and incubated after PBS washing with RPMI medium and 1 mL Collagenase Type II (Stock = 2000 U/mL in 1×PBS, Worthington, LS004204) overnight at 37°C. The next day, cells were plated in RPMI-1640 with 13% FBS/1× L-Glutamine/1× Pen/Strep. Cells were subjected to only two-three passages to allow maximum efficiency of metabolic labelling for PI measurement.

Phospholipid analysis

 Fibroblasts were labeled for 16 h in phosphate free DMEM (Invitrogen) containing 200 μCi/ml [32P]orthophosphate (Perkin Elmer). Lipids were extracted, separated on Silica gel G60 plates and analyzed by HPLC as described previously [42].

PtdIns5P was quantified by mass assay as described [43]. Briefly total lipids were extracted from duplicate or triplicate plates of DRG co-cultures from Mtmr2+/−− or Mtmr2−−/− knock-out mice and separated on Silica gel G60 plates. Monophosphorylated PIs were scraped, eluted from silica and assessed for PtdIns(4,5)P2 formation in vitro using the recombinant specific PIP4KIIalpha and [gamma-32P] ATP.

Antibodies

For western blot analysis and immunohistochemistry the following antibodies were used: rat anti-LAMP1 (Iowa Hybridoma bank), Guinea pig anti-P62 (Progen), rabbit anti-GFA (Sigma), rabbit anti-MAG (kindly provided by Dr. J. Salzer), rat anti-MBP (kindly provided by Dr. V. Lee), mouse anti-MBP (Covance), rabbit anti-NF-L (Chemicon), mouse anti-tubulin (Sigma), and mouse anti-FIG4 (NeuroMab).

Schwann cell/DRG neuron co-cultures

Myelinating Schwann cell/DRG neuron co-cultures were established from E13.5 mouse embryos as previously described [19, 44]. Myelination was induced by treatment for 15 days with ascorbic acid (final concentration, 50 μg/ml) (Sigma-Aldrich). Dissociated Schwann cell/DRG neuron co-cultures were established as described but DRGs were first incubated with trypsin (0.25%) for 45 min at 37°C. Cells were also mechanically dissociated and then plated at a concentration of one to two DRGs per glass coverslip. Isolated rat Schwann cells were prepared as reported previously and cultured using DMEM with 10% of fetal calf serum, 2 ng/ml recombinant human neuroregulin-1-b1 (R&D Systems), and 2 mM forskolin (Calbiochem).
YM201636 was provided by Symansis. A titration of the compound starting from 800 nM until 30 nM was performed on co-cultures to select the maximum amount of compound which did not affect myelination. As already described [27,28], 100 or 800 nM of compound provoked extensive cell vacuolization after overnight incubation. YM201636 was provided to co-cultures at 70 nM every other day together with ascorbic acid for 15 days to achieve full myelination.

Immunohistochemistry
Schwann cell/DRG neuron co-cultures 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% normal goat serum (Dako), 1% bovine serum albumin (BSA) (Sigma-Aldrich), and then incubated with primary antibody for 1 h. After extensive washing, the coverslips where incubated with the secondary antibody for 30 min, washed, and mounted. For double immunostaining with anti-NF-L and anti-MBP antibody, the coverslips were blocked with 1% BSA, 10% NGS for 20 min on ice, and primary antibodies were incubated overnight at 4°C. For LAMP1 staining, fibroblasts were permeabilized using 0.1% saponin after fixation. For immunolabeling, secondary antibodies included fluorescein-conjugated (FITC) and rhodamine (tetramethylrhodamine isothiocyanate) (Jackson ImmunoResearch). Coverslips were analyzed using TCS SP5 laser-scanning confocal (Leica) or Olympus BX (Olympus Optical) fluorescent microscope, and Zeiss Axiovert S100 TV2 with Hamamatsu OrcaII-ER.

Analysis of myelination
To quantify the amount of myelination, the number of MBP positive segments in each explant/cover slip was assessed. As myelination is also a function of the amount of neurites/axons and of the Schwann cell number in the culture, the network of NF-L positive filaments and the number of Schwann cells (DAPI) were also evaluated in each explant. To quantify MBP-positive fibers displaying myelin outfoldings, at least 200 MBP-positive myelinated fibers per explant/cover slip were evaluated, in at least ten different explants/cover slip. The percentage of MBP-positive fibers showing myelin outfoldings among the total number of MBP-positive fibers was counted.

Analysis of fibroblasts with enlarged late endosome/lysosomes
Fibroblasts were stained using LAMP1 antibody and images were acquired using a confocal microscope. Images were then processed using the Image J software and those cells displaying almost all LAMP1 positive endosomes bigger than 1.67 μm (only occasionally observed in wild-type cells) were considered as carrying enlarged late endosome/lysosomes.

Imaging and statistical analysis
Micrographs were acquired using a digital camera (Leica F300), and figures were prepared using Adobe Photoshop, version 7.0 and 8.0 (Adobe Systems). Statistical analysis was performed using the Student t test; two tails, unequal variants, and alpha = 0.005 were used. Error bars in the graphs represent SEM.

Lentiviral vector (LV) preparation
To downregulate PIKfyve expression, a shRNA cloned into pLKO.1 LV (human U6 promoter) without a GFP reporter was used (clone ID TRCN0000150881). Non-concentrated LVs were used for RNA interference. The transfer constructs were transfected into 293FT cells together with packaging plasmids Δ6.9 and pCMV-VSVG using Lipofectamine 2000 (Invitrogen). As control, a vector encoding a shRNA to a nonspecific sequence ( Luciferase) was used. Viral supernatants were collected 48 h after transfection, centrifuged at 3000 rpm for 15 min, and frozen at −80°C.

To check for PIKfyve depletion, freshly plated rat Schwann cells (104 cells per 100 mm plate) were incubated with the LV’s in DMEM, 10% FBS, and 2 mM L-glutamine plus forskolin and rhNRG-1 (EGF domain, R&D). Cells were expanded for an additional week and maintained in MEM, 10% FBS, 2 mM L-glutamine and 2 μM forskolin before use. A western blot using a anti-PIKfyve antibody (Santa Cruz) was performed. Using non-concentrated LV, transduction of Schwann cell/DRG neuron co-cultures was performed 4–5 days after dissection by incubating the cells with LV’s overnight. Cells were then supplemented with C-media, and myelination was induced after 2 days.

Glutathione S-transferase-binding assays
Glutathione S-transferase (GST) fusion proteins were expressed in Escherichia coli BL21 cells and purified directly from bacterial extract on glutathione-Sepharose 4 Fast Flow beads. Rat isolated Schwann cells and mouse brains were homogenated, and protein lyses were prepared using a binding buffer with 1% NP-40, 50 mM Tris buffer, pH 7.4, 10% glycerol, 100 mM NaCl, 10 mM NaF, 1 mM Na- vanadate. Equal amounts of protein lyses were incubated for 4 h at 4°C with immobilized GST fusion proteins and GST as control. After three washes with a buffer containing 0.5% NP-40, the pellets were dissolved in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting. To show the relative amount of GST fusion proteins used, beads were dissolved in SDS sample buffer and analyzed by SDS-PAGE, and the gel was stained with Coomassie.

Yeast analysis and phospholipid measurement in yeast
Yeast cells were labeled with SynaptopRed C2 (Biotium, Inc., CA). 0.1 units of cells (at 600 nm) were collected and resuspended in 250 μl fresh media. 6 μl of SynaptopRed C2 (10 μg/ml dissolved in dimethyl sulfoxide) was added to the cells and incubated at 24°C for 1 hour. Cells were then washed 2 times with fresh media and chased for 2.5 hours. Fluorescence and differential interference contrast (DIC) images were generated using a DeltaVision RT Microscope System (Applied Precision, WA). Images were processed using Softworx and Adobe Photoshop.

Measurement of phosphoinositide levels were performed as described previously [45]. Cells were grown in selective media to mid-log phase, harvested, washed, and resuspended in synthetic media lacking inositol. 1–4 x106 cells were inoculated into 5 ml of media lacking inositol containing 5 μCi of myo-[2-3H]-inositol. Cells were labeled for 18 h at 24°C, harvested by centrifugation, washed, and resuspended in 100 μl of inositol-free media. For hypertonic shock, an equal volume of 1.8 M NaCl was added to cells (for a final concentration of 0.9 M NaCl) and the resulting suspension was incubated at 24°C for the times indicated. 800 μl of ice cold 4.5% perchloric acid [46] was added to the cells. Cells were lysed in the presence of 0.5-nm zirconia beads (Biospec, Bartlesville, OK) on a Beadbeater (Biospec) for three cycles of 2 min at room temperature followed by 2 min on ice. Cell extracts were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed with 1 ml of 100 mM EDTA, centrifuged 14,000 rpm for 10 min at 4°C, and resuspended in 50 μl of sterile distilled deionized water.

Lipids were deacylated by treatment with methanolamine [47] 1 ml methanolamine reagent (10.7% methamine, 45.7% methanol, 11.4% n-butanol) was added to each sample and...
incubated at 55°C for 1 h. Samples were dried in a SpeedVac and the pellets were resuspended in 300 μl of sterile water, centrifuged at 14,000 rpm for 2 min and the supernatants were transferred to new Eppendorf tubes. 300 μl of butanol/ethyl ether/formic acid ethyl ester (20:4:1) was added to each. The samples were vortexed and centrifuged at 14,000 rpm for 2 min. The aqueous phase (bottom layer) was transferred to new tubes and the extraction was repeated. At the end of the second extraction the aqueous phase was dried in a SpeedVac. Samples were resuspended in 20 μl of sterile water and 15 μl of each was analyzed by HPLC using an anion exchange, Partisphere SAX (Whatman), column. The column was developed with a gradient of 1 M (NH₄)₂HPO₄, pH 3.8 (pH adjusted with phosphoric acid); 0% for 3 min, 1–2% over 15 min, 2% for 80 min, 2–10% over 20 min, 10% for 65 min, 10–80% over 40 min, 80% for 20 min and finally 80–0%; flow rate, 1.0 ml/min [48]. The value of each glycerol-inositol corresponding to PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,5),P and PtdIns(4,5)P₂ is reported as percent of total phosphoinositol, to normalize number of cells and incorporation of [³H] inositol.

**Supporting Information**

Figure S1  Semithin section analysis of the cerebellum from Mtmr2⁺⁺/+ and Mtmr2⁻⁻/Fig4⁻⁻/⁻ mice at P8. A disorganization of both the molecular and granular layer was observed in the cerebellum of Mtmr2⁺⁺/+ and Mtmr2⁻⁻/Fig4⁻⁻/⁻ mice. Middle panels show loss of Purkinje and basket cells which are not aligned at the border of the granular layer. Inset is showing a cell carrying vacuoles in the granular layer. Bar is 80 μm in (A–C); 50 μm in (A’–C’); 80 μm in (A”) and 50 μm in (B”, C”). (TIF)

Figure S2  Semithin section analysis of the cerebellum from Mtmr2⁺⁺/+ and Mtmr2⁻⁻/Fig4⁻⁻/⁻ mice at P20. The loss of Purkinje and basket cells is even more evident at P20 at the border of the granular layer. More vacuolated cells are present in the granular layer. Arrows indicate vacuolated cells. Bar is 80 μm in (A–C) and 50 μm in (A’–C’). (TIF)

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**Author Contributions**

Conceived and designed the experiments, as well as the averages (Avg) for each time point are presented. (DOC)

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