THE PHOSPHOINOSITIDE 3-PHOSPHATASE MTMR2 ASSOCIATES WITH MTMR13, A NOVEL MEMBRANE-ASSOCIATED PSEUДOPHOSPHATASE ALSO MUTATED IN TYPE 4B CHARCOT-MARIE-TOOTH DISEASE

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Running Title: Phosphatases mutated in Charcot-Marie-Tooth disease.

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Charcot-Marie-Tooth disease type 4B (CMT4B) is a severe, demyelinating peripheral neuropathy characterized by distinctive, focally folded myelin sheaths. CMT4B is caused by recessively inherited mutations in either myotubularin-related 2 (MTMR2) or MTMR13 (also called SBF2). MTMR2 encodes a member of the myotubularin family of phosphoinositide 3-phosphatases, which dephosphorylate phosphatidylinositol-3-phosphate (PI(3)P) and PI(3,5)P2. MTMR13 encodes a large, uncharacterized member of the myotubularin family. The MTMR13 phosphatase domain is catalytically inactive, as the essential Cys and Arg residues are absent. Given the genetic association of both MTMR2 and MTMR13 with CMT4B, we investigated the biochemical relationship between these two proteins. We find that the endogenous MTMR2 and MTMR13 proteins are associated in HEK293 cells. MTMR2-MTMR13 association is mediated by coiled-coil sequences present in each protein. We also examined the cellular localization of MTMR2 and MTMR13 using fluorescence microscopy and sub-cellular fractionation. We find that (i) MTMR13 is a predominantly membrane-associated protein, (ii) MTMR2 and MTMR13 co-fractionate in both a light membrane fraction and a cytosolic fraction, and (iii) MTMR13 membrane association is mediated by the segment of the protein that contains the pseudophosphatase domain. This work, which describes the first cellular or biochemical investigation of the MTMR13 pseudophosphatase protein, suggests that MTMR13 functions in association with MTMR2. Loss of MTMR13 function in CMT4B2 patients may lead to alterations in MTMR2 function, and subsequent alterations in 3-phosphoinositide signaling. Such a mechanism would explain the strikingly similar phenotypes of patients with recessive mutations in either MTMR2 or MTMR13.

INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is the most common inherited neurological disorder, affecting about 1 in 2000 in the United States1. The disease is a heterogeneous group of peripheral neuropathies that lead to progressive degeneration of the muscles of the extremities and loss of sensory function (1). Nerve conduction velocity tests are used to differentiate between the demyelinating forms of CMT (types 1, 3 and 4) and the axonal forms (type 2). CMT-causing mutations have been identified in 20 distinct human genes, and the cellular mechanisms by which these mutations lead to disease are under active investigation (2,3).

Type 4B CMT (CMT4B) is a severe form of demyelinating CMT with an autosomal recessive pattern of inheritance (4,5). Patients typically present with leg weakness during childhood and become wheelchair-bound in their 3rd decade (5). Nerve biopsies from CMT4B patients show a severe loss of large caliber axons and focally-folded myelin sheaths, the latter of which are considered the hallmark of CMT4B (4-6). Disease loci for CMT4B were mapped to chromosomal positions 11q22 (CMT4B1) and 11p15 (CMT4B2), which have recently been shown to represent the genes for myotubularin-related 2 (MTMR2) and MTMR13, respectively (7-11). The cellular and molecular mechanisms by
which mutations in \textit{MTMR2} or \textit{MTMR13} lead to CMT4B remain unclear.

The myotubularin proteins comprise a large family of protein tyrosine or dual specificity phosphatase (PTP/DSP)-like phosphatases; 14 members have been identified in the human genome (12-14). In addition to \textit{MTMR2} and \textit{MTMR13}, other myotubularin family members have been shown to play essential roles in mammalian physiology. Mutations in myotubularin (\textit{MTM1}) cause X-linked myotubular myopathy, a severe disorder in which skeletal muscle cells fail to maintain a properly differentiated state (15,16). In addition, \textit{Mtmr5} has been shown to be essential for spermatogenesis in mice (17).

\textit{MTMR2} homologs appear to be present in all eukaryotes (12-14). In addition to a phosphatase domain, MTMR proteins contain a PH-GRAM domain and sequences predicted to form coiled-coils (Fig. 1A) (18,19). Specific subclasses of MTMR proteins contain additional protein domains, such as the DENN and PH domains found in MTMR13 and MTMR5 (Fig. 1A). Intriguingly, 6 of the 14 human MTMR phosphatases appear to be catalytically inactive. MTMR5 and MTMRs 9 to 13 all lack the catalytically essential cysteine and arginine residues found in the Cys-x$_5$-Arg (Cx$_5$R) motif, which is the hallmark of the PTP/DSP superfamily (Fig. 1B). Such inactivating substitutions in MTMR pseudophosphatases are conserved amongst vertebrate and invertebrate species (Fig. 1C).

\textit{In vitro} studies have indicated that MTMR proteins function as PI 3-phosphatases specific for PI(3)P and PI(3,5)P$_2$ (20-28). PIs other than PI(3)P and PI(3,5)P$_2$ are very poor substrates for MTM1 and MTMR2, as are phosphoproteins (21,22,24). In addition, a number of \textit{in vivo} studies have indicated that MTMR proteins regulate PI(3)P or PI(3,5)P$_2$ (21,22,25,29-33). The specificity of MTMRs for PI(3)P and/or PI(3,5)P$_2$ suggests these enzymes may regulate endosomal trafficking, and several recent studies have described vesicular trafficking defects resulting from altered MTMR function (30-32,34). PI(3)P is primarily generated by class III PI 3-kinase (PI3K-III) or its yeast homolog Vps34p (35,36). In mammalian cells, PI(3)P is found on early endosomes and on the internal vesicles of multi-vesicular endosomes, and an analogous distribution of PI(3)P is observed in \textit{S. cerevisiae} (37,38). PI(3)P plays an essential role in the endosomal trafficking of both biosynthetic and endocytosed cargos, and is thought to function primarily by recruiting membrane proteins that contain PI(3)P-binding FYVE or PX domains (39-42). The other likely MTMR substrate, PI(3,5)P$_2$, is generated by the PI(3)P 5-kinases Fab1p (yeast) and PIKfyve (mammals) (43-48). In yeast, loss of PI(3,5)P$_2$ results in a defect in vacuolar membrane homeostasis and impairs the trafficking of some biosynthetic cargo to the vacuole (44,45). Consistent with studies of Fab1p, the expression of an inactive form of PIKfyve disrupts late endosome homeostasis in mammalian cells (49-51).

Although MTMRs have recently been shown to play essential roles in endosomal trafficking, the specific functions of these enzymes are unclear. For example, the cellular locations at which MTMRs regulate PI(3)P and/or PI(3,5)P$_2$ are unknown. In addition, the functions of the inactive, but highly conserved, MTMR pseudophosphatases are unknown. The multidomain, non-catalytic nature of large proteins such as MTMR13 and MTMR5 suggests these molecules might function as molecular scaffolds (Fig. 1). Consistent with this hypothesis, hetero-oligomerization of active and inactive MTMR proteins is emerging as a mechanism by which the active MTMR enzymes may be regulated. MTM1 has been shown to associate with the inactive MTMR12 (also called 3-PAP) (52). In addition, MTMR7 (active) has been shown to interact with MTMR9 (inactive) (26,32). Finally, MTMR2 has been shown to associate with MTMR5 (53). Inactive MTMR proteins have thus been proposed to regulate the localization or enzymatic activity of active MTMR PI 3-phosphatases (26,52,53).

Because of the genetic linkage of both MTMR2 and MTMR13 to CMT4B, we sought to determine if there is a biochemical relationship between the two proteins. Our results indicate that MTMR2 and MTMR13 form a stable complex in cultured mammalian cells. Subcellular fractionation experiments indicate that MTMR13 and MTMR2 are associated both in a particulate light membrane fraction and as a soluble complex. Thus, MTMR2 and MTMR13 may function
together as a membrane-associated complex to regulate PI(3)P and/or PI(3,5)P₂ levels.

**EXPERIMENTAL PROCEDURES**

**Phylogenetic analysis of Myotubularins** - Genbank Accession numbers for sequences used for alignments were as follows: human MTM1 (Q13496), MTMR1 (NP_003819), MTMR2 (Q13614), MTMR3 (NP_066576), MTMR4 (NP_004678), MTMR6 (NP_004676), MTMR7 (XP_044727), MTMR8 (NP_060147), MTMR5 (NP_002963), MTMR10 (NP_060232), MTMR11 (NP_002963), MTMR12 (NP_061934), mouse (M. musculus) MTMR5 (BAC98295) and MTMR13 (AAH15069), rat (R. norvegicus) MTMR5 (XP_217021) and MTMR13 (AAH15069), puffer fish (F. rubripes) MTMR5 (SINFRUP00000055664, SINFRUP00000055663) and MTMR13 (SINFRUP00000068834), worm (C. briggsae) MTMR5 (BAC98295) and MTMR13 (AAH15069), rat (M. norvegicus) MTMR5 (QP765643, QP765642), worm (C. elegans) MTMR5/13 (NP_508888), sea squirt (C. intestinalis) MTMR5/13 (ci0100143528), fly (D. melanogaster) MTMR5/13 (CG6939), and mosquito (A. gambiae) MTMR5/13 (EAA09662).

Alignments were performed using the ClustalW function of MacVector 7.7.2 (Accelrys, Inc.). Coiled-coil sequences were predicted using COILS version 2.2 (54).

**Plasmid constructs** - Expression vectors for FLAG epitope-tagged versions of human MTM1, MTMR1, MTMR2, MTMR2-Δ (Δ589-643), and MTMR6 have been described (22,24,53), as has the expression vector for EGFP-MTMR2 (24).

The sequence of the human MTMR13 cDNA used in this study is identical to the reported mRNA sequence (8,9) and consists of an open reading frame (ORF) of 5550 base pairs (bp) coding for a protein of 1849 amino acids (Genbank Accession NM_030962). To generate this construct, the KIAA1766 cDNA clone was obtained from the Kazusa DNA Research Institute (Chiba, Japan). A PCR product corresponding to bases 1-630 of the MTMR13 ORF was amplified from a human brain cDNA library using oligonucleotides FRO-243b (GCTCGGGTACCACCATGGCGCGCTGGCTGACTACTTCATCG) and FRO-208 (GCTGGAACAGGAGCCACACTATGGCCG) and digested with KpnI and SpeI and ligated to KIAA1766 plasmid that had been digested with KpnI and SpeI, yielding a construct named MTMR13-1-1150. A PCR product corresponding to bases 3275-5550 of the MTMR13 ORF was amplified from a human brain cDNA library using oligonucleotides FRO-209 (GATGAGAGTGAGCTCCCCACAACTGCC) and FRO-280 (GTCAGTCGGAGCTCCGTCTAGACTCAGGCTCGAGCGATCTGTTGTC).

This DNA fragment was digested with SacI and ligated to MTMR13-1-1150 that was digested with SacI. The resulting construct, MTMR13-5 KpnI-3 XbaI, contained the full-length MTMR13 ORF, flanked by 5′ KpnI and 3′ XbaI sites, between the KpnI and SacI sites of the pBluescript II SK(+) vector (Stratagene). To generate myc-MTMR13 (N-terminal tag), MTMR13-5 KpnI-3 XbaI was digested with KpnI and XbaI and the MTMR13 ORF was ligated to KpnI-XbaI-digested N-myc-CMV5-1. EGFP-MTMR13 was made by digesting MTMR13-5 KpnI-3 XbaI with KpnI and XbaI and ligation of the EGFP-MTMR13 ORF to KpnI-XbaI-digested pEGFP-C1 (Clontech). Myc-MTMR13 was made by digesting MTMR13-5 KpnI-3 XbaI with KpnI and XbaI and ligation of the MTMR13 ORF to KpnI-XbaI-digested pEGFP-C1 (Clontech). Myc-MTMR13 was made by digesting MTMR13-5 KpnI-3 XbaI with KpnI and XbaI and ligation of the MTMR13 ORF to KpnI-XbaI-digested pEGFP-C1 (Clontech). Myc-MTMR13 was made by digesting MTMR13-5 KpnI-3 XbaI with KpnI and XbaI and ligation of the MTMR13 ORF to KpnI-XbaI-digested pEGFP-C1 (Clontech). Myc-MTMR13 was made by digesting MTMR13-5 KpnI-3 XbaI with KpnI and XbaI and ligation of the MTMR13 ORF to KpnI-XbaI-digested pEGFP-C1 (Clontech).

Cell culture, transfection, immunoprecipitation and immunoblotting - HEK293 and HEK293A cells were cultured in DMEM (high glucose) containing 10% fetal bovine serum and penicillin/streptomycin and were ~80% confluent at the time of transfection. A 150 mm dish of cells was transfected with 30 µg of plasmid DNA using FuGENE6 (Roche) according to the manufacturers
instructions. Forty-eight hours after transfection, cells were washed with PBS and lysed in 3 ml of ice-cold lysis buffer (120 mM NaCl, 50 Tris mM (pH 8.0), 0.5 % Triton X-100, 100 mM NaF, 1 mM PMSF, 1 µg/ml Leupeptin, 10.5 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM benzamidine, 1 mM Na ortho-vanadate). Lysates were vortexed and cleared by centrifugation (17,000 g for 15 minutes at 4°C). Myc-tagged proteins were immunoprecipitated using anti-c-myc monoclonal antibody 9E10 (Roche) and protein A agarose (Invitrogen). FLAG-tagged proteins were immunoprecipitated using anti-FLAG-M2 affinity gel (Sigma). MTMR2 was immunoprecipitated using immuno or pre-immune serum from rabbit 119 and protein A agarose. Immunoprecipitations were incubated for 2 hours at 4°C and subsequently washed twice with 1 ml of lysis buffer, three times with 1 ml of lysis buffer containing 0.5 M NaCl and once more with 1 ml of lysis buffer. Immunoprecipitates were suspended in NuPAGE™ LDS sample buffer (Invitrogen). All immunoprecipitates and lysates were resolved in 4-12% or 10% NuPAGE™ Bis-Tris gels in MOPS buffer (Invitrogen), transferred to PVDF membranes and analyzed by immunoblotting.

Sub-cellular fractionation - HEK293 cells were washed with PBS, suspended in ice-cold hypotonic lysis buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors, and disrupted using a Dounce homogenizer. Samples were centrifuged at 1000g for 10 minutes at 4°C. The resulting post-nuclear supernatant was centrifuged at 14,000g for 15 minutes at 4°C to yield a heavy membrane pellet (P14) and a supernatant (S14). The S14 fraction was centrifuged at 100,000g for 1 hour at 4°C to a yield light membrane/vesicular pellet (P100) and a soluble fraction (S100). Equal proportions of the three fractions were analyzed by SDS-PAGE and immunoblotting. Densitometry was performed using a BioRad GS-800 densitometer and Quantity One 4.3.1 software. For each immunoblot, multiple X-ray films were scanned to ensure that a given exposure fell within the linear range of the film. Percoll density gradient fractionation of HEK293 cells was performed as described (55) and equal volumes of fractions were analyzed by SDS-PAGE and immunoblotting.

Antibodies - The anti-MTMR13 antibody (116-AP) was generated by immunizing rabbits with a recombinant protein consisting of the C-terminal PH domain from human MTMR13. Anti-MTMR13 116-AP antibodies were affinity-purified from the serum of rabbit 116 using a column of PHMTMR13 protein. The 2014 anti-MTMR13 antibody was generated by immunizing rabbits with a peptide corresponding to human MTMR13 residues 1062-1077 which was coupled to Keyhole Limpet Hemocyanin. Total serum IgG was purified using a protein A agarose column. The anti-MTMR2 antibody (119-AP) was generated by immunizing rabbits with full-length, recombinant, human MTMR2 containing an N-terminal His6 tag. Anti-MTMR2 antibodies were affinity-purified from the serum of rabbit 119 using a column of His6-MTMR2 protein. Commercial monoclonal mouse antibodies were obtained from Sigma (FLAG and Na+/K+ ATPase), BD Biosciences (LAMP1, GFP, Rab5, and EEA1), Ambion (GAPDH), Calbiochem (Rab9) and Roche (c-Myc). The Rab7 polyclonal rabbit antibody was a gift from Dr. Suzanne Pfeffer (Stanford University).

Fluorescence microscopy - HEK293A cells were cultured on lysine-coated covers slips and transfected with EGFP-MTMR2, EGFP-MTMR13 or EGFP control vector. About 48 hours later, cells were washed twice with PBS and fixed for 15 minutes at 37°C in a solution of 3.7% (v/v) formaldehyde. Coverslips/cells were washed twice with PBS, once with water, and were then mounted using Airvol 205 mounting medium containing 2.5% (w/v) DABCO (1,4-diazobicyclo[2.2.2]-octane). EGFP fluorescence was observed using a Leica HC microscope with a 100x oil objective (Leica 506042, PL APO, 1.40, PH3, ∞/0.17/D). Images were captured using a Hamamatsu C4742-95 digital camera and Openlab 4.0.1 software.

GST-MTMR13 coiled-coil pull down – HEK293 cells were transfected with FLAG-MTMR2 and lysates were prepared as described above. Twenty µg of GST or 25.5 µg of GST-CCMTMR13 protein was added to a 3 ml lysis derived from one 150 mm dish of HEK293 cells. This mixture was incubated overnight at 4°C, 20 µl of packed glutathione-agarose beads (Sigma) were added and
mixing continued for 45 minutes. Glutathione-agarose beads and associated proteins were washed as described above and analyzed by SDS-PAGE and immunoblotting.

RESULTS

CMT4B1 and CMT4B2 are both recessively inherited, strongly suggesting that it is a loss of function of either MTMR2 or MTMR13 that leads to demyelination (7-11). In accord with these genetic findings, loss of PI 3-phosphatase activity has been observed for all of the CMT4B-causing MTMR2 mutations that have been examined in vitro (23). Thus, it may well be the loss of MTMR2-mediated regulation of PI(3)P and/or PI(3,5)P2 that leads to CMT4B. How probable loss of function mutations in the catalytically inactive MTMR13 protein lead to a strikingly similar peripheral neuropathy phenotype is unknown. The genetic link between MTMR2 and MTMR13 led us to investigate potential biochemical links between the two proteins.

Association of MTMR2 and MTMR13 - To investigate the function of the uncharacterized MTMR13 protein, we considered whether MTMR13 might associate with, and potentially regulate MTMR2. In order to examine this question, we first prepared a full-length MTMR13 cDNA and also generated antibodies specific for both MTMR2 and MTMR13 (see Experimental Procedures). Both MTMR2 and MTMR13 were detected in immunoblots of HEK293 cell extracts, however such experiments suggested that MTMR13 is significantly less abundant than MTMR2 in this cell type (data not shown). To examine whether MTMR2 and MTMR13 might associate in vivo, we used an anti-MTMR2 serum to immunoprecipitate the endogenous MTMR2 protein from HEK293 cells (Fig. 2A). Anti-MTMR2 serum immunoprecipitated both endogenous MTMR2 and the associated MTMR13 from cell extracts, whereas pre-immune serum did not (Fig. 2A). In accord with this finding, association of MTMR2 and MTMR13 was also observed in immunoprecipitation experiments in which epitope-tagged versions of both MTMR2 and MTMR13 were over-expressed (Fig. 2B). Importantly, the anti-MTMR2 antibody used for immunoprecipitation failed to recognize either MTM1 or MTMR1, (the two MTMRs most closely related to MTMR2) indicating that this serum is specific for MTMR2 (Fig. 2C). These data indicate that the endogenous MTMR2 and MTMR13 proteins are associated in HEK293 cells.

MTMR2-MTMR13 association is mediated by coiled-coil sequences - We sought to characterize the MTMR2-MTMR13 complex by defining the sequences that mediate association of the proteins. Most MTMR phosphatases contain predicted coiled-coil sequences near their C-termini (Fig. 1A, Fig. 3A) (12-14), and in several cases, oligomerization of MTMR proteins has been found to require these sequences (26,53,56). Mutant MTMR13 proteins containing C-terminal deletions were expressed in HEK293 cells and tested for association with MTMR2 (Fig. 3A). Deletion of residues 1683-1849, which removed the PH domain and PDZ binding motif from MTMR13, had no impact on association with MTMR2 (Fig. 3B). However, deletion of residues 1630-1849, which removed the coiled-coil sequence, eliminated MTMR13 association with MTMR2 (Fig. 3B). In addition, an in-frame deletion of the MTMR13 coiled-coil sequence (Δ1630-1683; mutant ΔCC) abolished association with MTMR2. Notably, when performing immunoprecipitations of myc-MTMR13, we consistently observed that the co-expression of MTMR2 led to a dramatic increase in the recovery of MTMR13 (Fig. 3C, compare lanes 1 & 2), suggesting that the stability of MTMR13 is enhanced through association with MTMR2. This phenomenon likely accounts for the reduced abundance of the MTMR13 mutant proteins ΔCC-ΔPH and ΔCC, which are unable to associate with MTMR2 (Fig. 3B).

We also examined whether the coiled-coil segment of MTMR2 is required for association with MTMR13. Deletion of the PDZ binding motif from MTMR2 (mutant ΔPDZB; Thr641stop) had no effect on association with MTMR13 (Fig. 3D). However, deletion of both the coiled-coil and PDZ binding motifs of MTMR2 (mutant ΔCC; Δ589-643) abolished association with MTMR13, indicating that the coiled-coil sequence is essential for association (Fig. 3D). As mentioned above, failure to associate with MTMR2 resulted in a dramatically reduced recovery of the immunoprecipitated wild type myc-MTMR13.
protein (Fig. 3D, lane 3). Finally, we examined whether the coiled-coil interaction is sufficient to mediate association of MTMR2 and MTMR13. A recombinant protein, consisting of the MTMR13 coiled-coil sequence fused to GST, was used in an in vitro pull-down experiment. GST-CC<sub>MTMR13</sub> protein precipitated FLAG-MTMR2 from an HEK293 cell lysate, whereas GST control protein did not (Fig. 3E), indicating that the coiled-coil sequence of MTMR13 is sufficient to mediate association with MTMR2. Collectively, these data indicate that MTMR2-MTMR13 association is mediated by coiled-coil sequences.

**Oligomerization of MTMR2 and MTMR13**

MTMR2 has been shown to form homodimers via its C-terminal coiled-coil domain (53,56). Although the specific molecular function of MTMR2 homo-dimerization remains unclear, inhibition of dimerization (through deletion of the coiled-coil sequence) has been reported to lead to abnormal localization of over-expressed MTMR2 in COS cells (56). We sought to compare the propensity of MTMR2 to form homodimers versus hetero-oligomers with MTMR13. FLAG-MTMR2 was immunoprecipitated from transfected HEK293 cells that also expressed either EGFP-MTMR2 or EGFP-MTMR13. Although MTMR2 homo-dimerization was observed, hetero-oligomerization with MTMR13 was much more pronounced (Fig. 4A). Consistent with this interpretation is the finding that EGFP-MTMR13 was expressed at a very low level relative to EGFP-MTMR2 (Fig. 4A, lysate blots). Thus, MTMR2 homo-dimerization was observed, hetero-oligomerization with MTMR13 may be a higher affinity interaction than that of MTMR2 homo-dimerization. We also evaluated the specificity of MTMR2-MTMR13 association by examining whether MTMR13 might associate with several additional active MTMR phosphatases. FLAG-MTMR2 co-immunoprecipitated with myc-MTMR13, while FLAG-MTM1 and FLAG-MTMR6 did not (Fig. 4B) (MTM1 and MTMR6 are 64% and 45% identical to MTMR2, respectively), suggesting that MTMR13 associates specifically with MTMR2.

**Cellular localization of MTMR2 and MTMR13** - The subcellular localizations of active MTMR phosphatases will likely dictate which pools of PI(3)P and/or PI(3,5)P<sub>2</sub> these enzymes regulate, and may provide insight into the roles of MTMRs in membrane trafficking. Through their interactions with active MTMRs, inactive MTMR pseudophosphatases have been proposed to regulate the localization of PI 3-phosphatase activity (52,53). Toward testing this hypothesis, we sought to examine the cellular localizations of MTMR13 and MTMR2. EGFP fusions to MTMR13 and MTMR2 were over-expressed in HEK293A cells and examined by fluorescence microscopy. EGFP-MTMR13 fluorescence was very faint, consistent with a very low level of protein expression (data not shown and Fig. 4A). EGFP-MTMR13 was excluded from the nucleus and broadly distributed throughout the cytoplasm (Fig. 5B). EGFP-MTMR2 also exhibited a diffuse cytoplasmic staining pattern with some enrichment in the perinuclear zone (Fig. 5C), consistent with previous work involving over-expressed MTMR2 (24,29,56).

Although over-expressed MTMR2 and MTMR13 showed diffuse cytoplasmic localizations (Fig. 5B/C), we suspected that the endogenous proteins might associate with membranes, where phosphoinositide substrates are located. We therefore used subcellular fractionation to examine the distributions of the endogenous MTMR2 and MTMR13 proteins in HEK293 cells (see Experimental Procedures). Post-nuclear supernatants were fractionated by differential centrifugation, yielding soluble (S100), heavy membrane (P14) and light membrane/microsomal (P100) fractions, which were analyzed by immunoblotting and densitometry (see Fig. 5 legend). A majority of MTMR13 (~75%) was associated with membrane fractions, with the protein being the most abundant in the P100 fraction, which is enriched for vesicles and other light membranes (Fig. 5D). MTMR2 was most abundant in the soluble (S100) fraction, however about 25% of MTMR2 immunoreactivity co-fractionated with MTMR13 in the P100 fraction (Fig. 5D). Thus, significant fractions of both MTMR2 and MTMR13 are found in the vesicle-enriched P100 fraction, suggesting that these two proteins may form a complex that is associated with vesicles or other light membranes. MTMR2 and MTMR13 are likely also present as a cytosolic complex, as both proteins are found in the S100 fraction. Consistent with these findings, an MTMR2-MTMR13 complex can be
immunoprecipitated from both the S100 and the P100 fraction (data not shown).

We compared the sub-cellular distributions of MTMR2 and MTMR13 to those of proteins of the early endosome, where PI(3)P is found, and also to proteins of the late endosome/lysosome, where PI(3,5)P2 is likely localized. Unlike the membrane-associated MTMR2, the pelletable portions of the early endosome markers EEA1 and Rab5 were enriched in the P14 fraction (Fig. 5D). Thus, the membrane-associated portion of MTMR2 does not appear to be enriched on early endosomal membranes. We also compared the distributions of MTMR2 and MTMR13 to that of LAMP1, a marker of the late endosome and lysosome. LAMP1 was most abundant in the P14 heavy membrane fraction, and was present to a lesser extent in the P100 fraction, which contained MTMR2 and MTMR13 (Fig. 5D).

The finding that MTMR2 can dephosphorylate PI(3,5)P2 suggests that the enzyme might be localized to the late endosome, a site of PI(3,5)P2 function. Indeed, we did observe LAMP1 immunoreactivity in the P100 fraction, which contained MTMR2 and MTMR13 (Fig. 5D). To further examine this issue, we employed a distinct fractionation approach to examine whether the endogenous MTMR2 and MTMR13 proteins co-fractionate with markers of the late endosome and lysosome. HEK293 cells were fractionated by Percoll density gradient centrifugation (see Experimental Procedures). Under these conditions, the early endosome marker EEA1 was largely found in the lighter fractions, whereas late endosome markers (Rab7 and LAMP1) were enriched in the more dense fractions (Fig. 5E), consistent with previous reports (55,57). MTMR2 and MTMR13 largely co-fractionated and were enriched in the light fractions at the top of the gradient (fractions 1-4), which also contained EEA1 (Fig. 5E).

Importantly, the membrane-associated portions of MTMR2 and MTMR13 that were found in the P100 fraction (Fig. 5D) are likely too light to travel far into the Percoll gradient and are therefore found near the top of the gradient with soluble proteins (Fig. 5E, fractions 1-4). A significant fraction of MTMR13 is found in the more dense fractions (fractions 6-10), which are enriched for markers of the late endosome/lysosome and plasma membrane, but contain little MTMR2 (Fig. 5E). Fractions 5-13 correspond generally to the P14 heavy membrane fraction of Figure 5D. Our findings using Percoll density gradient centrifugation indicate that only a minor fraction of MTMR2 (or MTMR2-MTMR13 complex) co-fractionates with late endosomal membranes under these conditions. Although separation between early endosome membranes and the membrane-associated portion of the MTMR2-MTMR13 complex was observed using differential centrifugation (Fig. 5D), a similar separation was not achieved using this Percoll gradient profile (Fig. 5E).

Roles of specific MTMR13 sequences in membrane localization - The previously uncharacterized MTMR13 pseudophosphatase has a protein domain composition that suggests it may function as a molecular scaffold (Fig. 6A). We demonstrate here that ~75% of the endogenous MTMR13 protein is found in the membrane fractions of HEK293 cells (Fig. 5D). Membrane association of MTMR13 might play a role in localizing MTMR2 to specific membrane compartments, where phosphoinositide substrates are found. As MTMR13 is a large protein containing a unique and highly-conserved collection of protein domains (Fig. 6A), we considered which MTMR13 sequences might mediate localization to membranes. First, the C-terminal PH domain of MTMR13 contains a consensus motif required for binding to PI(3,4)P2 and/or PI(3,4,5)P3 (58). Second, the PDZ-binding motif at the C-terminus of MTMR13 might potentially localize the protein to membranes, as many PDZ domain-containing proteins reside in macromolecular complexes near the plasma membrane. Third, although the functions of DENN domains are not understood, several DENN domain-containing proteins have been shown to regulate or associate with Rab family GTPases, suggesting involvement in membrane trafficking (59). Finally, although the roles of MTMR family PH-GRAM domains in phosphoinositide binding or membrane localization remain unclear (18,34,56), Berger et al found the PH-GRAM domain of MTMR2 to bind in vitro to PI(4)P, PI(5)P, PI(3,5)P2 and PI(3,4,5)P3, suggesting that this domain might be involved in phosphoinositide binding (56).
We examined the essentiality of specific MTMR13 sequences for localization to membranes. Myc-tagged versions of wild type and mutant MTMR13 proteins were expressed in HEK293 cells and subcellular localization was examined using fractionation by differential centrifugation (Fig. 6). Over-expressed myc-MTMR13 was distributed amongst the soluble and membrane fractions in a manner largely similar to that of the endogenous protein (compare Fig. 5D and Fig. 6B). However, the fraction of myc-MTMR13 present in the S100 soluble fraction was somewhat increased relative to that of the P100 microsomal fraction (Fig. 6B). The MTMR13 deletion mutants Δ1-771, 772-1629 and 1028-1629 were each found distributed amongst the soluble (S100) and membrane fractions (P14 and P100) in a manner similar to that observed for full-length myc-MTMR13 (Fig. 6B). These findings indicate that the DENN, PH-GRAM, coiled-coil, PH and PDZB domains are not required for MTMR13 membrane targeting. Thus, residues 1028-1629, which contain the pseudophosphatase domain, are sufficient to target MTMR13 to membranes.

DISCUSSION

The MTMR phosphatases and pseudophosphatases comprise a large family of proteins likely to function in vesicular trafficking (12-14). Both the active MTMRs and the inactive MTMR pseudophosphatases are conserved between humans, flies and nematodes (Fig. 1). The genomes of the yeasts S. cerevisiae and S. pombe each contain a single active MTMR, but appear to lack inactive MTMRs. Presumptive loss of function mutations in a number of both active and inactive MTMRs lead to specific and distinct disease phenotypes in mammals (7-11,15,17), and to vesicular trafficking defects in C. elegans and S. cerevisiae (30-32). As PI 3-phosphatases acting to down-regulate PI(3)P and/or PI(3,5)P 2 (20-28), the active MTMRs play critical, but ill-defined roles in the regulation of endosomal trafficking (30-32,34). The cellular functions of the catalytically inactive, but highly conserved MTMR pseudophosphatases are less clear (26,32,52,53,60).

Recently, genetic studies have identified MTMR2 and MTMR13 as genes mutated in a distinctive form of Charcot-Marie-Tooth peripheral neuropathy (7-11). CMT4B1 (MTMR2) and CMT4B2 (MTMR13) are autosomal recessive forms of demyelinating CMT, distinguished from other forms by the presence of a distinctive focal out-looping of myelin within peripheral nerves (4,6,7). These genetic findings, combined with the growing awareness that active MTMR phosphatases may be regulated via the association of inactive MTMR pseudophosphatases (26,32,52,53), led us to investigate whether the MTMR2 and MTMR13 proteins might physically associate. Using a co-immunoprecipitation approach, we show here that the endogenous MTMR2 and MTMR13 proteins are associated in HEK293 cells. MTMR2-MTMR13 association is mediated by coiled-coil sequences present in each protein. The association of MTMR2 and MTMR13 is supported by the co-fractionation of the two proteins in subcellular localization experiments, where MTMR2 and MTMR13 are associated in both cytosolic and membrane fractions. We find also that MTMR13 is predominantly membrane-associated, raising the possibility that MTMR2 and MTMR13 function as a membrane-associated protein complex that regulates PI(3)P and/or PI(3,5)P 2.

Subcellular localization of MTMR2 and MTMR13 - PI(3)P and PI(3,5)P 2, the likely in vivo substrates of MTMR phosphatases, play essential roles in endosomal trafficking (35,36,44,61,62). In mammalian cells, PI(3)P is found on the cytoplasmic faces of early endosomes and the internal vesicles of multi-vesicular endosomes/bodies (MVEs/MVBs) (37). Analogously, in yeast, PI(3)P is found on intraluminal vesicles of both endosomes and vacuoles (37). The intracellular localization of PI(3,5)P 2 is somewhat less clear. PI(3,5)P 2 is generated from endosomal PI(3)P by the PI(3)P 5-kinases Fab1p (yeast) or PIKfyve (mammals) (44,48,61). Collectively, studies of Fab1p/PIKfyve in both yeast and mammalian cells largely indicate that PI(3,5)P 2 function is required in the portion of the endocytic pathway that includes the MVE/MVB, late endosome, and the limiting membrane of the vacuole/lysosome (40,50,51,63).

One possible scenario has MTMR phosphatases acting to regulate PI(3)P or PI(3,5)P 2 on the endomembranes where these phosphoinositides are normally formed. However, MTMRs have largely been found to be cytosolic
proteins, suggesting that these proteins are not constitutively associated with specific endosomal compartments, although many of these studies have involved over-expression (22,24,52,60,64,65). Several reports have documented partial localization of MTMRs to various membranes. For example, over-expressed MTM1 has shown a mixed cytoplasmic and plasma membrane localization (22,52,65). Tsujita et al have reported that over-expressed GFP-MTM1 moves from the cytosol to the late endosome following EGF treatment (34). Endogenous MTMR7 has also been reported to be partially membrane-associated in neuroblastoma cells and may be enriched in the Golgi or endosomes (26).

We examined the localization of the endogenous MTMR2 and MTMR13 proteins in HEK293 cells using two distinct sub-cellular fractionation approaches. In differential centrifugation experiments, MTMR2 and MTMR13 co-fractionate in both a vesicle-enriched, microsomal fraction (P100) and soluble fraction (S100). Little MTMR2 was found in the heavy membrane (P14) fraction, which was heavily enriched for markers of the plasma membrane, early and late endosome, and lysosome, although about 20% of MTMR13 immunoreactivity was found in this fraction (Fig. 5D). Thus, MTMR2 and MTMR13 are likely associated both as a soluble complex and as a membrane-associated complex that largely fails to co-fractionate with markers of endosomal compartments. A second experiment involved using a Percoll density gradient to examine the intracellular distributions of MTMR2 and MTMR13. Under these conditions, MTMR2 and MTMR13 largely co-fractionated and were enriched in the top portion of the gradient, whereas markers of heavier membrane compartments, such as late endosome, lysosome and plasma membrane, were found in the more dense fractions (Fig. 5E). Thus, in these experiments using HEK293 cells, the endogenous MTMR2 protein largely fails to co-fractionate with markers of the late endosome, where PI(3,5)P2 is thought to function. The further characterization of the membrane compartment(s) to which MTMR2 and MTMR13 are localized will likely provide insight into the functions of these proteins and perhaps shed light on the cellular etiology of CMT4B.

The molecular functions of MTMR pseudophosphatases are unknown. Due to their non-catalytic nature and their ability to associate with active MTMRs, it has been proposed that these proteins may regulate the localization of catalytically active MTMRs (52,53). We demonstrate that the endogenous MTMR13 protein is predominantly membrane-associated in HEK293 cells (Fig. 5D). Using a deletion analysis, we also find that the segment of MTMR13 containing the pseudophosphatase domain is sufficient to target MTMR13 to membranes. Our findings suggest that, at least in the case of MTMR13, the pseudophosphatase domain functions as a membrane localization sequence. Future studies will address the nature of MTMR13/MTMR2 association with membranes.

Biological implications of MTMR2-MTMR13 association - Five distinct CMT4B2-causing mutations in MTMR13 have been reported (8-11) (Fig. 1A). In addition to CMT4B2 peripheral neuropathy, patients homozygous for the nonsense mutations R482X, Q959X or R1196X also manifest early-onset glaucoma (9,10). In contrast, CMT4B2 patients homozygous for a small in-frame deletion within the DENN domain (Δ352-432) do not develop glaucoma (8). Mutations R482X, Q959X and R1196X are predicted to induce the nonsense-mediated mRNA decay response (66), which will likely lead to a complete loss of the MTMR13 protein, as suggested in (10). CMT4B2 patients homozygous for the Δ352-432 deletion express a mutant MTMR13 mRNA containing this deletion (8), suggesting that this deletion may affect MTMR13 at the protein rather than mRNA level. Thus, the Δ352-432 mutation may lead to a partially functional MTMR13 protein that is sufficient to prevent glaucoma, but unable to support normal peripheral nerve myelination, as suggested in (10). As early-onset glaucoma has not been reported in association with CMT4B1, this disease phenotype likely indicates a specific function for MTMR13 that does not involve MTMR2.

The physical association of MTMR2 and MTMR13, which we have described here, suggests that a complex of these two proteins may play a critical role in mammalian peripheral
nerves. The striking similarity between the focally-folded myelin observed in CMT4B1 and CMT4B2 patients (4,6,7) is in accord with this hypothesis. Recently, Bolino et al reported a description of an Mtmr2-deficient mouse, which exhibits a CMT4B1-like peripheral neuropathy (67). In addition, male Mtmr2-deficient mice display defects in spermatogenesis. Azoospermia has also been reported in a CMT4B1 patient (13). Loss of Mtmr2 appears to damage adherens junctions between Sertoli cells and developing spermatocytes, resulting in the shedding of immature spermatocytes into the lumen of seminiferous tubules (67). Defective spermatogenesis was also observed in Mtmr5-deficient mice (17). MTMR5 is 57% identical to MTMR13 and possesses the same collection of protein domains, including a pseudophosphatase domain (Fig. 1A, B). Within rodent seminiferous tubules, the Mtmr2 and Mtmr5 proteins are each selectively expressed in both Sertoli and germ cells (17,68) and our laboratory has shown that MTMR2 associates with MTMR5 (53). Thus, MTMR2 and MTMR5 may function as an essential complex within cells of the seminiferous epithelium, such as Sertoli cells. If this hypothesis were correct, loss of either member of the MTMR2-MTMR5 complex would result in a similar spermatogenesis defect.

The association of MTMR2 with both MTMR13 and MTMR5, combined with knowledge of the disease phenotypes manifested by humans and mice with loss of function mutations in these factors, suggests an intriguing biological paradigm. As mutation of either MTMR2 or MTMR13 leads to the distinctive CMT4B peripheral neuropathy, the MTMR2 and MTMR13 proteins may act as a complex to carry out an essential regulatory function in peripheral nerves. In the absence of MTMR13, MTMR2 PI 3-phosphatase activity may be misregulated, leading to altered levels of PI(3)P and/or PI(3,5)P2 and subsequent vesicular trafficking defects. By analogy, because mutation of either MTMR2 or MTMR5 leads to a similar defect in spermatogenesis, an MTMR2-MTMR5 complex may play an essential and related role within cells of the seminiferous tubule epithelium (13,17,67).

REFERENCES


FOOTNOTES

1 Charcot-Marie Tooth Association (charcot-marie-tooth.org).
2 Abbreviations are: MTMR, myotubularin related; PH, pleckstrin homology; GRAM, glucosyltransferases, Rab-like GTPase activators and myotubularins; DENN, differentially expressed in...
normal vs. neoplastic; P-loop, phosphate (binding) loop; CC, coiled-coil; PDZB, PSD95/Dlg/ZO-1 binding; FYVE, Fab1, YGL023, Vps27, and EEA1; PX, phagocyte NADPH oxidase; 3-PAP, 3-phosphatase adapter protein; SFB, SET binding factor 2; PI, phosphatidylinositol or phosphoinositides; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EEA1, early endosome antigen 1; LAMP1, lysosome-associated membrane protein 1.

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FIGURE LEGENDS

Fig. 1. (A) Domain compositions of the human MTMR2 and MTMR13 proteins. CMT4B2-causing mutations in MTMR13 are indicated above the protein schematic. The exon 32 splice donor (SD) site mutation (11) is predicted to alter the MTMR13 protein downstream of Val1482. (B) An alignment of the active site residues (P-loop; Cx5R) of the human MTMR phosphatases (protein name in green) and pseudophosphatases (protein name in red). The catalytic Cys and Arg residues present in all active protein tyrosine/dual-specificity phosphatases (PTPs/DSPs) are highlighted with orange and blue, respectively. MTMR pseudophosphatases contain evolutionarily conserved substitutions in the catalytic Cys and Arg residues. (C) An alignment of the active site residues of the MTMR5/13 orthologs/paralogs from humans and a number of other species. Vertebrate genomes contain both MTMR5 and MTMR13 orthologs, whereas invertebrate genomes contain a single member of this class of pseudophosphatase.

Fig. 2. Association of the endogenous MTMR2 and MTMR13 proteins in HEK293 cells. (A) Co-immunoprecipitation of the endogenous MTMR2 and MTMR13 proteins from HEK293 cells. MTMR13 was detected using the 116-AP anti-MTMR13 antibody. The association of MTMR2 and MTMR13 was further supported by an experiment in which a second anti-MTMR13 antibody (Ab 2014) was used to detect endogenous MTMR13 that immunoprecipitated in association with over-expressed FLAG-MTMR2 (data not shown). Representative data from three experiments are presented. (B) Specific immunoprecipitation of MTMR2 using the 119 anti-serum. Cells were either left untransfected or were transfected with constructs encoding FLAG or c-myc epitope tagged versions of the indicated proteins. After ~40 hours, cell lysates were prepared and analyzed by immunoprecipitation (IP), SDS-PAGE and immunoblotting (IB). The small amount of myc-MTMR13 visible in lane 3 is likely to have immunoprecipitated in association with endogenous MTMR2. A non-specific cross-reaction of the anti-FLAG antibody with a protein present in cell lysates is indicated (ns). (C) The anti-MTMR2 antibody 119 does not cross react with MTM1 or MTMR1.

Fig. 3. MTMR2-MTMR13 association is mediated by coiled-coil sequences. (A) The positions of predicted coiled-coil sequences within the C-terminal portions of MTMR2 and MTMR13. HEK293 cells were transfected with constructs encoding FLAG or c-myc epitope tagged versions of the indicated proteins. After 48 hours, cell lysates were prepared and analyzed by immunoprecipitation (IP), SDS-PAGE and immunoblotting (IB). A non-specific cross-reaction of the anti-FLAG antibody with a protein
present in cell lysates is indicated \( (ns) \). \( (B) \) The predicted coiled-coil sequence of MTMR13 is required for association with MTMR2. \( (C) \) Co-expression of FLAG-MTMR2 increases the recovery of immunoprecipitated myc-MTMR13 from HEK293 cells. \( (D) \) The coiled-coil sequence of MTMR2 is required for association with MTMR13. \( (E) \) The predicted coiled-coil sequence from MTMR13 is sufficient for association with MTMR2. Recombinant GST-CC\(_{MTMR13}\) protein (MTMR13 residues 1612-1680) precipitated FLAG-MTMR2 from an HEK293 cell lysate, whereas GST protein did not. Representative data from three experiments are presented.

**Fig. 4.** Homo- and hetero-oligomerization of MTMR2 and MTMR13. HEK293 cells were left untransfected or transfected with constructs encoding FLAG, e-myc or EGFP-tagged versions of the indicated proteins. After ~40 hours, cell lysates were prepared and analyzed by immunoprecipitation (IP), SDS-PAGE and immunoblotting (IB). \( (A) \) FLAG-MTMR2 immunoprecipitates EGFP-MTMR13 much more efficiently than it does EGFP-MTMR2. Note the very low expression level of EGFP-MTMR2. A non-specific cross-reaction of the anti-FLAG antibody with a protein present in cell lysates is indicated \( (ns) \). \( (B) \) Co-immunoprecipitation of MTMR13 with MTMR2, but not MTM1 or MTMR6. Representative data from one of two experiments are presented.

**Fig. 5.** Subcellular localizations of MTMR2 and MTMR13 in HEK293A and HEK293 cells. \( (A-C) \) HEK293A cells were transfected with EGFP vector, EGFP-MTMR13 or EGFP-MTMR2. After ~48 hours, cells were fixed and examined by fluorescence microscopy as described in Experimental Procedures. Representative data from one of three experiments are presented. \( (D) \) HEK293 cells were fractionated to yield soluble (S100), heavy membrane (P14) and microsomal (P100) fractions as described in Experimental Procedures. Equal proportions of fractions were analyzed by SDS-PAGE and immunoblotting (IB) with antibodies specific to MTMR13 (116-AP) and MTMR2. GAPDH served as an example of a cytosolic (C) protein and the Na+/K+ ATPase (\( \alpha \) subunit) served as a marker of the plasma membrane (PM). EEA1 and Rab5 served as markers for early endosomes (EE). Rab9 served as a marker of the late endosome (LE) and trans-Golgi network (TGN). LAMP1 served as a marker of both the late endosome and lysosome (L). Representative data from one of three experiments are presented. The relative abundance of MTMR13 and MTMR2 in subcellular fractions was determined by densitometry analysis of immunoblots. MTMR2 (S100: 73.3 ± 6.89%; P14: 23.8 ± 0.82%; P100: 24.4 ± 6.24%) and MTMR13 (S100: 27.9 ± 4.96%; P14: 18.3 ± 2.94%; P100: 53.8 ± 3.49%). Densitometry data are the average of three experiments and are presented with the standard deviation. \( (E) \) Subcellular fractionation of HEK293 cells by Percoll density gradient centrifugation. Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting (IB) with antibodies specific to MTMR13 (116-AP), MTMR2 and marker proteins. Rab7 served as a marker for late endosome membranes. Representative data from one of two experiments are presented.

**Fig. 6.** Roles of specific protein domain sequences in membrane localization of MTMR13. HEK293 cells were transfected with constructs encoding c-myc epitope-tagged versions of MTMR13 or the indicated MTMR13 truncation mutants \( (A) \). After 48 hours, cells were lysed and fractionated as in Figure 5D. \( (B) \) Equal proportions of fractions were analyzed by SDS-PAGE and immunoblotting (IB). Representative data from one of three experiments are presented.
Figure 2

A

IP: Preimmune anti-MTMR2
IB: anti-MTMR2
-64 kDa
IB: anti-MTMR13 (Ab 116)
-191 kDa

B

IP: Preimmune anti-MTMR2
myc-MTMR13
FLAG-MTMR2
IB: myc
MTMR13
IB: FLAG
MTMR2
Lysates
IB: FLAG
ns MTMR2

C

IP: FLAG
IB: anti-MTMR2
-64 kDa
IP: FLAG
IB: FLAG
64 kDa
Figure 3

A

MTMR2 (C-term.)

MTMR13 (C-term.)

ΔPH

ΔCC-ΔPH

ΔCC

B

myc-MTMR13

FLAG-MTMR2

IP: myc

IB: FLAG

IP: myc

IB: myc

Lysates

IB: FLAG

C

IP: myc

IB: myc

IP: myc

IB: myc

Lysates

IB: FLAG

D

FLAG-MTMR2

myc-MTMR13

IP: myc

IB: FLAG

IP: myc

IB: myc

Lysates

IB: FLAG

E

Pull-down:

GST

GST-CC13

Lysates

IB: FLAG

1 2

MTMR2

MTMR2

MTMR2

MTMR2

MTMR2

MTMR2
**Figure 5**

**A** EGFP  
**B** EGFP-MTMR13  
**C** EGFP-MTMR2

**D**

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**E**

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**Legend:**

- **A**: EGFP  
- **B**: EGFP-MTMR13  
- **C**: EGFP-MTMR2  
- **D**: Fractionation of proteins  
- **E**: Western blot analysis of proteins
Figure 6

(A) MTMR13

- DENN
- PH-GRAM
- Phosphatase (inactive)
- CC
- PH

- Δ1-771
- 772-1629
- 1028-1629

(B) IB: anti-myc (MTMR13)

- S100
- P14
- P100

- 191
- 97
- 64
The phosphoinositide 3-phosphatase MTMR2 associates with MTMR13, a novel membrane-associated pseudophosphatase also mutated in type 4B Charcot-Marie-tooth disease

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