Differential phosphorylation of the phosphoinositide 3-phosphatase MTMR2 regulates its association with early endosomal subtypes

Norah E. Franklin*, Christopher A. Bonham*, Besa Xhabija and Panayiotis O. Vacratsis‡

Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, N9B3P4, Canada

*These authors contributed equally to this work
‡Author for correspondence (vacratsi@uwindsor.ca)

Accepted 2 January 2013
Journal of Cell Science 126, 1333–1344
© 2013. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.113928

Summary
Myotubularin-related 2 (MTMR2) is a 3-phosphoinositide lipid phosphatase with specificity towards the D-3 position of phosphoinositol 3-phosphate [PI(3)P] and phosphoinositol 3,5-bisphosphate lipids enriched on endosomal structures. Recently, we have shown that phosphorylation of MTMR2 on Ser58 is responsible for its cytoplasmic sequestration and that a phosphorylation-deficient variant (S58A) targets MTMR2 to Rab5-positive endosomes resulting in PI(3)P depletion and an increase in endosomal signaling, including a significant increase in ERK1/2 activation. Using in vitro kinase assays, cellular MAPK inhibitors, siRNA knockdown and a phospho-specific-Ser58 antibody, we now provide evidence that ERK1/2 is the kinase responsible for phosphorylating MTMR2 at position Ser58, which suggests that the endosomal targeting of MTMR2 is regulated through an ERK1/2 negative feedback mechanism. Surprisingly, treatment with multiple MAPK inhibitors resulted in a MTMR2 localization shift from Rab5-positive endosomes to the more proximal APPL1-positive endosomes. This MTMR2 localization shift was recapitulated when a double phosphorylation-deficient mutant (MTMR2 S58A/S631A) was characterized. Moreover, expression of this double phosphorylation-deficient MTMR2 variant led to a more sustained and pronounced increase in ERK1/2 activation compared with MTMR2 S58A. Further analysis of combinatorial mutant (MTMR2 S58A/S631A) was characterized. Moreover, expression of this double phosphorylation-deficient MTMR2 variant led to a more sustained and pronounced increase in ERK1/2 activation compared with MTMR2 S58A. Further analysis of combinatorial phospho-mimetic mutants demonstrated that it is the phosphorylation status of Ser58 that regulates general endosomal binding and that the phosphorylation status of Ser631 mediates the endosomal shuttling between Rab5 and APPL1 subtypes. Taken together, these results reveal that MTMR2 compartmentalization and potential subsequent effects on endosome maturation and endosome signaling are dynamically regulated through MAPK-mediated differential phosphorylation events.

Key words: APPL1, ERK, MTMR2, Phosphoinositol 3-phosphate, PI(3)P, Endocytosis, Phosphorylation

Introduction
Although the majority of protein tyrosine phosphatases (PTPs) use phosphoryl tyrosine-containing proteins as substrates, the active myotubularin (MTM) family members dephosphorylate the lipid second messengers phosphoinositol 3-phosphate [PI(3)P] and PI(3,5)P2 (Blondeau et al., 2000; Kim et al., 2002; Taylor et al., 2000; Walker et al., 2001). These phosphoinositols mainly reside on endocytic structures and play key roles in membrane targeting, vesicular trafficking, and regulation of signal transduction pathways by interacting and recruiting distinct signaling proteins containing appropriate phosphoinositol-binding module(s) (Balla, 2005; Cullen et al., 2001; Vanhaesebroeck et al., 2001). The importance of regulating phosphoinositol phosphorylation is highlighted by the fact that loss of function mutations in three mtmr genes has been associated with distinct neuromuscular disorders (Boerkoel et al., 2002; Bolino et al., 2000; Laporte et al., 1996). For example, the gene encoding MTMR2 is mutated in Charcot–Marie-Tooth (CMT) disease 4B, an autosomal recessive demyelinating disorder, characterized by abnormally folded myelin sheaths, inadequate nerve signaling to muscles, and eventual muscle weakness and atrophy (Boerkoel et al., 2002).

Despite the fact that pathophysiological consequences resulting from loss of MTMR2 function are well established, how MTMR2 participates in trafficking events remains poorly understood. Intriguingly, under typical cell culture conditions, MTMR2 is not widely localized to the endosomal structures containing its substrates PI(3)P and PI(3,5)P2. Recently, our laboratory used mass spectrometry to identify a prominent phosphorylation site at Ser58 that is close to the PH-GRAM domain of MTMR2 (Franklin et al., 2011). Functional characterization of this modification demonstrated that phosphorylation dramatically regulates MTMR2 endosomal localization and thus access to its lipid substrates (Franklin et al., 2011). Specifically, mass spectrometry analysis suggested that the stoichiometry of phosphorylation at Ser58 was greater than 50%, providing evidence that MTMR2 Ser58 may be highly phosphorylated during normal cell growth conditions. A phosphorylation deficient variant (MTMR2 S58A) displays strong endosomal localization with Rab5-positive vesicles resulting in efficient depletion of PI(3)P and an increase in growth factor receptor signaling pathways, most notably the extracellular signal-regulated kinase (ERK). Therefore, reversible phosphorylation represents a critical mechanism regulating the endosomal targeting of MTMR2 and provides valuable insight into how MTMR2 activity toward its lipid substrates can be spatially and temporally controlled. However, the exact
The Rab5 GTPase is a master coordinator of early endosome maturation events including homotypic early endosome fusion as well as heterotypic early to late endosome fusion (Barr and Lambright, 2010; Spang, 2009). Furthermore, Rab5 collaborates with numerous peripheral endosome binding proteins to facilitate fusion of endosome membranes including Rab5 effectors whose endosome targeting requires association with endosomal PI(3)P (Gaullier et al., 2000; Merithew et al., 2003; Schnatwinkel et al., 2004; Zerial and McBride, 2001). This property reveals that the endosomal activities of Rab5 can be dramatically affected by PI(3)P levels. For example, the critical endosomal effectors Rabenosyn-5 and Early Endosomal Antigen 1 (EEA1) are essential tethering factors important for endosome fusion events (Merithew et al., 2003; Schnatwinkel et al., 2004; Zerial and McBride, 2001). These effectors require association with Rab5 and PI(3)P simultaneously to localize to early endosomes.

Recently it has been shown that endosomes rich in the adaptor protein containing a pleckstrin homology domain, phosphotyrosine binding domain and a leucine zipper motif (APPL1) represent a critical stage during attenuation of receptor signaling and initiation of early endosome maturation. It has been demonstrated that when PI(3)P is depleted using the PI(3)K inhibitor wortmannin, Rab5/EEA1-positive early endosomes are converted to Rab5/APPL1-positive early endosomes (Zoncu et al., 2009). Interestingly, the Rab5/APPL1 endosomes displayed a delay in endosomal maturation and enhanced growth factor endosomal signaling capability (Zoncu et al., 2009). In addition to functioning as a Rab5 effector and as a regulator of endosomal signaling, APPL1 serves as a stage marker for proximal early endosomes (Miaczynska et al., 2004). The shedding of APPL1 as vesicles move centrally coincides with increased levels of PI(3)P, recruitment of EEA1 and subsequent maturation into the canonical PI(3)P/Rab5-positive endosomes (Swan et al., 2010; Zoncu et al., 2009). Since APPL1 vesicles are known to play a functional role in the assembly of signaling complexes for MAPK and Akt pathways, increased levels of PI(3)P acts as a molecular switch to turn off growth factor endosomal signaling through the simultaneous loss of APPL1 on these endosomes.

In this study, we provide evidence that ERK1/2 is the Ser58 kinase and thus may regulate endosomal targeting of MTMR2 via a negative feedback loop. Moreover, we have characterized a C-terminal phosphorylation site at position Ser631 that regulates the targeting of MTMR2 to APPL1-rich endosomes presenting a mechanism through which MTMR2 can shuttle between different endosomal compartments to target different pools of PI(3)P.

**Results**

**ERK1/2 phosphorylates MTMR2 at position Ser58 in vitro**

Phosphorylation of Ser58 strongly correlates to sequestration of MTMR2 away from its physiological endosome substrate PI(3)P (Franklin et al., 2011). The ability to monitor phosphorylation levels of MTMR2 Ser58 would then reflect both the pseudo-inactive state of MTMR2 towards this endosomal PI(3)P, and also provide an indirect measure of the targeted endosomal PI(3)P levels. Furthermore, evidence of the responsible kinase(s) would be key information to understanding the mechanisms that underlie this critical regulatory event. To this end, we designed and generated a polyclonal phospho-specific antibody, and using immunoblot analysis, characterized its phosphospecificity towards MTMR2 Ser58 (Fig. 1A,B). The antibody readily detects wild type MTMR2 while treatment with alkaline phosphatase completely ablates the signal similar to that of MTMR2 S58A, emphasizing phosphate-dependent recognition. Interestingly, the phospho-specific antibody also detected MTMR2 S58E supporting our previous results that this phosphomimetic mutant of MTMR2 functionally behaves like phosphorylated MTMR2 (Franklin et al., 2011).

Ser58 resides in a so-called SP motif, suggesting that MTMR2 is regulated by proline directed kinases such as members of the mitogen activated protein kinase (MAPK) family. As phosphorylation deficient MTMR2 localizes to endosomes and enhances ERK1/2 activation (Franklin et al., 2011), our hypothesis is that activated ERK1/2 may regulate MTMR2 in a negative feedback loop by phosphorylating Ser58 to induce MTMR2 release from endosomes. As a first step towards testing this hypothesis, we employed in vitro kinase assays, and using our Ser58 phospho-specific antibody, examined the ability of MTMR2 to be phosphorylated by distinct MAPK family members.

Recombinant MTMR2 was purified from bacterial cell lysates and in vitro kinase assays using commercially available active MAPKs were performed. Following the kinase assay, the proteins were separated by SDS/PAGE and phosphorylation of MTMR2 at Ser58 was determined by immunoblot analysis using our phospho-Ser58 antibody (Fig. 1C). It was determined that both ERK1 and ERK2 could effectively phosphorylate MTMR2 at Ser58 (Fig. 1C, upper panel). In contrast, neither recombinant JNK1 nor p38α were able to detectably phosphorylate MTMR2 Ser58 in vitro.

While the kinase assays demonstrate direct phosphorylation in vitro, it was important to examine the phosphorylation status of MTMR2 Ser58 in cells whose expression levels of ERK1/2 have been reduced using small interfering RNA (siRNA). HEK293 cells transfected with ERK1/2 siRNA were grown under low serum conditions for 6 hours followed by a 10 minutes EGF stimulation before cell lysis. These conditions effectively knocked down total ERK1/2 protein levels and greatly reduced the levels of active phosphorylated ERK1/2 in the cells (Fig. 1D; for quantitation of the data, see supplementary material Fig. S1). This reduction in ERK1/2 levels significantly decreased the phosphorylation levels of MTMR2 at position Ser58 compared to control (~50% reduction), consistent with our in vitro kinase assay implementing ERK1/2 as the responsible kinase for Ser58 phosphorylation.

**Inhibition of ERK1/2 targets MTMR2 to endosomal structures**

To obtain insight into the potential of ERK1/2 as a regulator of MTMR2 endosomal localization, we performed immunofluorescence experiments in HeLa cells using various MAPK inhibitors to examine if attenuation of ERK1/2 activation would alter the localization of wild type MTMR2. This would be evident by the occurrence of a MTMR2 shift from the typical diffuse cytoplasmic pattern when phosphorylated (Berger et al., 2003; Franklin et al., 2011; Kim et al., 2003; Laporte et al., 2002), to an endosomal punctate pattern exhibited by unphosphorylated MTMR2 (Franklin et al., 2011). Consistent with the in vitro kinase assays, inhibitors to JNK and p38 did not alter the localization pattern of MTMR2 (Fig. 2F,H). However, when cells were treated with the MAPK/ERK kinase (MEK) inhibitor U0126, to inhibit the ERK1/2 pathway, this
dramatically re-localized MTMR2 to endosomal punctate structures, exhibiting a localization pattern analogous to MTMR2 S58A (Fig. 2D). Moreover, when we analyzed the localization of MTMR2 in ERK1/2 siRNA-treated HeLa cells, we observed a similar shift of MTMR2 localization to endosomal punctate structures (supplementary material Fig. S2). These results indicate that inhibition of the ERK1/2 pathway in vivo elevates MTMR2 endosomal targeting presumably by preventing Ser58 phosphorylation.

Phosphorylation of MTMR2 at Ser58 is greatly reduced following ERK1/2 inhibition

In response to various stimuli including growth factors such as epidermal growth factor (EGF), ERK1/2 becomes activated by phosphorylation (pERK1/2), which in turns leads to phosphorylation of downstream target substrates (Bardwell and Shah, 2006). To complement the immunofluorescence results, HEK293 cells expressing MTMR2 were treated with EGF and examined for the effects on Ser58 phosphorylation following MEK inhibition (Fig. 3). Under control conditions, MTMR2 displayed high levels of phosphorylation at position Ser58, which decreased following a 30-minute serum starvation period (Fig. 3A). EGF stimulation transiently activated ERK1/2, detected by phosphorylation levels of ERK1/2 (Fig. 3; supplementary material Fig. S3) and those of a downstream ERK1/2 substrate, S6 kinase (pS6K). Under these conditions, MTMR2 Ser58 phosphorylation levels also increased ~2-fold compared to the untreated samples (Fig. 3B). Importantly, this observed increase in Ser58 phosphorylation was strongly attenuated when EGF stimulated cells were pre-treated with the MEK inhibitor (Fig. 3).

These results, taken together with our in vitro kinase assay and the ERK1/2 knockdown studies, strongly implicate ERK1/2 as a kinase capable of phosphorylating MTMR2 at position Ser58 and potently regulating the endosomal localization of MTMR2.

Inhibition of JNK1 and ERK1/2 targets MTMR2 to APPL1 endosomes

Recent phosphoproteomic studies have mapped Ser631 as a phosphorylation site on MTMR2 (Dephoure et al., 2008). Interestingly, Ser631 also resides in a proline-directed consensus site suggesting that a MAPK family member may also regulate this phosphorylation event. Thus, we were interested in examining the endosomal localization pattern of MTMR2 in response to different MAPK inhibitors (Fig. 4; supplementary material Fig. S4). HeLa cells expressing MTMR2 were treated with various MAPK inhibitors and examined for colocalization with the early endosome marker Rab5. Untreated, MTMR2 displayed a punctate pattern with a subset of vesicles that were positive for both MTMR2 and Rab5 (Fig. 4A, middle panel). The extent of Rab5

Fig. 1. In vitro phosphorylation of recombinant MTMR2 by ERK1/2. (A) PhosphoSer58 and non-phosphoSer58 peptides were incubated with and without alkaline phosphatase and spotted on nitrocellulose membrane and probed with pSer58 antibody. (B) HEK293 cells were transfected with MTMR2 phospho-variants, FLAG immunoprecipitated and probed for total MTMR2 (IB: FLAG) and phosphorylated MTMR2 at Ser58 (IB: pSer58). UT, untreated. (C) Purified His<sub>6</sub> bacterial recombinant MTMR2 was the substrate in the in vitro MAPK kinase assay. Each reaction contained 5 μg of recombinant MTMR2, 100 ng of various kinases (top), 200 μM ATP and was incubated at 30°C for 30 minutes. The phosphorylation of Ser58 MTMR2 was detected using pSer58 antibody. Total MTMR2 levels were determined with α-MTMR2 antibody. Reactions were probed for total kinase levels, which served as additional loading controls. (D) HEK 293 cells expressing MTMR2 were transfected with control scramble siRNA or MTMR2 with ERK1/2 siRNA and cultured as described in Materials and Methods. Cells were stimulated with 5 ng/ml EGF for 10 minutes, lysed and analyzed by immunoblot analysis.
colocalization was similar to that observed with MTMR2 SS8A and Rab5 where the colocalized staining seem to be directly adjacent or on opposite sides of the same vesicle (Franklin et al., 2011). Combined inhibition of JNK1 and MEK1/2 displayed a pronounced MTMR2 punctate pattern. However, these vesicles were completely void of Rab5 colocalization and were noticeably enlarged (Fig. 4A, bottom panel; supplementary material Table S1). Additionally, combined inhibition of p38 and MEK1/2 resulted in a partial colocalization of MTMR2 and Rab5, a similar phenotype as the MEK inhibitor alone (supplementary material Fig. S5).

Recently, APPL1 containing vesicles were shown to define an early transient endosome subpopulation that resides downstream of clathrin-coated pits en route to PI(3)P-positive early endosomes. PI(3)P depletion at the early endosomes resulted in reversion of PI(3)P/Rab5 endosomes back to their preceding APPL1 endosomes with accompanying vesicle enlargement (Zoncu et al., 2009). Thus, we investigated whether the enlarged MTMR2-positive endosomes observed in response to double MAPK inhibition were positive for APPL1 using co-immunofluorescence. As shown in Fig. 4B, HeLa cells expressing MTMR2 strongly colocalized with APPL1 when the cells were treated with the MEK1/2 and JNK1 inhibitors. However, inhibition of the ERK1/2 pathway alone resulted in a punctate staining pattern that was devoid of APPL1 co-staining (Fig. 4B).

Fig. 2. Inhibition of the ERK1/2 pathway induces subcellular targeting of MTMR2 to punctate regions. (A–J) HeLa cells were transiently transfected with FLAG-MTMR2 or FLAG-MTMR2 SS8A and treated with the MEK inhibitor U0126 (ERK1/2) at 50 μM for 1 hour, SB203580 (p38) at 20 μM for 30 minutes and SP600125 (JNK) at 40 μM for 1 hour. Cells were probed for FLAG-MTMR2 (red) using Alexa Fluor 568. Boxes indicate regions of interest and are presented in expanded views. Images were collected using 63× objectives. Scale bars: 15 μm.

Fig. 3. Inhibition of the ERK1/2 pathway causes a decrease in Ser58 MTMR2 phosphorylation. (A) HEK293 cells were transiently transfected with FLAG-MTMR2 for 42 hours. Cells were serum starved (S.S) for 30 minutes then treated with the MEK inhibitor U0126 to downregulate ERK1/2 activity (where indicated), followed by stimulation with 5 ng/ml EGF for 5 or 30 minutes at 37°C. Cells were lysed and FLAG immunoprecipitated (IP), then probed for phosphorylation of Ser58 MTMR2. FLAG-MTMR2 immunoblotting (IB) of FLAG-IP was used to confirm equal transfections levels. Total ERK1/2, actin and FLAG lysates served as loading controls. (B) MTMR2 Ser58 phosphorylation was quantified by densitometry using ImageJ and normalized to total FLAG (IP). Representative western blots are shown in A. Means ± s.d. of the results of three independent experiments are given, with the values representing fold change relative to serum-starved control (s.s) without inhibitor. *P<0.05, **P<0.01 for the comparisons indicated.

Serum starvation promotes MTMR2 localization to Rab5 and APPL1 endosomes

In a variety cell types, serum starvation has also been shown to inhibit MAPK activation (Hu et al., 2003; Levin et al., 2010; Pirkmajer and Chibalin, 2011). Notably, we observed a significant decrease in MTMR2 Ser58 phosphorylation levels...
when cells were grown under low serum conditions that also exhibited low ERK1/2 activation (Fig. 3A). We also observed this pattern when we analyzed endogenous MTMR2 from serum starved cells (supplementary material Fig. S6). Since MAPK-mediated phosphorylation of MTMR2 impedes its targeting to early endosomes, we postulated that our serum starved conditions would enhance MTMR2 localization to endosomal compartments due to low MAPK activation. Interestingly, upon serum starvation conditions we observed significant colocalization of MTMR2 with Rab5 positive endosomes (Fig. 5A) (quantitation of the data, supplementary material Fig. S7). This observation was also recapitulated at the endogenous level (supplementary material Fig. S8). Furthermore, a subset of MTMR2 also colocalizes with APPL1 positive endosomes under these low serum conditions (Fig. 5B; supplementary material Fig. S7). Collectively, our results complement the MTMR2 phospho Ser58 immunoblot analysis performed under similar conditions while further supporting the hypothesis that MAPK-mediated phosphorylation regulates endosome targeting of MTMR2.

Phosphorylation-deficient MTMR2 at positions Ser58 and Ser631 results in APPL1 endosomal targeting

In an effort to complement our MAPK inhibitor treatments and determine the relationship with respect to the N-terminal and C-terminal phosphorylation sites of MTMR2, we generated MTMR2 double phosphorylation site mutants at Ser58 and Ser631 and examined their ability to colocalize with APPL1 or Rab5. Our previous work has shown that MTMR2 S58A colocalizes with Rab5 (Franklin et al., 2011), thus we initially analyzed if MTMR2 S58A could also colocalize with APPL1 (Fig. 6A top panel; supplementary material Fig. S9). Although we observed a significant amount of MTMR2 positive vesicles, MTMR2 did not display any significant colocalization with APPL1. In contrast, HeLa cells expressing the double phosphorylation-deficient mutant (S58A/S631A) displayed a punctate pattern that appeared to be localized just on the inside of the cell periphery but did not colocalize with Rab5 (Fig. 6B; supplementary material Fig. S7). Intriguingly, we instead observed that MTMR2 S58A/S631A strongly colocalized with APPL1, with the colocalized vesicles being enlarged in comparison to those only positive for APPL1 (Fig. 6B, enlarged views; supplementary material Table S1). Notably, upon mutation of the catalytic residue (S58A/C417S/S631A) this enlargement did not occur (supplementary material Fig. S10) and these vesicles were comparable in size to those of APPL1 alone (supplementary material Table S1B). Quantitative analysis of vesicle area revealed that those vesicles which were positive for both MTMR2 and APPL1 were enlarged only when MTMR2

![Fig. 4. Inhibition of ERK1/2 and JNK pathways targets MTMR2 to APPL1-positive endosomes. (A,B) HeLa cells were transiently transfected with FLAG-MTMR2 for 42 hours and analyzed by immunofluorescence microscopy. Cells were untreated (top panel) or treated with the following inhibitors: U0126 alone (middle panel) or combined with SP600125 (bottom panel). Cells were probed for FLAG MTMR2 (red) and endogenous Rab5 (green) (A) or endogenous APPL1 (B). Solid white arrows indicate regions of colocalization and open arrows indicate lack of vesicle colocalization and are presented in expanded views. Images were collected using 63× objectives. Scale bars: 15 μm.](image-url)
PI(3)P catalytic activity was intact (supplementary material Table S1). These suggestive findings are consistent with the hypothesis that the phosphatase activity of MTMR2 contributes to the observed enlargement of MTMR2/APPL1 positive vesicles.

Importantly, the double phosphorylation mutant recapitulated the observations seen with the double MAPK inhibitor treatment. Taken together, this is the first report of MTMR2 localizing to APPL1-positive early endosomes; an occurrence that seems to be mediated by dephosphorylation at position Ser631 of MTMR2.

Phosphorylation of Ser58 regulates general endosomal association while phosphorylation of Ser631 regulates endosomal shuttling

We have previously determined that dephosphorylation at Ser58 results in MTMR2 endosomal targeting; however, it is unclear if dephosphorylation at position Ser631 is sufficient for endosomal localization. As shown in Fig. 7, the S58E/S631A mutant of MTMR2 lacked any significant punctate pattern, suggesting that dephosphorylation of Ser631 is not sufficient to mediate endosomal targeting when Ser58 is phosphorylated. In contrast, the S58A/S631E mutant did display the characteristic punctate

Fig. 5. Serum starvation induces subcellular targeting of MTMR2 to Rab5 and APPL1-positive endosomes. (A,B) HeLa cells were transiently transfected with FLAG-MTMR2 for 42 hours and analyzed by immunofluorescence microscopy. (A) Untreated cells (top panel) and serum starved cells (lower panel) were probed for FLAG-MTMR2 (red) and Rab5 (green). Serum starvation resulted in an increase in the colocalization of FLAG-MTMR2 with Rab5-positive endosomes. (B) Cells were probed with FLAG-MTMR2 (red) and APPL1 (green). Serum starvation resulted in an increase in the colocalization of FLAG-MTMR2 with APPL1-positive endosomes. Merged images display regions of colocalization and are represented in expanded views. Images were collected using 40× oil objectives. Arrows indicate regions of colocalization. Scale bars: 15 μm.

Fig. 6. Dephosphorylation of Ser58 and Ser631 regulates MTMR2 localization to APPL1 endosomes. (A,B) HeLa cells were transiently transfected with FLAG-MTMR2 phospho-variants for 42 hours and analyzed by immunofluorescence microscopy. (A) Cells were transfected with S58A or S58A/S631A phospho-variants and were probed for FLAG-MTMR2 (red) and APPL1 (green). (B) Cells were transfected with S58A/S631A phospho-variant and probed for FLAG-MTMR2 (red) and endogenous Rab5 (green). Solid arrows indicate regions of colocalization on enlarged vesicles and open arrows mark smaller vesicles devoid of colocalization. Regions of interest are presented in expanded views. Images were collected using 40× oil and 63× objectives. Scale bars: 15 μm.
staining pattern and partial colocalization with Rab5 to an extent similar to that observed with the single point mutations at Ser58 (S58A) (Fig. 7A; supplementary material Fig. S11). As expected, we did not observe any regions of colocalization with APPL1 (Fig. 7B). These results argue that dephosphorylation of Ser58 is sufficient for targeting MTMR2 to endosomal structures.

We postulated that the partial colocalization with Rab5 may be due to the phosphatase activity of MTMR2 since many Rab5 effectors require PI(3)P for localizing to Rab5-positive endosomes (Gaullier et al., 2000; Merithew et al., 2003; Schnatwinkel et al., 2004; Zerial and McBride, 2001). Therefore we created a MTMR2 phosphatase inactive mutant in the S58A/S631E background and reassessed the ability to colocalize to Rab5-positive and APPL1-positive endosomes. As expected, this mutant also did not colocalize with APPL1 (Fig. 7B, bottom panel). However, we observed prominent colocalization between MTMR2 S58A/C417S/S631E and Rab5 compared to the catalytically active variant (Fig. 7A, bottom panel). Thus, these results further support the notion that Ser58 must be unphosphorylated for MTMR2 to localize to endosomal structures, while phosphorylation at position Ser631 seems to regulate the early endosome subtype accessible to MTMR2.

To further define the function of MTMR2 on APPL1-positive early endosomes, we examined if the S58A/S631A mutant was capable of depleting PI(3)P levels from endosomal structures. This was accomplished by analyzing the dispersion of a well characterized PI(3)P binding protein marker, EGFP-2×FYVE. This chimeric protein is thought to largely localize to Rab5-positive early endosomes and re-distributes to cytoplasmic structures when PI(3)P is depleted by lipid phosphatases or wortmannin treatment (Gillooly et al., 2000). MTMR2 S58A/S631E expression resulted in a significant loss of the characteristic punctate staining pattern for the PI(3)P marker similar to what we had observed previously for MTMR2 S58A (Fig. 8A, bottom panel) (Franklin et al., 2011). In contrast, co-expression of the MTMR2 S58A/S631A mutant with EGFP-2×FYVE revealed no effect on the localization of the PI(3)P marker as displayed by the punctate staining pattern and lack of any colocalization (Fig. 8A, top panel). These results suggest that Ser58 dephosphorylation and Ser631 phosphorylation is necessary for efficient PI(3)P depletion at Rab5 positive early endosomes.

It is possible that depletion of PI(3)P on APPL1-positive endosomes by MTMR2 S58A/S631A may have not been resolved due to the fact that the EGFP-2×FYVE marker is
enriched on Rab5-positive endosomes. Thus, we proposed that examining the phosphatase inactive MTMR2 variants would achieve greater sensitivity for establishing a functional relationship between MTMR2 and APPL1 (Fig. 8B; supplementary material Fig. S12). First we determined that APPL1 endosomes do contain detectable levels of PI(3)P accessible to the EGFP-2×FYVE marker, albeit at lower levels compared to Rab5-positive endosomes (supplementary material Fig. S13). Also, the inactive MTMR2 S58A/C417S/S631E mutant (which localizes to Rab5-positive endosomes) displayed nearly complete colocalization with the PI(3)P marker as expected (Fig. 8B lower panel). Intriguingly, S58A/C417S/S631A, which localizes to APPL1 endosomes (supplementary material Fig. S13), displayed a subset of vesicles showing colocalization with the PI(3)P marker (Fig. 8B; supplementary material Fig. S12). This colocalization pattern is consistent with our working hypothesis that MTMR2 may localize to APPL1-positive endosomes in order to deplete the low levels of PI(3)P within this proximal early endosome stage.

**MTMR2 localization to APPL1 endosomes increases ERK1/2 activation**

Previously we have determined that endosomal targeting of MTMR2 is mediated through dephosphorylation at Ser58 and subsequently results in enhanced signaling events particularly with respect to ERK1/2 (Franklin et al., 2011). Interestingly, it has been demonstrated that PI(3)P depletion prolongs residency of growth factor receptors within APPL1 endosomes, resulting in extended receptor signaling and activation of key signaling pathways including ERK1/2 (Zoncu et al., 2009). Considering that dephosphorylation at Ser58 and Ser631 targets MTMR2 to APPL1-positive endosomes, we postulated that expression of this double phosphorylation site mutant would further enhance endosomal signaling from these vesicles.

To test this hypothesis, we examined the activation of ERK1/2 in HEK293 cells expressing wild type MTMR2, MTMR2 S58A or MTMR2 S58A/S631A which were stimulated with EGF. Consistent with our previous findings, a reproducible increase in the phosphorylation of ERK1/2 was observed with cells expressing S58A as compared to wild-type (Fig. 9A). Densitometry analysis revealed an approximate 40% increase in ERK1/2 activation with S58A compared to wild-type (5-minute time period), which eventually tapered off back down to levels consistent with wild-type (Fig. 9B). Remarkably, cells transfected with S58A/S631A displayed further enhanced activation of ERK1/2 compared to S58A. More surprising however, was the duration of sustained ERK1/2 activation in cells expressing the double phosphorylation mutant of MTMR2 compared to the transient ERK1/2 activation seen with MTMR2 S58A. Accompanying densitometry analysis revealed that there was nearly a 50% and 80% difference at the 15–30 and 60-minute time periods, respectively, for cells expressing S58A/S631A compared to both wild-type and S58A (Fig. 9B). Altogether, these results suggest that the targeting of MTMR2 to APPL1 endosomes, mediated by dephosphorylation at Ser58 and Ser631, may enhance the endosomal signaling properties of APPL1-positive endosomes.

**Discussion**

In recent years, it has become apparent that early endosomes are heterogeneous in terms of both their protein and phosphoinositol lipid composition on the vesicle surface (Hayakawa et al., 2006; Lakadamyali et al., 2006). This presumably provides for functional diversity that likely contributes to differences in cargo destination, endosome maturation rates, and endosomal signaling. The finding in this study that phospho-isomers of MTMR2 localize to different endosomal subtypes illustrates the important role of reversible post-translational modifications in...
regulating and achieving early endosome heterogeneity. In the case of MTMR2, our data strongly suggests that Ser58 is the master regulator for the general endosomal binding capacity of MTMR2, as the phosphomimetic mutation (S58E) sequestered MTMR2 in the cytoplasm regardless of the phosphorylation status of Ser631. Meanwhile, phosphorylation of Ser631 seems to regulate early endosomal subtype destination; if phosphorylated, MTMR2 is targeted to PI(3)P-rich Rab5-positive endosomes, if unphosphorylated, MTMR2 is targeted to APPL1-positive endosomes. We are currently employing structural mass spectrometry studies to understand the mechanistic details of why and how phosphorylation of Ser58 so potently prevents MTMR2 endosomal localization. We are also interested in examining the mechanisms by which phosphorylation of the C-terminal Ser631 site mediates subtype specificity. Since MTMR2 possesses multiple protein–protein interaction domains in its C-terminus, we suspect that phosphorylation augments or attenuates the accessibility of these domains towards accessory molecules that are responsible for recruiting MTMR2 to specific endosome subtypes.

There are numerous reports that have described APPL1 and APPL2 as Rab5 effector molecules that colocalize on early endosomes (Chial et al., 2008; Miazynska et al., 2004; Zoncu et al., 2009). Moreover, the dissociation of APPL1 from early endosomes during the conversion of APPL1 endosomes into Rab5/EEA1 early endosomes is mediated by the accumulation of PI(3)P, and in turn, the recruitment of FYVE domain containing proteins including EEA1. Therefore, the generation and accumulation of PI(3)P is thought to function as the molecular switch that controls maturation into the canonical early endosomes. It has also been shown that EEA1 and APPL1 compete for a limited number of binding sites on Rab5-positive endosomes (Zoncu et al., 2009). When PI(3)P is depleted, the binding of APPL1 to Rab5-positive endosomes is dramatically increased, while in contrast, attenuation of APPL1 association to endosomes by PI(3)P generation allows for proficient stabilization of EEA1 binding to Rab5 endosomes. This competitive scenario is thus mediated by both PI(3)P kinases and phosphatases to ultimately regulate the signaling and maturation properties of early endocytic events transitioning through these particular stages. Moreover, this effect further transitions to downstream sorting machinery which utilize PI(3)P binding modules for activity. For example, we recently identified a novel PI(3)P binding protein, receptor-mediated endocytosis-8 (RME-8), involved in endosomal sorting decisions and retrograde transport (Xhabija et al., 2011). RME-8 association with early endosomes was dependent on PI(3)P levels and attenuated by MTMR2 lipid phosphatase activity exemplifying the importance of phosphoinositol levels in a diversity of endosomal events.

A surprising result from our study was that MTMR2 S58A/S631A or stimulated equivalents colocalized with APPL1 but not Rab5, suggesting that this doubly dephosphorylated MTMR2 localizes to APPL1 endosomes that are Rab5-negative. In all cases, this resulted in enlarged vesicle formation of MTMR2/APPL1-positive endosomes when compared to APPL-positive endosomes alone. Importantly, however, when catalytic activity of MTMR2 was impaired (S58A/C417S/S631A), vesicle size did not change, remaining comparable to sole APPL1 containing endosomes. One possibility is that MTMR2/APPL1 endosomes are a distinct endosomal subtype that functions as an initial platform for downstream signaling events regulated in a PI(3)P fashion. However, a more likely explanation is that the MTMR2/APPL1 endosomes represent a proximal endosomal stage that precedes Rab5/APPL1 early endosome formation, potentially as a means to spatially and temporally control PI(3)P levels which in part regulates the linked signaling events. The significant increase in ERK1/2 activation upon expression of MTMR2 S58A/S631A and the observed enlarged MTMR2/APPL1 endosomes supports this hypothesis. One possibility is that this effect is due to MTMR2-mediated PI(3)P depletion that leads to halting or retardation of endosomal maturation, a phenotype which others have shown to result in endosome enlargement and continued receptor signaling (Akbergenova and Bykhovskaia, 2009; Berlin et al., 2010; Kim et al., 2010). However, further testing will be needed, as our current results don’t directly demonstrate PI(3)P depletion on APPL1-positive endosomes. Nonetheless, our findings are reminiscent of an elegant study by De Camilli and colleagues who used a chimeric...
MTM1 inducible system to deplete PI(3)P from EE/A1/Rab5 vesicles causing reversion back into APPL1-positive endosomes. Importantly in this study, these PI(3)P-depleted APPL1 endosomes were also enlarged and displayed increased growth factor signaling, with enlargement also being dependent on the catalytic activity of MTM1 (Zoncu et al., 2009). Moreover, Larjani and colleagues, who were the first group to use the chimeric MTM1 inducible system, concluded that PI(3)P depletion resulted in endosomal tubulation rather than enlargement (Fili et al., 2006). Both of these studies, however, clearly demonstrated that depletion of PI(3)P on early endosome compartments autocatalytically compromises endosome maturation. Analogous to the inducible chimeric MTM1 system, our results highlight how controlling the PI(3)P phosphatase activity of MTMR2 via reversible phosphorylation serves as a potent mechanism to regulate the residency time of MTMR2 on these various early endosomal subtypes. MTM1 also possess potential phosphorylation sites within similar N-terminal and C-terminal regions. It will be interesting to determine if the subcellular targeting of MTM1 and other MTM family members are similarly regulated by reversible phosphorylation.

Our discovery that ERK1/2 regulates the phosphorylation of Ser58 is a compelling finding for a variety of reasons. For one, dephosphorylation of MTMR2 at position Ser58 leads to MTMR2-mediated depletion of PI(3)P on early endosomes resulting in an increase in ERK1/2 activation (Fig. 9), possibly due to a halt or slowing in endosomal maturation allowing for increased signaling from the PI(3)P-depleted endosomes. Thus, our working model is that Ser58 phosphorylation by ERK1/2 functions within a negative feedback loop mechanism that in part may be critical for achieving the appropriate level of endosomal signaling and maturation rate. Another interesting observation was the dramatic increase in the duration of ERK1/2 activation in cells expressing MTMR2 S58A/S631A (Fig. 9). A long standing dogma is that the signal duration can dynamically influence cellular fate (Ebisuya et al., 2005). Furthermore, sustained ERK activation is thought to illicit different biological outcomes compared to transient ERK activation. For example, differentiation of PC12 cells into sympathetic-like neurons requires sustained ERK activation, whereas transient ERK activation induces PC12 cell proliferation (Gotoh et al., 1990; Traverse et al., 1994). As is seen in PC12 cells, in most cell types, sustained ERK activation induces differentiation. However, in Schwann cells, sustained ERK activation triggers de-differentiation in response to a variety of environmental cues including neuronal injury (Harrissingh et al., 2004; Napoli et al., 2012). Schwann cell de-differentiation into a progenitor cell-like stage is critical for axonal re-growth and is highlighted by extensive demyelination (Napoli et al., 2012). Schwann cell demyelination is one of the main hallmarks of CMT diseases. Coupled with the fact that phosphorylation at position Ser58 results in a functionally inactive, cytoplasmic localized MTMR2, it will be important to investigate if sustained ERK1/2 mediated phosphorylation of MTMR2 plays a role in the pathophysiology of CMT disease.

**Materials and Methods**

**Plasmids, cell culture and transfections**

The synthesis of pCDNA3.1-NF and pET21a vectors containing wild type and mutant FLAG-MTMR2 or MTMR2-His6 constructs have been previously described (Soo-A Kim et al., 2002; Franklin et al., 2011). Generation of S631A and S631E mutant variants were preformed similar to that described for S58A, S68E and C417S (Franklin et al., 2011) using PCR based site-directed mutagenesis. The forward primer for the generation of S631A was 5’-GAGAGAGCCAGCGCTCCTGCACAGTG-3’ while the reverse primer was 5’-CACTGTCGAGGAGGCTCTCC-3’. The forward primer for the generation of S631E was 5’-CAAGAAAGCCGGAGGATCACCTGCTG-3’ while the reverse primer was 5’-GAACACAGTGAGGCTCTCC-3’. The mouse anti-FLAG (Yamada Science), S58A/S631A, S58A/S631E, S58E/S631A, S58E/S631E, S417A/S631S and S417A/C417S/S631S were verified by DNA sequencing (ACGT Corp. and Bio Basic Inc.). The human cell culture lines HEK293 (ATCC) and HeLa (ATCC) were cultured and transfected with constructs as described previously (Franklin et al., 2011). HEK293 cells were selected for reproducible transfections efficiency for the cell signaling experiments while HeLa cells were utilized for immunofluorescence microscopy studies to better resolve endosomal compartments. The pEF-GPM2/S631E construct was a generous gift from Harald Stenmark (Gilllooly et al., 2000).

**Production of MTMR2 Ser58 phospho-specific antibody**

The target peptide epitope was synthesized (NEO BioScience), resuspended in conjugation buffer (100 mM NaPO4, 150 mM NaCl, 5 mM EDTA, pH 7.3), reduced with immobilized TCEP and conjugated to maleimide-activated Keyhole limpet hemocyanin (Pierce Biotechnology) through an engineered thiol terminal. The formulated conjugate was then used for immunization of Gallus gallus (Amicus Biotech). Purified polyclonal chicken anti-pSer58 IgY antibodies were isolated by the water dilution method followed by subjective affinity chromatography against non-phosphorylated and phosphorylated peptide epitopes immobilized on (iodoacetamide-activated) agarose (Pierce Biotechnology) column bound with similar method described above. Antibody specificity was tested against both MTMR2 peptide and protein epitopes by standard dot and immunoblot procedures. Briefly, peptides or FLAG-MTMR2 immunoprecipitates (as described below) were treated with or without 2.5 U of calf intestinal alkaline phosphatase (Promega) for 4–6 hours at 25°C shaking, then spotted on nitrocellulose for peptide dot blot analysis or prepared for immunoblotting as described below and by (Franklin et al., 2011). Membranes were blocked with 5% BSA in TBST, probed with chicken anti-pSer58 IgY at 1:1000 (0.7 μg/μl stock) in 2% BSA TBST, washed, then probed with donkey anti-IgY HRP conjugate at 1:3000 (Gallus Immunotech) in 2% BSA TBST for subsequent imaging.

**In vitro kinase assay**

**In vitro kinase assays were performed using 5 μg of bacterial recombinant MTMR2-His6 proteins purified as described previously (Taylor and Dixon, 2004). In this study, the activated kinases ERK1, ERK2, p38α, JNK1 (Signal Chem) were tested with all assays being carried out in reaction buffer (25 mM HEPES, pH 7.2, 12.5 mM β-glycerol phosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT) containing 200 μM ATP and 100 ng of the specified kinase. Reactions were incubated shaking at 30°C for 30 minutes, terminated with 30 μl 2× SDS loading dye, boiled for 5 minutes, then 20 ng of protein substrate was separated on a 10% polyacrylamide gel and probed with chicken anti-pSer58 antibodies or FLAG-MTMR2 as described above, anti-MAPK (Sigma) at 1:10,000 in 1% skim milk TBST and rabbit anti-p38 (Cell Signaling) at 1:1000 in 5% BSA/TBST overnight 4°C with goat anti-rabbit HRP conjugate (Bio-Rad) at 1:5000 in 2.5% BSA/TBST, along with rabbit anti-JNK1 (Santa Cruz Biotechnology) at 1:1000 in 2.5% BSA/TBST overnight 4°C and mouse anti-MTM2 (Santa Cruz Biotechnology) at 1:1000 in 1% skimmed milk TBST with goat anti-mouse IgG HRP conjugate (Promega) at 1:5000 in 1% skim milk TBST.

**siRNA and serum starvation treatment**

HEK293 or HeLa cells were grown as described above and seeded into antibiotic-free medium 24 hours prior to transfection with either 35 nM SignalSilence® control siRNA or SignalSilence® p44/p42 MAPK (ERK1/2) siRNA (Cell Signaling Technology). Cells were also left untransfected for probing of endogenous MTMR localization and Ser58 phosphorylation in response to serum starvation. After ~30 hours, both treated and untreated cells were transfected with either FLAG-SS8A MTMR2 or MTMR2 and incubated for another ~30–36 hours. Cells were moved into low (0.5%) serum containing medium for another 6–10 hours, followed by a short serum starvation and 10 minutes of EGF stimulation as described below. Cells were then prepared for immunofluorescence microscopy as described below (siRNA and endogenous serum starved treatments) or washed, lysed and prepared for immunoblot analysis and/or immunoprecipitation (siRNA and endogenous serum starved treatments). Immunofluorescence of siRNA experiments were performed using primary antibodies rabbit anti-phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204; Cell Signaling Technologies) at 1:50 and mouse anti-FLAG (below) along with fluorescent horseradish peroxidase (Vector Laboratories) and Alexa Fluor 568 goat anti-rabbit (Molecular Probes, Invitrogen) secondary antibodies at 1:500. Endogenous studies were performed using rabbit anti-MTM2 (a generous gift from Gregory S. Taylor; University of Nebraska) and mouse anti-Rab5 (BD Biosciences) along with FITC-conjugated goat anti-rabbit (Vector Laboratories) and Alexa Fluor 568 goat anti-mouse secondaries as...
below. Microscopy image analysis and acquisition is described below. For immunoprecipitation, equal amounts of lysate were added to mouse anti-MTMR2 (Santa Cruz Biotechnology) immobilized on protein-A–agarose (Thermo Pierce; Invitrogen). Precipitation was allowed to go for approx. 6–8 hours, rotating at 4°C, followed by immunoblot analysis. Quantitative immunoblotting of both siRNA and serum starvation experiments was performed using chicken anti-pSer58 MTMR2 (1:200), mouse anti-FLAG (1:1000), mouse anti-phospho-p44/p42 MAPK (above; 1:200), rabbit anti-MAPK (1:10,000) and rabbit anti-actin (below; 1:1000) in either 2.5% BSA or skim milk. Secondary antibodies included donkey anti-IGG–HRP, goat anti-mouse–HRP, goat anti-rabbit (all above; 1:5000) and in either 1% or 2.5% skimmed milk. Acquired chemiluminescent images from at least n = 3 independent experiments were quantified through densitometry using ImageJ (NIH, http://image.nih.gov/). Resulting data was normalized to its respective actin loading control and subjected to either a one-way ANOVA with Tukey’s post test or a two-tailed, unpaired t-test statistical analysis both with 95% confidence intervals for relative comparison of related proteins (i.e. ERK1/2 versus pERK1/2 and FLAG-MTMR2 versus pSer58 FLAG-MTMR2) using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. Relative comparison among all experimental and control samples was performed, with significance differences among data sets being denoted by at least *P<0.05. Data are represented as the mean value ± s.e.m.

Cell treatment with kinase inhibitors
HEK293 or HeLa cells were treated with a JNK inhibitor SP600125 (LC laboratories) at 40 μM for 1 hour, a p38 inhibitor SB203580 (LC laboratories) at 20 μM for 30 minutes or a MEK inhibitor U0126 to inhibit ERK1/2 activation (LC laboratories) at 50 μM for 1 hour as indicated. For the phosphorylation studies, cells were left untreated or treated for 30 minutes by pre-incubation with U0126, then subsequently stimulated with EGF (5 ng/ml) for 5 or 30 minutes. Cells were lysed and samples purified by FLAG-IP, separated by SDS-PAGE and transferred to nitrocellulose membranes. MTMR2 construct was examined for dual-channel colocalization and values were calculated using the JACoP plug-in (Bolte and Cordelieres, 2006) within ImageJ (National Institutes of Health). A sample size of 30 cells for each PC values were calculated using the JACoP plug-in (Bolte and Cordelieres, 2006) within ImageJ (National Institutes of Health). A sample size of 30 cells for each

MTMR2 targets to APPL1 endosomes
This study was funded by Operating Grants from Canadian Institutes of Health Research (CIHR) [grant numbers MOP-89870, MOP-123141 to P.O.V.]; and by a NSERC Canadian Graduate Scholarship (to C.A.B.).

Supplementary material available online at http://jcscience.biologists.org/lookup/suppl/doi:10.1242/jcs.113928/-/DC1

References


**Fig. S1.** siRNA-mediated depletion of ERK1/2 protein levels reduces MTMR2 Ser\(^{58}\) phosphorylation. HEK 293 cells expressing MTMR2 were transfected with control scramble siRNA or ERK1/2 siRNA as described in Materials and Methods. Cells were cultured as described in Materials and Methods then stimulated with EGF for 10 min, lysed and analyzed by immunoblotting. Graphs represent normalized quantitative immunoblot densitometry data of each indicated antibody probe to their respective \(\alpha\)-actin loading control. Data represents mean and SEM values from at least \(n = 3\) independent experiments, with **\(P<0.01\)** or *\(P<0.05\).*

Franklin *et al.*, **Supplementary Figure 1**
**Fig. S2.** Depletion of ERK1/2 expression by siRNA induces subcellular targeting of FLAG-MTMR2 to punctate regions. (A). HeLa cells were transfected with Scramble siRNA and ERK1/2 siRNA for 72 h and with FLAG-MTMR2 for 42h and analyzed by immunofluorescence microscopy. Cells were probed for FLAG-MTMR2 (green) and pERK1/2 (red). The scale bar represents 15 μm. (B). Bar graph displaying the total number of puncta between Scramble siRNA and ERK1/2 siRNA using automated counting in ImageJ. Depletion of ERK1/2 caused a significant depletion in the phosphorylated ERK1/2 staining. Depletion of phosphorylated ERK1/2 resulted in approximately 3 fold increase in the total number of FLAG-MTMR2 puncta. (n=30), ** P<0.01, bars ±SD.
**Fig. S3.** Inhibition of ERK1/2 phosphorylation by U0126. Cells were first serum starved (ss) for 30 min then subsequently treated with U0126 (where indicated), followed by stimulation with 5 ng/ml EGF for 5 and 30 min at 37°C. Total ERK1/2 served as loading control to changes in ERK1/2 phosphorylation. Representative western blots are shown (in main text), bar graph is relative change in pERK1/2, means ± SD of the results from three independent experiments. **P ≤ 0.001**
**Fig. S4.** Localization analysis of wildtype MTMR2 following MAPK inhibitor treatment. Using JACoP analysis, scatter plots and correlation coefficients were determined. (A-D), representative scatter plots of the images in Figure 4. MTMR2 partially localizes with Rab5 following ERK1/2 inhibition, +U (A) with a PC of 0.547 while exhibiting no overlap when combined with JNK1 inhibition, +U+SP (B) with a PC of 0.200. MTMR2 exhibits no overlap with APPL1 following ERK1/2 inhibition, +U (C) with a PC of 0.185 while displaying significant co-localization when combined with JNK1, +U+SP (D) with a PC of 0.854. (E) Representative bar graph of Pearson’s correlation (PC) whole cell analysis from ten individual cells from three separate experiments. ($n = 30$), Error bars ± SD. * $P < 0.05$; ** $P < 0.01$
Fig. S5. Inhibition of ERK1/2 and p38α results in partial localization of MTMR2 to Rab5 endosomes. HeLa cells were transiently transfected with wt FLAG-MTMR2 for 42 h and analyzed by immunofluorescence microscopy. Cells were treated with the MEK inhibitor (ERK1/2) (+U) combined with the p38α (+SB) inhibitor and probed for wt FLAG MTMR2 (red) and endogenous Rab5 (green) (A) or endogenous APPL1 (B). Arrows indicate regions of interest and presented in expanded views. Images were collected using 63x objectives. The scale bar represents 15 μm.
Fig. S6. Endogenous MTMR2 Ser$^{58}$ phosphorylation is influenced by altering serum conditions. (A) HEK 293 cells were cultured and processed as described in Materials and Methods, following either serum (10 %; + Serum) or low serum (0.5 %; − Serum) treatment for approx. 12 h. Both lysate and α-MTMR2 immunoprecipitates were subjected to immunoblot analysis using the indicated antibodies, with graph (B) representing normalized quantitative densitometry data to the indicated loading control (y-axis). Data represents mean and ±S.D. values from 3 independent experiments.
**Fig. S7.** Localization analysis of FLAG-MTMR2 with Rab5 and APPL1 using JaCop scatter plots from ImageJ and Pearson correlation coefficient. (A) Scatter plot analysis of serum starved cells displaying a partial overlap of FLAG-MTMR2 with Rab5 with a PC of 0.520. (B) Representative bar graph of the Pearson’s Correlation between FLAG-MTMR2 and Rab5. (C) Scatter plot analysis of serum starved cells displaying a partial overlap of FLAG-MTMR2 with APPL1 with a PC of 0.586. (D) Representative bar graph of the Pearson’s Correlation between FLAG-MTMR2 and APPL1. (E) Bar graph displaying the total number of puncta between serum and serum starved cells using automated counting in ImageJ. Serum starved cells show a significant increase in the total number of puncta. All data was analyzed from 10 individual cells from 3 individual experiments. (n=30), **P<0.01**, bars ± SD.
Fig. S8. Serum starvation induces subcellular targeting of endogenous MTMR2 to Rab5 positive endosomes. (A) Untreated HeLa cells (top panel) or serum starved cells (lower panel) probed for endogenous MTMR2 (green) and endogenous Rab5 (red). Serum starvation resulted in an increase in the colocalization of MTMR2 with Rab5 positive endosomes. Merged images display regions of colocalization and are represented in expanded views. Images were collected using 40x oil objectives. The scale bar represents 15 μm. (B). Scatter plot analysis using JaCop scatter plots from ImageJ of serum starved cells which display a partial overlap of endogenous MTMR2 with Rab5 with a PC of 0.702. (C) Representative bar graph of the Pearson’s Correlation between endogenous MTMR2 and Rab5 of whole cell analysis of 10 individual cells from 3 individual experiments. (n=30), bars ± SD.
Fig. S9. Localization analysis of MTMR2 phospho-mutants and MAPK inhibitors with endogenous APPL1 and Rab5. Using JACoP analysis, scatter plots and correlation coefficients were determined. (A-C), representative scatter plots of the images shown in Figure 6. No overlap between S58A and APPL1 with a PC of 0.097 (A), and a significant degree of co-localization between S58A/S631A and APPL1 with a PC of 0.773 (B). No overlap between S58A/S631A and Rab5 with a PC of 0.172 (C). (D), Bar graph of Pearson’s correlation (PC) analysis from ten individual cells from three separate experiments. ($n = 30$), Error bars $\pm SD$. *** $P \leq 0.001$ compared S58A/S631A with S58A for APPL1 and S58A/S631A with APPL1 and Rab5.
**Fig. S10.** APPL1 vesicle enlargement is dependent on MTMR2 S58A/S631A catalytic activity. HeLa cells were transiently transfected with FLAG MTMR2 S58A/C417S/S631A (red) then immunostained for endogenous Rab5 or APPL1 (green). Closed arrow heads indicate regions of co-localization and open arrow heads mark vesicles devoid of co-localization. Images were collected using 63x objectives. Scale bar represents 15 µm.
Fig. S11. Localization analysis of MTMR2 active and inactive phospho-mutants with endogenous Rab5 and APPL1. Using JACoP analysis, scatter plots and correlation coefficients were determined. (A-D), representative scatter plots of the images in Figure 7. (E), Bar graph of Pearson’s correlation (PC) analysis from ten individual cells from three separate experiments. \( n = 30 \), Error bars \( \pm SD \). * \( P < 0.05 \) compared S58A/S631E with Rab5 and APPL1; ** \( P \leq 0.01 \) compared S58A/C417S/S631E with Rab5 and APPL1
Co-localization analysis of MTMR2 phospho-mutants with EGFP-2xFYVE. Using JACoP analysis, scatter plots and correlation coefficients were determined. (A-C), representative scatter plots of the images shown in Figure 8. MTMR2 S58A/S631A does not co-localize with EGFP-2xFYVE (PC of 0.086) (A), while S58A/C417S/S631A partially co-localizes with EGFP-2xFYVE (PC of 0.428) (B). MTMR2 S58A/C417S/S631E displays a high degree of co-localization with EGFP-2xFYVE (PC of 0.929) (C). (D) Bar graph of Pearson’s correlation (PC) whole cell analysis from ten individual cells from three separate experiments. (n = 30), Error bars ± SD. * P < 0.05 compared S58A/C417S/S631A with S58A/S631A; ** P ≤ 0.01 compared S58A/C417S/S631E with S58A/C417S/S631A.

Franklin et al., Supplementary Figure 12
**Fig. S13.** Subcellular Localization of PI(3)P on endosomes. HeLa cells were transfected with EGFP-2xFYVE (green) and immunostained for endogenous Rab5 or APPL1 (red). Merged images display regions of colocalization and are presented in expanded views. Closed arrow heads mark regions of colocalization and open arrow heads mark vesicles devoid of colocalization. Images were collected using 63x objectives. Scale bar represents 15 µm.
### Table S1.

Catalytic activity of MTMR2 results in enlargement of APPL1 vesicles when dephosphorylated at Ser\(^{58}\) and Ser\(^{631}\). HeLa cells were transfected with MTMR2 phospho-variants and analyzed by immunofluorescence microscopy. Cells were left untreated (S58A, S58A/S631A, S58A/S631E, S58A/C417S/S631A, S58A/C417S/S631E) or treated with MAPK inhibitors as shown. (A), The data indicates that subcellular localization of MTMR2 to APPL1 vesicles through dephosphorylation at sites Ser\(^{58}\) and Ser\(^{631}\) results in enlargement of MTMR2-APPL1 positive vesicles and is dependent on MTMR2 catalytic activity. (B), MTMR2 variants not localized to APPL1 does not affect vesicle area. The data is representative of the average area (\(\mu m^2\)) of 10 vesicles from individual cells from 3 independent experiments (n=30) ± S.D. Vesicle area determination was quantified using ImageJ (NIH). **** \(P < 0.0001\).

<table>
<thead>
<tr>
<th>Sample</th>
<th>MTMR2/APPL1 area ((\mu m^2))</th>
<th>APPL1 area ((\mu m^2))</th>
<th>Average APPL1 area ((\mu m^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt_+U+SP</td>
<td>1.01 ± 0.08</td>
<td>0.38 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>S58A_+SP</td>
<td>1.06 ± 0.07</td>
<td>0.41 ± 0.08</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>S58A/S631A</td>
<td>1.02 ± 0.06</td>
<td>0.43 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>S58A/C417S/S631A ****</td>
<td>0.43 ± 0.08</td>
<td>0.39 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>MTMR2 area ((\mu m^2))</th>
<th>APPL1 area ((\mu m^2))</th>
<th>Average APPL1 area ((\mu m^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>S58A</td>
<td>0.57 ± 0.13</td>
<td>0.46 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>S58A/S631E</td>
<td>0.54 ± 0.11</td>
<td>0.51 ± 0.10</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>S58A/C417S/S631E</td>
<td>0.48 ± 0.09</td>
<td>0.43 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>