MYOTUBULARIN RELATED - 2 REGULATES RECEPTOR MEDIATED ENDOCYTOSIS - 8, A NOVEL PI (3) P BINDING PROTEIN THAT CONTROLS EARLY ENOSMOLAL CLATHRIN DYNAMICS AND ENOSMOLAL RETROGRADE TRANSPORT PATHWAY THROUGH ITS N-TERMINAL PHOSPHOINOSITIDE BINDING MOTIF

Besa Xhabija

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MYOTUBULARIN RELATED - 2 REGULATES RECEPTOR MEDIATED ENDOCYTOSIS - 8, A NOVEL PI (3) P BINDING PROTEIN THAT CONTROLS EARLY ENDOSONAL CLATHRIN DYNAMICS AND ENDOSONAL RETROGRADE TRANSPORT PATHWAY THROUGH ITS N-TERMINAL PHOSPHOINOSITIDE BINDING MOTIF

By

Besa Xhabija

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
at the University of Windsor

Windsor, Ontario, Canada

2016

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"MYOTUBULARIN RELATED -2 REGULATES RECEPTOR MEDIATED ENDOCYTOSIS 8, A NOVEL PI (3) P BINDING PROTEIN THAT CONTROLS EARLY ENDOSONAL CLATHRIN DYNAMICS AND ENDOSONAL RETROGRADE TRANSPORT PATHWAY THROUGH ITS N-TERMINAL PHOSPHOINOSITIDE BINDING MOTIF"

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

**Chapter 2**- Collaboration of Besa Xhabija with Dr. Gregory S. Taylor (Nebraska Medical Center, University of Nebraska), Dr. Akemi Fujibayashi (Institute for Protein Research, Osaka University) and Dr. Kiyotoshi Sekiguchi Fujibayashi (Institute for Protein Research, Osaka University) under the supervision of Dr. Panayiotis Vacratsis at University of Windsor, Windsor, Canada. G.S.T provided the MTMR2 construct, whereas A.F and K.S the RME-8 constructs. B.X performed all the experiments. P.O.V and B.X wrote and edited the manuscript.

**Chapter 4**- Collaboration of Besa Xhabija with Dr. Christopher Bonham and Norah E. Franklin. B.X was the second author, whereas C.A.B and N.E.F were the co-first authors of the manuscript. The study was supervised by Dr. Panayiotis O. Vacratsis from the University of Windsor, Windsor, Canada. B.X. performed siRNA and serum starve immunofluorescence (IFA). C.A.B. designed and characterized the α-pSer58 MTMR2 antibody and optimized siRNA of ERK1/2 conditions, performed knock-down immunoblot (IB) experiments and endogenous serum starve immunoprecipitation (IP) and IBs. N.E.F. conducted the MAPK inhibition experiments. P.O.V. and N. E. F wrote the manuscript, C.A.B and B.X. performed revision experiments, writing and editing.
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ABSTRACT

Myotubularin related protein 2 (MTMR2) is a member of the myotubularin family of phosphoinositide lipid phosphatases whose subcellular localization is regulated by a phosphorylation event on Ser 58. Our laboratory has shown that the phosphorylation-mimetic mutant (S58E) targets MTMR2 to the cytoplasm, whereas the phosphorylation-deficient variant MTMR2 (S58A) targets it to Rab5-positive endosomes resulting in PI(3)P depletion. Although MTMR2 dephosphorylates PI(3)P and PI(3,5)P₂, the phosphoinositide binding proteins that are regulated by MTMR2 are poorly characterized. In this study, we identified RME-8 as a novel PI(3)P binding protein implicated in the translocation of Hsc70 to early endosomes for clathrin removal during retrograde transport. Remarkably, the depletion of PI(3)P by MTMR2 S58A attenuated RME-8 endosomal localization. Moreover, we have identified the amino acid determinants required for PI(3)P binding within a region predicted to adopt a pleckstrin homology-like fold in the N terminus of RME-8. The ability of RME-8 to associate with PI(3)P and early endosomes is largely abolished when residues Lys¹⁷, Trp²⁰, Tyr²⁴, or Arg²⁶ are mutated resulting in diffuse cytoplasmic localization of RME-8 while maintaining the ability to interact with Hsc70. We also provide evidence that RME-8 PI(3)P binding regulates the early endosomal clathrin dynamics and alters the steady state localization of the cation independent mannose 6-phosphate receptor.

In addition, once a phosphorylation-deficient variant (S58A) targets MTMR2 to Rab5-positive endosomes and depleting PI(3)P, it results in increased endosomal signaling, such as a significant increase in ERK1/2 activation. Using in vitro kinase assays, cellular MAPK inhibitors, siRNA knockdown and a phosphospecific-Ser58
antibody, we 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.

Taken together, our results suggest a model in which the localization of RME-8 to endosomal compartments is spatially mediated by PI(3)P binding and temporally regulated by MTMR2 activity and compartmentalization of MTMR2 and potential subsequent effects on endosome maturation and endosome signaling are dynamically regulated through MAPK-mediated differential phosphorylation events.
This thesis is dedicated to my mother, Lumturi.
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January 26, 2016
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Figure 3.7. RME-8 PI(3)P binding affects early endosomal clathrin dynamics. HeLa cells were transiently transfected with wild type RME-8 (blue) (A) and RME-8 W20A (blue) (B) and for 24 h. Cells were triple-stained to observe endogenous EEA1 (red) and endogenous endosomal clathrin (green). A, wild type RME-8 at EEA1 early endosomes is displayed as magenta colored puncta, and clathrin at EEA1 early endosomes is displayed as yellow puncta. EEA1-positive and RME-8-positive endosomes display reduced clathrin levels indicated by arrows, and early endosomes devoid of wild type RME-8 (shown by arrowheads) display strong co-localization between clathrin and EEA1. B, cells expressing RME-8 W20A display high co-localization between clathrin and EEA1. C, control cells are stained for endogenous RME-8 (blue), EEA1 (red), and clathrin (green). D and E, HeLa cells were transfected with siRNA targeting RME-8 (D) and SNX1 (E). Cells were stained for RME-8 (blue), EEA1 (red), and clathrin (green) to observe clathrin accumulation at EEA1 positive endosomes. F, Pearson’s correlation coefficient (PC) was utilized to quantify the extent of co-localization between clathrin and EEA1 in control, wild type RME-8, RME-8 W20A variant, RME-8 KD, and SNX1 KD samples. Means ±S.D. from three independent experiments (n = 30 cells) are shown. p values were calculated by the Student’s t test and compared with wild type RME-8. **, p < 0.01. The values of Pearson’s correlation coefficients of all the experiments were related and compared with RME-8 W20A set at 100% due to its largest statistical value. Solid white arrows, arrowheads, and expanded boxes represent regions of interest. Images were collected using a X40 objective. Scale bars, µ15m.

Figure 3.8. Alteration of CI-MPR cellular localization in RME-8 W20A expressing cells. A, HeLa cells expressing wild type RME-8 and RME-8 W20A were stained for endogenous CI-MPR. Cells were analyzed by confocal microscopy. Images were collected using a X40 objective. Scale bars, 15 µm. ImageJ was utilized to count the number of total punctate in negative control, wild type RME-8, and RME-8 W20A transfected cells. HeLa cells expressing RME-8 W20A exhibit a large decrease in the peripheral number of CI-MPR positive punctate displaying perinuclear localization. B, means ±S.D. of three independent experiments (n=30 cells) are shown. p values were compared with wild type RME-8. **, p < 0.01. C and D, both RME-8 and SNX1-depleted HeLa cells have been labeled with CI-MPR antibody and also displayed a decrease in the total number of CI-MPR positive punctate displaying perinuclear localization. E, bar graph representing the effect that RME-8 KD and SNX1 KD p values were compared with WT RME-8 in A and to negative control in C and D. All cells were analyzed by confocal microscopy. Means ± S.D. of three independent experiments (n = 30 cells) are shown. p values were calculated by Student’s t test and compared with wild type RME-8 and SNX1. ***, p < 0.001.
Figure 3.9. SNX1 expression affects RME-8 early endosome targeting. A–C, HeLa cells were transfected with the indicated constructs and analyzed by confocal microscopy. Co-localizations between RME-8 and SNX1 variants are shown with arrows depicting regions of co-localization. In the middle panel, HeLa cells were co-transfected with RME-8 W20A and wild type SNX1, and arrows display regions of co-localization. Images were collected using a X40 objective. Expanded boxes represent regions of interest. Scale bars, 15µm. D, quantitation of co-localization between RME-8 and SNX1. Means ± S.D. of three independent experiments (n = 30 cells) are shown. p values were calculated by Student’s t test and compared with wild type RME 8 and SNX1, **, p < 0.01; ***, p < 0.001.................................................................96

Figure 3.10. RME-8 expression regulates SNX1 tubulation. A, HeLa cells were transfected with RME-8 siRNA and SNX1 siRNA for 48 h. Knockdown of SNX1 protein expression does not affect RME-8 endosomal localization (middle panel). Knockdown of RME-8 protein expression results in the formation of SNX1- positive tubules (bottom panel). Images were collected using a X60 objective. Expanded boxes represent regions of interest. Scale bars, 15 _m. B, bar graph representation of the percentage of cells altering from punctate to tubules in response to knockdown of RME-8 expression. Means ±S.D. of three independent experiments (n = 30 cells). p values were calculated by Student’s t test and compared with control cells. *, p < 0.1.................................................................99

Figure 4.1. In vitro phosphorylation of recombinant MTMR2 by ERK1/2. (A) PhosphoSer58 and nonphosphoSer58 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 phosho-variants, FLAG immunoprecipitated and probed for total MTMR2 (IB: FLAG) and phosphorylated MTMR2 at Ser58 (IB: pSer58). UT, untreated. (C) Purified His6 bacterial recombinant MTMR2 was the substrate in the in vitro MAPK kinase assay. Each reaction contained 5 mg of recombinant MTMR2, 100 ng of various kinases (top), 200 mM 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 a-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.................................................................120

Figure 4.2. 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 S58A and treated with the MEK inhibitor U0126 (ERK1/2) at 50 mM for 1 hour, SB203580 (p38) at 20 mM for 30 minutes and SP600125 (JNK) at 40 mM 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 63X objectives. Scale bars: 15 µm.................................123

Figure 4.3. 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.

Figure 4.4. 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 63X objectives. Scale bars: 15 µm.

Fig. 4.5. 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 FLAGMTMR2 (red) and APPL1 (green). Serum starvation resulted in an increase in the colocalization of FLAGMTMR2 with APPL1-positive endosomes. Merged images display regions of colocalization and are represented in expanded views. Images were collected using 40X oil objectives. Arrows indicate regions of colocalization. Scale bars: 15 µm.

Figure 4.6. Dephosphorylation of Ser58 and Ser631 regulates MTMR2 localization to APPL1 endosomes. (A,B) HeLa cells were transiently transfected with FLAG-MTMR2 phosopho-variants for 42 hours and analyzed by immunofluorescence microscopy. (A) Cells were transfected with S58A or S58A/S631A phoshop-variants and were probed for FLAG-MTMR2 (red) and APPL1 (green). (B) Cells were transfected with S58A/S631A phoshop-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 40X oil and 63X objectives. Scale bars: 15 µm.

Figure 4.7. Rab5 endosomal targeting of MTMR2 is regulated by dephosphorylation of Ser58 and phosphorylation of Ser631. (A) HeLa cells were transfected FLAG
MTMR2 phospho-variants for 42 hours and probed for FLAG MTMR2 (red) and Rab5 (green). Loss of catalytic activity (S58A/ C417S/S631E) results in an increase in MTMR2-Rab5 colocalization. (B) Cells were probed for FLAG-MTMR2 phospho-variants (red) and endogenous APPL1 (green). Closed arrows indicate colocalized vesicles, open arrows indicate vesicles void of colocalization. Images were collected using 40X oil and 63X objectives. Scale bars: 15 µm.

Figure 4.8. Inducible PI(3)P depletion at early endosomes is dependent on MTMR2 catalytic activity. (A,B) HeLa cells were co-transfected with EGFP-2X FYVE (green) and FLAG-MTMR2 (red) phospho-variants for 42 hours and analyzed by immunofluorescence microscopy. (A) MTMR2 dephosphorylation at Ser58 and Ser631 (S58A/S631A) prevents localization to PI(3)P-rich endosomes (top panel). Expression of MTMR2 S58A/S631E induces PI(3)P depletion at early endosomes (lower panel). (B) Loss of catalytic activity (S58A/C417S/S631A) of dephosphorylated MTMR2 results in partial colocalization with PI(3)P endosomes (top panel). Phosphorylation at Ser631 (S58A/C417S/S631E) localizes to PI(3)P-rich early endosomes (lower panel). Closed arrows indicate colocalized vesicles, open arrow indicate vesicles void of colocalization. Images were collected using 40X oil and 63X objectives. Scale bars: 15 µm.

Figure 4.9. Dephosphorylation of Ser58 and Ser631 elicits sustained ERK1/2 activation. (A) HeLa cells were transfected with FLAG-MTMR2, S58A or S58A/S631A, serum starved for 30 minutes then treated with 5 ng/ml EGF for the indicated times. Lysates were immunoblotted (IB) for pERK1/2. Total ERK1/2 levels served as loading control. Lysates were immunostained with anti-FLAG to confirm equal protein expression. (B) Relative ERK1/2 phosphorylation levels were quantified by densitometry using ImageJ and normalized to total ERK1/2 levels. Representative western blots are shown in A. Means ± s.d. of the results of three independent experiments are shown. **P<0.01, ***P<0.001 for the comparisons indicated.

Figure 4.10. siRNA-mediated depletion of ERK1/2 protein levels reduces MTMR2 Ser58 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 α-actin loading control. Data represents mean and SEM values from at least n = 3 independent experiments, with ***P<0.001, **P<0.01 or *P<0.05.

Figure 4.11. 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. .......................................................... 147

**Figure 4.12. 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................................. 148

**Figure 4.13. 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................................. 149

**Figure 4.14. 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. .......................... 150

**Figure 4.15. Endogenous MTMR2 Ser<sup>58</sup> 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. ....... 151

**Figure 4.16. 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.

Figure 4.17. 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.

Figure 4.18. 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 ± SD. *** P ≤ 0.001 compared S58A/S631A with Rab5 and APPL1; ** P ≤ 0.01 compared S58A/C417S/S631E with Rab5 and APPL1.

Figure 4.19. 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.

Figure 4.20. 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 ± SD. * P < 0.05 compared S58A/S631E with Rab5 and APPL1; ** P ≤ 0.01 compared S58A/C417S/S631E with Rab5 and APPL1.

Figure 4.21. 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.

**Figure 4.22. 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 63× objectives. Scale bar represents 15 μm.

**Table 4.1** Catalytic activity of MTMR2 results in enlargement of APPL1 vesicles when dephosphorylated at Ser58 and Ser631. 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 Ser58 and Ser631 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 (μm²) 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.

**Figure 5.1. Sorting of the acid hydrolases via mannose 6-phosphate receptors during normal (left panel) and aberrant (right panel) endosomal retrograde transport from the early endosomes to the Trans- Golgi- Network.** Adapted from Bonifacino, J. S. & Hurley, J. H. (2008) Retromer, *Curr Opin Cell Biol.* 20, 427-36.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>Adaptor protein</td>
</tr>
<tr>
<td>APPL1</td>
<td>Adaptor protein, Ahosphotyrosine interaction, PH domain and Leucine zipper containing 1</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin–Amphiphysin–Rvs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coil</td>
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<tr>
<td>CCV</td>
<td>Clathrin Coated Vesicles</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
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<tr>
<td>CIP</td>
<td>Calf Intestinal alkaline Phosphatase</td>
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<tr>
<td>CMT4B1</td>
<td>Charcot-Marie-Tooth 4B1</td>
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<td>DENN</td>
<td>Differentially expressed in neoplastic versus normal cells Dlg-1 Disk large 1</td>
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<td>DUSP</td>
<td>Dual Specificity Phosphatase</td>
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<td>Early Endosome</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular Signal-Regulating Kinases 1 And 2</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein-Isothiocyanate</td>
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<tr>
<td>FYVE</td>
<td>Fab1p, YOTB, Vac1p and EEA1</td>
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<tr>
<td>GRAM</td>
<td>Glucosyl transferases, Rab-like GTPase activators and</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PI(3,5)P₂</td>
<td>Phosphatidylinositol-3,5-Bisphosphate</td>
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CHAPTER 1
CHAPTER 1
LITERATURE REVIEW

1.1. Protein Phosphorylation

One of the fundamental requirements of complex life is the need to respond quickly to internal and external cues. To do so, cells have adapted strict and flexible regulatory systems that are able to sense, transmit, store, and interpret information in order to respond in a coordinated, organized and timely manner. Post translational modifications (PTMs) are a major contributor to cellular responsiveness, due to their ability to modulate the function of a protein in a rapid and reversible manner [2].

In particular, protein phosphorylation is one of the most pervasive PTM because of its rapid reversibility and readily available phosphoryl group donor, ATP [3]. It is arguably the most ubiquitous modification and regulates most cellular processes. The addition and removal of a phosphoryl moiety on a protein is controlled by the action of two groups of enzymes, kinases and phosphatases (Fig 1.1). Protein kinases catalyze the covalent addition of a phosphate group to an amino acid in a peptide chain, while phosphatases catalyze the hydrolysis of phosphoproteins [2]. Interestingly, dephosphorylation or phosphorylation of an amino acid generates a new docking site for intra or intermolecular bond formation leading to changes in protein conformation. This action is reflected in alterations in protein-protein interactions, protein stability, enzyme activity, and subcellular localization (Fig 1.2), thereby acting as a quick molecular switch, capable of altering the behavior of its targets either directly or indirectly [2, 3].
On average about 30% of cellular proteins are phosphorylated [4, 5]. In mammalian cells, phosphorylation usually occurs on serine, threonine and tyrosine residues. However the majority of protein phosphorylation in eukaryotic cells occurs on serine or threonine residues at about 84% and 15%, respectively. Interestingly tyrosine phosphorylation is postulated to account for only 0.01% of the total protein phosphorylation and yet it is essential in controlling normal cellular growth, differentiation, metabolism, cell cycle, cell-cell communications, cell migration, gene transcription, the immune response, and survival [5, 6]. Aberrant tyrosine phosphorylation is linked with many human diseases such as diabetes, cancers, rheumatoid arthritis, and hypertension [5].

Figure 1.1. Protein phosphorylation and dephosphorylation causes structural changes of a protein and is regulated by the action of protein kinases and phosphatases. From Zhang, Z. Y. (2003) Chemical and mechanistic approaches to the study of protein tyrosine phosphatases, Accounts of chemical research. 36, 385-92 [7].
Figure 1.2. The rapid and reversible nature of protein phosphorylation serves as molecular switches mechanism modulating this way many aspects of protein function such as enzymatic activity, structural conformation, protein-protein binding, subcellular localization, protein turnover, crosstalk with other PTMs. From Humphrey, S. J., James, D. E. & Mann, M. (2015) Protein Phosphorylation: A Major Switch Mechanism for Metabolic Regulation, *Trends in endocrinology and metabolism: TEM* [2].
1.2. Protein Phosphatase Superfamily

Kinases have been thought to control the amplitude of a signaling response, whereas phosphatases to control its rate and duration. The concerted actions of these two classes of enzymes are tightly coordinated in order to regulate cellular signaling [8-10]. Interestingly, almost all protein kinases share the same 3D structure because they derive from one common ancestor [8, 11]. In contrast to kinases, protein phosphatases are a very diverse family of proteins and have evolved into quite discrete families. Members of the protein phosphatase family have adopted different 3D structure, with different active sites and mechanisms of hydrolysis [11]. Approximately, there are about 200 phosphatase genes [12], of which 100 are members of the Protein Tyrosine Phosphatase (PTP) family. Protein Tyrosine Kinases (PTK) and PTP gene families contain 90 and 100 genes respectively, which suggests that the level of complexity among these families is similar [8]. One of the most striking features of PTPs is that the majority of them contain combinations of regulatory modular domains other than the catalytic domain. Theses non-catalytic regulatory and targeting domains are usually attached to the N and C termini of the catalytic domain and generate a large diversity in the structural and functional context within the PTPs [13, 14]. These are mostly characterized by protein-protein or phospholipid binding modules [15].

Overall, there are 3 major families of protein phosphatases based on the sequence, structure and catalytic mechanism that they have adopted; Ser/Thr phosphatases, PTPs and Asp-based protein phosphatases [1]. All members of the PTP family contain the CXX3R signature motif within the active site and share a common catalytic mechanism,
where the initiation of the reaction mechanism starts with the cysteine residue within the signature motif acting as a nucleophile to attack the incoming phosphotyrosine of the substrate. The unique microenvironment within the active site greatly aids the catalysis reaction by lowering the pKa of the catalytic cysteine. Based on PTPs gene sequence and structure, they are further classified into 3 evolutionary separate families: class I-PTPs (cysteine based); class II-PTPs (cysteine based), class III-PTPs (cysteine based) PTPs, (Fig. 1.3) [1, 16], although the vast majority of protein phosphatases belong to Class I (cysteine based).

Based on secondary structure and sequence homology of the catalytic domain class I-PTPs, are further subdivided additionally into the classical PTPs (both receptor-like and non-receptor PTPs), and the VH1-like phosphatase group, also known as dual specificity phosphatases (DUSPs). The latter are not well conserved and have little sequence similarity, outside the signature motif. Although, they share the same catalytic mechanism as the classical PTPs, the active site cleft of DUSPs allows for the accommodation of dually phosphorylated substrates, pSer/ pThr residues as well as pTyr residues [8]. DUSPs consist of the following groups of enzymes: the MAP kinase phosphatases (MKPs), the atypical DUSPs, the slingshots, the PRLs, the CDC14s, the PTEN, and the myotubularins, (Fig. 1.3) [17-20]. Part of their heterogeneity originates because they can hydrolyze various substrates, where besides protein hydrolysis, they can also dephosphorylate RNA molecules as well as phosphatidylinositols [21, 22].
1.3. Catalytic mechanism of protein tyrosine phosphatases

PTPs catalyze phosphate hydrolysis via a covalent thiol-phosphate intermediate [7, 23]. The invariant cysteine residue of the CX$_5$R signature motif located at the base of the cleft is essential for catalysis. The unique microenvironment created within the active site pocket lowers the pKa of the invariant cysteine that acts as a nucleophile in the catalytic mechanism [5, 24, 25]. Besides the amino acids in the signature motif, other residues located on the P-loop, the WPD loop and the Q-loop of the active site are also critical for proper reaction. (Fig. 1.4) [25]. Structural studies of PTP1B, the first PTP isolated, revealed that following substrate binding its active site undergoes a large conformational change. In particular, WPD loop located on the sides of the active site, closes around the side chain like a “flap” of the pTyr residue of the substrate. Closure of the WPD loop positions an invariant aspartic acid residue (Asp181 in PTP1B), which now donates its proton to the tyrosyl leaving group (in PTP1B is an Asp 181). In this first step of the reaction, aspartic acid within the WPD loop acts as a general acid and forms a thiol-phosphate intermediate, (Fig. 1.4 and 1.5). Meanwhile, in the second step of the catalysis in PTP1B, an invariant glutamine (Gln 262 in PTP1B) located in the Q loop, mediates the coordination of a water molecule. Interestingly, in the second step of the reaction mechanism, the same invariant aspartic acid residue involved in step 1 (Asp 181 in PTP1B), now acts as a general base by activating a water molecule in the hydrolysis of the phosphoenzyme intermediate, which in turn induces the release of the phosphate group (Fig. 1.4).
Interestingly, the active site depth varies among different subfamilies of PTPs (Fig. 1.6). For instance the classical PTPs have a pronounced depth of the active site cleft, which contributes to the absolute specificity towards pTyr-containing substrates [26]. Smaller substrates such as phosphoserine and phosphothreonine residues cannot access the nucleophilic cysteine residue at the base of the cleft and as a result would not be targeted for hydrolysis.

The general features of this mechanism are conserved throughout all the PTPs, although the precise active site structural architecture changes slightly among subfamilies. For instance, unlike the active site of classical p-Tyr specific phosphatases that have a very deep cleft to accommodate long phosphotyrosine containing substrates, DUSPs contain a much shallower cleft to accommodate a dually phosphorylated substrate. Other members of the PTP family of proteins, such as in myotubularins, the active site cleft is similar in depth to the classical p-Tyr specific phosphatases, but is wider than both the classical p-Tyr specific phosphatases and DUSPs in order to accommodate the sugar head group of the inositol phospholipid, (Fig. 1.6) [27]. However, unlike the other members of the PTP family, myotubularins do not have the conserved acidic residue that resides in the WPD-loop of the classical PTPs. Instead, it has an aspartic acid within the active site that plays the function of the general acid in the first step of the catalysis [27].
Figure 1.4. General mechanism of dephosphorylation employed by PTPs. From Tonks, N. K. (2003) PTP1B: from the sidelines to the front lines!, *FEBS Lett.* 546, 140-8.
Figure 1.5. The architecture of the PTP1B active site. Upper panel displays in blue ribbon the secondary structure elements of the catalytic domain of PTP. Within the catalytic site the critical motifs are colored as the signature motif in yellow, the WPD-loop in red, the Q-loop in green and the P-loop in orange. Lower panel displays a zoomed in image of the active site and the critical amino acids necessary for catalysis. From Tonks, N. K. (2003) PTP1B: from the sidelines to the front lines!, FEBS Lett. 546, 140-8.
1.4. Myotubularin lipid phosphatases

The myotubularin (MTM) subfamily is one of the largest and conserved subfamily of protein tyrosine phosphatases from yeast to humans [28-32]. The human MTM family of phosphatases includes MTM1 and MTM-related proteins [28-30, 32]. The MTM family consists of 15 members that have the characteristic phosphatase domain. Nine of these family members possess a conserved active site motif CXXGWDR and hydrolyze the D3 position of the lipid second messengers, phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5- biphosphate, PI(3)P and PI(3,5)P2 respectively [22, 28, 31, 33-37]. The remaining six MTM proteins are catalytically inactive and have been shown to regulate the activities of active phosphatases, also known as the ‘pseudophosphatases’ [28].

The catalytically active MTM proteins are divided into four groups based on their structure: The MTM1 group consists of MTM1, MTMR1 and MTMR2; the MTMR3 group contains MTMR3 and MTMR4; the MTMR6 group contains MTMR6, MTMR7 and MTMR8; whereas the last group contains MTMR14 [27, 29]. All of the proteins within the MTM1, MTMR3 and MTMR6 groups contain a PH-GRAM (pleckstrin homology glucosyltransferases, Rab-like GTPase activators and myotubularins) domain at the N-terminus and a coiled-coil domain near the C-terminus (Fig. 1.7) [37]. Proteins within the MTM1 group contain a C-terminal PDZ-binding domain, whereas members of the MTMR3 subgroup possess a C-terminal FYVE domain. Meanwhile, MTMR14 contains only the phosphatase domain. Uniquely, certain MTMs contain a SID sequence [SET (Suvar3-9, Enhancer-of-zeste, Trithorax)-interacting domain] within the
phosphatase domain whose function is to mediate protein-protein interactions, (Fig. 1.7) [37]. Besides the inactive phosphatase domain, the six inactive members of the MTM family (MTMR5, MTMR9, MTMR10, MTMR11, MTMR12 and MTMR13) contain the same major architectural features as the active members such as the PH-GRAM domain and the C-terminal coiled-coiled domain.

1.5. Myotubularin related - 2

The loss of function mutation of the MTMR2 gene causes Charcot-Marie-Tooth disease type 4B1 (CMT4B1), which is an autosomal recessive demyelinating neuropathy, characterized by abnormally folded myelin sheaths [38, 39]. Studies in animal models have demonstrated that Mtmr2-null mice develop azoospermia, as well as progressive neuropathy particularly in the paranodes of Schwann cells [40]. Interestingly, the loss of either MTMR2 or MTMR13 causes Charcot-Marie-Tooth type 4B1 and 4B2 neuropathy, respectively [40-44]. In particular, MTMR2 is a ubiquitously expressed lipid phosphatase whose physiological substrates are both PI(3)P and PI(3,5)P_2. Studies have revealed that once mutated, the levels of PI(3)P as well as PI(3,5)P_2 are elevated [37, 45].

Both PI(3)P and PI(3,5)P_2 derive from phosphatidylinositol (PI) which is the basic building block for the intracellular inositol lipids in mammalian cells. They are composed of D-mylo-inositol-1- phosphate linked to 2 fatty acyls groups through a phosphate group [46]. The inositol ring of PIs can be reversibly phosphorylated D3, D4 or D5 positions and differential combination of phosphorylation on these positions can generate up to a total of seven different species of PIs [47]. The fundamental function of these secondary messengers is to recruit effector proteins with conserved modular protein domains to precise membranous location at a precise time [46-50]. This ability to spatially and temporally regulate location of effector proteins, makes these lipids absolutely critical regulators of cellular signaling. Interestingly, the interconvertability of these phospholipids is mediated and highly controlled by the action of lipid kinases and phosphatases, (Fig 1.8) [47]. In a typical eukaryotic cell, PIs represents less than 15% of
the total phospholipids. Since there is an abundance of PI(3)P compared to PI(3,5)P$_2$ in a typical cell (2-5% of PI(3)P vs 0.04% of PI(3,5)P$_2$ of total PIs), our studies concentrate on understanding the MTMR2 PI(3)P mediated cellular regulation. It is important to note that each of the seven phosphoinositides has a specific subcellular distribution within the cell. For instance, PI(3)P is predominantly located at the early endosomes, whereas PI(3,5)P$_2$ is enriched at the late endosomes [47]. Since CMT4B1 mutations cause a loss-of-function mutation by reducing MTMR2’s ability to dephosphorylate PI(3)P and PI(3,5)P$_2$, it is thought that the alterations of its substrates may cause a disruption of intracellular trafficking and membrane homeostasis, particularly in Schwann cells (ref).

Studies in our laboratory and others have demonstrated that phosphorylation of lipid phosphatases plays a significant role in their regulation. For example, a phosphorylation event on PTEN decreases its lipid phosphatase activity, which in turn inhibits access to its substrate [51, 52]. Our laboratory has discovered a phosphorylation event on Ser 58 of MTMR2 that is in close proximity to its PH-GRAM domain [53]. MTMR2 phosphorylation event alters its subcellular localization and thus access to its physiological lipid substrate. Once MTMR2 is phosphorylated on Ser 58 it is sequestered in the cytoplasm away from its physiological substrate. Conversely, dephosphorylated MTMR2 is localized in proximity to its substrate, at the early endosomes. Once there, MTMR2 triggers PI(3)P dephosphorylation and increases growth factor receptor signaling pathways, such as the extracellular signal-regulated kinase (ERK) pathway. This example demonstrates that phosphorylation serves as a molecular switch to control MTMR2 access to its lipid substrate, and in this manner, controls downstream endosome maturation events [53]. The role that MTMR2 plays in trafficking events remains poorly
understood, even though the pathophysiological consequences established from loss of the MTMR2 function is very well established. Hence the investigation of the MTMR2 phosphorylation mechanism is important for understanding its spatial and temporal functional regulation.

Figure 1.8. The metabolism of the seven isoforms of phosphatidylinositol. From Cullen, P. J., Cozier, G. E., Banting, G. & Mellor, H. (2001) Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking, Current biology : CB. 11, R882-93.
1.6. Endocytosis

Endocytosis is the process by which cells internalize the extracellular matrix, small molecules, macromolecules, ligands and receptors along with parts of their plasma membrane. Mammalian cells in particular, have adopted various mechanisms to internalize cargo from the cell surface and ultimately target them at a precise cellular location. Some of these mechanisms involve phagocytosis, pinocytosis, clathrin-dependent receptor-mediated endocytosis, and clathrin-independent endocytosis [54]. From all of the endosomal internalization mechanisms, receptor-mediated endocytosis via clathrin-coated pits is by far the best-characterized pathway [54-57]. The underlying mechanism of this process is becoming the focus of intense investigation in various biological fields, since its malfunction has been shown to be the cause of many medical conditions [58-61].

In receptor-mediated endocytosis, once ligands bind receptors, they concentrate in clathrin-coated pits at the plasma membrane. Interestingly, prior to forming clathrin-coated vesicles (CCV)s at the plasma membrane, clathrin and clathrin adaptor proteins are first recruited to mediate the assembly of clathrin into lattice-like structures, which in turn induces the inward curvature of the membrane [62]. This allows the pits to eventually bud off and form clathrin-coated vesicles (CCV) with receptors still bound to ligands. Following this, clathrin coat sheds off from the vesicles to fuse later on with the early endosomes (EEs), once CCVs internalize. An early endosome is a very dynamic vesicular structure, containing an extensive amount of tubules and is the site where receptor-ligand complex dissociate due to its slightly acidic internal environment (Fig
Following ligand-receptor dissociation, receptors selectively accumulate at tubules of the early endosome, so they can be targeted back to the plasma membrane through a recycling endosome. Typically, many of the receptors are re-used up to several hundred times, whereas ligands are degraded following the late endosome-to-lysosomes route [66-70]. In a similar manner, lysosomal enzymes accumulate at late endosomes and lysosomes by the same pathway in order to maintain proper lysosomal biogenesis [71]. Lysosomes are small vesicles, where ligands are targeted for degradation via the combined action of both lysosomal enzymes and acidic pH. Since, a plethora of medical conditions have arisen due to an aberrant lysosomal biogenesis, many laboratories are increasing their efforts to better understand the mechanism by which they are regulated [72-75].

In addition to originating from the plasma membrane, CCVs can also originate from the trans-Golgi network (TGN) and fuse with early endosomes. Some of the cargo delivered at the early endosomes via this route include the newly synthesized lysosomal enzymes which subsequently conclude their journey at the lysosomes [69]. Regardless of whether clathrin-coated vesicles originate at the plasma membrane or the TGN, they comprise a population of transport machineries that are specifically designed to fuse with endosomes once clathrin is uncoated [55]. Overall, the endocytic pathway contains functionally and physically discrete compartments, where the dissociation and sorting of receptor and ligands occurs at the early endosomes and the accumulation and digestion of macromolecules occurs at the late endosomes and lysosomes (Fig 1. 10) [69].
1.7. Endosomal Retrograde Transport Pathway

Following endocytosis, the majority of cargos accumulate at the early endosomes to then get sorted into their final intracellular destinations. Some cargo recycle back to the plasma membrane [70], whereas others are targeted for degradation to late endosomes/lysosomes [76]. Recent studies have started to characterize another endosomal vesicular transport pathway, called the endosomal retrograde transport. It can selectively target certain cargo to escape the early endosome to-lysosome degradation pathway and translocate to the trans-Golgi network (TGN). This pathway in particular was first discovered in 1998 and to this day remains a poorly explored cellular process [77, 78]. Some of the cargo that get sorted by the retrograde transport pathway include sortilins, wntless receptors, signaling molecules such as EGF receptor, viruses and pathogenic products from bacteria such as toxins. Nonetheless, the best characterized retrograde transport cargo proteins are the cation-independent mannose 6-phosphate receptors (CI-MPR)s [79].

CI-MPRs are type I transmembrane proteins that recognize the newly synthesized acid hydrolases tagged with a mannose 6-phosphate group at the TGN. Once CI-MPRs bind mannose 6-phosphate tagged acid hydrolases, clathrin adaptor proteins such as GGA and API-1 signal adaptor proteins at the TGN bind the cytosolic tail of CI-MPRs [80]. These interactions allow for the capture of the CI-MPR-ligand complexes into clathrin-coated vesicles, which then fuse with an early endosomes once clathrin is uncoated. The acidic pH of the early endosomes induced the release of the acid hydrolases into the early
endosomes lumen, from where they are transported back to the late endosomes and eventually to the lysosomes. In contrast to the ligands, the unoccupied CI-MPR receptors embedded in the early endosomes membranes recycle back to TGN via retrograde transport for further rounds of acid hydrolase sorting (Fig. 1.10) [79].

The retrieval of CI-MPRs from the early endosomes to TGN via retrograde transport is mediated through the action of a heteropentameric protein complex that associates with the cytosolic face of endosomes called retromer [81]. The mammalian retromer complex is composed of two major subunits, the sorting nexin dimer composed of SNX1/SNX2 and trimer composed of Vps26, Vps29 and Vps35, which serve as the cargo recognition unit. Recent studies have implicated retromer in a broad range of physiological, developmental and pathological processes, highlighting the critical nature of retrograde transport mediated by this complex [82].

In addition to retromer coat, there is a growing body of evidence that suggests clathrin also has a role in retrograde trafficking, particularly in retromer biology [83-87]. A valuable tool in understanding the role of retromer and clathrin in retrograde trafficking is the trafficking of the bacterial protein toxin Shiga toxin (STx) and CI-MPR [83]. For instance, STx is capable of taking over the cells endocytic and retrograde machinery to allow efficient uptake and retrograde trafficking to the TGN via retromer [84]. Once the function of clathrin is perturbed, STxB results in failure of the B subunit of STx (STxB) to traffic to the TGN, instead it is targeted on subdomains of early endosome and gets stalled [84]. Besides clathrin, it has also been shown that perturbations of retromer subunit VPS26 results in a nearly complete block of STxB
retrograde trafficking [84], indicating that retromer and clathrin coats act on a similar pathway.

Recently, it has been demonstrated that retromer regulates endosomal clathrin dynamics via the DnaJ domain protein RME-8/Hsc70 chaperone complex [83, 86]. Although, the mechanism by which RME-8/Hsc70 dissociates clathrin is unknown, other DnaJ containing proteins such as auxilins are known to play a role in uncoating of CCVs at both the plasma membrane and TGN (48). Following direct binding to clathrin, they recruit Hsc70 to CCVs enabling the uncoating of CCVs by breaking clathrin heavy chains interactions [83, 86, 88].

RME-8 was first discovered in 2001 utilizing a genetic screen in *C. elegans* to be involved in the process of endocytosis [89]. Following this, studies in *Drosophila* discovered RME-8 to be specifically involved in the receptor-mediated endocytosis and predominantly found to be present at the early endosomes [62, 90]. Remarkably, RME-8 co-localizes and directly binds quite strongly to SNX1 at early endosomes [83, 86]. The importance of RME-8’s function in endosomal vesicular traffic was brought to light once it was observed to be involved in retromer-mediated retrograde trafficking of cargo such as CI-MPR, STxB and wntless receptors [83, 86]. To note is the fact that RME-8 and Hsc70 roles in retrograde transport are tightly associated with each other; for example perturbation of either Hsc70 or RME-8 resulted in the same phenotype [83, 86]. Moreover, the knock down of SNX-1, RME-8 or Hsc70 alters clathrin dynamics, translocating it from the cytoplasm to the endosomes leading to an endosomal clathrin accumulation [87].
1.8. Objectives

The objective of my thesis is to study mechanistic aspects of MTMR2 phosphorylation and effects on downstream endosomal maturation and signaling. In particular, a major focus of my work has been centered in discovering MTMR2 targets whose function depends on PI(3)P levels and thus would represent potential novel biomarkers or therapeutic targets for CMT4B1 disease.

Specifically we aim to:

1. Identify and characterize novel PI(3)P binding proteins that are regulated by MTMR2.

2. Identify the mechanism by which MTMR2 reversible phosphorylation regulates endosomal trafficking events.
1.9. References


CHAPTER 2
CHAPTER 2
RECEPTOR MEDIATED EDOCYTOSIS 8 IS A NOVEL PI(3)P BINDING PROTEIN REGULATED BY MYOTUBULARIN RELATED -2

2.1. Introduction

The phosphatidylinositol phosphate (PIP) isoforms PI(3)P and PI(3,5)P$_2$ are known to serve as membrane targeting ligands for proteins that are essential for cellular membrane trafficking processes. These lipid-protein interactions function in translocating signaling proteins to discrete membrane locations where they can properly respond to extracellular stimuli [47]. The signaling pathways are subsequently turned off through the dephosphorylation of the PIPs.

The MTMR family of lipid phosphatases, composed of active and inactive subgroups, represents the largest protein tyrosine phosphatase subfamily conserved from yeast to humans [29, 32]. Initially, MTM1 was the first family member shown to dephosphorylate the D3 position of PI(3)P in vitro and in cellula [22, 91]. Subsequently, all active members tested have been shown to also be PI(3)P phosphatases [34, 36]. Furthermore, there is accumulating evidence that MTMs can also utilize PI(3,5)P$_2$ as a physiologic substrate [36, 92, 93]. Therefore, MTMs are thought to antagonize effector molecules that utilize PI(3)P or PI(3,5)P$_2$ as targeting ligands and/or allosteric activators.
Mutations in mtmr2, on chromosome 11q22, have been shown to cause the neurodegenerative disorder, Charcot-Marie-Tooth disease 4B1 (CMT4B1) [40]. CMT4B1 is an autosomal recessive aggressive form of CMT, characterized by abnormally folded myelin sheaths, inadequate nerve signaling to muscles, and eventual muscle weakness and atrophy [44]. While the pathophysiological consequence resulting from the loss of MTMR2 function is well established, how MTMR2 participates in trafficking events remains poorly understood. Recently we have characterized an N-terminal MTMR2 phosphorylation site at position Ser^{58} that dramatically regulates MTMR2 endosomal localization and thus access to its lipid substrates [53]. A phosphorylation deficient variant (MTMR2 S58A) displays strong endosomal localization and an enhanced ability to deplete PI(3)P from endosomal vesicles signifying that reversible phosphorylation is a critical mechanism regulating the activities of MTMR2.

The biological pathways affected by MTMR proteins remain inadequately characterized. This task has proven difficult due to the poor understanding of PIP binding domains that specifically interact with the PIP isoforms that MTMRs target [47]. In this study, we utilized a proteomic approach using immobilized PIP isoforms to identify novel PIP binding proteins. Herein, we describe the identification of receptor mediated endocytosis 8 (RME-8) as a novel PI(3)P binding protein whose lipid binding activity is affected by MTMR2.
2.2. **Materials and Methods**

2.2.1. **Identification of novel PIP binding proteins by mass spectrometry**

Immobilized PIP isoforms (Echelon) were prepared for affinity chromatography according to the manufacturer’s protocol. RT4-D6P2T Schwann cells (6 x 10^7) were grown to 80% confluency, lysed and the soluble lysate filtered. The cellular lysates was pre-cleared with PI control beads followed by the loading of 10 mg of total protein to either PIP, PI(3)P, PI(3,5)P_2, or PI(5)P resin. The lipid pulldown was performed by batch method overnight at 4° C. Following rigorous washing conditions the bound proteins were eluted in SDS/PAGE loading buffer and analyzed by SDS/PAGE and silver staining. Protein bands of interest were excised and in-gel digested with trypsin. The extracted pools of tryptic peptides were then analyzed by MALDI-TOF mass spectrometry (MS) for protein identification as described previously [94].

2.2.2. **Cell culture and transfection**

HeLa and HEK293 cells were utilized for their reproducible transfection efficiency and were maintained in DMEM/ F-12 supplemented with 10 % FBS, 1% penicillin/streptomycin and maintained at 37° C with 5 % CO_2. Cells were transiently transfected with expression vectors encoding FLAG tagged-MTMR2 S58A [53] and GFP-RME-8 variants [90] using FuGene HD Transfection Reagent (Roche ) according to the manufacturer’s protocol. The DsRed-FYVE construct was created by PCR amplification of the sequence encoding the FYVE domain of EEA1 followed by cloning
into the pDsRed expression vector (Clonetech). Where indicated, wortmannin treatment (1 µM) was for 1h at 37 °C.

2.2.3. PIP pulldown

HEK293 cells were transiently transfected with GFP-RME-8 variants for 24 hrs and lysed in modified RIPA buffer (50mM Tris-HCl pH 7.4, 1% Nonidet P-40, 76 mM NaCl, 2 mM EGTA, 10% Glycerol) supplemented with protease inhibitors PMSF (1 mM) and aprotinin (1 mM). Cell lysates were incubated with PI(3)P, PI(3,5)P₂ and PIP conjugated resin (Echelon Research Laboratories) overnight at 4 °C. The samples were washed 3 times with 10 mM HEPES pH 7.4, 150 mM NaCl, 0.25% NP-40 and resuspended in SDS/PAGE loading dye. The protein samples were separated on an 8% SDS/PAGE, transferred to a PVDF membrane and immunoblotted with goat anti-GFP (Rockland) as the primary antibody and rabbit-anti goat HRP (Rockland) as the secondary antibody. Proteins were visualized using Super Signal West Femto reagent (Thermo scientific).

2.2.4. Immunoflourescence and image acquisition

Following transient transfection, Hela cells were fixed at room temperature with 3.7% paraformaldehyde in PBS. Cells were then permeabilized with 0.15% Triton X-100 in PBS at room temperature for 2 min and blocked for 1 hr in 5% BSA (Sigma). Cells were incubated with mouse anti-FLAG antibody (Sigma Aldrich) or rabbit anti-EGFR
(Santa Cruz Biotechnology) in TBST. Following washing, cells were incubated with either Alexa 568 goat anti-mouse, Alexa 350 donkey anti-rabbit (Invitrogen) or fluorescein goat anti-rabbit (Vector laboratories) secondary antibodies. All the incubations were performed at room temperature for 1 hr followed by three 5 min washes in TBST. Hoechst 33342 (Molecular Probes) was used to stain the nuclei and Slowfade Antifade kit was used to mount the slides (Molecular Probes) according to the manufacturer’s protocol. Images were captured with a Q-imaging CCD camera on a Leica DMIRB microscope using the Northern Eclipse software and Adobe Photoshop 7.0.

2.3. Results and Discussion

2.3.1. Isolation of the RME-8/HSC70 complex from Schwann cells using PI(3)P affinity chromatography.

Knowledge of proteins affected by the lipid phosphatase activity of MTMR2 is poorly defined. To identify putative targets of MTMR2, a pull down assay was performed using disease relevant Schwann cells and conjugated PI(3)P and PI(3,5)P$_2$ beads. Beads conjugated with the enzymatic products of MTMR2, PI and PI(5)P, were also included. The PIP beads were incubated with equal concentrations of cellular lysate from rat RT4-D6P2T Schwann cells followed by extensive washing. Following elution in SDS/PAGE loading dye, the proteins were separated by SDS/PAGE and detected by silver staining (Fig. 1). Several protein bands appeared specific to the PIP isoforms including two protein bands observed at molecular weights of approximately 205 kDa and 70 kDa that were specific for PI(3)P and PI(3,5)P$_2$ (indicated by asterisks).
Figure 2.1. **PIP lipid pull down from Schwann cells.** Cell lysates were prepared from rat RT4-D6P2T Schwann cells (6 x 10⁷). Approximately 10 mg of total protein was subjected to affinity chromatography on the indicated immobilized PIP resin. Following stringent washing conditions, the samples were resolved on a 12% SDS-PAGE gel and silver stained. Asterisks indicate the protein bands of interest which were excised and identified by mass spectrometry. Protein bands of interest were excised and in-gel digested with trypsin. The extracted pools of tryptic peptides were then analyzed by MALDI-TOF MS/MS for protein identification. The 205 kDa band was identified as receptor-mediated endocytosis 8 (RME-8) and the 70 kDa band was identified as heat shock cognate 70 (Hsc70) (Fig. 2). At least 10 peptides from each trypsin digest were subjected to MS/MS analysis to obtain unambiguous identification. The high sequence coverage was particular important in the case of Hsc70 in order to distinguish from closely related homologues. Notably, the 200 kDa band present in the PI(5)P sample (Fig. 1) was identified as pyruvate decarboxylase (data not shown).
Figure 2.2. Identification of putative PIP binding proteins by MALDI-MS/MS. Following trypsin digestion the peptides were analyzed by MALDI-MS/MS for protein identification. (A) Shown is a MS/MS spectrum for the precursor ion 1957 m/z. Fragment ion series analysis resulted in identification of a sequence found in RME-8. (B) Shown is a MS/MS spectrum for precursor ion 1691 m/z. Fragment ion series analysis resulted in identification of a sequence found in Hsc70.
The identification of RME-8 and Hsc70 is intriguing since these two proteins in a variety of organisms and cell types have been shown to function in a complex and regulate receptor-mediated and fluid-phase endocytosis [62, 89, 95]. Furthermore, Drosophila RME-8 and Hsc70 have been shown to interact in vitro [62], while human RME-8, through its J-domain, has been shown to directly bind to Hsc70 in an ATP dependent manner [95].

2.3.2. PI3P is required for the association between RME-8 and early endosomes

It has been demonstrated that RME-8 can localize to membrane vesicles including early endosomes [83, 90]. Our current proteomic results suggest that RME-8 can also interact with the substrates of MTMR2, such as PI(3)P, which are present on membrane vesicles such as endosomes. To test if PI(3)P is required for its endosomal localization, we examined the ability of RME-8 to co-localize with a well characterized reporter for PI(3)P enriched endosomes, DsRed-FYVE [96].
Figure 2.3. Rme-8 colocalizes with vesicles containing PI(3)P. Hela cells were co-transfected with GFP-RME-8 and DsRed-FYVE expression vectors and analyzed by immunofluorescence microscopy. Localization pattern of GFP-RME-8 without wortmannin treatment (A) and with wortmannin treatment (B) is shown. The merged images are depicted in the right panel showing partial overlap of GFP-RME-8 and DsRed-FYVE localization without wortmannin treatment and dispersed localization with wortmannin treatment. Images were taken with 40X objective using a fluorescence microscope. Bars, 15 µm.
As shown in Fig. 2.3A, RME-8 displayed strong co-staining with DsRed-FYVE confirming that RME-8 positive vesicles contain PI(3)P. Cells were then treated with the phosphoinositide 3-kinase (PI(3)K) inhibitor wortmannin to investigate the requirement of PI(3)P for the membrane vesicle localization of RME-8. Wortmannin-induced PI(3)P depletion led to a strong reduction in the endosomal staining pattern of RME-8. Thus, in addition to interacting with PI(3)P beads in vitro, RME-8 associates with PI(3)P containing vesicles in cellula.

RME-8 possesses a DnaJ domain in the C-terminal half of the protein that is responsible for interacting with Hsc70 [62, 89]. The C-terminal region also mediates the reported interaction between RME-8 and sorting nexin 1 [83]. On the other hand, the N-terminal region has been shown to mediate the membrane localization of RME-8 [90]. Likewise, PIP pull down assays were performed with RME-8 deletion constructs to determine which RME-8 region was responsible for the observed PI(3)P and PI(3,5)P₂ binding. The results showed that full length RME-8 and a variant encompassing all but the last 425 amino acids were able to associate with PI(3)P and PI(3,5)P₂. In contrast, deletion of the first 453 amino acids completely abolished binding to PI(3)P and PI(3,5)P₂ (Fig. 2.4). Taken together, our results strongly suggest that RME-8 is a novel PI(3)P binding protein both in vitro and in cells.
Figure 2.4. Deletion of the N terminal region of RME-8 abolishes RME-8-PI(3)P association. Hek 293 cellular lysates expressing GFP-RME-8 variants were subjected to a PI(3)P pulldown (A) or a PI(3,5)P₂ pulldown (B) as described in Materials and Methods and analyzed by immunoblotting using a GFP antibody. Immunoblots representative of three independent trials are shown. GFP-RME-8 variants included full length GFP-RME-8, GFP-RME-8·N453 and GFP-RME-8·C425.
2.3.3. MTMR2 attenuates the association of RME-8 with the early endosome

We have recently discovered that MTMR2 is highly phosphorylated on Ser\(^{58}\) which sequesters MTMR2 in the cytoplasm and limits access to its lipid substrates. Conversely, a phosphorylation deficient mutant, MTMR2 S58A, is capable of localizing to endosomal vesicles and depleting PI(3)P. Therefore, to address whether the PI(3)P binding ability of RME-8 could be affected by MTMR2, RME-8 was co-expressed with MTMR2 S58A and the integrity of the RME-8 endosomal pattern was analyzed by immunofluorescent microscopy. When overexpressed alone, GFP-RME-8 and MTMR2 S58A both exhibited the characteristic punctate endosomal localization pattern (Fig. 2.5A, B, left panels). Strikingly, co-expression of MTMR2 S58A resulted in significant loss of the RME-8 endosomal localization pattern (Fig. 2.5A, upper right panel), as did treatment with wortmannin (Fig. 2.5B, right panel). Furthermore, expression of a catalytically inactive variant (MTMR2 S58A.C417S) failed to disrupt the endosomal localization pattern of RME-8 (Fig. 2.5A, lower right panel), suggesting that the association of RME-8 with PI(3)P rich vesicles can be disrupted by the phosphatase activity of MTMR2.
Figure 2.5. MTMR2 S58A alters the endosomal localization pattern of RME-8. (A) HeLa cells were transiently transfected with FLAG-MTMR2 S58A or FLAG-MTMR2 S58A.C417S and GFP-RME-8 and analyzed by immunofluorescence microscopy. Alteration of the localization pattern of GFP-RME-8 is observed when co-expressed with FLAG-MTMR2 S58A (A, upper panel) or when cells are treated with wortmannin (B). Images were collected using 63× objectives. The scale bar represents 10 µm.
RME-8 has been previously shown to regulate the endosomal sorting of a variety of cargo at the early to late endosome interface, including the epidermal growth factor receptor (EGFR) [83, 90, 95, 97]. Similarly, in HeLa cells that were stimulated with EGF, GFP-RME-8 displayed significant co-localization with EGFR (Fig. 2.6A). To ascertain the compartment where RME-8 and EGFR co-localize we utilized triple staining immunofluorescence with the early endosome marker Rab5 (Fig. 2.6B) and the late endosome/lysosomal marker LAMP1 (Fig. 2.6C). Although EGFR was found to partially co-localize with both of these markers, vesicles containing EGFR and RME-8 only co-stained with Rab5 and not LAMP1 suggesting that RME-8 co-localizes with EGFR on early endosomes. Meanwhile, expression of MTMR2 S58A disrupted the ability of RME-8 to co-localize with the EGFR (Fig. 2.6D). Again, this disruption required the phosphatase activity of MTMR2 as RME-8 was capable of co-localizing to EGFR positive vesicles in the presence of the catalytically inactive mutant of MTMR2 (Fig. 2.6E). Collectively, these results demonstrate that MTMR2 regulates the PI(3)P dependent targeting of RME-8 to EGFR-positive early endosomes.
Figure 2.6. GFP-RME-8 co-localization with EGFR on early endosomes is disrupted by MTMR2 S58A. HeLa cells were transiently transfected with GFP-RME-8, serum starved for two hours and then stimulated with EGF (20 ng/ml) for 15 min. Cells expressing GFP-RME-8 alone (A–C) or co-expressing MTMR2 S58A (D), or MTMR2 S58A.C417S (E) were analyzed by immunofluorescence microscopy. Cells were probed for endogenous EGFR and GFP-RME-8 co-localization with the early endosome marker Rab5 (B) or late endosome/lysosomal marker LAMP1 (C). Arrowheads represent vesicles displaying significant co-localization. Images were collected using 63X objectives. The scale bar represents 15 µm.
In conclusion, our results have elucidated a novel mechanism for regulating RME-8 endosomal targeting. As RME-8 in a variety of organisms has clearly demonstrated to be a critical regulator of endosomal processing, determining the temporal and spatial details of how PI(3)P regulates the docking of RME-8 to endosomal vesicles will deepen our understanding of the specific role of RME-8 in vesicular trafficking. Seeing that RME-8 is expressed in a variety of mammalian tissues [95], it will also be interesting to investigate if other MTMRs can regulate RME-8 to the same extent as MTMR2. Finally, as CMT4B1 is caused by loss of a functional MTMR2, our findings open the door to examining if RME-8 is overactive and contributes to the demyelination phenotypes in CMT 4B1.

2.4. References


CHAPTER 3
CHAPTER 3

RECEPTOR MEDIATED ENDOCYTOSIS 8 UTILIZES AN N-TERMINAL PHOSPHOINOSITIDE-BINDING MOTIF TO REGULATE ENDOSSMAL CLATHRIN DYNAMICS

3.1. Introduction

Endocytosis is a highly regulated and diverse process of plasma membrane and extracellular matrix internalization that participates in a variety of cellular processes [54-56]. Recent work in the field has revealed that endocytic vesicles are heterogeneous macromolecular complexes, making it critical to further identify and fully characterize factors that associate and regulate endocytic events such as endosomal signaling, endosomal maturation, cargo sorting, cargo recycling, and cargo degradation. Receptor-mediated endocytosis 8 (RME-8) was first discovered utilizing genetic screens in Caenorhabditis elegans, where rme-8 mutants exhibited defects in receptor-mediated yolk endocytosis in the oocyte and fluid phase endocytosis in the coelomocyte [89]. Likewise, studies of rme-8 mutants in Drosophila displayed blockage in the internalization of the Bride of Sevenless receptor causing the formation of the rough eye phenotype [62]. RME-8 has not only been shown to be highly conserved in the animal kingdom [90, 95], but it is also present in plants, where studies in Arabidopsis thaliana have demonstrated that RME-8 mutants exhibit gravitropism defects and associate with endosomal structures [98]. Finally, a recent study has determined that a gain of function mutation in RME-8 correlates with Parkinson disease [99], highlighting the importance of studying RME-8 biology in more detail. RME-8 is a large protein composed of more than
2000 amino acids. It contains four IWN repeats of unknown function and a DnaJ binding domain located between the second and the third IWN repeat that has been shown to associate with heat shock protein Hsc70 in a variety of species[62, 86, 90, 95]. DnaJ protein family members act as coupling factors to stimulate ATP hydrolysis by its partner heat shock protein and thus they function as co-chaperones [100]. The pleiotropic Hsc70 has a well established role in the disassembly of clathrin [101]. Clathrin is crucial for vesicle formation at the plasma membrane during clathrin mediated endocytosis and for protein sorting from early endosomes [102]. In the case of endocytosis, the DnaJ domain protein auxillin recruits Hsc70 to release clathrin coats from clathrincoated vesicles by binding to the terminal domain of clathrin heavy chain [88, 101, 103]. Clathrin-coated vesicles are also featured on early endosomes and are the target of the RME-8/ Hsc70 complex where they are employed to sort cargo from early endosomes to the trans-Golgi network (TGN) during retrograde transport [62, 86, 104, 105]. In addition to binding Hsc70, RME-8 has also been shown to associate and co-localize with the endosome membrane remodeling component SNX1 [83, 86]. SNX1 when complexed with SNX2 recruits the Vps26-Vps29-Vps35 retromer trimer to form the heteropentameric coat, also known as retromer [81]. SNX1 contains a phox (PX) domain that binds specifically to phosphatidylinositol 3-phosphate (PI(3)P). Subsequently, the Vps26-Vps29-Vps35 trimer is recruited and recognizes the transmembrane cargo to be sorted from early endosomes to the trans-Golgi network during retrograde transport [81, 106, 107]. In mammals, a well characterized transmembrane protein that is sorted by a retromer is the cation-independent mannose 6-phosphate receptor (CI-MPR) [81, 106]. Generally, newly synthesized acid hydrolase precursor proteins are recognized by the CI-MPRs at the TGN
membranes and are later sorted at early endosomes. After acid hydrolases reach the lumen of early endosomes, they dissociate from the receptor due to the acidic environment. They are then directed to lysosomes to degrade biological material, whereas the acid hydrolase receptors escape the early-to-late endosomal degradation pathway and are transported to the TGN through the retrograde transport pathway [71, 79, 81]. A prominent mechanism during endosomal processing events is the recruitment of target effector proteins through association with PI(3)P on the surface of early endosomes. We have recently discovered that RME-8 associates with PI(3)P containing early endosomes in a myotubularin-related-2-dependent manner [108]. Here, we have now identified critical residues mediating PI(3)P binding within the N terminus of RME-8. We have characterized this PI(3)P binding region in terms of its biochemical properties and examined its requirement for RME-8 activities at the early endosome.

3.2. Materials and Methods

3.2.1. Plasmid Constructs

The plasmids encoding GFP-RME-8 were a kind gift from Drs. Fujibayashi and Sekiguchi from Osaka University, Osaka, Japan [90]. Site-directed mutagenesis was performed to generate RME-8 point mutants. The forward and reverse primers for the GFP-RME8 K8A mutant are 5’-TAATTAGGGAAAATGCGGATCTGGCATGTT-3’ and 5’-AACATGCCAGATCCGCATTTTCCCTAATTA-3’. The forward and reverse primers for generation of the GFP-RME-8 K17A mutant are 5’-TTCTACACAACAGCACATTCATGGAG-3’ and 5’-
CTCCATGAATGTGCTGTTGTGTAGAA-3’. To generate the GFP-RME-8 W20A mutant, the forward primer used was 5’-CAACAAAAACATTCAAGCGAGGGGAAGTATA-3’, whereas the reverse primer was 5’-TATACTTCCCCCTCGCTGAATGTTTGTGTTG-3’. The forward primer for GFP-RME-8 Y24A was 5’- CATGGAGGGGAAGGCTAAGCGTGTCTTTT-3’, and the reverse was 5’-AAAAGACACGCTTAGCCTTCCCCCTCCCATG-3’. The forward primer for the generation of GFP-RME-8 K25A was 5’-GGGAAGTATGCAGCGTGTCTTTTC-3’, and the reverse was 5’-AAGACACGCGCATACTTTCCCCCT-3’. The forward primer for the generation of GFP-RME-8 R26A was 5’-GGGAAGTATAAGGCTGTCTTTTCAGTT-3’, and the reverse was 5’-AACCTGAAAAGACAGCCTTATACTTCCC-3’. All the constructs generated were verified by automated DNA sequencing (ACGT Corp). FLAG-Hsc70 and mRFP-SNX1 constructs were the kind gifts from Dr. Frank R. Sharp (University of California at Davis) and Dr. Peter J. Cullen (University of Bristol), respectively. Site directed mutagenesis was performed to generate the mRFPSNX1 K214A point mutant. The forward and reverse primers for mRFPSNX1 K214A are 5’-CCGCCGCGAGGGAAGCCTCATAGGGA-3’ and 5’-TCCCTATGAGGCTTGCTCCGGGGCGG-3’.

3.2.2. Bioinformatics Analysis

RME-8 sequences were obtained by NCBI (www.ncbi.nlm.nih.gov), and orthologue sequences were aligned utilizing ClustalW multiple sequence alignment
The three-dimensional structure prediction model of RME-8 was obtained by submitting the sequence of the first 100 amino acids of RME-8 to Protein Homology/AnalogY Recognition Engine Version 2.0 (Phyre2) [110, 111]. The predicted three-dimensional structure of RME-8 generated from Phyre2 was superimposed onto the structure of the FERM domain of talin (Protein Data Bank code d1mixa2) using the Swiss-PDB Viewer (Deep View) [112, 113].

3.2.3. Cell Culture, Transfection, and Cell Lysis

HeLa (ATCC) and HEK293 (ATCC) cells were maintained in DMEM/F-12 with 10% FBS, 2 mM L-glutamine and supplemented with 1% penicillin/streptomycin antibiotics at 37 °C and 5% CO2. Cells were seeded 24 h before transfection. Following transient transfection with 5 µg of DNA using FuGENE HD (Roche Applied Science) using the manufacturer’s protocol, cells were lysed with lysis buffer (50mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 76 mM NaCl, 2 mM EGTA, 10% glycerol) supplemented with protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin. Knockdown of endogenous RME-8 and SNX1 protein expression was performed using ON-TARGET siRNA SMARTpools (Dharmacon). HeLa cells were transfected with RME-8 and SNX1 siRNAs to a final concentration of 25 nM using DharmaFECT 1 transfection reagent following the manufacturer’s protocols. Cells were then incubated with antibiotic-free media for 48 h before analysis.
3.2.4. Phosphatidylinositol Pulldown

HEK293 were transiently transfected with GFP-RME-8 variants and lysed as described above. Cellular lysates were incubated overnight at 4 °C with various phosphatidylinositol (PI) phosphates conjugated to resin (Echelon Biosciences), including control PI beads, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3. Following three washing steps with washing/binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% Nonidet P-40), protein samples were subjected to SDS-PAGE analysis. Afterward, the samples were transferred onto a PVDF membrane and incubated with goat anti-GFP (Rockland) and anti-goat HRP (Rockland) antibodies at room temperature for 1 h. Proteins were visualized using SuperSignal West Femto Reagent (Thermo Scientific).

3.2.5. Liposomal Floatation Assay

40 and 30% Opti-Prep density gradient medium (Sigma) was prepared in washing/binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% Nonidet P-40). HEK293 cells were lysed with 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 76 mM NaCl, 2 mM EGTA, 10% glycerol lysis buffer. Cellular lysates were incubated with control PI or PI(3)P-polymerized liposomes (PolyPIPosomes, Echelon Biosciences) for 2 h at room temperature. Following incubation, 60% Opti-Prep density gradient medium (Sigma) was added to obtain a final 40% Opti-Prep density gradient. The protein/liposome mixtures were added to thick-walled polycarbonate tubes (Beckman
Coulter). 30% Opti-Prep density gradient medium was then overlaid on top of the 40% layer. Finally, wash(binding) buffer was added as the top layer. Samples were spun in an ultracentrifuge at 50,000 rpm for 3 h at room temperature. After centrifugation, SDS-PAGE loading dye was added, and protein samples were analyzed by immunoblotting.

3.2.6. Co-immunoprecipitation

For the RME-8/Hsc70 co-immunoprecipitation, we co-transfected HEK293 cells with GFPRME-8 variants and FLAG-Hsc70 for 24 h, and cells were lysed as described above. Cellular lysates were incubated with FLAG protein A-agarose beads (Sigma) overnight at 4 °C. Immunoprecipitates were washed three times with 50mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 0.1% SDS and analyzed by immunoblotting using anti-FLAG (Sigma), goat anti-GFP (Rockland), rabbit anti-actin (Sigma), and secondary antibodies anti-mouse HRP (Promega), anti-goat HRP (Rockland), and anti-rabbit HRP (Vector Laboratories). For RME-8/SNX1 coimmunoprecipitations, we transfected cells with GFP-RME-8 variants for 24 h. We pre-bound the protein A-agarose beads with 1 μg of goat anti-GFP antibody for 45 min at room temperature. Following washing steps, we incubated cellular lysates with the conjugated beads overnight at 4 °C. Samples were washed three times as described above and analyzed by immunoblotting. Anti-SNX1 (BD Transduction Laboratories) was used to detect endogenous SNX1.
3.2.7. Immunofluorescence and Image Analysis

HeLa cells were seeded on four-chamber slides (BD Biosciences) 24 h prior to transient transfection with 0.5 μg of DNA using FuGENE HD. Slides were fixed for 15 min with 3.7% paraformaldehyde at room temperature. Cells were then permeabilized with 0.15% Triton X-100 for 2 min and blocked with 5% BSA for 1 h at room temperature. Primary antibodies, including mouse anti EEA-1 (BD Biosciences), rabbit anti-EEA-1 (Sigma), goat anticalathrin (Santa Cruz Biotechnology), mouse anti-CI-MPR (Abcam), and rabbit anti-SNX1 were incubated for 1 h. Rabbit anti-RME-8 antibody was a kind gift from Dr. Peter McPherson (McGill University, Canada). Following washes, cells were incubated with the appropriate Alexa secondary antibodies (Life Technologies, Inc.) for 1 h at room temperature. Cells were washed, stained with Hoechst stain (0.5 mg/ml) (Invitrogen) for 2 min, and mounted on the slide. Confocal fluorescence microscopy was utilized to capture the images using a x 40 oil and a x 60 water objective. To determine the extent of co-localization between EEA1-positive endosomes and RME-8, Pearson’s correlation coefficients were measured. Pearson’s correlation coefficient values were calculated using ImageJ software via JACoP plugin (National Institutes of Health) [114]. Data in the bar graphs represent the average of 30 randomly selected cells examined for co-localization.
3.3. Result and Discussion

3.3.1. Identification of the RME-8 PI(3)P-Binding Motif

Earlier studies have shown that RME-8 associates with early endosomes [83, 90, 95], and recently our laboratory has discovered RME-8 to be a novel PI(3)P-binding protein that is regulated by the PI(3)P phosphatase MTMR2 [108]. Because RME-8 does not contain a classical PI(3)P binding domain (e.g. FYVE domain, PX domain) we sought to localize the PI(3)P-binding motif in RME-8. To guide this process, the primary sequence of RME-8 was analyzed by the Phyre2 protein folding prediction program [110, 111]. The result of this analysis was a high confidence score predicting the presence of a pleckstrin homology (PH)-like binding fold present in the first 100 amino acids of RME-8. Along with predicting homology with PH domains from a variety of proteins, the highest homology was with the PH domain of the PI(4,5)P2-binding protein Talin (94% confidence) (Fig. 3.1, A and B) [115, 116]. Further homology modeling using the Swiss-PDB Viewer and the three-dimensional structure of the PH domain of Talin (Protein Data Bank code d1mixa2) was performed (Fig. 3.1A). The resulting structural model revealed strong alignment with a portion of the PH fold of Talin, complementing the Phyre2 prediction program result. Generally, most PH domain binding interfaces reside on loop structures that link β-strands [50]. Examining these regions on the RME-8/Talin model revealed residues in the extreme N terminus of RME-8 predicted to reside on such loop structures (Fig. 3.1A). Moreover, when examining this N-terminal region in RME-8, it was determined that many of these residues are highly conserved among orthologues (Fig. 4.1C), leading us to hypothesize their involvement in PI(3)P binding (Fig. 3.1, C
and D). This region contains many positively charged residues and several bulky hydrophobic residues conserved among orthologues. Such residues have been shown to be functionally critical in PH domains [50] as well as other PI(3)P binding domains such as PX and FYVE domains [117, 118].
Figure 3.1. RME-8 contains a putative phosphoinositide-binding motif. A, structural homology model depicting RME-8 (red) aligned with the solved structure of Talin’s FERM third subdomain (blue) (27). The first 100 amino acids of RME-8 sequence were first submitted to the Phyre2 server for homology detection followed by Swiss-PDB Viewer to produce the structural model (see “Experimental Procedures”). Solvent-exposed amino acids of interest (Lys17, Trp20, Tyr24, and Arg26) in RME-8 that potentially represent a phosphoinositide-binding interface are shown in green. B, alignment of RME-8 and FERM’s PH-like subdomain (d1mixa2). Sequences are aligned based on the secondary structure homology predicted by the Phyre2 server. Blue arrows represent β-strands, and the amino acids in bold represent amino acid identity. C, multiple sequence alignment of the N-terminal region of RME-8 orthologues. The RefSeq accession numbers used were the following: Homo sapiens, AAV41096.1; Mus musculus, NP_001156498.1; Drosophila melanogaster, NP_610467.1; C. elegans, AF372457_1; A. thaliana, AEC07904.1; Desmodus rotundus, JAA50105.1; Leishmania donovani, CBZ36243.1; Ixodes ricinus, JAB76328.1; Capsaspora owczarzaki, EFW44108.1; Apis mellifera, XP_394533.4; and Chlamydomonas reinhardtii, EDP05338.1. Based on the ClustalW alignment, the putative signature phosphoinositide-binding motif of RME-8 to is KXSW(K/R)G(K/R)YXR. D, schematic presentation of the human RME-8 protein with its IWN repeats (gray boxes) and its DnaJ domain (black box) shown. The proposed RME-8 PI(3)P-binding motif targeted for subsequent mutagenesis studies is highlighted.
PH domains have been shown to bind to a variety of phosphoinositide isoforms, in particular isoforms with vicinal phosphates such as PI(3,4)P₂ and PI(4,5)P₂ [50]. Therefore, it was important to examine the phosphoinositide specificity of RME-8. To accomplish this, HEK293 cells expressing wild type RME-8 were lysed and incubated with each of the seven phosphatidylinositol phosphates (PIP) conjugated to resin, and binding specificity was assessed via immunoblotting (Fig. 3.2). Consistent with earlier studies [108], RME-8 associates with PI(3)P and PI(3,5)P₂ while failing to associate with the products of MTMR2, PI or PI(5)P. Furthermore, RME-8 shows no appreciable association with PI(4)P, PI(3,4)P₂, or PI(4,5)P₂. Interestingly, RME-8 did associate with PI(3,4,5)P₃ to a similar extent as PI(3)P and PI(3,5)P₂. Although the finding that RME-8 can associate with resin functionalized with PI(3,5)P₂ and PI(3,4,5)P₃ is potentially biologically relevant (see discussion below), the focus of this study was regulation of RME-8 at early endosomes. Thus, subsequent analysis focused on delineating residues important for association with PI(3)P.
Figure 3.2. RME-8 binds PI(3)P, PI(3,5)P2, and PI(3,4,5)P3 in vitro. A, HEK293 cells were transiently transfected with wild type RME-8 for 24 h. Cells were then lysed as described under “Experimental Procedures” and subjected to pulldowns with the following lipid groups: PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3. Following elution, immunoblots (IB) were probed with anti-GFP antibody to detect GFP-RME-8. Whole cell lysates were analyzed by GFP and actin immunoblotting to confirm equal GFP RME-8 and total protein levels. B, lipid pulldown assay was quantified by densitometry using ImageJ software and normalized to actin levels. Means ± S.D. of three independent experiments are shown (n = 3). p values were calculated by the Student’s t test comparing each sample to GFP-RME-8. **, p < 0.01; *** p < 0.001.
As noted above, the predicted PH-like fold region in the N terminus of RME-8 contains several highly conserved residues with the potential to mediate PI(3)P binding. Therefore, we examined the effect of substituting a variety of positively charged and bulky hydrophobic residues within this region for PI(3)P binding using the PIP pulldown assay (Fig. 3.3). Lysates generated from HEK293 cells expressing RME-8 variants were incubated with resin containing PI or PI(3)P. Following washing and SDS-PAGE separation, the PI(3)P binding activity of RME-8 variants was determined by immunoblotting. As expected, wild type RME-8 associates with PI(3)P. Moreover, RME-8 K8A and RME-8 K25A variants also display no significant difference in their ability to interact with PI(3)P. In contrast, the RME-8 variants K17A, W20A, Y24A, and R26A show markedly reduced capacity to associate with PI(3)P in this in vitro pulldown assay (Fig. 3.3, A and B). These results demonstrate that Lys\textsuperscript{17}, Trp\textsuperscript{20}, Tyr\textsuperscript{24}, and Arg\textsuperscript{26} represent possible determinants for an N-terminal PI(3)P-binding motif within RME-8.
Figure 3.3. Lys$^{17}$, Trp$^{20}$, Tyr$^{24}$, and Arg$^{26}$ are involved in RME-8 PI(3)P binding in vitro. Continued
Figure 3.3. Lys$^{17}$, Trp$^{20}$, Tyr$^{24}$, and Arg$^{26}$ are involved in RME-8 PI(3)P binding in vitro. Continued
Figure 3.3. Lys$^{17}$, Trp$^{20}$, Tyr$^{24}$, and Arg$^{26}$ are involved in RME-8 PI(3)P binding in vitro. A, HEK293 cells were transiently transfected with wild type RME-8, RME-8 K8A, RME-8 K17A, RME-8 W20A, RME-8 Y24A, RME-8 K25A, and RME-8 R26A constructs for 24 h. Cells were lysed as described under “Experimental Procedures” and subjected to PI and PI(3)P lipid pulldowns. Subsequent immunoblots were probed with anti GFP to detect GFP-RME-8 variants. Whole cell lysates were analyzed by GFP immunoblotting (IB) to confirm equal GFP-RME-8 expression levels. A representative blot from three independent experiments is shown (n=3). B, lipid pulldown assays quantified by densitometry using ImageJ software and normalized to actin levels. Means ± S.D. of three independent trials are shown. p values were calculated by the Student’s t test and compared with wild type RME-8, **, p<0.01; ***, p<0.001. C, HEK293 cells expressing wild type RME-8 (wt) and RME-8W20A were subjected to liposomal flotation assays. Lysates incubated with PI liposomes and PI(3)P liposomes were separated using an Opti-Prep gradient and ultracentrifugation. Gradient fractions were then analyzed by immunoblot analysis. Representative blots from three independent experiments are shown (n=3). D, liposome flotation assay quantified by densitometry using ImageJ software. Means ± S.D. of three independent trials are shown. p values were calculated by the Student’s t test and compared with wild type RME-8. ***, p < 0.001.
To complement the bead-based pulldown assay, we also examined the ability of wild type RME-8 and a representative binding mutant RME-8 W20A to associate with liposomes containing PI(3)P using liposome flotation assays [119]. HEK293 cells expressing wild type RME-8 or RME-8 W20A were incubated with PI or PI(3)P containing liposomes followed by the addition of an Opti-Prep density gradient solution. Samples were subjected to ultracentrifugation upon which the PI(3)P liposomes (along with any bound RME-8) migrate to the top portion of the gradient. Gradients were then fractionated and analyzed by immunoblotting (Fig. 3.3, C and D). We reproducibly observed a significant portion of wild type RME-8 fractionate with the top PI(3)P liposome containing fraction but not with liposomes loaded with PI. Moreover, RME-8 W20A was defective at fractionating with the PI(3)P liposomes suggesting that RME-8 can associate with PI(3)P liposomes and that Trp20 is required for PI(3P) binding activity.

3.3.2. PI(3)P-binding Mutants Attenuate RME-8 Association with PI(3)P-rich early endosomes

It has been demonstrated that RME-8 displays a punctate localization pattern due to its association with early endosomal structures [68, 83, 89, 95, 108] that can be disrupted by the PI(3)-kinase inhibitor wortmannin and expression of the PI(3)P phosphatase MTMR2 [108]. Therefore, to complement the above biochemical assays, we screened the various RME-8 mutants for subcellular localization changes from punctate to a diffused cytoplasmic pattern. As shown in Fig. 3.4, wild type RME-8, RME-8 K8A, and RME-8 K25A variants display a punctate pattern consistent with functional endosomal localization. In contrast RME-8 K17A, RME-8 W20A, RME-8 Y24A, and
RME-8 R26A significantly lost the RME-8 punctate pattern, instead displaying largely diffuse cytoplasmic localization.
Figure 3. RME-8 K17A, W20A, Y24A, and R26A alter the subcellular localization of RME-8. HeLa cells were transiently transfected with wild type RME-8, RME-8 K8A, RME-8 K17A, RME-8 W20A, RME-8 Y24A, RME-8 K25A, and RME-8 R26A and analyzed by confocal microscopy as described under “Experimental Procedures.” Images were collected using a X40 objective. Scale bars, 15 µm.
To test whether these residues would be involved in dictating RME-8 PI(3)P-dependent early endosomal localization in cellula, we utilized immunofluorescence microscopy to examine co-localization of RME-8 variants with endogenous EEA-1, which is a well characterized PI(3)P early endosome marker (Fig. 3.5) [118, 120, 121]. Consistent with our pulldown experiments, RME-8 K8A and RME-8 K25A co-localize with EEA1 to a similar extent as wild type RME-8 (Fig. 3.4, A and C), indicating that these Lys residues likely are not critical for PI(3)P binding. On the contrary, RME-8 K17A, RME-8 W20A, RME-8 Y24A, and RME-8 R26A failed to associate with PI(3)P-rich EEA1 early endosomes as indicated by loss of co-localization with EEA-1 along with diffuse cytoplasmic localization (Fig. 3.5, B and C).
Figure 3.5. RME-8 early endosome localization requires Lys$^{17}$, Trp$^{20}$, Tyr$^{24}$, and Arg$^{26}$. Continued
Figure 3.5. RME-8 early endosome localization requires Lys\textsuperscript{17}, Trp\textsuperscript{20}, Tyr\textsuperscript{24}, and Arg\textsuperscript{26}. Continued
Figure 3.5. RME-8 early endosome localization requires Lys\textsuperscript{17}, Trp\textsuperscript{20}, Tyr\textsuperscript{24}, and Arg\textsuperscript{26}. A and B, HeLa cells were transfected with wild type RME-8, RME-8 K8A, RME-8 K17A, RME-8 W20A, RME-8 Y24A, RME-8 K25A, and RME-8 R26A (green) for 24 h and stained for EEA1 (red). Co-localization was analyzed by confocal microscopy. A, wild type RME-8, RME-8 K8A, and RME-8 K25A co-localize with EEA1-positive early endosomes. B, absence of co-localization between RME-8 K17A, RME-8 W20A, RME-8 Y24A, and RME-8 R26A with EEA1 at early endosomes. Solid white arrows show regions of co-localization. Expanded boxes represent regions of interest. Images were collected using a X40 objective. Scale bars, µ15m. C, Pearson’s correlation coefficient (PC) was utilized to quantify the extent of co-localization between RME-8 and EEA1-positive endosomes. Means ±S.D. of three independent experiments (n = 30 cells) are shown. p values were calculated by the Student’s t test and compared with wild type RME-8, *, p < 0.1; **, p < 0.01; ***, p < 0.001.
Taken together, our data indicate that RME-8 binds PI(3)P biochemically in vitro and on early endosomes \textit{in cellula} through a novel N-terminal binding motif that is predicted to reside within a PH-like folded domain. Moreover, residues Lys$^{17}$, Trp$^{20}$, Tyr$^{24}$, and Arg$^{26}$ were identified as key determinants for competent PI(3)P association. Akin to other PI(3)P-binding proteins, RME-8 utilizes a combination of hydrophobic and positive charge functional groups for association with PI(3)P. In particular, the PX domain also possesses invariant Lys and Tyr residues to drive PI(3)P binding [122, 123]. It is interesting to note that analogous to RME-8, PX-containing proteins have also been shown to associate with PI(3,5)P$_{2}$ [79, 122-125]. This is in contrast to the FYVE domain, whose structurally distinct zinc coordination and small positively charged pocket is postulated to be too small to accommodate PI(3,5)P$_{2}$ [126]. Another distinguishing feature of RME-8’s PIP specificity is the interaction with PI(3,4,5)P$_{3}$ in the PIP pulldown assay (Fig. 3.2). Further structure-function analysis will be required to experimentally determine whether the N-terminal region of RME-8 truly adopts a PH-like fold and to examine whether RME-8’s association with PI(3,5)P$_{2}$ and PI(3,4,5)P$_{3}$ is biologically relevant. However, it is interesting to note that in addition to early endosomes, RME-8 has been reported to localize to late endosomes and recycling endosomes, which are known to contain PI(3,5)P$_{2}$ and PI(3,4,5)P$_{3}$, respectively [89, 95, 127]. Thus, it will be interesting to assess whether these PIP isoforms localized on distinct endosome subtypes regulate subcellular localization of RME-8.
3.3.3. RME-8 does not require its PI(3)P-binding for association with Hsc70

Hsc70 binds DnaJ domain-containing cofactor proteins and is involved in ATP-dependent clathrin dissociation from clathrin-coated vesicles [128]. Studies in Drosophila, C. elegans, and humans have shown that RME-8 binds the ATPase domain of Hsc70 through its DnaJ domain [62, 83, 86, 95, 128]. Intriguingly, RME-8 is the only known PI(3)P-binding protein that recruits Hsc70 to early endosomes via its DnaJ domain. Because the mechanism by which these two molecules regulate clathrin is not well characterized, it was of interest to explore whether the RME-8/Hsc70 interaction was maintained in RME-8 PI(3)P-binding mutants. Co-immunoprecipitation experiments were conducted using HEK293 cells expressing GFP-RME-8 variants and FLAG-Hsc70 followed by GFP immunoprecipitation and immunoblot analysis (Fig. 3.6 A). All of the RME-8-binding mutants that were found to disrupt PI(3)P association were able to co-immunoprecipitate Hsc70 to a similar extent when compared with wild type RME-8. The GFPRME-8 ΔC1165 truncation mutant, which is devoid of its DnaJ domain (6), was also analyzed as a negative control, and as expected it was largely defective at co-immunoprecipitating Hsc70. The data strongly indicate that the RME-8/Hsc70 interaction is not perturbed by the ability of RME-8 to bind PI(3)P. The notion that the PI(3)P binding activity of RME-8 is segmented from its protein-protein binding activity was confirmed using another interacting partner, SNX1. As shown in Fig. 3.6 B, we observed RME-8 W20A to co-immunoprecipitate endogenous SNX1 to a similar extent as wild type RME-8. In addition to indicating that mutating residues in the N-terminal PI(3)P binding region does not disrupt the functional fold of other RME-8 domains, this finding also signifies that RME-8 may potentially associate with Hsc70 or SNX1 away from...
endosomal structures.

It remains unclear how Hsc70 is distributed between auxillin and RME-8 during endocytosis and endosome maturation, respectively. One possibility is that due to the relative high concentration of Hsc70 in the cell, there are sufficient amounts of Hsc70 protein to partition within the two complex pools independently. Alternatively, PI(3)P levels may play a role in signaling the cell toward forming the RME-8/Hsc70 complex for sorting processing. In either case, our results signify that the endosomal clathrin uncoating activity of Hsc70 may potentially be indirectly regulated by PI(3)P levels via its interaction with RME-8.
Figure 3.6. Wild type RME-8 and PI(3)P-binding mutants associate with Hsc70 and SNX1. A, HEK293 cells were transiently co-transfected with GFP-RME-8 and FLAG-Hsc70 constructs for 24 h. Cellular lysates were then incubated with anti-FLAG protein-A-agarose beads. Following extensive washing steps, RME-8 bound to FLAG-Hsc70 was analyzed via immunoblotting (IB) using an anti-GFP antibody. IP, immunoprecipitation. Actin served as a cell number control. B, HEK293 cells were transiently transfected with wild type GFP-RME-8 or GFP-RME-8 W20A for 24 h. Lysates were subjected to anti-GFP immunoprecipitation and probed for endogenous SNX1 using immunoblot analysis. Actin served as a cell number control (Neg. Cont). A representative blot from three independent experiments is shown (n = 3).
3.3.4. Functional PI(3)P binding is required for RME-8 mediated early endosomal clathrin regulation

One of the hallmarks of membrane trafficking is cargo recognition and sorting from one cellular compartment to the other. Following cargo concentration into patches, the membrane undergoes deformation into smaller coated vesicles containing the cargo of interest [87]. One of these coats is clathrin, which has been shown to regulate the trafficking of retrograde transport receptors at early endosomes [83, 84, 86, 87] in addition to its role in the endocytosis of receptors at the plasma membrane. Recent studies have revealed that the retromer complex controls endosomal clathrin levels through its incorporation of the RME-8/Hsc70 tandem [83, 86]. Interestingly, the loss of either RME-8 or Hsc70 results in early endosomal clathrin accumulation [62, 95]. Having established that the RME-8/Hsc70 association is preserved in RME-8 PI(3)P-binding mutants, we hypothesized that early endosomal clathrin levels would be perturbed in RME-8 PI(3)P-binding mutants due to the inability of Hsc70 to access the endosomal clathrin structures. To examine this hypothesis, HeLa cells were transiently transfected with wild type or the RME-8 W20A variant. We chose the RME-8 W20A variant as the representative binding mutant for this and all subsequent experiments due to its extensive conservation among RME-8 orthologues and based on the structural model that predicts Trp20 of residing on an exposed loop that could potentially serve as a strong binding interface (Fig. 3.1). Following fixation, cells were triple-labeled for endogenous EEA-1 (red), endogenous clathrin heavy chain (green), and GFP-RME-8 (blue) (Fig. 3.7). The extent of co-localization between clathrin (green) and EEA-1 (red) was calculated as a measure of co-localization between the two channels (Fig. 3.7 E). Of interest were RME-8 decorated early endosomes that were positive for PI(3)P (EEA-1). Analysis of these
structures revealed a clear pattern where vesicles that were positive for RME-8 and PI(3)P (EEA-1) were largely devoid of clathrin (Fig. 3.7A). Interestingly, early endosomes that were lacking RME-8 (shown by arrowheads, Fig. 3.7A ) displayed strong co-localization between PI(3)P (EEA-1) and clathrin. These results emphasize the function that RME-8 plays at the early endosomes and its involvement in clathrin regulation. Conversely, HeLa cells transfected with siRNA targeting RME-8 expression resulted in an endosomal clathrin phenotype opposite of overexpressing wild type RME-8 with increased co-localization between clathrin and the PI(3)P marker EEA-1 (Fig. 3.7D). We observed an analogous phenotype by knocking down the protein expression of SNX1 by siRNA (Fig. 3.7E). Interestingly, similar to the siRNA-treated samples, cells expressing RME-8 W20A displayed a statistically significant increase in co-localization of early endosomal clathrin on EEA-1-positive early endosomes compared with wild type RME-8 expressing cells (Fig. 3.7, B and F). In concert with RME-8 W20A failing to associate with PI(3)P-positive endosomes, the clear increased accumulation of clathrin at EEA1-positive structures compared with wild type and control cells points to RME-8 W20A possibly behaving as a dominant negative mutant. RME-8 W20A can still associate with Hsc70 (Fig. 3.6A) and thus likely competes with endogenous RME-8 for Hsc70, resulting in clathrin remaining at early endosomes. Thus, our results suggest that RME-8 binding to PI(3)P through its PI(3)P-binding motif plays a critical mechanistic role in regulating early endosomal clathrin levels in collaboration with its binding partner Hsc70.
Figure 3.7. RME-8 PI(3)P binding affects early endosomal clathrin dynamics. Continued.
Figure 3.7. RME-8 PI(3)P binding affects early endosomal clathrin dynamics. Continued.
Figure 3.7. RME-8 PI(3)P binding affects early endosomal clathrin dynamics. Continued.
Figure 3.7. RME-8 PI(3)P binding affects early endosomal clathrin dynamics. HeLa cells were transiently transfected with wild type RME-8 (blue) (A) and RME-8 W20A(blue) (B) and for 24 h. Cells were triple-stained to observe endogenous EEA1 (red) and endogenous endosomal clathrin (green). A, wild type RME-8 at EEA1 early endosomes is displayed as magenta colored puncta, and clathrin at EEA1 early endosomes is displayed as yellow puncta. EEA1-positive and RME-8-positive endosomes display reduced clathrin levels indicated by arrows, and early endosomes devoid of wild type RME-8 (shown by arrowheads) display strong co-localization between EEA1 and clathrin. B, cells expressing RME-8 W20A display high co-localization between clathrin and EEA1. C, control cells are stained for endogenous RME-8 (blue), EEA1 (red), and clathrin (green). D and E, HeLa cells were transfected with siRNA targeting RME-8 (D) and SNX1 (E). Cells were stained for RME-8 (blue), EEA1 (red), and clathrin (green) to observe clathrin accumulation at EEA1 positive endosomes. F, Pearson’s correlation coefficient (PC) was utilized to quantify the extent of co-localization between clathrin and EEA1 in control, wild type RME-8, RME-8 W20A variant, RME-8 KD, and SNX1 KD samples. Means ±S.D. from three independent experiments (n = 30 cells) are shown. p values were calculated by the Student’s t test and compared with wild type RME-8. **, p < 0.01. The values of Pearson’s correlation coefficients of all the experiments were related and compared with RME-8 W20A set at 100% due to its largest statistical value. Solid white arrows, arrowheads, and expanded boxes represent regions of interest. Images were collected using a X40 objective. Scale bars, µ15m.
3.3.5. Perturbation of mannose 6-phosphate receptor localization by the RME-8 W20A PI(3)P-binding mutant

Clathrin and retromer coats have been implemented in the retrograde transport of transmembrane proteins from early endosomes to the trans-Golgi network (TGN) [84]. The action of these two coats working in a coordinated sequential fashion is facilitated by the RME-8/Hsc70-mediated clathrin uncoating process at early endosomes [83, 84, 86, 105]. RME-8 is a component of the retromer coat, and it has been shown to be important for the retrograde transport of transmembrane proteins such as the CIMPR [81, 83, 86]. The sorting of the acid hydrolase precursors starts at the TGN, and their delivery to early endosomes is mediated by their binding to CI-MPR embedded in the membrane followed by retrograde recovery of CI-MPR back to the TGN [81, 106, 129]. Proper functioning of the retrograde transport is regulated by RME-8. Knockdown of RME-8 expression has been shown to mis-sort retrograde transport receptors such as MIG-14 in *C. elegans* and CI-MPR in mammalian cells [83, 86, 95]. Because RME-8 regulates CI-MPR transport through clathrin disassembly [86], we investigated whether CI-MPR sorting would be affected in cells expressing RME-8 PI(3)P binding mutants. Therefore, HeLa cells expressing wild type RME-8 and RME-8 W20A were stained for endogenous CIMPR. In control cells as well as in cells expressing wild type RME-8, CI-MPR was observed to display mainly a perinuclear localization pattern with moderate staining at dispersed cytoplasmic punctate consistent with previous reports on the steady state localization of CI-MPR to TGN and endosomal structures [129-131]. However, in RME-8 W20A-expressing cells, CI-MPR localization on cytoplasmic punctate structures was significantly diminished and was predominantly found in the perinuclear region (Fig. 3.8,
The observed localization shift of CI-MPR was similarly reported in studies where RME-8 expression was knocked down using siRNA [83, 95]. Analogous results have also been reported when other members of retromer components were knocked down in mammalian cells [106, 129]. In these studies, it was concluded that disruption of retrograde transport of CI-MPR from endosomes to TGN results in rapid degradation of CI-MPR due to re-routing of CI-MPR from the endosome to the lysosome. This results in the loss of CI-MPR localization on endocytic punctate structures and the observed perinuclear localization pattern [83, 95, 106, 129]. Thus, as a control, we examined HeLa cells treated with siRNA targeting RME-8 and SNX1 (Fig. 3.8,C-E). Under these conditions, we also observed a significant decrease in CI-MPR localized to punctate structures, confirming previous studies and supporting the RME-8 W20A result. Overall, our results demonstrate that PI(3)P association is critical for RME-8 cellular activities, as disrupting the PI(3)P binding ability of RME-8 yields a phenotype resembling knocking down RME-8 or SNX1 protein levels.
Figure 3.8. Alteration of CI-MPR cellular localization in RME-8 W20A expressing cells. Continued.
Figure 3.8. Alteration of CI-MPR cellular localization in RME-8 W20A expressing cells. Continued.
Figure 3.8. Alteration of CI-MPR cellular localization in RME-8 W20A expressing cells. A, HeLa cells expressing wild type RME-8 and RME-8 W20A were stained for endogenous CI-MPR. Cells were analyzed by confocal microscopy. Images were collected using a X40 objective. Scale bars, 15 µm. ImageJ was utilized to count the number of total punctate in negative control, wild type RME-8, and RME-8 W20A-transfected cells. HeLa cells expressing RME-8 W20A exhibit a large decrease in the peripheral number of CI-MPR positive punctate displaying perinuclear localization. B, means ±S.D. of three independent experiments (n=30 cells) are shown. p values were compared with wild type RME-8. **, p < 0.01. C and D, both RME-8 and SNX1-depleted HeLa cells have been labeled with CI-MPR antibody and also displayed a decrease in the total number of CI-MPR-positive punctate. E, bar graph representing the effect that RME-8 KD and SNX1 KD p values were compared with WT RME-8 in A and to negative control in C and D. All cells were analyzed by confocal microscopy. Means ± S.D. of three independent experiments (n = 30 cells) are shown. p values were calculated by Student’s t test and compared with wild type RME-8 and SNX1. ***, p < 0.001.
3.3.6. SNX1 rescues RME-8 W20A dispersed cellular localization

The retromer complex is important in recognizing the cytosolic tail of MPRs for efficient transport from endocytic structures [129]. The retromer constituent SNX1 is a PX domain containing PI(3)P-binding protein [105, 125] that has been shown to play a role in sensing and driving membrane curvature [83, 86, 105]. RME-8 associates with SNX1 through a region C-terminal to its DnaJ domain, containing the third IWN repeat and the linker region between the third and the fourth IWN repeat [86]. To examine the relationship between the PI(3)P binding ability of RME-8 and its association with SNX1, we co-transfected HeLa cells with wild type SNX1/RME-8 and variants of both that are defective in PI(3)P association. As expected, wild type RME-8 and SNX1 strongly co-localize on peripheral structures consistent with endocytic vesicles (Fig. 3.9B). Surprisingly, cells co-expressing both RME-8 W20A and wild type SNX1 exhibited a drastic reversal in the RME-8 W20A subcellular localization pattern from diffused cytoplasmic to punctate (Fig. 3.9, A and B). Moreover, the rescued RME-8 W20A mutant strongly co-localizes with wild type SNX1. We next examined whether wild type RME-8 could rescue a well characterized PX domain point mutant of SNX1 (K214A) that disrupts PI(3)P binding [124, 132]. As shown in Fig. 3.9B, SNX1 K214A remains diffusely localized throughout the cytoplasm when co-expressed with wild type RME-8, indicating that RME-8 is not able to recover the PI(3)P-mediated localization of SNX1 to endocytic structures when its PX domain is compromised. More striking, however, was the observation that when co-expressed, SNX K124A induced significant loss of endocytic localization of wild type RME-8 (Fig. 3.9B). One possible interpretation of this result is that RME-8 requires SNX1 to localize to endocytic structures. To test this
hypothesis, we utilized a C-terminal deletion mutant of RME-8 (RME-8 Δ C1165) that lacks the SNX1 binding region and thus is unable to associate with SNX1 (6). However, this deletion construct possesses the N-terminal PI(3)P-binding motif. When analyzed for localization, RME-8 ΔC1165 was fully competent at targeting to peripheral punctate structures (Fig. 3.9, A and C). Moreover, overexpression of SNX1 K124A failed to disrupt localization of RME-8 ΔC1165 indicating that the PI(3)P-binding motif is sufficient for localization to endocytic vesicles. Interestingly, RME-8 ΔC1165 and wild type SNX1 strongly co-localized to these punctate structures suggesting that their interaction is not required for targeting to these vesicles. To test this hypothesis without the use of overexpressed proteins, we examined endogenous RME-8 localization in cells treated with SNX1 siRNA (Fig. 3.10). We observed no significant alteration in the RME-8 localization pattern in SNX1-depleted cells, confirming that RME-8 does not require SNX1 for endosomal localization. We also investigated the effect of knocking down RME-8 protein levels on SNX1 subcellular localization. As shown in Fig. 3.10, although SNX1 was still observed on endocytic structures, there was a significant increase in SNX1 tubulation in RME-8 depleted cells. An increase in SNX1 tubulation in response to RME-8 depletion has been reported by others and is thought to represent stalled cargo sorting [83, 133], indicating the importance of RME-8 to retromer-mediated trafficking events.

The above results are intriguing as they reveal that although RME-8 endosomal targeting is mediated through its PI(3)P binding motif, its interaction with SNX1 can also affect its endosomal association. A possible explanation for these observations is that the SNX1/RME-8 interaction could be simply stronger than the PI(3)P/RME-8 association,
which could explain why the PX mutant of SNX1 was able to remove wild type RME-8 from endocytic structures, whereas wild type SNX1 was able to maintain RME-8 W20A at these vesicles (Fig. 3.9B). Additionally, the SNX1 interaction may stabilize RME-8 at the early endosome or perhaps serve an allosteric role during clathrin disassembly. Although further studies are required, our results indicate the intriguing possibility that regulatory mechanisms acting on SNX1 PI(3)P association (post-translational modifications and protein/protein interactions) will also potentially regulate RME-8 and subsequently Hsc-70-mediated clathrin disassembly.
Figure 3.9. SNX1 expression affects RME-8 early endosome targeting.
Continued.
Figure 3.9. SNX1 expression affects RME-8 early endosome targeting. Continued.
Figure 3.9. SNX1 expression affects RME-8 early endosome targeting. A–C, HeLa cells were transfected with the indicated constructs and analyzed by confocal microscopy. Co-localizations between RME-8 and SNX1 variants are shown with arrows depicting regions of co-localization. In the middle panel, HeLa cells were co-transfected with RME-8 W20A and wild type SNX1, and arrows display regions of co-localization. Images were collected using a X40 objective. Expanded boxes represent regions of interest. Scale bars, 15 μm. D, quantitation of co-localization between RME-8 and SNX1. Means ± S.D. of three independent experiments (n = 30 cells) are shown. p values were calculated by Student’s t test and compared with wild type RME 8 and SNX1, **, p < 0.01; ***, p < 0.001.
Figure 3.10. RME-8 expression regulates SNX1 tubulation. A, HeLa cells were transfected with RME-8 siRNA and SNX1 siRNA for 48 h. Knockdown of SNX1 protein expression does not affect RME-8 endosomal localization (middle panel). Knockdown of RME-8 protein expression results in the formation of SNX1-positive tubules (bottom panel). Images were collected using a X60 objective. Expanded boxes represent regions of interest. Scale bars, 15 μm. B, bar graph representation of the percentage of cells altering from punctate to tubules in response to knockdown of RME-8 expression. Means ±S.D. of three independent experiments (n = 30 cells). p values were calculated by Student’s t test and compared with control cells. *, p < 0.1.
In conclusion, we have shown that RME-8 associates with PI(3)P-positive endosomes through an N-terminal PI(3)P binding motif predicted to adopt a PH-like fold and characterized by the presence of conserved basic and aromatic residues. The data support the notion that RME-8 at PI(3)P-rich early endosomes facilitates the process of clathrin removal, and the failure to do so may alter retrograde transport of cargo such as CI-MPR. Our results are consistent with a previous study reporting that RME-8 is located in SNX1-positive, clathrin negative subdomains [105]. This study by McGough and Cullen [105] elegantly showed that clathrin is not required for SNX1-BAR tubulation during retrograde transport as SNX1-BAR tubules start forming at RME-8-positive, SNX1-positive, and clathrin-negative subdomains of the early endosomes. Because RME-8 has not been found to be present in the tubules themselves, it indicates that it likely plays a proximal role mainly at the endosomal level when clathrin is present. Collectively, our data extend this model by which RME-8 utilizes PI(3)P binding in its role in shedding off clathrin before SNX1-BAR tabulation takes place in the retrograde transport pathway.
3.4. References


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CHAPTER 4
CHAPTER 4
DIFFERENTIAL PHOSPHORYLATION OF THE PHOSPHOINOSITIDE 3-
PHOSPHATASE MTMR2 REGULATES ITS ASSOCIATION WITH EARLY
ENDOSOMAL SUBTYPES

4.1. Introduction

Although the majority of protein tyrosine phosphatases (PTPs) use phosphotyrosine-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)P$_2$] [22, 33, 34, 36]. 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) [47, 134, 135]. 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 [38, 40, 136]. 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 [38]. 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)P$_2$. 
Recently, our laboratory used mass spectrometry to identify a prominent phosphorylation site at Ser58 that is close to the PH-GRAM domain of MTMR2 [53]. Functional characterization of this modification demonstrated that phosphorylation dramatically regulates MTMR2 endosomal localization and thus access to its lipid substrates [53]. 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 mechanism of MTMR2 phosphorylation, including identification of the responsible kinase, remains to be elucidated.

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 [137, 138]. 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 [139-142]. 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 [139-142]. 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 [143]. Interestingly, the Rab5/APPL1 endosomes displayed a delay in endosomal maturation and enhanced growth factor endosomal signaling capability [143]. 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 [144]. 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 [143, 145]. 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.
4.2. Materials and Methods

4.2.1. 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 [34, 53]. Generation of S631A and S631E mutant variants were preformed similar to that described for S58A, S58E and C417S [53] using PCR based site-directed mutagenesis. The forward primer for the generation of S631A used was 5’-GAGAGAGCCAGCGCTCCTGCACAGTG-3’ while the reverse primer was 5’-CACTGTGCAGGAGCGCTGGCTCTCTCTC-3’. The forward primer for the generation of S631E used was 5’- CAGAGAGAGCCAGCGAGCCTGCACAGTGTGTC-3’ while the reverse primer was 5’- GACACACTGTGCAGGCTGGCTCTCCTCTCTG-3’. The constructs generated include S58A/S631A, S58A/ S631E, S58E/S631A, S58E/S631E, S58A/C417S/S631A and S58A/C417S/S631E and 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 [53]. 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 pEFGP- 2XYVE construct was a generous gift from Harald Stenmark [146].
4.2.2. 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 carrier 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 subtractive affinity chromatography against non-phosphorylated and phosphorylated peptide epitopes immobilized on iodoacetamide-activated agarose (Pierce Biotechnology) conjugated by 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 [53]. Membranes were blocked with 5% BSA in TBST, probed with chicken antipSer58 IgY at 1:1000 (0.7 mg/ml 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.

4.2.3. In vitro kinase assay

In vitro kinase assays were performed using 5 mg of bacterial recombinant
MTMR2-His$_6$ proteins purified as described previously [147]. In this study, the activated kinases ERK1, ERK2, p38$\alpha$, JNK1 (Signal Chem) were tested with all assays being carried out in reaction buffer (25 mM HEPES, pH 7.2, 12.5 mM $\beta$-glycerol phosphate, 25 mM MgCl$_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT) containing 200 mM ATP and 100 ng of the specified kinase. Reactions were incubated shaking at 30°C for 30 minutes, terminated with 30 ml 2XSDS 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 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-MTMR2 (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.

4.2.4. 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 Signal Silence H control siRNA or SignalSilenceH p44/42 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 S58A 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) (Thr20Tyr204; Cell Signaling Technologies) at 1:50 and mouse anti-FLAG (below) along with fluorescein horse anti-mouse (Vector Laboratories) and Alexa Fluor 568 goat anti-rabbit (Molecular Probes, Invitrogen) secondary antibodies at 1:500. Endogenous studies were performed using rabbit anti-MTMR2 (a generous gift from Gregory S. Taylor; University of Nebraska) and mouse anti-Rab5 (BD BioSciences) along with FITC-conjugated goat antirabbit (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-MTMR2 (1:1000), mouse anti-FLAG (1:3000), rabbit antiphospho-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-IgY-HRP, goat anti-mouse-HRP, goat anti-rabbit (all above; 1:5000) in either 1% or
2.5% skimmed milk. Acquired chemiluminescent images from at least n53 independent experiments were quantified through densitometry using ImageJ (NIH, http://imagej.nih.gov/ij). 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 (ie. 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.

4.2.5. Cell treatment with kinase inhibitors

HEK293 or HeLa cells were treated with a JNK inhibitor SP600125 (LC laboratories) at 40 mM for 1 hour, a p38 inhibitor SB203580 (LC laboratories) at 20 mM for 30 minutes or a MEK inhibitor U1026 to inhibit ERK1/2 activation (LC laboratories) at 50 mM for 1 hour as indicated. For the phosphorylation studies, cells were first serum starved for 30 minutes followed 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 subjected to immunoblotting analysis as described [53]. Immunoblotting was performed to probe for both loading controls and to confirm ERK1/2 inhibition using primary antibodies rabbit anti-MAPK (Sigma) at
1:10,000 and PathScanH Multiplex Western Cocktail I (Cell signaling technology) at 1:200, mouse anti-FLAG (Sigma) at 1:3000 and rabbit anti-actin (Sigma) at 1:3000 as described previously [53]. Phosphorylation at Ser58 of MTMR2 was determined using primary chicken anti-pSer58 MTMR2 as described above and was quantified from three independent experiments by densitometry using ImageJ, and normalized to FLAG (IP) levels.

4.2.6. Immunofluorescence

For immunofluorescence, HeLa cells were fixed, permeabilized, pre-treated, stained and imaged as previously described [53]. Cell treatment with kinase inhibitors was preformed as described as above and as indicated. Primary antibodies included mouse anti-FLAG (Sigma) at 1:500, rabbit anti-Rab5 (Santa Cruz) at 1:80, rabbit anti-Rab7 (Sigma) at 1:100 and rabbit anti-APPL1 (Santa Cruz) at 1:80. Secondary antibodies included Alexa Fluor 568 goat antimouse (Molecular probes) at 1:500 and FITC-conjugated goat anti-rabbit (Vector Laboratories) at 1:500. Images were acquired with a Zeiss Axiovert 200M inverted fluorescent microscope with FITC/TRITC/DAPI filter cubes and LD Plan-APONeofluar 636 0.75 or EC Plan-Neofluar 406 1.3 oil objectives equipped with 103061300 monochrome CCD camera (Axiovision MRM) was also used. Captured pictures from both microscopes were processed with Northern Eclipse software 6.0 (Empix, Mississauga, ON) with contrast/brightness and exposure image corrections performed using program processing application software in addition to Adobe Photoshop 7.0.
4.2.7. Image analysis

To quantify the colocalization of selected proteins, Pearsons’s correlation coefficient (PC) values were calculated using the JACoP plug-in [114] within ImageJ (National Institutes of Health). A sample size of 30 cells for each MTMR2 construct was examined for dual-channel colocalization and values representative of the average from three independent experiments (n=30). A crosscorrelation analysis by shifting the green image in x-direction pixel per pixel relative to the red image and calculating the respective PC was used to analyze the colocalization of MTMR2 point mutants to APPL1, Rab5 or EGFP 2X-FYVE. This analysis was not performed on images in which the localization pattern was largely diffuse to avoid reporting false colocalization. This method does not depend on the relative intensities of the two channels and therefore alleviates the interpretation of true partial or complete colocalization to intermediate PC values (range from -1 to 1: negative values not used for colocalization). All images before automatic colocalization analysis from raw images were corrected post-acquisition using mean background subtraction [148, 149].

4.3. Result

4.3.1. ERK 1/2 phosphorylates MTMR2 at position Ser58 in vitro

Phosphorylation of Ser58 strongly correlates to sequestration of MTMR2 away from its physiological endosomal substrate PI(3)P [53]. The ability to monitor phosphorylation levels of MTMR2 Ser58 would then reflect both the pseudoinactive 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. 4. 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 [53].

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 [53], 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.
4.1C). It was determined that both ERK1 and ERK2 could effectively phosphorylate MTMR2 at Ser58 (Fig. 4.1C, upper panel). In contrast, neither recombinant JNK1 nor p38α were able to detectably phosphorylate MTMR2 Ser58 in vitro.
Figure 4. In vitro phosphorylation of recombinant MTMR2 by ERK1/2. (A) PhosphoSer58 and nonphosphoSer58 peptides were incubated with and without alkaline phosphatase and probed 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 His6 bacterial recombinant MTMR2 was the substrate in the in vitro MAPK kinase assay. Each reaction contained 5 mg of recombinant MTMR2, 100 ng of various kinases (top), 200 mM 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 a-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.
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. 4.1D; for quantitation of the data, see Fig. 4.10). 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.

4.3.2. 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 [28, 31, 53, 150], to an endosomal punctate pattern exhibited by unphosphorylated MTMR2 [53]. Consistent with the in vitro kinase assays, inhibitors to JNK and p38 did not alter the localization pattern of MTMR2 (Fig. 4.2F,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. 4.2D). 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 (Fig. 4.11). These results indicate that inhibition of the ERK1/2 pathway in cellula elevates MTMR2 endosomal targeting presumably by preventing Ser58 phosphorylation.
Figure 4. 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 S58A and treated with the MEK inhibitor U0126 (ERK1/2) at 50 mM for 1 hour, SB203580 (p38) at 20 mM for 30 minutes and SP600125 (JNK) at 40 mM 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 63X objectives. Scale bars: 15 µm.
4.3.3. 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 [151]. 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. 4.3). Under control conditions, MTMR2 displayed high levels of phosphorylation at position Ser58, which decreased following a 30-minute serum starvation period (Fig. 4.3A). EGF stimulation transiently activated ERK1/2, detected by phosphorylation levels of ERK1/2 (Fig. 4.3; Fig. 4.12) 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. 4.3B). Importantly, this observed increase in Ser58 phosphorylation was strongly attenuated when EGF stimulated cells were pre-treated with the MEK inhibitor (Fig. 4.3).

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.
Figure 4. 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.
Recent phosphoproteomic studies have mapped Ser631 as a phosphorylation site on MTMR2 [152]. 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. 4.4; Fig. 4.13). HeLa cells expressing MTMR2 were treated with various MAPK inhibitors and examined for colocalization with the early endosome marker Rab5. Untreated, MTMR2 displayed the characteristic cytoplasmic staining pattern with greater intensity in the perinuclear region and lack of any Rab5 localization (Fig. 4.4A, upper panel). When cells were treated with the MEK inhibitor, MTMR2 displayed a punctate pattern with a subset of vesicles that were positive for both MTMR2 and Rab5 (Fig. 4.4A, middle panel). The extent of Rab5 colocalization was similar to that observed with MTMR2 S58A and Rab5 where the colocalized staining seem to be directly adjacent or on opposite sides of the same vesicle [53]. 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. 4.4A, bottom panel; Table 4.1). 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 (Fig. 4.14).
Figure 4.4. 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 63X objectives. Scale bars: 15 µm.
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 [143]. 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).

4.3.5. 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 [153-155]. 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. 4.3A). We also observed this pattern when we analyzed endogenous MTMR2 from serum starved cells (Fig. 4.15). 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. 4.5A) (quantitation of the data, Fig. 4.16). This observation
was also recapitulated at the endogenous level (Fig. 4.17). Furthermore, a subset of MTMR2 also colocalizes with APPL1 positive endosomes under these low serum conditions (Fig. 4.5B; Fig. 4.16).

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.
Fig. 4. 5. Serum starvation induces subcellular targeting of MTMR2 to Rab5 and APPL1-positive endosomes. (A,B) HeLa cells were transiently transfected with FLAGMTMR2 for 42 hours and analyzed by immunofluorescence microscopy. (A) Untreated cells (top panel) and serum starved cells (lower panel) were probed for FLAGMTMR2 (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 FLAGMTMR2 (red) and APPL1 (green). Serum starvation resulted in an increase in the colocalization of FLAGMTMR2 with APPL1-positive endosomes. Merged images display regions of colocalization and are represented in expanded views. Images were collected using 40Xoil objectives. Arrows indicate regions of colocalization. Scale bars: 15 µm.
4.3.6. 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 [53], thus we initially analyzed if MTMR2 S58A could also colocalize with APPL1 (Fig. 4.6A top panel; Fig. 4.18). 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. 4.6B; Fig. 4.18). 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. 4.6B, enlarged views; Table 4.1). Notably, upon mutation of the catalytic residue (S58A/C417S/S631A) this enlargement did not occur (Fig. 4.19) and these vesicles were comparable in size to those of APPL1 alone (Table 4.1 B). Quantitative analysis of vesicle area revealed that those vesicles which were positive for both MTMR2 and APPL1 were enlarged only when MTMR2 PI(3)P catalytic activity was intact (Table 4.1). 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.
Figure 4. 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 40X oil and 63X objectives. Scale bars: 15 µm.
4.3.7. 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. 4.7, 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 staining pattern and partial colocalization with Rab5 to an extent similar to that observed with the single point mutations at Ser58 (S58A) (Fig. 4.7A; Fig. 4.20). As expected, we did not observe any regions of colocalization with APPL1 (Fig. 4.7B). 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 [139-142]. 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. 4.7B, bottom panel). However, we observed prominent colocalization between MTMR2 S58A/C417S/S631E and Rab5 compared to the catalytically active variant (Fig. 4.7A, 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.
Figure 4.7. Rab5 endosomal targeting of MTMR2 is regulated by dephosphorylation of Ser58 and phosphorylation of Ser631. (A) HeLa cells were transfected FLAG MTMR2 phospho-variants for 42 hours and probed for FLAG MTMR2 (red) and Rab5 (green). Loss of catalytic activity (S58A/C417S/S631E) results in an increase in MTMR2-Rab5 colocalization. (B) Cells were probed for FLAG-MTMR2 phospho-variants (red) and endogenous APPL1 (green). Closed arrows indicate colocalized vesicles, open arrows indicate vesicles void of colocalization. Images were collected using 40X oil and 63X objectives. Scale bars: 15 µm.
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-2XFYVE. 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 [146]. 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. 4.8A, bottom panel) [53]. In contrast, coexpression of the MTMR2 S58A/S631A mutant with EGFP-2XFYVE 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. 4.8A, 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-2XFYVE 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. 4.8B; Fig. 4.21). First we determined that APPL1 endosomes do contain detectable levels of PI(3)P accessible to the EGFP-2XFYVE marker, albeit at lower levels compared to Rab5-positive endosomes (Fig. 4.22). 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. 4.8B lower panel). Intriguingly, S58A/C417S/S631A, which localizes to APPL1 endosomes (Fig. 4.22), displayed a subset of vesicles showing colocalization with the PI(3)P marker (Fig. 4.8B; Fig. 4.21). 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.
Figure 4. 8. Inducible PI(3)P depletion at early endosomes is dependent on MTMR2 catalytic activity. (A,B) HeLa cells were co-transfected with EGFP-2X FYVE (green) and FLAG-MTMR2 (red) phospho-variants for 42 hours and analyzed by immunofluorescence microscopy. (A) MTMR2 dephosphorylation at Ser58 and Ser631 (S58A/S631A) prevents localization to PI(3)P-rich endosomes (top panel). Expression of MTMR2 S58A/S631E induces PI(3)P depletion at early endosomes (lower panel). (B) Loss of catalytic activity (S58A/C417S/S631A) of dephosphorylated MTMR2 results in partial colocalization with PI(3)P endosomes (top panel). Phosphorylation at Ser631 (S58A/C417S/S631E) localizes to PI(3)P-rich early endosomes (lower panel). Closed arrows indicate colocalized vesicles, open arrow indicate vesicles void of colocalization. Images were collected using 40X oil and 63X objectives. Scale bars: 15 µm.
4.3.8. 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 [53]. 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 [143]. 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. 4.9A). 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. 4.9B). 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. 4.9B). 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.
Figure 4.9. Dephosphorylation of Ser58 and Ser631 elicits sustained ERK1/2 activation. (A) HeLa cells were transfected with FLAG-MTMR2, S58A or S58A/S631A, serum starved for 30 minutes then treated with 5 ng/ml EGF for the indicated times. Lysates were immunoblotted (IB) for pERK1/2. Total ERK1/2 levels served as loading control. Lysates were immunostained with anti-FLAG to confirm equal protein expression. (B) Relative ERK1/2 phosphorylation levels were quantified by densitometry using ImageJ and normalized to total ERK1/2 levels. Representative western blots are shown in A. Means ± s.d. of the results of three independent experiments are shown. **P< 0.01, ***P< 0.001 for the comparisons indicated.
Figure 4.10. 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 α-actin loading control. Data represents mean and SEM values from at least n = 3 independent experiments, with ***P<0.001, **P<0.01 or *P<0.05.
Figure 4.11. 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 42 h 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.
Figure 4.12. 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. ** $P \leq 0.001$
Figure 4.13. 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
Figure 4.14. 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.
Figure 4.15. Endogenous MTMR2 Ser\textsuperscript{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.
Figure 4.16. 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.
Figure 4.17. 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.
Figure 4.18. 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 ± SD. *** P ≤ 0.001 compared S58A/S631A with S58A for APPL1 and S58A/S631A with APPL1 and Rab5.
Figure 4.19. 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.
Figure 4.20. 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 ± SD. * P < 0.05 compared S58A/S631E with Rab5 and APPL1; ** P ≤ 0.01 compared S58A/C417S/S631E with Rab5 and APPL1.
Figure 4.21. 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; ** \( p \leq 0.01 \) compared S58A/C417S/S631E with S58A/C417S/S631A.
Figure 4.22. 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 4.1 Catalytic activity of MTMR2 results in enlargement of APPL1 vesicles when dephosphorylated at Ser58 and Ser631. 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 Ser58 and Ser631 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 (μm²) 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.

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<th>Average APPL1 area (μm²)</th>
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4.4. 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 [156, 157]. 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 [143, 144, 158]. 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 [143]. 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 [108]. 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 these 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 [159-161]. 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 EEA1/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 [143]. Moreover, Larijani 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 [162]. Both of these studies however, clearly demonstrated that depletion of PI(3)P on early endosome compartments acutely 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. 4.9), 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. 4.9). A long standing dogma is that the signal duration can dynamically influence cellular fate [163]. 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 [164, 165]. As is seen in PC12 cells, in most cell types, sustained ERK activation induces differentiation. However, in Schwann cells, sustained ERK activation triggers dedifferentiation in response to a variety of environmental cues including neuronal injury [166, 167]. Schwann cell de-differentiation into a progenitor cell-like stage is critical for axonal re-growth and is highlighted by extensive demyelination [166]. 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.

4.5. References


CHAPTER 5
CHAPTER 5
GENERAL CONCLUSIONS, OUTLOOK AND FUTURE WORK

5.1. Identification and characterization of novel PI(3)P binding proteins regulated by MTMR2

CMT4B1 is a severe demyelinating neuropathy caused by a loss-of-function mutation on the mtmr2 gene. Unfortunately, the mechanistic details by which the disease is caused are largely unknown. This task has proven difficult partly due to the poor understanding of PIP binding domains that specifically interact with the PIP isoforms targeted by MTMR2. In this study, we utilized a proteomic approach using immobilized PIP isoforms to identify novel PIP binding proteins. Herein, we identify RME-8 as a novel PI(3)P binding protein regulated by MTMR2. In particular, our data revealed that MTMR2 mediated PI(3)P depletion induced attenuation of RME-8 endosomal localization, suggesting a model in which the localization of RME-8 to endosomal compartments is spatially mediated by PI(3)P binding and temporally regulated by MTMR2 activity.

RME-8 is a poorly characterized protein whose function is thought to mediate the translocation of Hsc70 to early endosomes for clathrin removal during retrograde transport. Since, RME-8 association with early endosomes occurs in a PI(3)P-dependent fashion, we further identified the amino acids necessary for PI(3)P binding. The ability of RME-8 to associate with PI(3)P and early endosomes is largely abolished when residues Lys17, Trp20, Tyr24, or Arg26 are mutated, resulting in diffused cytoplasmic localization of RME-8 while maintaining the ability to interact with Hsc70. These amino acids reside within a region predicted to adopt a pleckstrin homology-like fold located in the N
terminus of RME-8. More importantly, we also discovered that RME-8 PI(3)P binding regulates early endosomal clathrin dynamics in addition to altering the steady state localization of the CI-MPR. These results further emphasize the significance of PI(3)P in the RME-8-mediated organizational regulation of various endosomal trafficking events, such as the retrograde transport.

More investigation needs to be done to further study the effect of myotubularins in endocytic vesicular traffic, and in particular endosomal-to-TGN retrograde transport. Studies in C. Elegans describe that MTM-6 and MTM-9 lipid phosphatases act as regulators of MIG-14/Wls trafficking, whose receptors follow the endosome-to-TGN retrograde transport pathway [168]. Under normal conditions, prior to PI(3)P dephosphorylation, MTM-6 and MTM-9 form a heterodimer. The direct evidence that myotubularins regulate retrograde transport was observed in genetic mutants of mtm-6 or mtm-9 in C. Elegans, which caused severe defects in retrograde-dependent Wnt signaling [168]. Our results demonstrate that MTMR2 directly regulates the retromer component of RME-8, which in turn also controls the traffic of acid hydrolase receptors via PI(3)P binding (Fig 5.1). Thus it would be very insightful to further investigate whether MTMR2 plays a role in retrograde transport regulation of acid hydrolase receptors, as this would likely point to a role in regulation of lysosomal biogenesis. Discovering a link between CMT4B1 and lysosomal biogenesis may allow researchers in the field to determine if aberrant lysosomal activation contributes to myelin degradation in the disorder.
Figure 5.1. Sorting of the acid hydrolases via mannose 6-phosphate receptors during normal (left panel) and aberrant (right panel) endosomal retrograde transport from the early endosomes to the Trans-Golgi-Network. Adapted from Bonifacino, J. S. & Hurley, J. H. (2008) Retromer, *Curr Opin Cell Biol.* 20, 427-36.
Moreover, there are numerous studies suggesting that clathrin and retromer act in a coordinated successive manner during retrograde transport sorting pathway [84]. Of note, the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) has been shown to associate with both clathrin and the retromer subunit SNX-1 [169, 170]. Once Hrs binds PI(3)P via its FYVE domain, it helps recruit clathrin to the early endosomes to form Hrs/clathrin domains, whereby ubiquitinated cargo concentrates prior to being transferred to the endosome-associated complex required for transport (ESCRT) machinery for degradation soon after it fuses with lysosomes [169, 171]. Since both Hrs and RME-8 associate with SNX-1 it is possible that a competition mechanism exists for access to SNX-1. It is presumed that SNX-1/Hrs and SNX-1/RME-8/Hsc70 complexes act in an antagonist manner, with the SNX-1/RME-8/Hsc70 complex disassembling clathrin and promoting cargo sorting via retrograde transport, whereas the SNX-1/Hrs complex accumulating cargo and promoting degradation via lysosomal degradation.

Furthermore, the competition between Hrs and RME-8 for binding to SNX-1 may allow switching from clathrin recruitment (SNX-1/Hrs) to clathrin uncoating (SNX-1/RME-8/Hsc70), determining the fate of cargo, either to degrade or recycle through retrograde transport [83]. Hrs and SNX-1 both bind PI(3)P. We are therefore interested in investigating whether endosomal localization of SNX-1 or Hrs is regulated by MTMR2. Our preliminary results suggest that while Hrs endosomal localization is not affected by MTMR2 phosphatase activity, SNX-1 endosomal punctate pattern of localization is largely abolished in MTMR S58A expressing HeLa cells (data not shown). This data suggests that MTMR2 targets a pool of PI(3)P rich endosomes that are positive for SNX-1 and RME-8, while not affecting the PI(3)P rich endosomes that are Hrs positive. Our
working model has MTMR2 targeting complexes involved in clathrin uncoating (SNX-1/RME-8/Hsc70) rather than clathrin accumulation (SNX-1/Hrs). We thus hypothesize a role for MTMR2 in cargo recycling via retrograde transport rather than degradation. While more research is needed to validate these results, we conclude that MTMR2 reversible phosphorylation may serve as a molecular switch to rescue and direct cargo at a specific subcellular localization by controlling a particular endocytic vesicular traffic pathway.

5.2. Identification of the mechanism by which MTMR2 reversible phosphorylation regulates endosomal trafficking events

MTMR2 is a 3-phosphoinositide lipid phosphatase with specificity towards the D-3 position of PI(3)P and PI(3,5)P_2 lipids enriched on endosomal structures. Recently, we have shown that phosphorylation of MTMR2 on Ser58 is responsible for its cytoplasmic sequestration, whereas dephosphorylation targets it to Rab5-positive endosomes. This results in PI(3)P depletion and an increase in endosomal signaling, in particular ERK1/2 activation. Moreover, we provide evidence that ERK1/2 is the kinase responsible for phosphorylating MTMR2 at position Ser58, indicating 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 endosomal shuttling was recapitulated when a double phosphorylation-deficient mutant (MTMR2 S58A/S631A) was characterized. 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 endosomal maturation and signaling are dynamically regulated through MAPK-mediated differential phosphorylation events.

Although mtmr2 gene mutations cause the severe demyelinating neuropathy CMT4B1, the mechanism by which these mutations produce demyelinating phenotypes in the peripheral nervous system is still a matter of intense investigation. A major mechanism active in demyelinating neuropathies is dedifferentiation of myelinating Schwann cells [172]. During this process, Schwann cells dedifferentiate into an immature state in response to losing contact with the axon. During this stage, Schwann cells turn off the myelin program, re-enter the cell cycle and proliferate [172]. A similar phenomenon occurs following axonal damage, Schwann cells dedifferentiate into immature cells and start proliferating [172]. Then, they begin to re-differentiate and remyelinate the newly regenerated axons to finalize the regeneration process. It is presumed that it is the instability of the differentiating and the dedifferentiating signals that lead to demyelinating neuropathies [172]. One of the key signaling molecules that induce Schwann cell dedifferentiation and demyelination is ERK1/2 [167, 173-175].

Moreover, studies in mice deficient for the myelin component P0, which is a model of a CMT subtype, have revealed an increase in the recruitment of demyelinating macrophages at the nerves. Moreover, it was observed that the accumulation of demyelinating macrophages was mediated by ERK1/2 dependent MCP-1 chemokine upregulation, suggesting that ERK signaling pathway plays a significant role not solely in
triggering the demyelination process, but maintaining it as well. These results are very intriguing and might explain the mechanistic details behind the demyelinating neuromuscular physiopathology [176, 177].

In conclusion, there is accumulating evidence indicating a role of ERK1/2 as a driver of the dedifferentiation and demyelination process in Schwann cells. Moreover, our results suggest that ERK1/2 down regulates MTMR2’s function in the cell. Therefore, we believe that it would be imperative to determine the MTMR2 phosphorylation state at different stages of Schwann cell dedifferentiation to gain insight about MTMR2’s function during this pivotal process. Determining if aberrant ERK signaling or MTMR2 function hurdles the process of redifferentiation and/or remyelination might provide crucial insight of our understanding not only of the regenerative process, but also the signaling pathways that promote inherited peripheral demyelinating neuropathies.

5.3. References


REFERENCES


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and epidermal growth factor in PC12 cells. Identity with the mitogen-activated MAP kinase of fibroblastic cells, European journal of biochemistry / FEBS. 193, 661-9.


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