Doctoral Thesis

Structural and functional characterization of interactions of myotubularin-related proteins

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Structural and Functional Characterization of Interactions of Myotubularin-Related Proteins

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for degree of

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presented by

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The Myotubularin-Related Proteins (MTMRs) represent one of the largest and most conserved subfamily of protein tyrosine/dual specificity phosphatase-like phosphatases (PTPs). Out of the 14 family members identified in the human genome, six family members contain substitutions in the phosphatase domain rendering them catalytically inactive. Mutations in the catalytically active members Myotubularin (MTM1) and Myotubularin-Related-Protein-2 (MTMR2) are associated with the physiological distinct human diseases X-linked myotubular myopathy (XLMTM), a recessive congenital muscle disorder, and Charcot-Marie-Tooth disease Type 4B1 (CMT4B1), a hereditary demyelinating neuropathy, respectively. Mutations in the inactive member MTMR13/Set-Binding-Factor-2 (SBF2) cause CMT Type 4B2. It is still unclear how mutations in MTM1 and MTMR2 or SBF2 can lead to such distinct human diseases.

In this work I followed two different approaches to elucidate the specificity of MTMRs: (1) the identification of novel PDZ-domain containing proteins that interact with MTMRs and (2) the structural and functional characterization of complexes between active and inactive MTMRs. I identified Pdzk2 as specific interaction partner of Mtm1. Pdzk2 is a PDZ-domain containing scaffold protein composed of four PDZ domains which has been shown to interact through its PDZ domains with ion channels, receptors, transporters and signaling molecules. Thereby it regulates the transport activity and subcellular localization of transporters and channels especially in kidney and intestine. The interaction of Pdzk2 with Mtm1 represents a possible mechanism to modulate cell surface expression of receptor/Pdzk2/Mtm1 complexes. Mtmr12/3-Pap is an inactive phosphatase and it interacts like Sbf2 with Mtmr2. The characterization of Mtmr2/3-Pap complex revealed that it exists as heterodimer, which is distinct to the tetrameric Mtmr2/Sbf2 complex. Furthermore, we could show that under hypo-osmotic conditions 3-Pap, in contrast to Sbf2, does not influence the subcellular localization of Mtmr2. Based on these structural and functional findings we suggest that the dead MTMR phosphatases can be classified into
two groups. Thereby, the differential combinations of the different classes of inactive MTMRs with one specific active MTMR might have opposite effects on membrane identity and receptor sorting. In addition, we observed that Mtmr2 and 3-Pap localized along the recycling axis (Rab5-Rab4-Rab11) and promoted sorting of the EGF receptor (EGFR) to Rab11 positive recycling endosomes. We propose that Mtmr2 phosphatase activity influences the local composition of phosphoinositides at these membranes. This has the consequence that EGFR is redirected into the recycling pathway and is subsequently not degraded.
Zusammenfassung

3 Zusammenfassung


In dieser Arbeit verfolgte ich zwei verschiedene Ansätze um eine Spezifität unter den MTMRs aufzudecken: (1) die Identifizierung von neuen PDZ-Domänen enthaltenden Proteinen die mit MTMRs interagieren und (2) die strukturelle und funktionale Charakterisierung von Komplexen zwischen aktiven und inaktiven MTMRs. Ich identifizierte Pdzk2 als spezifischen Interaktionspartner von Mtm1. Pdzk2 is ein PDZ-Domänen enthaltendes Verbindungsprotein, das aus vier PDZ-Domänen besteht und durch seine PDZ Domänen mit Ionenkanälen, Rezeptoren, Transportern und Signalmolekülen interagiert. Dadurch reguliert es die Transportaktivität und die subzelluläre Lokalisation der Transporter und Kanäle speziell in der Niere und im Darm. Die Interaktion zwischen Pdzk2 und Mtm1 zeigt einen möglichen Mechanismus zur Regulierung der Zelloberflächenexpression von Rezeptor/Pdzk2/Mtm1 Protein-Komplexen auf. Mtmr12/3-Pap ist wie Sbf2 eine inaktive Phosphatase, die mit Mtmr2 interagiert. Die Charakterisierung des Mtmr2/3-Pap Komplexes zeigte, dass er als Heterodimer existiert, der sich vom tetrameren Mtmr2/Sbf2 Komplex
Zusammenfassung

unscheidet. Darüber hinaus konnten wir zeigen, dass 3-Pap unter hypo-
osmotischen Bedingungen, im Gegensatz zu Sbf2, die subzelluläre Lokalisation
von Mtmr2 nicht beeinflussen konnte. Basierend auf diesen Erkenntnissen,
schlagen wir eine Einteilung der toten MTMR Phosphatasen in zwei Gruppen
vor. Unterschiedliche Kombinationen von inaktiven MTMRs mit einem
spezifischen aktiven MTMR könnten gegensätzliche Auswirkungen auf die
Membran-Identität und die Rezeptor-Entsorgung haben. Ausserdem
beobachteten wir, dass Mtmr2 und 3-Pap entlang der Rezyklierungsachse
(Rab5-Rab4-Rab11) lokalisierten, was die Entsorgung vom EGF Rezeptor
(EGFR) in die Rab11 positiven Rezyklierungs-Endosomen förderte. Wir schlagen
vor, dass die Phosphatase-Aktivität von Mtmr2 die lokale Zusammensetzung von
Phosphoinositiden an diesen Membranen beeinflusst. Dies hat zur Folge, dass
EGFR in den Rezyklierungsweg umgelenkt wird und deshalb nicht abgebaut
wird.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3-PAP</td>
<td>3-Phosphatase Adapter Protein</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
</tr>
<tr>
<td>EE</td>
<td>Early Endosome</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complexes Required for Transport</td>
</tr>
<tr>
<td>GST</td>
<td>Gluthatione-S-Transferase</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney 293 cells</td>
</tr>
<tr>
<td>HMSN</td>
<td>Hereditary Motor and Sensory Neuropathies</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot</td>
</tr>
<tr>
<td>ILV</td>
<td>Intralumenal Vesicle</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LE</td>
<td>Late Endosome</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MTM1</td>
<td>Myotubularin 1</td>
</tr>
<tr>
<td>MTMR2</td>
<td>Myotubularin-Related-Protein-2</td>
</tr>
<tr>
<td>MTMRs</td>
<td>Myotubularin-Related-Proteins</td>
</tr>
<tr>
<td>MVB</td>
<td>Multi-Vesicular Body</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NaPi-IIa</td>
<td>Na⁺- dependent inorganic Phosphate cotransporter type IIa</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NHERF</td>
<td>Na⁺/H⁺ Exchanger Regulatory Factor</td>
</tr>
<tr>
<td>OK</td>
<td>Opossum Kidney cells</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95, DLG1, ZO-1</td>
</tr>
<tr>
<td>PDZ-BD</td>
<td>PDZ-Binding Domain</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology domain</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PI3K</td>
<td>PI-3 Kinase</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>PTP</td>
<td>Phosphatase-like Phosphatases</td>
</tr>
<tr>
<td>RE</td>
<td>Recycling Endosome</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SAP97</td>
<td>Synapse-Associated Protein 97</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-Angle X-ray Scattering</td>
</tr>
<tr>
<td>SBF2</td>
<td>Set-Binding-Factor-2</td>
</tr>
<tr>
<td>SNXs</td>
<td>Sorting Nexins</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi Network</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular Endothelial Growth Factors receptor-2</td>
</tr>
<tr>
<td>VPS</td>
<td>Vacuolar Protein Sorting</td>
</tr>
<tr>
<td>XLMTM</td>
<td>X-linked Myotubular Myopathy</td>
</tr>
</tbody>
</table>
5 Introduction

5.1 The Myotubularin family
The Myotubularin-Related Proteins (MTMRs) represent one of the largest and most conserved family of protein tyrosine/dual specificity phosphatase-like phosphatases (PTPs). They are conserved from yeast to human (Wishart et al, 2002; Laporte et al, 2001). A hallmark of PTP domains is the Cys-X$_5$-Arg motif, comprising the catalytically essential residues. Interestingly, out of the 14 family members identified in the human genome, six family members contain substitutions in the cysteine and arginine residues, rendering them catalytically inactive (Laporte et al, 2003). Inactive PTPs were found in vertebrates and invertebrates but not in yeast (Robinson et al, 2006). Several studies have shown that MTMRs do not only homodimerize with themselves, but also heterodimerize with their active or inactive homologues (Schaletzky et al, 2003; Berger et al, 2003; Dang et al, 2004; Kim et al, 2003; Mochizuki et al, 2003; Nandurkar et al, 2003; Lorenzo et al, 2005). Although inactive MTMRs by themselves are “dead phosphatases”, they can influence the enzymatic activity and the subcellular localization of their binding partner by associating with an active family member (Kim et al, 2003; Mochizuki et al, 2003; Nandurkar et al, 2003; Berger et al, 2006a) (Nandurkar et al, 2003). It has also been speculated that these inactive phosphatases act as substrate traps protecting the substrate. Substrate-trapping mutants for MTM1 and MTMR2 have been described, and the putative catalytic acids were identified as Asp278 and Asp320, respectively (Blondeau et al, 2000). Structural information of Mtmr2 however, revealed that Asp320 is completely buried in the hydrophobic core of the protein and that its mutation to alanine would disrupt the protein core, which speaks against the substrate-trapping hypothesis (Begley et al, 2003). In addition to the phosphatase domain, all Myotubularin-Related Proteins contain a phosphoinositide-binding PH-GRAM (pleckstrin homology glucosyltransferases, Rab-like GTPase activators and myotubuarins) domain and all MTMRs also contain a C-terminal coiled-coil motif. Furthermore, individual family members contain a DENN (differentially expressed
in neoplastic versus normal cells), a PH, a FYVE zinc-finger or a C-terminal PDZ-binding motif. The phosphoinositides phosphoinositol-3-phosphate (PI-3-P) and PI-3,5-P$_2$ are the major enzymatic substrates of MTMRs (Blondeau et al, 2000; Berger et al, 2002; Schaletzky et al, 2003). Both, PI-3-P and PI-3,5-P$_2$ are mainly localized on early and late endosomes and are key players in mediating vesicular trafficking and membrane transport (Corvera et al, 1999; Odorizzi et al, 2000). Three members of the myotubularin family have been associated with human diseases (see also 5.1.1). 

Myotubularin (MTM1) is the founding member of the family and was originally identified as the disease-causing gene in X-linked myotubular myopathy (XLMTM) (Laporte, 1996). Myotubularin-Related-Protein-2 (MTMR2) and MTMR13/Set-Binding-Factor-2 (SBF2) are the disease causing genes of Charcot-Marie-Tooth disease Type (CMT) 4B1 and 4B2, respectively (Bolino, 2000; Azzedine et al, 2003; Senderek et al, 2003).

5.1.1 Myotubularins in human diseases

5.1.1.1 MTM1 and X-linked myotubular myopathy

X-linked myotubular myopathy (also known as centronuclear myopathy, CNM) is a recessive congenital muscle disorder, where skeletal muscle development and/or regeneration is impaired, leading to severe hypotonia and generalized muscle weakness in newborn patients (reviewed in (Romero, 2010)). The nuclei are abnormally localized to the center of the small rounded myofibers, making muscle biopsies initially a valuable tool for detection of XLMTM. Pathological hallmark of mild forms of XLMTM with late onset are necklace-fibers. Additionally to the recessive form caused by mutations in the MTM1 gene (Laporte, 1996), CNMs can also be caused by mutations in dynamin2 (DNM2) gene (Bitoun et al, 2005) and amphyphisin2 (BIN1) gene (Nicot et al, 2007). Mutations in DNM2 cause the classical autosomal CNM with mild, moderate or severe phenotype, whereas mutations in BIN1 cause an autosomal recessive form with moderate to severe phenotype. Interestingly, DNM2 mutations result also in CMT neuropathy with intermediate or normal nerve conduction velocities (Bitoun et al, 2005;Claeys et al, 2009; Fabrizi et al, 2007). Furthermore, MTM1, BIN1 and DNM2 are
all essential for clathrin-mediated endocytosis (Dowling et al, 2008). Mtm1 knockout mice recapitulate the skeletal muscle phenotype of XLMTM patients: generalized and progressive myopathy, with amyotrophy and accumulation of central nuclei in skeletal muscle fibers (Buj-Bello et al, 2002b). Since muscle differentiation occurred normally in Mtm1-deficient mice, a failure to maintain muscle cell structure was suggested. It was also shown that Myotubularin overexpression in muscle of mice induced the accumulation of packed membrane saccules and presence of vacuoles that contained markers of sarcolemma and T-tubules, suggesting Mtm1 to be involved in plasma membrane homeostasis of myofibers (Buj-Bello et al, 2008). Also in mammalian cells MTM1 overexpression lead to altered cell shape including plasma membrane projections and ruffles (Laporte et al, 2002a; Kim et al, 2002).

5.1.1.2 MTMR2 and SBF2 in CMT4B1 and CMT4B2
Charcot-Marie-Tooth (CMT) disease comprises hereditary motor and sensory neuropathies (HMSN) affecting myelinated axons of the peripheral nervous system (Niemann et al, 2006; Berger et al, 2006b). CMT is characterized by progressive distally accentuated muscle weakness and atrophy and can be grouped into axonal and demyelinating forms. Axonal forms, including CMT2, are characterized by a loss of myelinated axons resulting in a reduction of the compound muscle action potential (CMAP) amplitude (Zuchner et al, 2006). CMT4 subtypes B1 and B2 belong together with CMT1 and CMT3 to the demyelinating forms, characterized and diagnosed by reduced nerve conduction velocity caused by demyelination of axons. CMT4B1 and CMT4B2 are severe demyelinating peripheral neuropathies caused by recessive mutations in MTMR2 (Bolino, 2000) and SBF2 (Azzedine et al, 2003). Both types share the CMT disease typical features including a demyelinating neuropathy associated with slowed nerve conduction and focally folded myelin sheaths (Previtali et al, 2007; Quattrone et al, 1996). Generally the disease onset is in early infancy and severeness proceeds with aging, why wheelchair is needed from late childhood on. In some families early onset of glaucomas was associated with CMT4B2 (Azzedine et al, 2003; Senderek et al, 2003; Hirano et al, 2004). Similar to
CMT4B1 patients, Mtmr2-deficient mice showed complex myelin infoldings and outfoldings. However, signs of demyelination like thinly myelinated large caliber axons or onion bulb formation were not detected (Bonneick et al, 2005). Mtmr2 mutant mice were also reported to show impaired spermatogenesis (Bolino et al, 2004). Sbf2-deficient mice developed peripheral neuropathy similar to CMT4B2 patients (Tersar et al, 2007; Robinson et al, 2008), but showed minor signs of reduced nerve conduction velocity and no obvious evidence for primary demyelination and remyelination (Tersar et al, 2007). No significant differences were observed between the double deficient Mtmr2/Sbf2 mice and the Sbf2-deficient animals (Tersar et al, 2007).

5.1.2 Structural aspects of MTMR domains and their function
All MTMRs have a main core in common, which is composed of a GRAM-PH domain, a phosphatase and a coiled-coil motif (Figure 5-1). MTM1, MTMR2, MTMR3, and MTMR6 were shown to dephosphorylate both PI-3,5-P$_2$ and PI-3-P at position D3 suggesting that this substrate specificity is common to all active family members (Walker et al, 2001; Berger et al, 2002; Schaletzky et al, 2003). The phosphatase domain is N-terminally flanked by a RID (Rac-induced recruitment domain) and C-terminally by a SID (SET-interacting domain) motif (Cui et al, 1998; Begley et al, 2003; Robinson et al, 2006). The RID domain was proposed to be necessary for the recruitment of myotubularins (MTMR2, MTMR3 and MTMR9) to the plasma membrane (Laporte et al, 2002b; Laporte et al, 2002a) and to mediate protein interaction with neurofilament light chain (NF-L) in the case of MTMR2 (Previtali et al, 2003). SBF1 interacts through its SID domain with the SET-domain containing protein HRX, thereby modulating growth control (Cui et al, 1998). The SID domain of MTM1 was shown to be important for interaction with MTMR12/3-PAP, pointing towards a role in protein-protein interaction (Nandurkar et al, 2003). The PH-GRAM domain was reported to be essential in mediating membrane association by binding to phosphoinositides, mainly to PI-3-P and PI-3,5-P$_2$, which are also the major substrates of MTMRs (Schaletzky et al, 2003; Berger et al, 2003; Tsujita et al, 2004; Lorenzo et al, 2005). Several studies have shown that the coiled-coil motifs of MTMRs enable
homodimerization and heterodimerization with their active or inactive homologues (Schaletzky et al, 2003; Berger et al, 2003; Dang et al, 2004; Kim et al, 2003; Mochizuki et al, 2003; Nandurkar et al, 2003; Lorenzo et al, 2005). The coiled-coil motif of MTMR2 mediates homodimerization, and promotes thereby indirectly membrane association and localization (Berger et al, 2003). Mutations in the GRAM domain of MTM1 lead to XLMTM, underscoring the significance of the GRAM domain for cellular function (de Gouyon et al, 1997; Laporte et al, 1998). Localization studies of MTMR4 with endosomal markers EEA1 and Hrs, both FYVE (Fab1, YGL023, Vps27 and EEA1) domain containing proteins that localize to endosomes through interactions with PI-3-P (Gaullier et al, 1998; Urbe et al, 2000) showed clear colocalization (Lorenzo et al, 2006), suggesting a function in endosomal membrane localization. MTMR13/SBF2 and MTMR5/SBF1 contain an N-terminal DENN (differentially expressed in normal and neoplastic cells) domain. Several DENN domain proteins have been shown to interact with Rab GTPases (Levivier et al, 2001), which are important effectors of membrane trafficking. The DENN domains of SBF1 and SBF2 were reported to have GEF activity toward Rab28 (Yoshimura et al, 2010), which in fact interacts with the retromer and the ESCRT complex in the endosomal pathway (Lumb et al, 2011). This provides potential function of the DENN domains of MTMRs for vesicular trafficking and signaling processes. Loss of the dDENN domain (one of three subdomains of the DENN domain) due to a mutation in MTMR13/SBF2 resulted in CMT4B2 disease (Senderek et al, 2003). Additionally, MTMR13/SBF2 contains a classical PI-3,4,5-P\(_3\) binding PH-domain (Berger et al, 2006a). In the case of MTMR5/SBF1 it was shown to have regulatory function on cell growth (Firestein et al, 2001). PDZ domain binding motif (PDZ-BD) and coiled-coil motif mediate both protein-protein interactions and are discussed in more detail in chapter 5.1.3. The PDZ-BD is usually a short stretch of 3-7 amino acids at the C-terminus recognizing PDZ-domain containing proteins.
Figure 5-1: The Myotubularin family

Depicted is the phylogenetic tree of the Myotubularin-Related-proteins (MTMRs) and their structural composition. All share a main core consistent of a GRAM-PH (blue), a phosphatase (yellow), and a coiled-coil (orange) motif. MTM1, MTMR1, MTMR2, MTMR5 and MTMR13 harbour a carboxy-terminal PDZ binding motif (grey). In addition, MTMR3 and MTMR4 contain a FYVE domain (red). MTMR5 and MTMR13 contain a supplemental amino-terminal DENN domain (green), an uncharacterized region with no homology to other proteins (purple) and a C-terminal PH domain (light yellow). Inactive phosphatase domains are crossed out. Figure adapted from (Clague et al, 2005).

A crystal structure of MTMRs has only been solved of human MTMR2, whereas MTMR2 structures in complex with PI-3-P and PI-3,5-P₂ were proposed based on deuterium-exchange mass spectrometry (DXMS) results (Begley et al, 2003; Begley et al, 2006). Although the crystal structure has provided valuable new insight including the structural information of the PH-GRAM and the phosphatase domain as well as the phosphoinositide binding active site, the last C-terminal 57 amino acids, including the coiled-coil motif and the PDZ binding domain are missing (see Figure 7-18). To fully understand, how homodimeric or heterodimeric MTMR complexes are build and how complex formation can influence substrate affinity and membrane association, a crystal structure of the full length protein in complex would be needed.
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5.1.3 MTMR-protein interactions

5.1.3.1 MTMR-PDZ protein interactions

The name PDZ derives from the founding members: the postsynaptic density-95 (PSD-95/SAP90), *Drosophila* tumor suppressor protein discs-large-1 (DLG1, also known as SAP97) and the epithelial tight junction protein zona occludens-1 (ZO-1). PDZ-domain containing proteins are scaffolding proteins that mediate protein-protein interactions. They play an important role in the regulation, clustering and formation of multimeric protein complexes including membrane transporters and ion channels at surfaces and specific subcellular sites. In scaffolding proteins they can occur in one or multiple copies. In general, the PDZ domain containing proteins are localized to specific subcellular sites, such as synapses; intercellular contact sites; or the apical, basal, or lateral cell surface (reviewed in (Fanning et al, 1999)). PDZ domains are recognized by short C-terminal ~5-7 residue long peptide motifs or rarely by internal sequences, structurally mimicking a terminus. To date, four types of PDZ recognition motifs have been classified: class I (S/T-x-Φ), class II (Φ-x-Φ), class III (Ψ-x-Φ) and class IV (D-x-V), where x is any amino acid, Φ is a hydrophobic residue (V, I, L, A, G, W, C, M, F) and Ψ is a basic, hydrophilic residue (H, R, K) (Songyang et al, 1997). The C-terminal PDZ-BD motifs of MTM1 (-VQTHF), MTMR1 (-VHTSV), MTMR2 (-VQTVV), MTMR5/SBF1 (-CLSDA) and MTMR13/SBF2 (-CISDA) belong therefore to the class I motifs. Yet, only few PDZ-interactions with myotubularins have been reported: MTMR1 and TIP-15 (Fabre et al, 2000), Mtmr2 and Sap97/Dlg1 (Bolino et al, 2004; Bolis et al, 2005), Mtmr2 and Psd-95/Sap90 (Lee et al, 2010). An interaction of Mtmr2 with Sap97 has been reported to be essential for membrane homeostasis and cell junction integrity in Schwann cells (Bolis et al, 2009) and interaction with PSD95/Sap90 to be important for maintenance of excitatory synapses by modulating endosomal trafficking (Lee et al, 2010). Furthermore, SAP97 was shown to interact with minus-end directed actin motor myosin IV, suggesting a scenario in which myosin VI is involved in the trafficking of vesicle-associated SAP97 and AMPA receptor (Wu et al, 2002). Both, MTMRs and PDZ-domain containing proteins are
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essential players in membrane homeostasis, endosomal trafficking and sorting of membrane proteins. But little is known about their action as complex on these actions. The identification of novel PDZ-interacting proteins with MTMRs are therefore of high interest.

5.1.4 MTMR-MTMR interactions
Up to now, several interactions between MTMR family members have been reported through identification of coimmunoprecipitation and/or yeast-two hybrid-screens (see Table 1 in (Lorenzo et al, 2006); simplified and updated here in Table 5-1). Self-associations were described for the following proteins: MTM1 (Caldwell et al, 1991; Schaletzky et al, 2003; Lorenzo et al, 2006); MTMR2 (Berger et al, 2003); MTMR3 and MTMR4 (Lorenzo et al, 2006); MTMR6 (Zou et al, 2009); MTMR9 (Zou et al, 2009), MTMR12 (Lorenzo et al, 2006) and MTMR13 (Berger et al, 2006a). The only heteromeric interaction between two active MTMRs has been reported between MTMR3 and MTMR4, which was coiled-coil dependent (Lorenzo et al, 2006). Several heteromeric interactions between active and inactive myotubularins have been characterized as follows: the inactive MTMR12/3-PAP with the active phosphatase MTM1 or MTMR2 (Nandurkar et al, 2003); the active MTMR2 additionally interacts with the inactive proteins MTMR5/SBF1 (Kim et al, 2003) and a MTMR2 homodimer forms a coiled-coil dependent complex with a MTMR13/SBF2 homodimer (Berger et al, 2006a); inactive MTMR9/STYX interacts with the active proteins MTMR6 (Zou et al, 2009), MTMR7 (Mochizuki et al, 2003) or MTMR8 (Lorenzo et al, 2006); inactive MTMR10 with the active members MTM1 or MTMR2 (Lorenzo et al, 2006). Interestingly, heteromerization between active and inactive MTMRs resulted in several changes: increased catalytic activity of the active member (Kim et al, 2003; Nandurkar et al, 2003; Mochizuki et al, 2003; Berger et al, 2006a; Zou et al, 2009), relocalization of the protein complex to specific subcellular sites (Nandurkar et al, 2003; Kim et al, 2003; Lorenzo et al, 2006) and modified substrate specificity (Nandurkar et al, 2001). These specific interactions between active and inactive MTMR family members seem to follow a general rule
with functional significance. Furthermore, they suggest differential regulated function upon tissue specific complex formation.

**Table 5-1: Protein interactions within the MTMR family**

Catalytically active MTMRs are represented with light blue background, and inactive members with grey background. Self-associations are labeled with an asterisk. For references, consult discussion above. n.d. = no data

<table>
<thead>
<tr>
<th>MTMRs</th>
<th>Interacting MTMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTM1</td>
<td>MTM1*, MTMR12/3-PAP, MTMR10</td>
</tr>
<tr>
<td>MTMR1</td>
<td>n.d</td>
</tr>
<tr>
<td>MTMR2</td>
<td>MTMR2*, MTMR5/STYX, MTMR12/3-PAP, MTMR13/STYX</td>
</tr>
<tr>
<td>MTMR3</td>
<td>MTMR3*, MTMR4</td>
</tr>
<tr>
<td>MTMR4</td>
<td>MTMR4*, MTMR3</td>
</tr>
<tr>
<td>MTMR5/STYX</td>
<td>MTMR12/3-PAP, MTMR10</td>
</tr>
<tr>
<td>MTMR6</td>
<td>MTMR6*, MTMR9/STYX</td>
</tr>
<tr>
<td>MTMR7</td>
<td>MTMR9</td>
</tr>
<tr>
<td>MTMR8</td>
<td>MTMR8*, MTMR9/STYX</td>
</tr>
<tr>
<td>MTMR9/STYX</td>
<td>MTMR9/STYX*, MTMR6, MTMR7, MTMR8</td>
</tr>
<tr>
<td>MTMR10</td>
<td>MTM1, MTMR2</td>
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<tr>
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<td>n.d</td>
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<tr>
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<td>MTMR12/3-PAP*, MTM1, MTMR2</td>
</tr>
<tr>
<td>MTMR13/STYX</td>
<td>MTMR13/STYX*</td>
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5.1.5 Other MTMR-protein interactions

Myotubularin-related-proteins and MTMR-interacting proteins are involved in a variety of CMT forms and myopathies. Yeast two-hybrid screenings revealed that MTMR2 interacts with the class IV intermediate filament (IF) neurofilament light chain (NF-L) in Schwann cells and neurons (Previtali et al, 2003). Neurofilaments are formed by a trimeric complex composed of NF-L, NF medium chain (NF-M) and NF heavy chain (NF-H), whereby NF-L functions as scaffolding protein (Ching et al, 1993; Lee et al, 1993). Mice lacking either NF-L, NF-M, NF-H or both NF-M and NF-H lost up to 20% of their motor axons and were subjected to disturbances in axonal maturation (reviewed in (Lariviere et al, 2004)). These findings were interesting as such, that mutations in NF-L gene are associated with the dominant axonal CMT type 2E and dominant demyelinating CMT type 1F form (Perez-Olle et al, 2005), supporting the nerve-specific pathogenesis of CMT4B1. Recently, also the class III intermediate filament proteins vimentin, GFAP, desmin and peripherin as well as the class V IF lamin A were identified as MTMR2 interactors (Tersar, 2008). Besides Mtmr2, MTM1 was also reported to interact with desmin and was shown to regulate desmin cytoskeleton and mitochondria homeostasis in skeletal muscle (Hnia et al, 2011). Mutations in
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Desmin cause myopathies in skeletal and cardiac muscle (Paulin et al, 2004; Schroder et al, 2007). Cardiac involvement however, is not a common sign of adult XLMTM patients (Herman et al, 1999). This is supported by the finding that in cardiac muscles of mice no interaction between Mtm1 and desmin was observed and colocalization was rather weak (Hnia et al, 2011). Nevertheless, it is intriguing that alterations of MTM1 or desmin affect skeletal muscles, whereas alterations of MTMR2 and NF-L specifically affect peripheral neurons and Schwann cells. MTMR2 also functionally interacts with the phospholipid phosphatase FIG4/SAC3 in both Schwann cells and neurons (Vaccari et al, 2011). Mutation of FIG4 in human and mice is involved in autosomal recessive demyelinating CMT type 4J and a subset of patients present a late-onset phenotype that resembles amyotrophic lateral sclerosis (ALS) (Chow et al, 2007; Zhang et al, 2008). Furthermore, Fig4 deficiency in mice affects motor neurons differently from sensory neurons by mechanisms involving excessive retention of molecules in lysosomes or disruption of vacuolated organelles (Katona et al, 2011). MTMR4 was shown to interact with the ubiquitin ligase Nedd4, which was associated with skeletal muscle atrophy (Koncarevic et al, 2007). It becomes very clear that MTMRs and their interaction partners build a complex network of proteins with cell relevant functions in intracellular trafficking and are key regulators in the peripheral nervous system (Berger et al, 2006b; Suter, 2007).

Surprisingly, MTMR phosphatases were also found in complex with kinases. In testis, MTMR2 interacts with the nonreceptor protein tyrosine kinase c-Src and together they regulate Sertoli-germ cell adherens junction dynamics (Zhang et al, 2005). In mammalian cells, both MTM1 and MTMR2 were reported to interact with the lipid kinase complex hVps15/hVps34 on endosomes, thereby modulating Rab5-Rab7 dependent endosome maturation (Cao et al, 2007; Cao et al, 2008; Poteryaev et al, 2010) (see also 5.2.5.1). Receptor mediated endocytosis 8 (RME-8), another protein interacting with MTMR2, was just recently identified as a novel PI-3-P binding protein, whose localization is regulated by MTMR2 (Xhabija et al, 2011). Another interaction with MTMR4 was found for phosphorylated R-Smads. MTMR4 dependent dephosphorylation of R-Smads,
reduced R-Smad translocation to the nucleus and resulted in preventing overactivation of TGFβ signaling (Yu et al, 2010).

5.1.6 Function and localization
One of the most intriguing questions in the field of disease relevant MTMRs is: Although MTMRs share substrate specificity, high sequence homology and are ubiquitously expressed, how does it come that MTMRs are not redundant and have unique functions within cells? How comes that MTM1 and MTMR2 mutations lead to two human diseases affecting different cell types? One possible mechanism for this could be the differential interaction with proteins like PDZ-domain containing proteins, inactive MTMRs or other proteins. Dependent on the interaction not only the place of action could be altered but also the catalytical activity. Active MTMRs dephosphorylate both PI-3,5-P$_2$ and PI-3-P at position D3 of the inositol ring to PI-5-P and phosphatidylinositol (PtdIns), respectively. Thereby, they regulate specific pools of PI-3-P and/or PI-3,5-P$_2$, which in turn are anchor sites on membranes for effector proteins of early and late phases of the endocytic process. Besides the function of MTMRs in cell shape and plasma membrane homeostasis (see 5.1.1.1), they have also been shown to regulate receptor sorting. Overexpression of both MTMR2 and MTM1 resulted in blocked degradation of epidermal growth factor receptor (EGFR) (Tsujita et al, 2004; Berger et al, 2009). Furthermore, Berger et al. could show that the adaptor unit MTMR13/SBF2 could counteract this effect, without affecting prolonged Akt activation. Since PI-3-P and/or PI-3,5-P$_2$ levels interfere with the maturation of endosomes, their reduction might explain the observed block of EGFR degradation. PI-3-P is synthesized by the PI3 kinase Vps34 on early endosomes and displays a recruitment site for FYVE-domain containing Rab5 effectors such as EEA1 and Hrs (Simonsen et al, 1998; Christoforidis et al, 1999; Gaullier et al, 1998). PI-3,5-P$_2$ is generated from PI-3-P by PIKfyve, which was found to localize to late endosomes (Sbrissa et al, 2002). Effector proteins binding PI-3,5-P$_2$ were reported for sorting nexins (SNXs), Vps24 and Svp1p (Whitley et al, 2003; Dove et al, 2004; Seet et al, 2006). Interestingly, by inhibiting the PI3 kinase with wortmannin, endocytosed EGFR failed to reach late
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endosomes and was trapped in early/recycling endosomes (Petiot et al, 2003). Additionally they suggested that PI-3-P signaling controls the sorting of receptor in early endosomes presumably by Hrs. Also knockdown of Vps24 or overexpression of dominant-negative forms of Vps24 blocked EGFR degradation (Yan et al, 2005; Bache et al, 2006).

5.2 Endosomes, trafficking and effector proteins including MTMRs

5.2.1 Overview: Endosomes, Rab GTPases and trafficking pathways

Endocytosis and membrane trafficking between organelles is essential for protein and membrane homeostasis of the cell and is summarized in Figure 5-2. Here we focus on the internalization of plasma membrane receptors and their subsequent sorting. Dependent on signaling and effector molecules at the endocytic compartments the fate of the internalized receptors can vary: (1) as part of the signaling process; (2) recycling back to the plasma membrane for continuous action; (3) propagation to the lysosome for degradation. Large particles like cell debris and fluids usually enter the cell by phagocytosis and macropinocytosis, respectively. Other material including membrane receptors are taken up in clathrin- or caveolin-coated vesicles or tubular structures, which are also known as clathrin- and dynamin-independent carriers (CLICs), which include GEEC-, and ARF6-dependent pathways (Mayor et al, 2007). All internalized protein first converges at the apical early endosomes (EEs), which are characterized by several markers, such as PI-3-P, EEA1, PI-3 kinase Vps34 and small Rab GTPase Rab5 (Christoforidis et al, 1999; Zerial et al, 2001; Behnia et al, 2005). PI-3-P, Rab5 and their binding effector proteins play an important role in endosome maturation, which includes Rab5 to Rab7 and PI-3-P to PI-3,5-P_2 conversion (Rink et al, 2005; Poteryaev et al, 2010). The structure of individual EEs with tubular and vacuolar domains is heterogeneous. Inward vesiculation to form intraluminal vesicles (ILVs) happens already at the stage of EEs and is an important process during late endosome (LE) and lysosome maturation.
Activated, internalized membrane receptors are still able to signal, as long as their C-terminal tail is facing the cytosolic phase. One essential step to inactivate these signaling receptors is their ubiquitination and subsequent removal from the endocytic membranes by inclusion into ILVs (Raiborg et al, 2002). Recruitment of ubiquitinated cargo to EEs is regulated by the ubiquitin interacting motifs on hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and components of the endosomal sorting complexes required for transport (ESCRT) complex (Slagsvold et al, 2006). ILVs follow together with the cargo destined for degradation the degradative pathway: from early endosomes (EEs) through late endosomes (LEs) to endolysosomes/lysosomes, whereby the endosomes move towards the perinuclear space along microtubules (MT). The fusion of LEs with lysosomes is unidirectional, whereby endolysosomes are generated, in which the actual degradation is taking place (Luzio et al, 2007). Retrograde transport between endosomes and trans-Golgi network (TGN) occurs between all levels of endocytic pathway and is essential for the supply of lysosomal and removal of endosomal components. Thereby, the retromer, an oligomeric protein complex, as well as the small GTPases Rab7 and Rab9 are important regulators (van Weering et al, 2011). The retromer is composed of the trimeric vacuolar protein sorting (Vps) subcomplex (Vps26,-29,-35) and heterodimeric phosphoinositide and receptor binding sorting nexins (SNXs, SNX1 and SNX2 or SNX5 and SNX6) (McGough et al, 2011). From the TGN cargo is sorted through Rab8 vesicles either directly to the plasma membrane or indirectly through the recycling endosome (RE) (De Matteis et al, 2008; Cramm-Behrens et al, 2008). Endocytosed protein, which has arrived in EEs has been initially proposed to escape the degradative pathway by recycling back to the plasma membrane in two different ways (Maxfield et al, 2004): via the Rab11 dependent trafficking through the RE (also known as slow recycling); or by leaving the EE directly in Rab4 vesicles (fast recycling). Thereby, membrane proteins such as the transferrin receptor (TfR) are sorted away from soluble proteins via the formation and fission of Rab11 positive narrow tubules from the EE to the RE. This is regulated by a subset of proteins including SNX4, which has been reported to
coordinate the long-range translocation by binding to the minus end-directed microtubule motor dynein (Shinozaki-Narikawa et al, 2006; Traer et al, 2007; Cullen, 2008).
Figure 5-2: Overview of vesicle transport - from recycling to degradation

Material is transported to EEs in clathrin- or caveolin-coated vesicles or in clathrin- and dynamin-independent carriers (CLICs), including GEEC- and ARF6-dependent pathways. Rab5 vesicles fuse with the early endosomes (EEs), where the endocytosed cargo accumulates before sorting. The structure of EEs is characterized by tubular and vacuolar domains with few intralumenal vesicles (ILVs). Hallmarks of EEs on the protein level are the small GTPases Rab5 and Rab4, the PI3 kinase Vps34, its product PI-3-P and PI-3-P effector proteins like EEA1. From the EE the internalized cargo, including membrane proteins, is either sorted for degradation to lysosomes or recycled back to the plasma membrane. By entering the degradative pathway, the endosomes are converted from EEs to late endosomes (LEs) and fuse with lysosomes to form endolysosomes. Thereby, the vesicles are moving towards the perinuclear space along microtubules (MT) and undergo structural changes ranging from growing in size by continuous inward vesiculation to changes in protein components. Typical hallmarks for LE maturation is the Rab5-Rab7 switch, as well as the conversion of PI-3-P to PI-3,5-P$_2$ with the subsequent effector proteins such as the ESCRT machinery. During LE maturation membrane proteins still can escape the degradative pathway by retrograde transport through the trans-Golgi network (TGN). PI-4-P is localized at the TGN. The retrograde transport to the TGN is regulated by the SNX-Bar retromer, typically composed of SNX1/2, SNX5/6 and Vps26, Vps29 and Vps35. Through Rab8 vesicles cargo is sorted from the TGN to the recycling endosome (RE), which is characterized by Rab11. Via Rab11/FIP2/myosinVb protein complex, Rab11 vesicles can move along the actin skeleton back to the plasma membrane. Recycling from EEs can be divided into “slow” and “fast” and proceeds in a sequence-dependent manner. The slow recycling involves formation and fission of Rab11 positive narrow tubules from the EE to the RE. Sequence-dependent recycling involves membrane proteins with a PDZ-binding motif. PDZ domain containing proteins such as GIPC or ERM proteins, and the transition along actin-stabilized recycling tubules positive for Rab4 and Rab11.

5.2.2 Phosphoinositides

5.2.2.1 Structure and turnover

Phosphoinositides (PIs) are essential components of eukaryotic cell membranes and regulate fundamental biological processes including cell growth, survival, membrane trafficking and cytoskeletal dynamics (Roth, 2004; Lemmon, 2008). Phosphatidylinositol (PtdIns) belongs to the glycerophospholipids and is composed of two fatty acid tails, which anchor the molecule into cellular membranes and are linked through a glycerol backbone and an inorganic phosphate to the polar inositol head group (Figure 5-3, A). PtdIns is the most abundant PI in mammalian cells (~15% of total phospholipids found in eukaryotic cells) (Di et al, 2006). Phosphorylated products of phosphatidylinositol are called phosphoinositides and are less abundant (by one order of magnitude). Reversible phosphorylation of the inositol ring at position D3, D4 or D5 generates seven possible phosphoinositides, which are enriched on subcellular compartments and serve as docking sites for signaling effectors (Roth, 2004; Di et al, 2006). PI-4,5-P$_2$ (often called PIP$_2$) and PI-3,4,5-P$_3$ (also known as PIP$_3$)
are both formed and localized to the plasma membrane (see Figure 5-2). PI-4,5-P$_2$ is the principal substrate of receptor-stimulated phospholipase C and comprises only about 1% of total phospholipids in the whole cell (Falkenburger et al, 2010). PI-4-P was mainly found at the TGN. Because of continuous sequential phosphorylation/dephosphorylation reactions by specific kinases and phosphatases, PtdIns, PI-4,5-P$_2$ and PI-4-P are kept in a steady-state level in the cell membrane. PI-3-P and PI-3,5-P$_2$ are concentrated on early and late endosomes, respectively. PI-3-P is constitutively present at low but rather stable levels, whereas PI-3,5-P$_2$ and PI-5-P levels can increase upon cell stress/activation (Dove, 1997; Zonia et al, 2004; Sbrissa et al, 2005). Interconversions between different PIs are regulated by PI kinases and phosphatases, leading to temporal and spatial regulation of membrane budding, motility and fusion. The importance of the different PI levels and their catalyzing enzymes is supported by the occurrence of various human diseases generated by mutations in these metabolizing enzymes (Vicinanza et al, 2008; Nicot et al, 2008; Liu et al, 2010) (see Figure 5-3, B; modified from (Vicinanza et al, 2008)).
5.2.2.2 Recruitment and binding of PIs by specific domains

Phosphoinositides are bound to effector proteins, whereby individual effector proteins can interact with several PIs (Kutateladze, 2010). Up to date 12 PI-binding modules have been identified: (1) pleckstrin homology (PH) domain, (2) AP180 N-terminal homology (ANTH) domain, (3) conserved region-2 of protein kinase C (C2) domain, (4) epsin N-terminal homology (ENTH), (5) 4.1, ezrin, radixin, moesin (FERM) domain, (6) Fab1, YOTB, Vac1 and EEA1 (FYVE) domain, (7) Golgi phosphoprotein 3 (GOLPH3) domain, (8) PDZ domains, (9) β-propellers that bind PIs (PROPPINs) domain, (10) phosphotyrosine binding (PTB) domain, (11) Phox homology (PX) domain and (12) Tubby modules (Figure 5-4, adapted from Kutateladze, 2010). FYVE domain-containing proteins recognize PI-3-P with high specificity and affinity and are characterized by a zinc-binding finger (Gaullier et al, 1998). They are involved in the regulation of endocytic trafficking, fusion of endosomal membranes and maturation of late endosomes including formation of MVBs (Gillooly et al, 2001). PX domains were initially found to bind PI-3-P. Thereby, SNXs represent the largest group of PX domain-containing proteins (Seet et al, 2006). They are involved in endosomal sorting and recycling, in internalization and in membrane tubulation (see 5.2.1).

Less than 10% of all identified PH domains bind PIs with high affinity, such as PI-4,5-P$_2$, PI-3,4-P$_2$ and PI-3,4,5-P$_3$ (Lemmon, 2007). PH-domain containing proteins were associated not only with membrane budding and trafficking, but also with essential processes such as cell growth, proliferation, migration and metabolism. PH, PTB and PDZ domain structures are markedly similar and share a structural core consisting of several antiparallel β strands and an α-helix (Doyle et al, 1996; Balla, 2005).
5.2.3 Conversion of PI-3-P to PI-5-P

5.2.3.1 PI interacting effectors and their role

PI-3-P and PI-3,5-P$_2$, as well as their catalyzing enzymes, play an important role in endosome identity, maturation and trafficking between plasma membrane, Golgi and lysosome (Odorizzi et al, 2000). PI-3 kinase complex Vps34/p150 is recruited to EEs by binding to Rab5 and generates PI-3-P from PtdIns (Schu, 1993; Christoforidis et al, 1999). As soon as PI-3-P is synthesized, it attracts various effector proteins. Recruitment of early endosome antigen 1 (EEA1) in concert with SNARE proteins promotes vesicle fusion and endocytosis (Simonsen et al, 1998; Lawe et al, 2000). The ESCRT complexes I and II bind PI-3-P through Vps27 and Vps36, respectively (Katzmann et al, 2003; Teo et al, 2006). Thereby, the ESCRT machinery regulates MVB formation and sorting of ubiquitinated cargo. Hrs of the ESCRT-0 complex is recruited to EEs and MVBs by Rab5 and PI-3-P, where it controls receptor sorting and internalization within the MVBs (Lloyd et al, 2002; Raiborg et al, 2002; Raiborg et al, 2001). PIKfyve is a PI 5 kinase which converts PI-3-P into PI-3,5-P$_2$ and is implicated in membrane homeostasis, MVB/ILV formation and endosome maturation (Nicot et al, 2006). The PX-domain containing SNXs, which have been shown to modulate endosome-to-TGN trafficking, interact with PI-3-P, PI-3,5-P$_2$ and/or PI-5-P. These include SNX3 or the SNX-Bar retromer components SNX1/2 and SNX5/6 (Cozier et al, 2002; Carlton et al, 2005; Wassmer et al, 2007; Wassmer et al, 2009;
Harterink et al, 2011). Besides MVB formation, Hrs is involved in Smad signaling through cooperation with Smad anchor for receptor activation (SARA), another FYVE-domain containing PI-3-P binding protein (Miura et al, 2000). Furthermore, SARA has been shown to regulate TGF-β/Smad signaling (Itoh et al, 2002), whereas MTMR4 was reported to act as a negative regulator of TGF-β/Smad signaling (Yu et al, 2010). Autophagy-linked FYVE protein (Alfy), another PI-3-P binding FYVE domain-containing protein, was found to colocalize with autophagic membranes and was suggested to target cytosolic protein aggregates for autophagic degradation (Simonsen et al, 2004). Interestingly, also the PI-3-P producing kinase Vps34 and the phosphatase MTMR3 were both associated with autophagy (Matsunaga et al, 2009; Taguchi-Atarashi et al, 2010).

Overall, PI-3-P level and its effectors regulate cellular functions covering endocytic membrane traffic, autophagy, degradative pathway, recycling through retrograde endosome-to-TGN transport and receptor signaling (Figure 5-5).

PI-3,5-P\(_2\) is substrate for FIG4 and MTMRs (MTM1, MTMR2), which generate PI-3-P and PI-5-P, respectively. Effectors of PI-3,5-P\(_2\) were mostly identified and characterized in yeast. Atg18p/Svp18p, a member of the PROPPIN family (for \(\beta\)-propeller(s) that bind(s) PPIn), binds PI-3,5-P\(_2\) with high affinity and participates in retrograde membrane traffic from the vacuole in yeast (Dove et al, 2004). The epsin-like proteins Ent3p and Ent5p as well as the ESCRT-III component Vps24 and the ESCRT-II component Vps36 bind to PI-3,5-P\(_2\) and were associated with MVB sorting (Whitley et al, 2003; Friant et al, 2003; Eugster et al, 2004; Teo et al, 2006). Functional MVB formation has been reported to be required for successful envelopment/egress of the following virus infections: herpes simplex virus (HSV), non-enveloped picornavirus echovirus 1 (EV1), old world arenavirus Lassa virus (LASV) (Calistri et al, 2007; Karjalainen et al, 2011; Pasqual et al, 2011). Furthermore, the entry of LASV into the cell was reported to be dependent on PI-3-P and PI3K activity. Given that MVB membranes serve as platforms for viral envelopment/egress, thereby representing a critical step in viral infection, MVB involved factors like Vps24 may represent putative drug targets to treat viral infections. A recent publication showed that PI-3,5-P\(_2\) activates the
endolysosomal calcium channel TRPML1 by directly interacting with the latter (Dong et al., 2010). Furthermore, they proposed that TRPMLs regulate membrane trafficking by transducing information regarding PI-3,5-P$_2$ levels into changes in juxtaorganellar Ca$^{2+}$, thereby triggering membrane fusion/fission events. In summary, PI-3,5-P$_2$ as well as its binding effectors are mainly involved in MVB/ILV formation including subsequent cargo degradation and LE maturation.

In mammals, PI-5-P is produced from PI-3,5-P$_2$ by active MTMRs (Tronchere et al., 2004). In *Shigella flexneri* infected mammalian cells the phosphatase IpgD takes over this part, whereby it uses PI-4,5-P$_2$ as substrate (Rameh et al., 1997). Injection of IpgD by *S. flexneri* into the host cell leads to reorganization of the host cell membrane morphology and promotes the uptake of the bacterium into the host (Niebuhr, 2002). Interestingly, IpgD infection leads to EGFR activation which is required for PI-5-P induced Akt activation (Ramel et al., 2009; Ramel et al., 2011). Furthermore, they analyzed the impact of PI-5-P on endosome formation and EGFR trafficking in HeLa cells. Thereby they revealed that PI-5-P increased the numbers of EEs with activated EGFR, prolonged EGFR signaling and blocked general vesicular transport from early to late endosomes (Ramel et al., 2011). These findings are interesting as such that overexpression of MTMR2 also resulted in sustained Akt activation and blocked EGFR degradation. Furthermore, overexpression of Sbf2 could counteract the blocked EGFR degradation (Berger et al., 2009). The PX domain of SNX5 specifically binds to PI-3,5-P$_2$, PI-4-P and PI-5-P, but not PI-3-P as SNX1 does (Liu et al., 2006). Few effectors of PI-5-P have been lately identified: the plant homeodomain (PHD) finger of inhibitor of growth protein-2 (ING2), a candidate tumor suppressor protein (Gozani et al., 2003); the PH-domain containing downstream of tyrosine kinase (Dok) proteins Dok-1 and Dok-2, which are involved in T-cell homeostasis maintenance (Guittard et al., 2009); PX domain of phospholipase D1 (PLD1), which is also an effector of PI-3-P and is involved in macroautophagy and amino acid sensing (Du et al., 2003; Dall'Armi et al., 2010; Yoon et al., 2011); Nox organizing protein 1 (NOXO1), which is involved in NADPH oxidase regulation...
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(Cheng et al, 2004). All together, the collected data point to a function of PI-5-P in recycling and signaling.

Figure 5-5: Conversion of PI-3-P to PI-5-P and PI effectors
Phosphoinositide binding effectors are encircled and colored corresponding to their cellular function in endocytosis (brown), retrograde transport (petrol), MVB formation and trafficking (green), autophagy (orange) and signaling (yellow). PLD1 binds PI-3-P and PI-5-P, the function in autophagy however has been shown in relation to PI-3-P. The unknown function of PLD1 with respect to PI-5-P is depicted in grey. PI metabolizing enzymes are represented in red.

5.2.3.2 PI metabolizing proteins and their function in cell biology
In mammalian cells three classes of PI-3 kinases (PI3Ks) exist. They phosphorylate phosphatidylinositol or phosphoinositides at the D3 position of the inositol ring, thereby generating PI-3-P, PI-3,4-P<sub>2</sub>, PI-3,4,5-P<sub>3</sub> or PI-3,5-P<sub>2</sub>. Class I PI3K subtype IA is activated by receptor tyrosine kinases (RTKs) and subtype IB by G-protein coupled receptors (GPCRs). Especially kinases of the subtype IA were associated with oncogenesis. The generation of PI-3,4,5-P<sub>3</sub> from PI-4,5-P<sub>2</sub> leads to the activation of downstream pathways that involve AKT and PTEN. The oncogene AKT encodes a serine/threonine kinase that triggers a cascade of responses including cell growth, proliferation, survival and motility, all driving tumour progression (Vivanco et al, 2002). In contrast, PTEN encodes a phosphatase which converts PI-3,4,5-P<sub>3</sub> back to PI-4,5-P<sub>2</sub> and therefore acquires
tumour-suppressor function. PI-3 kinase/AKT pathway is dysregulated in a variety of human cancers, including breast, colon, ovarian, pancreatic, lymphoid and prostate cancers. Class II PI3K enzymes include PI3K-C2α, PI3K-C2β, and PI3K-C2γ, which use PtdIns as substrate to produce PI-3-P and PI-3,4-P₂ and are characterized by a PX and C2 domain. In COS-1 cells the PX and the C2 domain of PI3K-C2α were shown to mediate binding to lipid membranes of the TGN and clathrin-coated vesicles (Domin et al, 2000). Although some reports point to a activation by external stimuli similarly to class I PI3Ks, there is still relatively little understanding of their role in cellular function (Falasca et al, 2007). The first PI3K coding gene Vps34 was found in Saccharomyces cerevisiae and was characterized to be required for vacuolar protein sorting and vacuole segregation (Herman et al, 1990; Schu, 1993). Vps34 is the sole PI3K that produces only PI-3-P from PtdIns in vivo and was therefore classified as PI3K class III. The human homologue hVps34/PIK3C3 is regulated by Vps15/p150 and localizes to EEs by binding to Rab5 (Schu, 1993; Stack et al, 1995; Christoforidis et al, 1999). Together, they are part of two distinct multimeric protein complexes: complex I (hVps34/PIK3C3, Vps15/p150, Beclin1, and Atg14L) which activates autophagy and complex II (hVps34/PIK3C3, Vps15/p150, Beclin1, and UVRAG/Vps38) which regulates trafficking at late endosomes (Simonsen et al, 2001; Lindmo et al, 2006; Backer, 2008; Itakura et al, 2008). PI-3-P is also recognized by the FYVE domain of PIKfyve, a kinase that phosphorylates PI-3-P at position D5 to form PI-3,5-P₂ (Sbrissa et al, 1999). Overexpression of a dominant-negative form which is unable to synthesize PI-3,5-P₂ (PIKfyve-K1999E) induces endomembrane swelling and vacuolation (Ikonomov et al, 2001). Swollen vacuoles were also observed in yeast, when fab1, the yeast orthologue of PIKfyve was deleted (Yamamoto et al, 1995). PI-3,5-P₂ contributes less than 0.1% of the total phosphoinositide pool but it is transiently upregulated under hypo-osmotic conditions in COS cells, in T-lymphocytes upon stimulation with interleukin-2, or in COS cells upon stimulation with EGF (Dove, 1997; Jones et al, 1999; Tsujita et al, 2004). Initially, it has been shown that mouse PIKfyve associates with late endosomes in mammalian cells (Shisheva et al, 2001).
Human PIKfyve localizes to vesicles which are positive for early endosomal markers like EEA1 and Hrs. PIKfyve localizes on these vesicles to microdomains which contain little EEA1, Hrs, and PI-3-P (Cabezas et al, 2006). This indicates that recruitment of PIKfyve to PI-3-P rich microdomains of early endosomes leads to the local conversion of PI-3-P to PI-3,5-P$_2$ which is then probably followed by the recruitment of PI-3,5-P$_2$ effector proteins. The antagonistic enzyme of PIKfyve is the phosphatase FIG4/Sac3, which converts PI-3,5-P$_2$ to PI-3-P. Both enzymes are regulated by binding to Vac14/ArPIKfyve (Sbrissa et al, 2007). FIG4/Sac3 plays a dual role in this complex. FIG4 not only catalyzes PI-3,5-P$_2$ turnover but also promotes PI-3,5-P$_2$ synthesis, by facilitating the association of PIKfyve and ArPIKfyve (Sbrissa et al, 2007; Sbrissa et al, 2008).

Deletion of Fig4 in yeast lead to elevated PI-3,5-P$_2$ levels, enlarged vacuoles and impaired retrograde traffic to the late endosomes, similar to knockdown of mammalian Fab1/PIKfyve kinase knockdown phenotype (Gary et al, 1998; Bonangelino et al, 2002; Michell et al, 2005). SiRNA-mediated knockdown of endogenous FIG4 in 3T3L1 adipocytes resulted also in a slight but significant elevation of PI-3,5-P$_2$ level (Ikonomov et al, 2009). Furthermore, it was shown that FIG4 activity towards PI-3,5-P$_2$ was markedly reduced upon acute insulin stimulation and its down-regulation subsequently increased insulin responsiveness. In yeast, Vac14 promotes the localization of Fig4 to the site of PI-3,5-P$_2$ synthesis (Dove et al, 2002; Rudge et al, 2004). Deletion of Vac14 resulted in enlarged vacuoles and reduced PI-3,5-P$_2$ levels (Bonangelino et al, 2002).

PI-3,5-P$_2$ binding Vps24 protein of the ESCRT-III complex controls recycling to the Golgi and degradation pathway (Raiborg et al, 2008). In yeast, Vps24 was additionally identified to be involved in pH-responsive Rim101 pathway (Hayashi et al, 2005). Especially to mention here, is the influence of Vps24 on stimulated degradation of membrane proteins. Depletion of Vps24 resulted in accumulation of EGF/EGFR complex in acidified EEs and was not further degraded (Bache et al, 2006). Also the degradation of monoubiquitinated potassium channel, which is encoded by the human ether-a-go-go-related gene (hERG) was blocked in
depleted Vps24 HEK cells (Sun et al, 2011). Vps24 dependent MVB formation, which is a component of the degradative pathway, plays an important role in viral host cell infection and will be discussed in the proximate chapter. Function of PI binding MTMR phosphatases in cellbiological processes was extensively discussed in chapter 5.1.

5.2.4 PI metabolizing enzymes and the relevance of their correct function in vivo

Plakin3C3 gene encodes the PI3 kinase VPS34. Pik3c3\textsuperscript{flox/flox} mice with sensory neuron-specific deletion of Pik3c3 developed increasing difficulties in coordinating movement and maintaining body postures from postnatal day 5–6 (P5–P6) and died within 2 weeks of age. This pathological feature was the result of neurodegeneration, which was based on drastic defects in the endo-lysosomal pathways but not autophagy (Zhou et al, 2010). Substrate trap experiment in Schizosaccharomyces pombe suggest that human MTM1 regulates VPS34 activity by dephosphorylation of VPS34 and by decreasing the PI-3-P pool (Blondeau et al, 2000). Such dual activity, comprising protein phosphatase and lipid phosphatase function, has also been suggested for the tumor suppressor and PI-3 phosphatase PTEN (Di et al, 2000). Pik3c3 null mice embryos are poorly developed with no evidence of mesoderm formation, suffer from severe reduced cell proliferations and are embryonic lethal (Zhou et al, 2011). Furthermore, it was shown that amino acid activated mTOR signaling is indeed drastically reduced in Pik3c3 mutant embryos. PIK3C3/VPS34 is therefore not only important in autophagy or trafficking at late endosomes, but also absolutely required for signaling during developmental processes.

Heterozygous mutations in Plakin3fye cause François-Neetens fleck corneal dystrophy (CFD), an autosomal dominant syndrome clinically manifested by the presence of enlarged/swollen vesicles (speckles) within keratocytes (Li et al, 2005). Deletion of Plakin3fye (Fab1) in yeast was not lethal and caused aberrant nuclear division and growth defects, characterized by aberrant vacuolation (Yamamoto et al, 1995). Pikfyve null mutants of C. elegans or Drosophila had developmental defects and were embryonic/pupae lethal, respectively (Nicot et
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al, 2006; Rusten et al, 2006). Recently, the first mouse model was published with global deletion of the Pikfyve gene using the Cre-loxP approach (Ikonomov et al, 2011). PIKfyveKO/KO mutant embryos died before the 32–64-cell stage, a result of defective cell division. Heterozygous PIKfyveWT/KO mice however, developed normally even though their PIKfyve levels were lower than those of wild-type mice.

PI-3,5-P2 is substrate for the phosphatase FIG4. Missense mutation FIG4 I41T combined with a FIG4 null allele in humans causes Charcot-Marie-Tooth 4J disease, a recessive neurodegenerative disorder that accounts for ~0.2% of CMT (Chow et al, 2007). CMT4J is characterized by slow nerve conduction velocities, axonal loss, thinly myelinated nerve fibres and evidence of de- and re-myelination. Fig4−/− mice (designated as “pale tremor” mice) displayed similar features including defects in PNS myelination. Additionally, the pale tremor mice exhibited a dramatic reduction of myelin in the brain and spinal cord, which however could be rescued by neuronal expression of the human FIG4 I41T mutant protein (Winters et al, 2011). In contrast to the Fig4/Sac3 knockdown mutants in yeast and adipocytes, the PI-3,5-P2 levels in the Fig4 null mice were reduced (Chow et al, 2007). Fig4 and Vac14 mutant mice are blocked in autophagy, supporting the evidence that PI-3,5-P2 pool in the CNS plays an essential role in autophagy pathway (Ferguson et al, 2009). Vac14−/− knockout mice displayed enlarged endolysosomes and suffered from neurodegeneration (Zhang et al, 2007). Cultured fibroblasts from missense mutant Vac14 mouse showed reduced level of PI-3,5-P2 and similar pathological features as observed from Fig4−/− fibroblasts (Ferguson et al, 2010): severe neurodegeneration, reduced number of myelinated axons in sciatic nerve, loss of neurons from sensory and autonomic ganglia and lethality by 6 weeks of age. Mutations in MTM1, MTMR2 and SBF2 and their causing diseases XLMTM, CMT4B1 and CMT4B2, respectively, were described earlier (5.1.1).
5.2.5 Rab GTPases and their influence on transport

The role of Rab GTPases in membrane traffic, vesicle transport and endosome maturation has been extensively reviewed (see small selection for review (Zerial et al, 2001; Schwartz et al, 2007; Lee et al, 2009; Stenmark, 2009; Hutagalung et al, 2011; Huotari et al, 2011). Some Rab GTPases have been introduced in chapter 5.2.1 and are depicted in Figure 5-2. More than 60 members of Rab GTPases were identified in humans, which localize to distinct subcellular membranes or distinct microdomains of the same vesicles (Zerial et al, 2001; Schwartz et al, 2007). They exist in an inactive GDP bound stage and an active GTP bound form, whereby the interconversion is catalyzed by guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP). Cytosolic inactive Rab is associated with GDP dissociation inhibitor (GDI), stabilizing the GDP bound Rab (Matsui et al, 1990). Assisted by GDI dissociation factor (GDF) Rab GTPase is inserted into the appropriate membrane (Sivars et al, 2003). Active Rabs recruit specific effectors to restricted membrane microdomains, thereby specifying membrane identity. The most cited example is the recruitment of class III PI3K by Rab5 and subsequent production of PI-3-P, the hallmark of early endosomal membranes (Christoforidis et al, 1999). Through interaction with miscellaneous effectors, Rabs regulate multiple processes of vesicular traffic. These are listed here by means of few examples (reviewed in (Stenmark, 2009; Hutagalung et al, 2011)): (1) Rab5 in complex with GDI is essential for assembly of clathrin-coated pits and clathrin-mediated endocytosis/vesicle budding at the plasma membrane (McLauchlan et al, 1998); (2) Rab5 together with rab5 exchange factor RME-6 coordinate regulation of AP2 uncoating from CCVs (Semerdjieva et al, 2008); (3) Rab5 recruits EEA1, which interacts with the endosomal SNAREs syntaxin 6 and syntaxin 13 to drive vesicle fusion (Simonsen et al, 1999; McBride et al, 1999); (4) Rab11a in complex with FIP2 binds to the motor protein myosin Vb, thereby connecting Rab11a positive vesicles with the actin filaments and facilitating recycling along the cytoskeleton back to the plasma membrane (Hales et al, 2001; Hales et al, 2002).
5.2.5.1 Rab5 to Rab7 conversion/switch
Like the PI conversion of PI-3-P to PI-3,5-P₂ plays an important role in membrane identity of EEs, LEs and their maturation, so does the Rab conversion of Rab5 to Rab7 (reviewed in (Huotari et al, 2011)). The degradative pathway of cargo destined to LEs and lysosomes starts in Rab5 positive microdomains of EEs, which can undergo conversion to Rab7 and subsequent maturation (Rink et al, 2005; Hutagalung et al, 2011). Rab5 gets activated by the GEF Rabex-5 (Horiuchi et al, 1997). Activated, GTP-bound Rab5 promotes then binding of further Rab5 through interaction with Rabex-5 and Rabaptin-5 (Lippe et al, 2001). Inhibition of this positive feedback loop is the first step in Rab5/Rab7 switch and needs the dissociation of Rabex-5 from the membrane (Huotari et al, 2011). This is initiated by Mon1/SAND-1 complexed with Ccz1, which bind to Rab5, PI-3-P and Rabex-5. Increasing PI-3-P level was suggested to localize on “elderly” maturing microdomains of the EEs, and thereby driving SAND-1 binding specifically to these sites (Poteryaev et al, 2010). Inactivation of GTP-Rab5 by GAP, which hydrolyses GTP to GDP, is followed by removement of Rab5 from the membrane. Mon1/SAND-1 complexed with Ccz1 plays not only a critical role in recruitment and activation of Rab7, but also in mediating membrane tethering by binding to the homotypic fusion and protein sorting complex (HOPS: Vps11, Vps16, Vps18, Vps33, Vps39, and Vps41) (Wurmser et al, 2000; Nickerson et al, 2009). As soon as Rab7 is activated, it recruits its own effectors, such as RILP/ORP1L which regulates endo-lysosomal morphogenesis and enables transport of the maturing endosome along the minus-end directed microtubule (Cantalupo et al, 2001; Johansson et al, 2005; Johansson et al, 2007; Wang et al, 2011).

5.2.5.2 Rab GTPases and their influence on receptor trafficking
Ligand-induced activation of many hormone and growth factor receptors leads to intracellular signaling, internalization and trafficking to lysosomes for degradation. Receptor signaling is regulated through endocytosis, recycling, ubiquitination, inclusion into ILVs and degradation, whereby Rab GTPases play a significant role (Raiborg et al, 2002; von et al, 2007; Stenmark, 2009). The influence of
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Rab4 and Rab11 on recycling and therefore also signaling of β2AR, TfR and VEGFR-2 is discussed in detail in the chapter 8.1. Rab5 controls intracellular trafficking through receptor endocytosis and through endosomal dynamics (Zerial et al, 2001). Based on the findings that activation of RTKs lead to the formation of actin-based membrane ruffles through the activation of the small GTPases Ras and Rac (Bar-Sagi et al, 2000), Lanzetti et al. (2004) showed that Rab5 is the critical regulator in this pathway. Platelet derived growth factor (PDGF) induces the formation of circular ruffles, which however was inhibited by expression of a dominant negative Rab5 mutant. Rab5 activity was therefore required in this PDGF originated signaling pathway leading to circular ruffles. Rab7 regulates transport of receptors from early to late endosomes. Nerve growth factor (NGF) activated TrkA receptors are important for the mediation of neurite outgrowth. Rab7 was reported to control endosomal trafficking and neuritogenic signaling by directly interacting with TrkA. Inhibition of Rab7 activity resulted in accumulation of TrkA in endosomes, augmented TrkA signaling, potentiated phosphorylation of Erk1/2 and enhanced neurite outgrowth (Saxena et al, 2005). Missense mutations in Rab7 cause the autosomal dominant peripheral neuropathy Charcot-Marie-Tooth disease type 2B (CMT2B). Interestingly, Rab7 mutant causing CMT2B was shown to interact with TrkA similarly as Rab7 WT, but enhanced TrkA phosphorylation significantly in response to NGF stimulation and increased Erk1/2 phosphorylation, which was triggered on signaling endosomes (BasuRay et al, 2010). These observations let suggest a mechanistic link between Rab7 CMT2B mutants and altered TrkA and Erk1/2 signaling from endosomes. Mutations in Rab23 were associated with the neurological disease Carpenter syndrome in humans and the embryonically lethal open brain (opb) mutant phenotype in mice (Gunther et al, 1994; Jenkins et al, 2007). Rab23 acts as a negative regulator of sonic hedgehog (shh) signaling during dorsoventral development of the mouse spinal cord (Eggenschwiler et al, 2001). Rab40 was also reported to act in a signaling pathway by recruiting components of the ubiquitination machinery to regulate Wnt signaling and normal gastrulation in Xenopus (Lee et al, 2007).
5.2.6 Influence of Rabs and PIs on EGFR signaling and trafficking

The epidermal growth factor receptor (EGFR, also known as HER1 or ErbB1) is a member of the human epidermal growth factor receptor (HER)-erbB family of receptor tyrosine kinases (RTKs) and plays an important role in cell growth, proliferation and development. Ligand-binding triggers EGFR homo- or heterodimerization with its family members HER2 (ErbB2), HER3 (ErbB3) and/or HER4 (ErbB4). This induces autophosphorylation of the intracellular domain through intrinsic tyrosine kinase activity and activates downstream signaling pathways like Ras/Raf mitogen activated protein kinase (MAPK), the PI3K/AKT, the signal transducer and activator of transcription (STAT) and the phospholipase Cγ/protein kinase C (PKC) pathways. Overexpression or constitutive activation of EGFR is characteristic for many tumors with increased cell growth, proliferation and survival, making EGFR an attractive target for anticancer therapy (Hynes et al, 2009). Frequently used EGFR inhibitors in clinics are the monoclonal antibodies (1) Cetuximab and (2) Panitumumab and the less selective small-molecules (3) Erlotinib and (4) Gefitinib, all of which inhibit EGFR activation (reviewed in (Ciardiello et al, 2008)). The length of signaling is highly dependent on ligand-stimulated endocytosis, receptor ubiquitination and subsequent lysosomal degradation as well as on the rate of recycling (Roepstorff et al, 2009; Sorkin et al, 2009; Sorkin et al, 2009). A good overview, summarizing the regulation of Rab proteins on EGFR endocytoc trafficking, was published by Ceresa et al. (2006).

Rab5: Barbieri et al. (2000) reported that EGF stimulation of NR6 cells induced Rab5a activation and enhanced Rab5 effector EEA1 translocation from cytosol to early endosomes, which concomitantly activated endocytosis and internalization of EGFR. EGF stimulation was also shown to regulate cell proliferation. By inducing release of Rab5 effector APPL1 from the membrane, APPL1 translocated to the nucleus, where in complex with APPL2 interacted with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1 (Miaczynska et al, 2004).
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**Rab4:** Not much is known about the role of Rab4 in EGFR trafficking and signaling. McCaffrey et al. (2001) reported that expression of a dominant negative mutant Rab4 (Rab4AS22N) in HeLa cells lead to a significant reduction in both recycling and degradation of EGF.

**Rab11:** Rab11-family interacting proteins (FIPs) include FIP1, FIP2, FIP3/Efrin, FIP4, FIP5/Rip11 and RCP, all of which have been identified to potentially mediate Rab11 effects in recycling endosomes. Rab11-FIP2 associates with the α-adaptin subunit of AP-2 complexes, which are known to recruit receptors into clathrin-coated vesicles (Cullis et al, 2002). Rab11a-FIP2 has been reported to form a ternary complex with the myosin Vb, representing a possible sorting mechanism back to the plasma membrane (Hales et al, 2001; Hales et al, 2002). However, overexpression of FIP2 suppresses the internalization of EGFR, favoring Rab11-FIP2 function in endocytosis and subsequent sorting of receptor-containing vesicles in endosomes (Cullis et al, 2002). *In vitro* studies of immortal, nontumorigenic MCF10A breast cells transfected with Rab11a or dominant negative Rab11a resulted in accelerated or postponed EGFR recycling, respectively (Palmieri et al, 2006). In contrast to FIP2, these results clearly support a function of Rab11a in recycling. Furthermore, they could show that Rab11a modulates EGFR recycling, promotes the proliferation but inhibits the motility of immortal MCF10A breast cells.

**Rab7:** Similar to the effect of Rab7 on TrkA trafficking (see 5.2.5.2), expression of inactivated Rab7 mutant in HeLa cells lead to the accumulation of the EGFR/EGF complex in LEs. Furthermore, expression of dominant negative Rab7 slowed the rate of EGFR degradation, suggesting that functional Rab7 is required for the degradation EGFR/EGF complex by the lysosome (Ceresa et al, 2006).

**Rab22B:** The small GTPase Rab22B was found to be highly enriched in astroglia lineage and likely has a role in TGN-endosome transport (Rodriguez-Gabin et al, 2001). Ng et al. (2009) showed that Rab22B interacts with EGFR in a GTP-dependent manner. Furthermore, they reported that Rab22B silencing prevented
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or delayed EGFR trafficking to late endosomal compartments and reduced EGF degradation.

**PI-3-P:** By inhibition of PI3 kinase with wortmannin, EGFR was trapped in early/recycling endosomes, showing that PI-3-P controls the sorting of EGFR in early endosomes (Petiot et al, 2003). Hrs and SNX1 both bind to PI-3-P and in HeLa colocalize to early endosomes, but not cytosol (Chin et al, 2001). Furthermore, overexpression of Hrs, SNX1, Hrs/SNX1 complex or the SNX1-binding domain of Hrs inhibited ligand-induced degradation of EGFR (Kurten et al, 1996; Chin et al, 2001).

**PI-3,5-P₂:** PIKfyve is a PI 5 kinase and converts PI-3-P to PI-3,5-P₂. In transitional carcinoma cells (TCCSUP) PIKfyve was shown to interact with cytoplasmic EGFR and to mediate EGFR translocation to the nucleus. Transfection of wild-type PIKfyve increased the level of nuclear-resident EGFR, whereas silencing of PIKfyve by siRNA inhibited nuclear accumulation of EGFR significantly (Kim et al, 2007). In HeLa cells siRNA knockdown of PIKfyve alone produced no defect in EGFR degradation, however a combined knockdown with Vac14 revealed a modest inhibition of EGFR degradation. This suggested that a low threshold of PI-3,5-P₂ is necessary and sufficient for this pathway (de et al, 2009). Additionally, pharmacological inhibition of PIKfyve resulted in a profound block to the lysosomal degradation and accumulation of EGFR in the interior of a swollen, EEA1 positive endosomes. Additionally to PI-3-P, MTM1 and MTMRs bind to PI-3,5-P₂ and hydrolyzes it to PI-5-P. Overexpression of MTM1 has been shown to inhibit EGFR degradation and to induce large endosomal vacuoles (Tsujita et al, 2004). Furthermore, overexpression of MTMR2 was shown to inhibit EGFR degradation, whereas coexpression of SBF2 counteracted this effect (Berger et al, 2009). Additionally, Berger at al. observed sustained Akt activation upon MTMR2 overexpression, which was not affected by coexpression of SBF2. The PX domain of SNX5 specifically binds to PI-3,5-P₂, PI-4-P and PI-5-P, but not PI-3-P as SNX1 does (Liu et al, 2006). Overexpression of SNX5 was shown to inhibit the degradation of EGFR, whereas overexpression of SNX1 was able to attenuate this effect. SNX5 and SNX1 were therefore suggested to
play antagonistic roles in regulating endosomal trafficking of the receptor, similar to MTMR2 and Sbf2. It should also be mentioned, that no direct interaction between SNX5 and EGFR was detected. However, direct interactions were reported for SNX1 and EGFR, as well as for SNX1 and SNX5, pointing to a regulatory function of SNX1 (Chin et al, 2001; Liu et al, 2006).

**PI-4,5-P$_2$ (PIP$_2$):** EGFR activation starts a signaling cascade that includes direct tyrosine phosphorylation of phospholipase C$_\gamma$. Subsequent PI-4,5-P$_2$ hydrolysis to inositol-1,4,5-P$_3$ and diacylglycerol leads to Ca$^{2+}$ mobilization and PKC activation, respectively (Rhee, 2001). Recently Michaelidis et al. (2011) reported, that in oocytes PI-4,5-P$_2$ down-regulation by PI kinase inhibitor Wortmannin severely impaired EGFR activation, whereas up-regulation of PI-4,5-P$_2$ by overexpression of PIP5K1a potentiated EGF-mediated activation of the receptor.
5.3 Aim of the thesis

Although MTM1 and MTMR2 are expressed in the same tissues and although they share the same substrate specificity and a high sequence similarity (~64%), it is still unclear how mutations in these enzymes can lead to such distinct human diseases. Interaction of MTM1 and MTMR2 with different adaptor proteins including scaffolding proteins and other MTMR family members, could lead to differential localization and action as well as differential sorting and signaling of membrane proteins. The first part of this study is dedicated to the screening, verification and characterization of novel PDZ-domain containing proteins interacting with MTMRs. The identification of new PDZ-domain containing proteins gives the opportunity to study the influence of MTMRs on the trafficking and sorting of yet unidentified PDZ-dependent protein and membrane receptor complexes. The second part of this thesis focuses on the structural analysis of the protein complexes Mtmr2/Sbf2, Mtmr2/Sap97 and Mtmr2/3-Pap using analytical methods such as immunoprecipitation, size exclusion chromatography, multi-angle light scattering and small-angle X-ray scattering. For detailed analysis of the Mtmr2/3-Pap protein complex, the subcellular localization of these proteins in cultured cells will be specified by help of coexpression with endosomal markers. To elucidate the functional aspects of 3-Pap and Mtmr2/3-Pap complex on endocytic EGFR trafficking, the localization of the individual proteins will be monitored after different timepoints after EGF stimulation using light microscopy.
Experimental Procedures

6 Experimental Procedures

6.1 Screen for novel binding partners of MTMRs

6.1.1 Cloning

6.1.1.1 GST-MTMR constructs for screening of the PDZ domain array

The PDZ domain proteomic array is an excellent tool to screen for 96 different PDZ domains of PDZ-domain containing proteins (Fam et al, 2005). All PDZ domains spotted on the membrane are His- and S-tagged fusion proteins (see also chapter 6.1.6). The PDZ array is overlaid with GST fused to the carboxy-terminus of the bait protein. To assess the binding of myotubularins (MTMRs), the carboxy-terminal 25 amino acids of each MTMR (named C-term), had to be expressed as GST-fusion proteins. From human or mouse genomic DNA or appropriate cDNA the C-termini were amplified by PCR using Phusion High-Fidelity DNA polymerase (Finnzymes) and forward primers containing an *N*<sup>col</sup> restriction site and reverse primers containing a *Xho* restriction site (listed in Table 6-1). PCR products were digested with *N*<sup>col</sup> and *Xho* and ligated using T4-ligase (Fermentas) into *N*<sup>col</sup>/*Xho* cut pET42a-TEV vector (modified vector with an N-terminal TEV cleavage site downstream of the GST coding sequence). The ligation mixture was transformed into electro-competent *Escherichia coli* (E. coli) DH10β-cells for amplification and selection. All constructs were verified by restriction digest and DNA sequencing.

Table 6-1: Primers used for cloning of MTMRs into the expression vector pET42a-TEV.

Restriction sites *N*<sup>col</sup> in forward primers (For) and *Xho* in reverse primers (Rev) are underlined.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer sequence 5'-3'</th>
<th>Primer Name</th>
</tr>
</thead>
</table>
| pET42a-TEV C-term mSbf1 | For: 5'-GATCCCATGGGATACAACCTTCTGTGCCAGACCC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | mSbf1GSTfor  
                       mSbf1GSTback |
| pET42a-TEV C-term mSbf2 | For: 5'-GATCCCATGATAACCTTCTGTGCCAGACTCGC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | mSbf2GSTfor  
                       mSbf2GSTback |
| pET42a-TEV C-term mMtm1 | For: 5'-GATCCCATGGGATACCTTCGTGCCAGACCC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | mMtm1GSTfor  
                       mMtm1GSTback |
| pET42a-TEV C-term hMTMR1 | For: 5'-GATCCCATGGGATACCTTCGTGCCAGACCC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | hMTMR1GSTfor  
                       hMTMR1GSTback |
| pET42a-TEV C-term mMtmr1 | For: 5'-GATCCCATGGGATACCTTCGTGCCAGACCC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | mMtmr1GSTfor  
                       mMtmr1GSTback |
| pET42a-TEV C-term mMtmr2 | For: 5'-GATCCCATGGGATACCTTCGTGCCAGACCC3'  
                      Rev: 5'-GATCTTCAGTCAGCAGCCACAGGAC3' | mMtmr2GSTfor  
                       mMtmr2GSTback |
6.1.1.2 Constructs for PDZ domain mapping of Sap97

The coding regions of Sap97-PDZ1, Sap97-PDZ2, Sap97-PDZ3, Sap97-PDZ1-2, and Sap97-PDZ1-3 were amplified from mouse cDNA pCMV-Sport6-Sap97 (accession number: NM_007862.2) by a two-step PCR using the primers listed in Table 6-2. All Sap97 PDZ fragments were generated without the L27, SH3 and MAGUK domains. Restriction sites SalI and NotI were introduced for insertion into the MCS1 of pFBDM His-Mtmr2 vector (Berger et al, 2006a), resulting in dual expression vectors pFBDM His-Mtmr2_HA-Sap97-PDZ. PCR products and target backbone plasmid pFBDM His-Mtmr2 were digested with SalI and NotI and ligated using T4-ligase (Fermentas). Recombined plasmids were transformed into electro-competent E. coli DH10β-cells for amplification and selection. All constructs were verified by restriction digest and DNA sequencing.

Table 6-2: Primers used for amplification of Sap97 PDZ domains

Coding regions were amplified from pCMV-Sport6-Sap97 using the listed primers. Restriction sites SalI (in SAP97 univ for) and NotI (in all reverse primers) are underlined.

<table>
<thead>
<tr>
<th>Coding Region/Primer Name</th>
<th>Primer sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP97 PDZ1 Sap97 univ for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1 for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1 rev</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ2 Sap97 univ for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ2 for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ2 rev</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ3 Sap97 univ for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ3 for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ3 rev</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1-2 Sap97 univ for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1 for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1-2 rev</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
<tr>
<td>SAP97 PDZ1-3 Sap97 univ for</td>
<td>5’-GATCGTCGACATGTACCCATACGATGTTCCAGATTACGCTGATTACGATATCCCAACGACCGAGAACCTCTACTTCCAGGGCGCCGATGCAGATTATGAATATG-3’</td>
</tr>
</tbody>
</table>
6.1.1.3 Cloning of pcDNA3.1 RGS-His Mtm1-ΔPDZ
RGS-His-tagged Mtm1-ΔPDZ construct, devoid of the last five amino acids, was generated by PCR-subcloning. (Geiser et al, 2001) By using pcDNA3.1 zeo(+) RGS-His-Mtm1 (obtained from Philipp Berger) as template, RGS-His-Mtm1 was amplified and simultaneously the PDZ-BD was looped out by using the following primers: oligo408 (5'-GCCCCATTGACGCAAATGG-'3) and mMtm1 c-del2 rev (5'-GGCCCTCTAGACTCGACG GCCGCTCAATGGGGCACCATCTG TGACG-3'). The first PCR product was purified and subsequently served as primer for a second PCR step to amplify the plasmid backbone linked to the insert. pcDNA3.1 zeo(+) RGS-His-Mtm1-ΔPDZ was verified by restriction digest and sequencing.

6.1.2 Protein expression
6.1.2.1 Protein expression of GST-tagged MTMR C-termini and His-tagged PDZ domains in E. coli
For the expression of GST-tagged MTMR C-termini (listed in Table 6-1) and His- and S-tagged PDZ domains (listed in Table 6-3, generous gift from Randy Hall, Emory University, Atlanta) electro-competent E. coli Acella cells (EdgeBio, #42649) were transformed and grown in LB medium at 37°C, containing 50 µg/ml kanamycin, to an OD$_{600}$ of 0.6. Protein expression was induced by addition of isopropyl beta-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation (10'000 x g, 10 min, 4°C) 4 hours after IPTG induction and the pellet was stored at -80°C.

<table>
<thead>
<tr>
<th>Table 6-3: pET30a PDZ domain plasmids used for expression in E.coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of plasmids for the expression of His- and S-tagged PDZ-domain containing proteins. Plasmids were obtained from Randy Hall (Emory University, Atlanta).</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>pET-30a MAGI-3 PDZ1</td>
</tr>
<tr>
<td>pET-30a NHERF-1 PDZ2</td>
</tr>
<tr>
<td>pET-30a NHERF-2 PDZ2</td>
</tr>
<tr>
<td>pET-30a PSD-95 PDZ3</td>
</tr>
<tr>
<td>pET-30a nNOS PDZ</td>
</tr>
<tr>
<td>pET-30a SAP97 PDZ1+2</td>
</tr>
<tr>
<td>pET-30a SAP97 PDZ3</td>
</tr>
<tr>
<td>pET-30a ERBIN PDZ</td>
</tr>
</tbody>
</table>
Experimental Procedures

<table>
<thead>
<tr>
<th>pET-30a C2PA PDZ</th>
<th>His-S-tag-C2PA PDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-30a GIPC</td>
<td>His-S-tag-GIPC</td>
</tr>
<tr>
<td>pET-30a DENSIN-180</td>
<td>His-S-tag-DENSIN-180</td>
</tr>
<tr>
<td>pET-30a MUPP PDZ8</td>
<td>His-S-tag-MUPP PDZ8</td>
</tr>
<tr>
<td>pET-30a PTPN13 PDZ4+5</td>
<td>His-S-tag-PTPN13 PDZ4+5</td>
</tr>
<tr>
<td>pET-30a PDZK1 PDZ3</td>
<td>His-S-tag-PDZK1 PDZ3</td>
</tr>
<tr>
<td>pET-30a PDZK2 PDZ1</td>
<td>His-S-tag-PDZK2 PDZ1</td>
</tr>
<tr>
<td>pET-30a PDZK2 PDZ3</td>
<td>His-S-tag-PDZK2 PDZ3</td>
</tr>
<tr>
<td>pET-30a LNX PDZ3</td>
<td>His-S-tag-LNX PDZ3</td>
</tr>
</tbody>
</table>

6.1.2.2 Protein expression of His-tagged Mtmr2 and HA-tagged SAP97 PDZ domains in Sf21 insect cells

The generation of baculovirus DNA, virus production, Sf21 insect cell transfection and protein expression was performed as described (Fitzgerald et al, 2006). Sf21 insect cells were infected with baculovirus encoding His-Mtmr2 together with HA-Sap97-PDZ1, Sap97-PDZ2, Sap97-PDZ3, Sap97-PDZ1-2 or Sap97-PDZ1-3. The cells were grown in Lonza serum-free Insect-XPRESS™ medium with L-glutamine (Lonza, #BE12-730Q) at 27°C, kept at a cell density of 0.5 x 10^6 cells/ml and harvested 5 days after infection. After centrifugation (900 x g, 10 min, RT) the cell pellets were stored at -80°C.

6.1.2.3 Protein expression of Mtmr2/Sbf2 complex in Sf21 insect cells

Sf21 insect cells were infected with baculovirus, which was earlier produced by Philipp Berger (Berger et al, 2006a). The plasmids pFBDM His-Mtmr2/CBP-Sbf2, pFBDM His-Mtmr2/CBP-Sbf2-MT+PH and pFBDM His-Mtmr2/CBP-Sbf2-MT were used for the generation of baculovirus DNA and virus production. The cells were grown in Lonza serum-free Insect-XPRESS™ medium with L-glutamine (Lonza, #BE12-730Q) at 27°C, kept at a cell density of 0.5 x 10^6 cells/ml and harvested 5 days after infection. After centrifugation (900 x g, 10 min, RT) the cell pellets were stored at -80°C.

6.1.3 Protein purification

6.1.3.1 Protein purification of GST-tagged MTMR C-termini

Harvested cell pellets were thawed on ice and resuspended in resuspension buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 2 mM PMSF, 3mM DTT, Roche protease inhibitor cocktail). The suspension was sonicated with 50 pulses on ice and the soluble protein fraction was isolated by
Experimental Procedures

centrifugation (20’000 x g, 10 min, 4°C). The cleared lysate was then passed through a 0.22 µm filter unit. The GST-tagged proteins were bound on a GST Trap™ FF column (GE Healthcare), washed with washing buffer with increasing sodium chloride concentrations (50 mM Tris-HCl pH 7.5, 100-600 mM NaCl, 0.1% Triton X-100), and eluted in elution buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 10 mM reduced glutathione). Purified proteins were stored at -20°C.

6.1.3.2 Protein purification of His-tagged PDZ-domain proteins
Harvested cell pellets were thawed on ice and resuspended in resuspension buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 2 mM PMSF, 3 mM DTT, 10 mM imidazole, Roche protease inhibitor cocktail). The suspension was sonicated with 50 pulses on ice and the soluble protein fraction was isolated by centrifugation (20’000 x g, 10 min, 4°C). Cleared lysate was then passed through a 0.22 µm filter unit. The His-tagged proteins were bound on a nickel charged HiTrap™ Chelating HP column (GE Healthcare), washed with binding buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 0.01% Tween-20), and eluted in elution buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.01% Tween-20, 0.5 M imidazole). Purified proteins were stored at -20°C.

6.1.3.3 Protein purification of His-Mtbr2/HASap97-PDZ1-2 complex
Harvested cell pellets were thawed on ice and resuspended in resuspension buffer (100 mM Tris-HCl pH 8.6, 200 mM NaCl, 0.01% Tween-20, 2 mM PMSF, 3mM DTT, 10 mM imidazole, Roche protease inhibitor cocktail). The complex was purified with a nickel charged HiTrap™ Chelating HP column (GE Healthcare). The column was equilibrated and washed with washing buffer (100 mM Tris-HCl pH 8.6, 200 mM NaCl, 10 mM imidazole, 0.01% Tween-20) and the complex was eluted in elution buffer (100 mM Tris-HCl pH 8.6, 200 mM NaCl, 0.01% Tween-20, 0.5 M imidazole). The eluate of the NiNTA column was loaded on a Superdex 200 16/60 gel filtration column for size exclusion chromatography (SEC) and eluted in 100 mM Tris-HCl pH 8.6, 200 mM NaCl and
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1 mM DTT. NiNTA and SEC eluted samples were analyzed by Coomassie stained SDS-PAGE.

6.1.3.4 Protein purification of His-Mtmr2/CBP-Sbf2 complexes
Harvested cell pellets were thawed on ice and resuspended in buffer (previously tested in a solubility screen) as follows: His-Mtmr2/CBP-Sbf2 (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM imidazole, Roche protease inhibitor cocktail); His-Mtmr2/Sbf2-MT and His-Mtmr2/Sbf2-MT+PH (100 mM Bis-Tris pH 6.5, 200 mM NaCl, 10 mM imidazole, Roche protease inhibitor cocktail). The complex was purified with a nickel charged HiTrap™ Chelating HP column (GE Healthcare). The column was equilibrated and washed with washing buffer (same as the according resuspension buffer, but without protease inhibitors) and the complex was eluted in elution buffer (same as the according washing buffer, but with 0.5 M imidazole instead). The eluate of the NiNTA column was purified with a Superdex 200 16/60 column (GE Healthcare) equilibrated in gelfiltration buffer (same as the according washing buffer, but without imidazole). NiNTA and SEC eluted samples were analyzed by Coomassie stained SDS-PAGE.

6.1.4 Protein crystallization
All protein samples were concentrated with Vivaspin (Sartorius) centrifugal protein concentrators with a molecular weight cut-off (MWCO) of 50 Kilo Dalton (KDa) and filtered with a 0.22 µm centrifugal filter device (Millipore) to the final protein concentrations listed in Table 6-4. Protein crystallization screens were set up in Innovaplate™ SD-2 (Innovadyne Technologies, Inc.) using the Crystal PHOENIX dispenser (Art Robbins Instruments). Crystallization drops were dispensed in protein:precipitant ratios of 1:1 and the screens were incubated at 20°C. His-Mtmr2/CBP-Sbf2 was manually screened in a 24-well plate (Greiner) with buffer conditions ranging from pH 6.0-8.6 and polyethyleneglycole with a size of 35 KDa (PEG 35'000) of 2%, 2.5%, 2.75% and 3%. The reservoir contained 500 µl screening solution and the protein was pipetted in a protein:precipitant ratio of 1:1.
**Table 6-4: Applied crystallization screens for His-Mtmr2/CBP-Sbf2 complexes**
The protein complex analyzed for crystal growth is represented with the used protein concentration, the applied crystal screen and additives treatment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. [mg/ml]</th>
<th>Screen</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-Mtmr2/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBP-Sbf2-MT</td>
<td>2.4; 10.0</td>
<td>Nextal- The Classics Lite Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4; 10.0</td>
<td>Nextal- The Classics Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4; 10.0</td>
<td>Nextal - The JCSG+ Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.4; 10.0</td>
<td>Nextal - The PEGs Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5; 10.3</td>
<td>Nextal - The pHClear Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5; 10.3</td>
<td>Nextal - The pHClear II Suite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5; 10.3</td>
<td>Qiagen - The JCSG Core Suite I-IV</td>
<td>0.3 mM Pi-3-P</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>Qiagen - The JCSG Core Suite I-IV</td>
<td>0.3 mM Pi-3,5-P_2</td>
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<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His-Mtmr2/</td>
<td>3.7; 7.4</td>
<td>Nextal - The pHClear Suite</td>
<td></td>
</tr>
<tr>
<td>CBP-Sbf2-MT+ PH</td>
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<td>Qiagen - The JCSG Core Suite I-IV</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.3 mM Pi-3,5-P_2</td>
</tr>
<tr>
<td>His-Mtmr2/</td>
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<td>24-well manual screen:</td>
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</tr>
<tr>
<td>CBP-Sbf2</td>
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<td>100 mM MES pH 6.0 and 6.5</td>
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<td></td>
<td></td>
<td>100 mM HEPES pH 7.0 and 7.5</td>
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<tr>
<td></td>
<td></td>
<td>100mM Tris-HCl pH 8 and 8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEG35'000 2%-3%</td>
<td></td>
</tr>
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</tr>
</tbody>
</table>

### 6.1.5 Mass spectrometry

To analyze the amino acid composition of the purified GST-tagged proteins, the peptides were analyzed by mass spectrometry (MS) at PSI (Alain Blanc, Center for Radiopharmaceutical Sciences). The identification was performed using a high-pressure liquid chromatography (HPLC, Waters 2796 Separations module) coupled to an electrospray ionization mass spectrometer (ESI-MS, Waters Micromass LCT-Premier, ESI-TOF, Waters Corporation, Milford, MA). The algorithm software Maxent I Electrospray was used for deconvolution of the data.

### 6.1.6 Proteomic PDZ-domain array overlay with GST-tagged MTMRs

To identify novel PDZ-domain containing proteins interacting with the C-terminal PDZ-binding domain (PDZ-BD) motif of the GST-tagged MTMRs, we made use of a PDZ array based screen (Fam et al, 2005). Our collaborator (Randy Hall, Emory University, Atlanta) kindly provided us 10 nylon membranes (SuperCharge 96-grid, Schleicher & Schuell BioScience, #10416257) spotted with 96 different His- and S-tagged class I PDZ domain fusion proteins (~1µg/bin) (**Table 6-5**). Class I PDZ domains are preferentially bound by carboxyl-terminal PDZ-BD motifs consisting of S/T-x-§ (where § represents a hydrophobic residue).
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### Table 6-5: List of the His- and S-tagged PDZ fusion proteins spotted on the PDZ array, delivered by Randy Hall

<table>
<thead>
<tr>
<th>Bin</th>
<th>PDZ Domain</th>
<th>Bin</th>
<th>PDZ Domain</th>
<th>Bin</th>
<th>PDZ Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>MAGI-1 PDZ1</td>
<td>C9</td>
<td>ERBIN PDZ</td>
<td>F5</td>
<td>MUPP1 PDZ12</td>
</tr>
<tr>
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<td>C10</td>
<td>ZO-1 PDZ1</td>
<td>F6</td>
<td>MUPP1 PDZ13</td>
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<tr>
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<td>C11</td>
<td>ZO-1 PDZ2</td>
<td>F7</td>
<td>PTPN13 PDZ1</td>
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<td>C12</td>
<td>ZO-1 PDZ3</td>
<td>F8</td>
<td>PTPN13 PDZ3</td>
</tr>
<tr>
<td>A5</td>
<td>MAGI-2 PDZ1</td>
<td>D1</td>
<td>ZO-2 PDZ1</td>
<td>F9</td>
<td>PTPN13 PDZ4+5</td>
</tr>
<tr>
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<td>MAGI-2 PDZ2</td>
<td>D2</td>
<td>ZO-2 PDZ2</td>
<td>F10</td>
<td>PDZK1 PDZ1</td>
</tr>
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<td>F11</td>
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<tr>
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<td>PDZ-GEF1 PDZ</td>
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<td>SPINOPHILIN PDZ</td>
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<td>MUPP1 PDZ1</td>
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<td>SHANK1 PDZ</td>
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<td>MUPP1 PDZ6</td>
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<td>F3</td>
<td>MUPP1 PDZ8</td>
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<td>F4</td>
<td>MUPP1 PDZ10</td>
<td>H12</td>
<td>PAR-3 PDZ3</td>
</tr>
</tbody>
</table>

The membranes were blocked in blocking buffer (2% milk powder, 0.1% Tween-20, PBS) for 1 hour at room temperature (RT). The PDZ arrays were overlaid with the purified GST-tagged MTMR fusion proteins and incubated on a shaker for 1 hour with (25 nM GST-MTMR in blocking buffer). After incubation with GST fusion proteins, the membranes were washed three times for 5 min with blocking buffer. For detection the PDZ arrays were incubated with blocking buffer containing the following antibody dilutions: goat anti-GST (Amersham, #27-4577-01, 1:5000) and horse radish peroxidase (HRP) coupled
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Secondary rabbit anti-goat antibody (Southern Biotech, #4010-05, 1:10'000). Prior incubation with secondary antibodies the membranes were washed in blocking buffer and after incubation washed in 0.1% Tween-20/PBS. Binding of GST-MTMRs was visualized with ECL Plus western blotting detection kit (Amersham, #RPN2132).

The PDZ-array results were visually evaluated by Philip Berger and Katharina Schmid and summarized in a 96-grid scheme, reflecting the PDZ array. The signal strength was related to the overall signals and rated from no signal (=0) to strongest signal (=4) (Figure 7-3).

6.1.7 Pulldown experiments

6.1.7.1 GST pulldown experiments
Five microgramm purified GST-tagged MTMR protein and 0.5 µg purified His-tagged PDZ-domain protein was subjected with 20 µl glutathione beads slurry (50% in PBS) and the final volume was adjusted to 0.5 ml with lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 2 mM PMSF, 3 mM DTT, Roche protease inhibitor cocktail). The protein/beads suspension rotated overnight at 4°C. After incubation, the beads were washed 2 times with lysis buffer and bound protein was eluted with 30 µl elution buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.01% Tween-20, 10 mM glutathione). Eluted supernatant was mixed with SDS PAGE Laemmli buffer and boiled for 5 min at 95°C. The protein samples were separated on a 12% SDS-PAGE and identified by immunoblotting using mouse anti-penta-His (Qiagen, #34660, 1:1000) and mouse anti-S-tag (Novagen, #71549-3, 1:5000) as primary antibodies and alkaline phosphatase (AP) coupled secondary antibodies goat anti-mouse (Southern Biotech, #1031-04, 1:10’000). Lumi-Phos™ WB (Thermo Scientific) was used as AP substrate.

6.1.7.2 Immunoprecipitation of His-Mtm1 and Myc-Pdzk2 in HEK293 cells
The plasmid pcDNA3.1 hygro Myc-Pdzk2 for mammalian expression of Pdzk2 was kindly provided by our collaborator Jürg Biber (Institute of Physiology, University of Zürich). The plasmids pcDNA3.1 zeo(+) RGS-His-Mtm1 and
pcDNA3.1 zeo(+) RGS-His-Mtmr2 were cloned by Philipp Berger and pcDNA3.1 zeo(+) RGS-His-Mtm1-ΔPDZ was cloned using pcDNA3.1 zeo(+) RGS-His-Mtm1 as template (see 6.1.1.3). HEK293 cells with a confluency of 80% were transiently transfected by the calcium phosphate transfection method. For 10 cm dishes, 600 µl solution A (250 mM CaCl$_2$) containing 30 µg DNA were mixed with 600 µl solution B (140 mM NaCl, 50 mM HEPES, 1.5 mM Na$_2$HPO$_4$, adjusted to pH 7.05). The mixture was incubated for 1 minute and then added to the cells. The cells were maintained at 37°C in 5% CO$_2$ and grown in DMEM medium (BioConcept, #1-26F03-I) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The medium was changed after 16 hours and 40 hours after transfection the cells were washed with PBS and removed from the plates with 4 ml lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 2 mM PMSF, Roche protease inhibitor cocktail). The cell suspension was sonicated with 6 pulses and cell debris was removed by centrifugation (10'000 x g, 10 min, 4°C). Two milliliter of the supernatant was incubated for 16 h with 60 µl protein A Sepharose CL-4B beads slurry (50% in PBS, GE Healthcare) and mouse anti-penta-His antibodies (Qiagen, #34660, 1:1000). After incubation the mixture was centrifuged (500 x g, 5 min, 4°C) and the beads were washed two times with lysis buffer. Bound protein was eluted by boiling the beads in 30 µl Laemmli SDS-PAGE sample buffer for 5 min at 95°C. The protein samples were separated on a 12% SDS-PAGE gel and identified by immunoblotting using the following primary antibodies: mouse anti-Myc 9E10 (selfmade, 1:100) and mouse anti-penta-His (Qiagen, #34660, 1:2000). As secondary antibodies alkaline phosphatase coupled goat anti-mouse AP (Southern Biotech, #1031-04, 1:10'000) was used. Lumi-Phos™ WB (Thermo Scientific) was used as AP substrate.

6.1.7.3 Immunoprecipitation of Mtm1 and Pdzk2 in mouse tissues
Tissue of wildtype mice were obtained from Dr. Michaela Miehe (Lab of Prof. Ueli Suter, Institute of Cell Biology, ETH Zurich) and weighted (Table 6-6).
### Experimental Procedures

**Table 6-6: List of analyzed mouse tissues and total amount used for the immunoprecipitation experiment**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
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<td>Liver</td>
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</tr>
<tr>
<td>Kidney</td>
<td>0.14 g</td>
</tr>
<tr>
<td>Heart</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.18 g</td>
</tr>
<tr>
<td>Lung</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Cortex</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.17 g</td>
</tr>
<tr>
<td>Muscle (hind leg)</td>
<td>0.24 g</td>
</tr>
</tbody>
</table>

Tissue samples with a weight of 0.14-0.25 g were homogenized in 4 ml lysis buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 2 mM PMSF, Roche protease inhibitor cocktail) using a polytron. Cell debris was separated from soluble protein by centrifugation (4500 x $g$, 20 min, 4°C). The supernatant was then incubated with 30 µl protein A Sepharose CL-4B (GE Healthcare) beads for 1 h at 4°C, to clear the supernatant from endogenous mouse IgGs. The pre-cleared lysate was then incubated with 30 µl protein A Sepharose and 0.5 µg mouse anti-Mtm1 (Abnova, #H00004534-M01) antibody for 16 h at 4°C. Beads were centrifuged (500 x $g$, 5 min, 4°C) and washed two times with lysis buffer and bound protein was eluted by boiling the beads in 30 µl Laemmli SDS-PAGE sample buffer for 5 min at 95°C. The protein-samples were separated on an 8% SDS-PAGE gel and identified by immunoblotting as follows: rabbit anti-Pdzk2 (Thermo Scientific, #PA3-16819, 1:2000) and mouse anti-Mtm1 (Abnova, #H00004534-M01, 1:2000). Alkaline phosphatase coupled goat anti-rabbit AP (Southern Biotech, #4030-04, 1:10'000) and goat anti mouse AP (Southern Biotech, #1031-04, 1:10'000) were used as secondary antibodies for chemiluminescence detection. Lumi-Phos™ WB (Thermo Scientific) was used as AP substrate.

**6.1.7.4 Identification of Sap97 PDZ domains interacting with Mtmr2**

Sf21 insect cells were distributed in a 6-well plate with a final density of 1.5 x 10$^6$ cells/well and infected with 1 µl of baculovirus V1 each. Five days after
infection Sf21 insect cells expressing His-Mtmr2 and HA-Sap97 PDZ domains (PDZ1, PDZ2 PDZ3, PDZ1-2 or PDZ1-3) were harvested and resuspended in lysis buffer (100 mM Tris-HCl pH 8.6, 200 mM NaCl, 0.01% Tween-20, Roche protease inhibitor cocktail). The suspension was sonicated and cell debris was removed by centrifugation (10'000 x g, 10’, 4°C). The cleared lysate was incubated for 16 h with 60 µl 50% protein A Sepharose CL-4B bead slurry (GE Healthcare) and mouse anti-penta-His antibody (Qiagen, #34660, 1:100) at 4°C. The beads were centrifuged (500 x g, 5 min, 4°C) and washed two times with lysis buffer and boiled in Laemmli SDS-PAGE sample buffer for 5 min at 95°C. Eluted proteins were separated on a 12% SDS-PAGE and identified by immunoblotting as follows: rat anti-HA (3F10, Roche, #11867423001, 1:1000) and mouse anti-penta-His (Qiagen, #34660, 1:1000). Alkaline phosphatase-coupled goat anti-mouse (Southern Biotech, #1031-04, 1:10’000) or rabbit anti-rat (Southern Biotech, #6180-01, 1:10’000) were used as secondary antibodies, followed by chemiluminescence detection. Lumi-Phos™ WB (Thermo Scientific) was used as AP substrate.

6.1.8 Cell culture and immunofluorescence microscopy
HEK293, COS and OK cells were maintained at 37°C in 5% CO₂. HEK293 and COS-7 cells were cultured in DMEM (BioConcept, #1-26F03-I) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. OK cells were cultured in 43.5% DMEM (BioConcept, #1-26F03-I), 43.5% HAM’s F-12 (BioConcept, #1-14F03-I), 10% FBS, 2% HEPES pH 7.4, 100 units/ml penicillin and 100 µg/ml streptomycin. For transient transfection the cells were transfected in 3.5 cm dishes with FugeneHD (Roche) according manufacturer’s recommendations. For Mtm1 and Pdzk2 colocalization studies, HEK293 and COS-7 cells were transiently transfected with the following expression constructs: pcDNA3.1 Myc-Pdzk2 with pcDNA3.1 RGS-His-Mtm1, pcDNA3.1 RGS-His-Mtm1 C375S, pcDNA3.1 RGS-His-Mtm1-ΔPDZ or pcDNA3.1 RGS-His-Mtmr2. For the localization studies of Mtm1 and Pdzk2 with endosomal markers, HEK293 cells were transiently transfected with expression constructs for: RGS-His-Mtm1, Myc-Pdzk2 and YFP-Rab4-Q67L, GFP-Rab5A-Q79L, EGFP-Rab7-Q67L or
YFP-Rab11-Q70L. For the localization studies in OK NaPi-IIa cells, the expression constructs pcDNA3.1 Myc-Pdzk2 and pcDNA3.1 RGS-His-Mtm1 were transfected into polarized OK cells. The cells were fixed with 2% paraformaldehyde in PBS 48-72 hours after transfection. After permeabilization in buffer (1% NP-40/PBS) for 10 min the cells were incubated with primary antibodies for 3 hours and with secondary antibodies for 1.5 hours. Primary antibodies were diluted in PBS as follows: mouse anti-RGS-His (Qiagen, #34610, 1:200), mouse anti-Mtm1 (Abnova, #H00004534-M01, 1:200), rabbit anti-Pdzk2 (Thermo Scientific, #PA3-16819, 1:500). Secondary antibodies were labeled with Cy3, Cy5 or Alexa-488 (Invitrogen, Jackson ImmunoResearch Laboratories). Samples were mounted in Citifluor AF1 (Citifluor). For the analysis a confocal laser scanning microscope (Leica SP5) was used and single sections with a thickness of 0.5 µm were obtained.

6.2 Characterization of Mtmr2/3-Pap complex

6.2.1 Plasmids and cloning

6.2.1.1 Plasmids for transient and stable expression in HEK293 cells
The pcDNA3.1 plasmids encoding RGS-His-tagged Mtmr2, RGS-His-tagged Mtmr2 P589X and HA-tagged Sbf2 were previously described (Berger et al, 2006a; Berger et al, 2003). The construct for the expression of 3-Pap/Mtmr12 was kindly provided by H. Nandurkar (Nandurkar et al, 2001). His-tagged and V5-tagged 3-Pap cDNAs were amplified by PCR from pCMV-Sport6 3-Pap (mouse, Geneservice, IMAGE: 3661020) and cloned into the pcDNA3.1 zeo(+) expression vector using ligase independent cloning as described previously (Geiser et al, 2001). His-tagged and V5-tagged 3-Pap G654X missing the C-terminal predicted coiled-coil motif were generated by mutating glycine 654 into a stop codon. Using the templates pcDNA3.1 His-3-Pap and pcDNA3.1 V5-3-Pap and by using the oligonucleotides 3-Pap-G654X-for (5’-ccagaagcccaaatcttgtgagtcgagtctaggg-3’) and 3-Pap-G654X-rev (5’-ccctctagactgactcagagtttggtgtttgg-3’) the respective DNAs were amplified
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by PCR. All PCR-generated constructs were verified by restriction digest and DNA sequencing.

6.2.1.2 Plasmids for baculovirus-based insect cell expression
The construct pFBDM His-Mtmr2 for expression in Sf21 cells was previously described (Berger et al, 2006a). His- and Strep-tagII (named from now on “Strep”) – tagged 3-Pap constructs were generated by two-step PCR from pCMV-Sport6 3-Pap. Thereby an N-terminal TEV cleavage site and the restriction sites EcoRI and SalI (Table 6-7) were introduced. EcoRI/SalI digested PCR-fragments were ligated into EcoRI/SalI cut pFL vector (Fitzgerald et al, 2006) resulting in pFL His-3-Pap and pFL Strep-3-Pap. The constructs were verified by restriction digest and DNA sequencing.

Table 6-7: Primers used for amplification of His-3-Pap and Strep-3-Pap from pCMV-Sport6-3-Pap
Restriction sites EcoRI (in His_3PAP_for and Strep_3PAP_for) and SalI (in 3PAP_rev) are underlined.

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<td>His_3PAP_for</td>
<td>5'-GATCGAGAATCTTTATTTTCAGGGGCGAGACTGGGGAAAGGGGG-3'</td>
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<tr>
<td>TEV_for</td>
<td>5'-GATCGTCGACTCACGTCCCCTAGG-3'</td>
</tr>
<tr>
<td>3PAP_rev</td>
<td>5'-GATCGTCGACTCACGTCCCCTAGG-3'</td>
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<tr>
<td>Strep-3-Pap</td>
<td>5'-GATCGAATTCTATGTCTGACACACCATCACATCAACCAGTTTTATTCTTTTTTGAGGCC-3'</td>
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<td>5'-GATCGTCGACTCACGTCCCCTAGG-3'</td>
</tr>
<tr>
<td>3PAP_rev</td>
<td>5'-GATCGTCGACTCACGTCCCCTAGG-3'</td>
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6.2.2 Generation of stable cell lines and cell culture
Stable HEK293 FlpIn cell lines expressing His-tagged Mtmr2, HA-tagged Sbf2 or His-tagged Mtmr2 together with HA-tagged Sbf2 were previously described (Berger et al, 2006a; Berger et al, 2009). The HEK293 FlpIn cell line coexpressing His-tagged Mtmr2 and His-tagged 3-Pap was obtained by integrating pcDNA3.1 zeo(+) His-3-Pap into the stable HEK293 His-Mtmr2 using FugeneHD (Roche) as transfection reagent according manufacturer’s recommendations followed by selection with hygromycin (Roche, 50 µg/ml) and zeocin (Invitrogen, 100 µg/ml). HEK293wt cells were transfected with pcDNA3.1 zeo(+) His-3-Pap and selected with zeocin (Invitrogen, 100 µg/ml) to generate
HEK293 cells expressing His-3-Pap. HEK293 and COS cells were maintained at 37°C in 5% CO₂. Both cell lines were cultured in DMEM (BioConcept, #1-26F03-I) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.

6.2.3 Coimmunoprecipitation of Mtmr2 and 3-Pap from transiently and stably transfected HEK293 cells
HEK293 wildtype cells were transiently transfected by the calcium phosphate transfection method as previously described in 6.1.7.2. The medium was changed after 16 hours and 40 hours after transfection the cells were washed with PBS and removed from the plates with lysis buffer (0.5% Triton X-100/100 mM NaCl/50 mM Tris-HCl pH 7.5/10 mM β-mercaptoethanol/Roche protease inhibitor cocktail). HEK293 cells stably expressing Mtmr2 and 3-Pap were removed from the plate and lysed as described above. Cells were sonicated and cell debris was removed by centrifugation. The supernatant was incubated for 2 hours or overnight with 30 µl protein A Sepharose CL-4B (GE Healthcare) and with the corresponding antibodies: 1 µg rabbit anti-3-Pap (Sigma, #SAB2104232); 1 µg mouse anti-Mtmr2 (Tersar, 2008); 1 µg mouse anti-penta-His (Qiagen, #34660); 1 µg mouse anti-V5 (Invitrogen, #R960-25). The beads were washed three times with lysis buffer and boiled in Laemmlli SDS-PAGE sample buffer for 5 min at 95°C. Eluted proteins were separated on an 8% SDS-PAGE gel and identified by western blotting with mouse anti-Mtmr2 (Tersar, 2008; 1:5), rabbit anti-3-Pap (Sigma, #SAB2104232, 1:5000), mouse anti-penta-His (Qiagen, #34660, 1:1000) or mouse anti-V5 (Invitrogen, #R960-25, 1:5000). Alkaline phosphatase-coupled goat anti-mouse (Southern Biotech, #1031-04, 1:10’000) or goat anti-rabbit (Southern Biotech, #4030-04, 1:10’000) were used as secondary antibodies, followed by chemiluminescence detection.

6.2.4 Protein expression of Mtmr2 and 3-Pap in Sf21 insect cells
The construct pFBDM His-Mtmr2 for expression in Sf21 cells was previously described (Berger et al, 2006a). The plasmids for the expression of His-tagged or Strep-tagged 3-Pap were cloned into a EcoRI/SalI cut pFL vector (Fitzgerald et al, 2006) resulting in pFL His-3-Pap and pFL Strep-3-Pap (6.2.1.2). The
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generation of baculovirus DNA, virus production, Sf21 insect cell transfection and protein expression was performed as described (Fitzgerald et al, 2006). Sf21 insect cells were infected with baculovirus coding for His-Mtmr2 together with Strep-3-Pap or with His-3-Pap and His-Mtmr2 alone. The cells were grown in Lonza serum-free Insect-XPRESS™ medium with L-glutamine (Lonza, #BE12-730Q) at 27°C and harvested by centrifugation 3-5 days after infection.

6.2.5 Analytical protein purification of His-Mtmr2, Strep-3-Pap and His-Mtmr2/Strep-3-Pap complex

Sf21 insect cell pellets were sonicated in lysis buffer as follows: His-Mtmr2 alone or together with Strep-3-Pap (100 mM Tris-HCl pH 8.6, 200 mM NaCl, 0.01% Tween-20, 10 mM imidazole, Roche protease inhibitor cocktail); His-3-Pap (50 mM Tris-HCl pH 8.6, 350 mM NaCl, 10 mM imidazole, Roche protease inhibitor cocktail). His-Mtmr2 and His-3-Pap lysates were purified independently from each other with a NiNTA column, washed with lysis buffer and eluted with increasing concentrations of imidazole. The eluate of the NiNTA column was loaded on a Superdex 200 16/60 gelfiltration column and eluted in 100 mM Tris-HCl pH 8.6 and 200 mM NaCl. His-Mtmr2/Strep-3-Pap lysate was first purified with a StrepTrap column, washed in lysis buffer and eluted with lysis buffer containing 2.5 mM D-desethiobiotin. The eluate of the StrepTrap column was loaded for size exclusion chromatography on a Superdex200 16/60. The molecular weight (MW) was estimated by comparing the elution spectra with the BioRad gelfiltration standard. Fractions containing His-Mtmr2 and Strep-3-Pap were pooled and additionally purified with a NiNTA column. The eluted proteins and complexes of all performed purification runs were either analyzed with Coomassie stained 8% SDS-PAGE or immunoblotting. The proteins were detected with mouse anti-penta-His (Qiagen, #34660, 1:1000) and mouse anti-Strep-tagII (IBA, #2-1507-001, 1:1000). As secondary antibody alkaline phosphatase coupled goat anti-mouse (Southern Biotech, #1031-04, 1:10'000) was used.
6.2.6 Multi-angle light scattering (MALS) measurements and mass spectrometry analysis
The multi-angle light scattering (MALS) experiments were performed at 20°C on an Agilent 1100 HPLC-system (Agilent Technologies) coupled to an analytical-grade Superdex 200HR 10/30 column (GE Healthcare) and connected to the Wyatt miniDAWN Tristar (Wyatt Technologies) system. The column was equilibrated in buffer as follows: for His-Mtmr2 in 100 mM Tris-HCl pH 8.6 and 200 mM NaCl; for His-3-Pap in 50 mM Tris-HCl pH 8.6 and 350 mM NaCl. For each MALS measurement, 100 µg of concentrated His-Mtmr2 or His-3-Pap protein was loaded onto the SEC column. The elution profiles were recorded as UV-absorbance at a wavelength of 280 nm and as the intensity of Rayleigh scattering at three different angles. To calculate the average molecular mass the ASTRA™ software (Wyatt Technologies) was used.

To analyze the amino acid composition of the purified His-tagged Mtmr2 and His-tagged 3-PAP proteins, the peptides were analyzed by mass spectrometry as previously described (see 6.1.5).

6.2.7 Small-Angle X-ray Scattering (SAXS)

6.2.7.1 SAXS analysis
Small-angle X-ray scattering (SAXS) experiments were performed at the cSAXS beamline X12SA at the Swiss Light Source (SLS) in Villigen, Switzerland. A wavelength of λ=1 Å was used and the scattered intensities were collected with a Pilatus 2M detector. The scattering vector range was 0.008 [1/Å] – 0.400 [1/Å]. The length of the scattering vector is defined as q = 4πsinθ/λ, where 2θ is the scattering angle. A standard Silver Behenate sample was used to calibrate the q-range (Huang et al, 1993). The purified protein samples and the corresponding buffer solutions were measured in quartz capillaries of 1 mm diameter (Hilgenberg GmbH, Germany). Two series of measurements were necessary to subtract background scattering from the actual protein scattering. First each capillary filled with buffer was measured. After data collection the capillaries were emptied and the second measurement series was performed on the corresponding protein sample. The data was recorded at several positions along
the capillary with an exposure time of 0.5 s per position. For each protein sample three different concentrations were measured.

6.2.7.2 SAXS data analysis and evaluation
Each data set is composed of a subset of frames. Individual frames were checked for radiation damage and radiation damage-free frames were integrated and averaged using the in-house MATLAB macros programmed by Dr. John Missimer and Dr. Kaisa Kisko. The buffer backgrounds were subtracted from the protein using the program PRIMUS from the ATSAS package (Konarev et al, 2006; Konarev et al, 2003). Kratky-plots were calculated to analyze the proper folding of the measured protein. The radius of gyration (Rg) was calculated from the data in the Guinier region. The distance distribution function P(r) was calculated using the program AUTOGNOM and was used as input for the \textit{ab-initio} shape reconstruction program DAMMIN (Konarev et al, 2006). For each protein concentration 20 independent DAMMIN reconstructions were calculated, checked for consistency and averaged with DAMAVER (Konarev et al, 2006). Generated models were illustrated in PyMol (www.pymol.org). Analysis of the macromolecular interfaces and assemblies of the PDB files of Mtmr2 (1LW3 and 1M7R) was performed with the interactive online tool PISA (Krissinel et al, 2007). The Mtmr2 and 3-Pap \textit{ab-initio} shape reconstruction models were manually superimposed with the crystal structure of Mtmr2 monomer (PDB: 1LW3) or with the PISA evaluated Mtmr2 dimer (PISA-dimer) using PyMol.

6.2.7.3 Protein crystallization of His-3-Pap, Strep-3-Pap and His-Mtmr2/Strep-3-Pap complex
All protein samples were treated as previously described in chapter 6.1.4. His-3-Pap, as indicated in Table 6-8, was incubated 30 min on ice with 1:100 or 1:1000 (w/w) \(\alpha\)-chymotrypsin prior distribution into the crystallization plate.
6.2.8 Immunofluorescence microscopy

6.2.8.1 Immunostainings of Mtmr2/3-Pap cotransfected HEK293 cells under steady-state and hypo-osmotic conditions

HEK293 cells were transfected in 3.5 cm dishes with FugeneHD (Roche) according manufacturer’s recommendations. Equal amounts of pcDNA3.1 RGS-His-Mtmr2 and pcDNA3.1 V5-3-Pap were used for the transfection reaction. One day after transfection, the cells were starved overnight in 1% BSA/DMEM and fixed 48 hours after transfection with 2% paraformaldehyde in PBS (steady-state condition). Hypo-osmotic conditions were applied as previously described (Berger et al, 2003; Berger et al, 2006a). Cells were permeabilized in buffer (1% NP-40/PBS) for 10 min. Permeabilized cells were incubated with primary antibodies for 3 hours and with secondary antibodies for 1.5 hours. Primary antibodies were diluted in PBS as follows: mouse anti-RGS-His (Qiagen, #34610, 1:200), mouse anti-V5 (Invitrogen, #R960-25, 1:200), rabbit anti-3-Pap (Sigma, #SAB2104232, 1:200). Secondary antibodies were labeled with Cy3 and Alexa-488 (Invitrogen, Jackson ImmunoResearch Laboratories). Samples were mounted in Citifluor AF1 (Citifluor) or gelvatol. A confocal laser scanning microscope (Leica SP5) was used for the analysis and to obtain single sections with a thickness of 0.5 µm.
6.2.8.2 Immunostainings of EGF stimulated COS and HEK293 cells

Cells were transfected in 3.5 cm dishes with FugeneHD (Roche) according to manufacturer’s recommendations. COS cells were cotransfected with 0.7 µg pcDNA3.1 RGS-His-Mtmr2, 0.7 µg pcDNA3.1 V5-3-Pap and 0.6 µg fluorescently tagged YFP-Rab4-Q67L, GFP-Rab5A-Q79L, EGFP-Rab7-Q67L or YFP-Rab11-Q70L. One day after transfection the cells were starved overnight in 1% BSA/DMEM and stimulated with 200 ng Alexa647 conjugated EGF (Invitrogen, #E35351). HEK293 cells were cotransfected with pRC-hEGFR, pcDNA3.1 RGS-His-Mtmr2 and/or pcDNA3.1 V5-3-Pap and/or constructs for the expression of fluorescently tagged endosomal markers (same as above). One day after transfection the cells were starved overnight in 1% BSA/DMEM and stimulated with 200 ng Alexa647 conjugated EGF (Invitrogen, #E35351). Quadruple transfected cells were stimulated with 30 ng/ml unlabeled recombinant human EGF (rhEGF, Lonza, #CC-4107). The cells were fixed and permeabilized as described above (6.2.8.1). Antibodies were diluted in PBS as follows: mouse anti-RGS-His (Qiagen, #34610, 1:200), mouse anti-V5 (Invitrogen, #R960-25, 1:200), rabbit anti-3-Pap (Sigma, #SAB2104232, 1:200). Secondary antibodies were labeled with Cy3, Cy5 and Alexa-488 (Invitrogen, Jackson ImmunoResearch Laboratories). Samples were mounted in Citifluor AF1 (Citifluor) and analyzed by using the confocal laser scanning microscope (Leica SP5) as described above.
7 Results

7.1 Identification of new PDZ binding partners
To identify novel putative PDZ-domain containing proteins interacting with the C-terminus of the proteins Mtm1, Mtmr1, MTMR1, Mtmr2, Sbf1, Sbf2, NRP1, NRP2 and Pten, we decided to made use of the well-established proteomic PDZ array based screen (Fam et al, 2005). All PDZ domain proteins spotted on the membrane are His- and S-tagged fusion proteins (see also chapter 6.1.6). To assess the binding of the above mentioned MTMRs and proteins, the carboxy-terminal 25 amino acids of each MTMR (named C-term), were fused C-terminally to a GST-tag by cloning into the pET42a expression vector.

7.1.1 Expression and purification of recombinant GST-tagged MTMR C-term proteins
Small scale expression tests showed that all recombinant GST-fusion proteins could be expressed upon induction with 1 mM IPTG in E. coli Acella cells (EdgeBio, #42649). The expression maximum was reached after 4 hours of induction (data not shown). GST-tagged MTMRs that were expressed and purified are listed in Table 7-1.

Table 7-1: List of expressed and purified GST-tagged proteins

<table>
<thead>
<tr>
<th>Construct</th>
<th>Species</th>
<th>Expected MW [Da]</th>
<th>MW based on MS [Da]</th>
</tr>
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<tr>
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<td>30908</td>
<td>30909</td>
</tr>
<tr>
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<td>30894</td>
<td>31810</td>
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<td>32430</td>
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<td>32105</td>
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<tr>
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<td>31215</td>
<td>32132</td>
</tr>
<tr>
<td>GST-NRP2 C-term</td>
<td>human</td>
<td>31324</td>
<td>31324</td>
</tr>
<tr>
<td>GST-Pten C-term</td>
<td>mouse</td>
<td>31331</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Results

Pelleted cells were lysed, sonicated and centrifuged. The soluble protein fraction was loaded on a GST Trap™ column. Bound protein was eluted with reduced glutathione after column washing. Figure 7-1 (A) shows the purification of GST-Mtm1 C-term and is representative for all GST purifications. The GST-tagged proteins were analyzed for their amino acid integrity by mass spectrometry. The corresponding MS-chromatogram is represented in Figure 7-1 (B). Expected molecular mass of GST-Mtm1 C-term is 30909 Da, which could be confirmed by MS. Part of the protein missed the C-terminal methionine or was glutathionylated at one cysteine, leading to a decrease of 131 Da or increase of 305 Da in molecular mass, respectively. However, concluding from the MS chromatogram no further amino acid substitutions could be observed at the N-terminus or the C-terminus. Therefore, purified GST-Mtm1 C-term protein was expected to harbour the PDZ-binding motif. MS chromatograms of the remaining purified GST-tagged proteins can be found in the appendix chapter 9.1.

Figure 7-1: Purification and MS analysis of GST-Mtm1 C-term
(A) Recombinant GST-tagged Mtm1 C-term was expressed in E. coli Acella cells and purified with a GST Trap column. Eluted fractions were analyzed with Coomassie stained SDS-PAGE. (B) Mass spectrometry result of purified GST-Mtm1 C-term shows a main signal at 30908 Da, corresponding to the full length protein (expected MW: 30909 Da). Some protein fraction misses the methionine (orange), leading to a final mass of 30770 Da or got glutathionylated at one cysteine, leading to an increase of the molecular weight of 305 Da (red) to 31214 Da.
7.1.2 PDZ-domain array - Overlay results

Our collaborator Randy Hall provided ten PDZ-array membranes. We were therefore restricted to test in maximum ten different GST-tagged C-termini. By following the protocol and using for the overlay experiment 500 nM purified protein, resulted in a strong unspecific background signal (data not shown). After having adjusted the overlaying protein concentrations to 25 nM the signal output was qualitatively acceptable, showing reduced background signals. The fine-tuning of the experimental procedure wasted 2 membranes, why were left with 8 PDZ array membranes for the actual analysis. To be able to test most of the generated GST-fusion proteins, we decided to omit performing the experiments in triplicates and to leave the negative control, the GST protein itself, out. The PDZ array membranes were individually overlaid with 25 nM of the following purified GST fusion proteins: GST-Mtm1 C-term, GST-MTMR1 C-term, GST-Mtmr1 C-term, GST-Mtmm2 C-term, GST-Sbf1 C-term, GST-Sbf2 C-term, GST-NRP1 C-term, GST-NRP2 C-term and GST-Pten C-term. The overlay with GST-NRP1 C-term and GST-NRP2 C-term resulted in no detectable signals (data not shown). The result of the overlay with GST-Mtm1 C-term is represented in Figure 7-2 (A) and positive signals are listed and highlighted in black (B). The overlay results of GST-MTMR1 C-term, GST-Mtm1 C-term, GST-Mtmr2 C-term, GST-Sbfl C-term, GST-Sbf2 C-term and GST-Pten C-term can be found in the appendix (9.1).
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Figure 7-2: PDZ array overlaid with GST-Mtm1 C-term
(A) The PDZ array spotted with 96 class I PDZ domains (listed in B) was overlaid with 25 nM purified GST-Mtm1 C-term. Positive interactions appear as dark spots. (B) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.

<table>
<thead>
<tr>
<th>Bin</th>
<th>PDZ Domain</th>
<th>Bin</th>
<th>PDZ Domain</th>
<th>Bin</th>
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</tr>
</tbody>
</table>
Results

The strength of the interactions was rated independently by me and by Philipp Berger in an arbitrary manner, by considering the darkness of the spots. Figure 7-3 represents the summary of my own rating. The rating of Philipp Berger can be found in the appendix (Supplementary Figure 9-7). Comparing both ratings, they were congruent with respect to the categorization into Mtm1 specific, MTMR specific and unspecific signals. However, both ratings differed in the strength of the rating of the individual signals, which especially affected the categorization of signals which are MTMR1 but not Mtmr1 specific (and vice versa). To summarize the overlay results of myotubularins only, the evaluation of the overlay of GST-Pten C-term was ignored in this scheme. For simplification, the following discussion refers to the rating represented below, in Figure 7-3. Interestingly the bins B4, B11, D11 and F3 (depicted with a yellow background) were strongly positive for all conducted overlays, and a general unspecific binding for these particular PDZ domains cannot be excluded. For GST-Mtm1 C-term, strong interactions (dark spots) appeared for MAGI-3 PDZ1, NHERF1, NHERF2, MUPP1 PDZ8, PTPN13 PDZ4+5 and PDZK2 PDZ3. Especially to mention is the strong interaction for PDZK2 PDZ3 (G4), which was unique for GST-Mtm1 C-term (depicted with blue background). As expected, Mtmr2 C-term interacted with SAP97 and PSD95. Interestingly the interaction with SAP97 PDZ 1-2 (C2) and SAP97 PDZ3 (C3) seemed to be specific for Mtmr1/MTMR1 and Mtmr2, but not for Mtm1 (colored with brown background). The C-terminal amino acid sequence composition between human MTMR1 (-ATSVHTSV) and mouse Mtmr1 (-ATPVHTSV) is similar for the last 5 residues, but deviates at the sixth last position. The PDZ overlay revealed that Mtmr1 and MTMR1 binding differed in at least 8 cases including MAGI3-PDZ1, PSD95 PDZ3, PDZ-GEF1 PDZ, CAL PDZ, ZO2-PDZ1, ZO3-PDZ2, HARMONIN PDZ2, PAPIN1, MUPP1 PDZ1 and PTPN13 PDZ1 (colored with green background). Whether the sixth last amino acid proline in the carboxy-terminus of Mtmr1 might have an effect on the overall structure of the PDZ-BD motif and therefore on the binding would have to be investigated.
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Figure 7-3: Arbitrary rating of the PDZ array results
Depicted is a scheme representing the PDZ array with the 96 different spotted PDZ domains listed in Table 6-5 and Figure 7-2, B. Positive signals for GST-Mtm1 C-term, GST-MTMR1 C-term, GST-Mtmr1 C-term, GST-Mtmr2 C-term, GST-Sbf1 C-term and GST-Sbf2 C-term were weighted in an arbitrary manner considering the darkness of the spot and are illustrated as green dots: weak signal (one green dot) to strong signal (four green dots). Mtm1 and MTMR specific interactions are colored with blue or brown background, respectively. Signals which differed between human MTMR1 and mouse Mtmr1 are colored with a grey background.

7.1.3 Verification of the PDZ array results by GST pulldown experiments
Our collaborator Randy Hall kindly provided a subset of plasmids for the expression of S-tag and His-tagged PDZ domains in E. coli (see Table 6-3). Sequencing of the plasmids (data not shown) with T7 primer (Microsynth) revealed that a point mutation in the sequence coding for GIPC lead to an early stop codon and the plasmid pET30a ERBIN PDZ was indeed pET30a Rho GEF PDZ. Sequence alignment with nNOS was negative. All other plasmid sequences were correct. The positive plasmids were transformed into E. coli Acella cells for protein expression. NHERF-1 PDZ2 and PDZK2 PDZ1 were not expressable. All other PDZ domains were purified with a NiNTA column. The GST pulldown experiments were performed with purified GST-tagged protein together with crude lysate of transformed E. coli expressing the PDZ domains or NiNTA purified PDZ domain protein. GST alone was used as negative control. GST pulldown results are summarized in Figure 7-4. The Mtm1 specific signal for PDZK2 PDZ3 found in the PDZ array overlay was confirmed by GST pulldown (Figure 7-4, A). Protein interactions specific for Mtmr1/MTMR1 and Mtmr2, but
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not for Mtm1, were found for the PDZ domains SAP97 PDZ1+2, PTPN13 PDZ4+5 and PSD95 PDZ3 (Figure 7-4, B). Furthermore, in the case of PTPN13 PDZ4+5 mouse Mtm1 and mouse Mtmr2 seem to interact stronger with PTPN13 PDZ4+5 compared to human MTMR1, consistent with the rating of the PDZ-array. In the case of SAP97 PDZ3, Mtm1 was not observed to interact in the PDZ-array overlay. However, in the corresponding GST pulldown Mtm1 coprecipitated SAP97 PDZ3 (Figure 7-4, C). Comparing the precipitated amount of SAP97 PDZ3 between GST-Mtmr1 and GST-MTMR1, the SAP97 PDZ3 coprecipitated with higher amount in the GST-MTMR1 pulldown. Additionally, the two negative controls, Mtmr2-ΔPDZ and GST, were able to precipitate SAP97 PDZ3. Due to these findings this pulldown was classified as “unspecific or no interaction” (Figure 7-4, C). MAGI-3 PDZ1 signal was negative for MTMR1 in the PDZ-array overlay. Examining the GST-pulldown, MAGI-3 PDZ1 coprecipitated strongly with all, including the negative control GST, except for Mtmr2-ΔPDZ, Sbf1 and Sbf2. Since these interactions could be MTMR specific as well as unspecific, they were also classified as “unspecific or no interaction”. Considering the PDZ-overlay results, MUPP1 PDZ8 was rated as unspecific. In the GST-pulldown experiments, MUPP1 PDZ8 coprecipitated with all GST fusion protein, confirming this observation. Nonetheless, it should be mentioned that MUPP1 PDZ8 coprecipitated in higher amounts with MTMR1, Mtmr2-ΔPDZ, Sbf2 and NRP1 (Figure 7-4, D). PDZK1 PDZ3 was expected to coprecipitate only with Mtm1, but precipitated with all GST fusion proteins. DENSIN-180 and NHERF2 PDZ2 were expected to coprecipitate with some GST proteins, but did not coprecipitate at all.
Results

7.1.4 Characterization of Pdzk2 as binding partner of Mtm1

The PDZ array and the GST pulldown results showed that the last 25 amino acids of Mtm1 fused to GST are able to interact with the third PDZ domain of Pdzk2. This interaction was specific for Mtm1. Next we wanted to verify, whether this protein-protein interaction indeed takes place through the last 5 C-terminal amino acids of Mtm1 and whether this interaction also occurs with full length proteins expressed in vivo. Therefore we performed immunoprecipitation experiments from transiently transfected HEK293 cells and mouse tissues.

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Figure 7-4: Overview of GST pulldowns

Indicated purified GST-tagged MTMRs were bound to glutathione sepharose and incubated with indicated purified PDZ domain proteins or the corresponding crude lysates. Precipitated protein was analyzed with SDS-PAGE and visualized with immunoblotting (IB) using anti S-tag antibody. (A) S-tag-PDZK2 PDZ3 coprecipitated only with GST-Mtm1 C-term. (B) S-tagged SAP97 PDZ1+2, PTPN13 PDZ4+5 and PSD95 PDZ3 coprecipitated with GST-Mtrmr1 C-term, GST-MTMR1 C-term and GST-Mtmr2 C-term. (C) SAP97 PDZ3, MAGI-3 PDZ1, MUPP1 PDZ8 and PDZK1 PDZ3 coprecipitated with all GST fusion proteins including the GST control, rendering the interactions unspecific. DENSIN-180 and NHERF2 PDZ2 did not coprecipitate with any GST fusion proteins.
7.1.4.1 Pdzk2 coimmunoprecipitates with Mtm1 in HEK293 cells and mouse tissues

Human embryonic kidney 293 cells (HEK293) were transiently cotransfected with expression vectors for full length Myc-Pdzk2 and His-Mtm1, His-Mtm1-ΔPDZ or His-Mtmr2. Cells were harvested and cell extracts were subjected to immunoprecipitation with anti-penta-His antibodies. Precipitated protein was analyzed by immunoblotting using anti-Myc and anti-penta-His antibodies. Pdzk2 coprecipitated with Mtm1, but not with Mtm1-ΔPDZ, lacking the last 5 amino acids consisting the PDZ-BD motif (Figure 7-5, A). Therefore, full length Pdzk2 and Mtm1 interact in HEK293 cells and protein-protein interaction is clearly dependent on the last 5 carboxy-terminal amino acids.

To test, whether Mtm1 and Pdzk2 also interact in vivo, a set of mouse tissues was obtained from wildtype mice and homogenized in lysis buffer. Crude cell extract was subjected to immunoprecipitation with anti-Mtm1 antibody and the associated Pdzk2 was analyzed with Western blot using anti-Pdzk2 antibody. Here we show for the first time, that Mtm1 interacts with Pdzk2 in mouse tissues (Figure 7-5, B). Mtm1 and Pdzk2 were not detectable in crude lysates by immunoblotting, therefore the loading control was missing. We suggested that the expression of Mtm1 and Pdzk2 in the analyzed tissues was very low. Pdzk2 was also named intestinal and kidney-enriched PDZ protein (IKEPP) based on its mRNA distribution in human cells (Scott et al, 2002). Our pulldown results showed that Pdzk2 protein is additionally present in other tissues. Interestingly, Pdzk2 seemed to coprecipitate in higher amounts from cerebellum, muscle, stomach, heart, spleen and liver compared to kidney and intestine.
Results

Figure 7-5: Mtm1 interacts with Pdzk2 in HEK293 cells and in mouse tissues
(A) HEK293 cells were transiently transfected with Myc-tagged Pdzk2 and His-tagged Mtm1, His-tagged Mtm1-ΔPDZ or His-tagged Mtmr2. As negative controls the cells were transfected with Myc-Pdzk2, His-Mtm1, His-Mtm1-ΔPDZ or His-Mtmr2 alone. His-tagged proteins were immunoprecipitated from crude lysates with anti-penta-His antibody. Immunoblots were probed with anti-Myc and anti-penta-His antibodies. Myc-Pdzk2 associated specifically with His-Mtm1 but not with the PDZ-BD deletion mutant His-Mtm1-ΔPDZ. (B) Crude mouse lysates of obtained tissues were incubated with sepharose beads and anti-Mtm1 antibody. Immunoblots were probed with Mtm1 and Pdzk2 specific antibodies. The interaction Mtm1 with Pdzk2 can be observed in all tested mouse tissues, especially in cerebellum, muscle, stomach, heart, spleen and liver.

7.1.4.2 Colocalization of Mtm1 and Pdzk2 in COS-7 and HEK293 cells
Previous reports have shown that Mtm1 transfected cells displayed staining in the nucleus, the cytosol and at the plasma membrane (Nandurkar et al, 2003; Kim et al, 2002; Laporte et al, 2002a). Overexpression of Mtm1 resulted in membrane projections, extensive filopodia and localization in membrane ruffles (Laporte et al, 2002a; Kim et al, 2002). This phenotype however, was disrupted in cells coexpressing Mtm1 with the inactive adaptor unit 3-Pap (Nandurkar et al, 2003). In vivo immunostainings of Pdzk2 predominantly showed staining in the subapical compartment in mouse renal proximal tubules (Gisler et al, 2001). To analyze, whether Mtm1 and Pdzk2 colocalize in human cells, we transiently transfected COS-7 (African green monkey kidney) cells and HEK293 cells with
Results

the expression vectors for His-Mtm1, His-Mtm1 C375S, His-Mtm1-ΔPDZ and Myc-Pdzk2.

In COS-7 cells, Mtm1 staining was observed in the cytosol as vesicular structures and at the plasma membrane (Figure 7-6, A). Pdzk2 showed similar staining, and colocalized with Mtm1 (Figure 7-6, A: indicated by arrows and visible in yellow in Merge). Myotubularin mutants Mtm1 C375S (Figure 7-6, B) and Mtm1-ΔPDZ (Figure 7-6, C) showed similar staining compared to their wildtype (Figure 7-6, A) and colocalization with Pdzk2 was not disturbed. The mutations did therefore not affect the localization of Mtm1. Interestingly, while using Mtmr2 as control, we observed that Pdzk2 also colocalized with Mtmr2 vesicles (Figure 7-6, D). Since Mtm1 and Mtmr2 were reported to localize at endosomal compartments, localization of Pdzk2 at these vesicles suggests a scaffolding function at these membranes.
Results

Figure 7-6: Pdzk2 colocalizes with Mtm1 in transiently transfected COS-7 cells. COS-7 cells were transiently cotransfected with Pdzk2 and RGS-His-tagged Mtm1, Mtm1 C375S, Mtm1-ΔPDZ or Mtmr2 (A-D). Cells were probed with Mtm1, Pdzk2 or RGS-His specific primary antibodies. Merged images are shown on the right panels, where yellow staining indicated by arrows show colocalization of Pdzk2 (red) with MTMR protein (green). White boxes indicate regions which are represented as magnified views on the right. Scale bar = 10 µm.

To verify these results, we additionally transfected HEK293 cells and repeated the immunostainings. All MTMRs localized to the cytosol in vesicular structures, as previously seen in COS-7 cells. Immunostaining of Pdzk2 resulted in staining of vesicular, punctuate structures in the cytosol and structures below the plasma membrane. These results agree with the COS-7 immunostainings of Pdzk2. Pdzk2 colocalization was observed with Mtm1 and Mtmr2 (Figure 7-7, A and D) but not with Mtm1 C375S or Mtm1-ΔPDZ (Figure 7-7, B and C).
Results

Figure 7-7: Pdzk2 colocalizes with Mtm1 in transiently transfected HEK293 cells

HEK293 cells were transiently cotransfected with Pdzk2 and RGS-His-tagged Mtm1, Mtm1 C375S, Mtm1-ΔPDZ or Mtmr2 (A-D). Cells were probed with Mtm1, Pd zk2 or RGS-His specific primary antibodies. Merged images are shown on the right panels, where yellow staining indicated by arrows show colocalization of Pd zk2 (red) with MTMR protein (green). White boxes indicate regions, represented as magnified views on the right. Scale bar = 10 µm.

The substrate specificity of Mtm1 for PI-3,5-P$_2$ and PI-3-P suggest a function in endocytosis, sorting and degradation. PI-3,5-P$_2$ is generated from PIKfyve, which localizes to endosomes (Gaullier et al, 1998; Simonsen et al, 1998). Localization studies of endogenously expressed Mtm1 as well as of overexpressed Mtm1 and its phosphatase inactivated mutant Mtm1 C375S, showed colocalization with Rab5 and Rab7 positive vesicles in BHK cells (Cao et al, 2007). For a more detailed localization study of Mtm1 and Pd zk2, HEK293 cells were transiently
Results

cotransfected with the constitutively active endosomal markers Rab4-Q67L, Rab5A-Q79L, Rab7-Q67L and Rab11-Q70L. Mtm1 clearly colocalized with Pdzk2 along the plasma membrane and on vesicles, depicted in the merged figures as magenta (Figure 7-8). However, no colocalization of Mtm1 or Pdzk2 with any of the four tested endosomal markers could be observed.

Figure 7-8: Intracellular localization of Mtm1 and Pdzk2
HEK293 cells were transiently cotransfected with Pdzk2, RGS-His-tagged Mtm1 and constitutive active endosomal markers YFP-Rab4-Q67L, GFP-Rab5A-Q79L, EGFP-Rab7-Q67L and YFP-Rab11-Q70L. Cells were probed with Mtm1, Pdzk2 or RGS-His specific primary antibodies. Merged images are shown on the right panels. Colocalization of Pdzk2 (blue) with Mtm1 (red) is visible in magenta. White boxes indicate regions, represented as magnified views on the right. Scale bar = 10 µm.
7.1.4.3 Localization studies of Mtm1, Pdzk2 and the sodium phosphate co-transporter NaPi-IIa in opossum kidney cells

Yeast two-hybrid screen against the C-terminal, cytosolic tail of the type II Na\(^+\)-dependent inorganic phosphate (NaPi-IIa) cotransporter revealed the interaction with the NHERF protein family members NHERF1, NHERF2, PDZK1 and PDZK2 (Gisler et al, 2001). NaPi-IIa cotransporter plays an important role in renal phosphate reabsorption and homeostasis at the brush border membrane. Parathyroid hormone (PTH) regulates phosphate homeostasis and was shown to induce internalization and mediate increased lysosomal degradation of NaPi-IIa (Pfister et al, 1998; Pfister et al, 1997). Opossum kidney cells (OK) are a well-established model system to study PTH dependent regulation of NaPi-IIa cotransporter (Malmstrom et al, 1986). Since Mtm1 has a regulatory effect on the trafficking and degradation of EGF receptor, we were wondering whether binding of Mtm1 to Pdzk2 might have a similar influence on the PTH induced degradation of NaPi-IIa. To analyze that, we first had to test, whether OK cells stably transfected with NaPi-IIa (kind gift of H. Murer and N. Hernando, University of Zurich) were suitable for transient transfections and subsequent monitoring of PTH induced NaPi-IIa trafficking.

Immunostaining of polarized OK cells stably expressing V5-tagged NaPi-IIa cells transiently transfected with His-Mtm1 and Myc-Pdzk2 are depicted in Figure 7-9. Consistent with previous localization studies (Blaine et al, 2009; Pfister et al, 1997; Pfister et al, 1998) NaPi-IIa clustered at the apical plasma membrane (Figure 7-9, A) and could be observed in microvilli (Figure 7-9, B and C). Pdzk2 localized subapically of the plasma membrane in the cytoplasm (Figure 7-9, C transsections). Mtm1 localized in vesicular structures distributed in the cytosol (Figure 7-9, B and C). No colocalization of Mtm1 and Pdzk2 was observed.
Results

Figure 7-9: Immunostaining of NaPi-IIa, Mtm1 and Pdzk2 in OK cells
Opossum kidney cells stably expressing V5-tagged NaPi-IIa were transiently transfected with His-Mtm1 and Myc-Pdzk2. His-Mtm1 was stained with anti-RGS-His. V5-NaPi-IIa was detected with anti-V5 antibodies. Pdzk2 was stained with anti-Myc antibodies. (A) V5-NaPi-IIa, false colored in red, clustered at the apical plasma membrane and (B) in microvilli (arrow). (C) Pdzk2 (blue) localized to the subapical cytoplasm and Mtm1 (green) in vesicular structures. Single sections with a thickness of 0.5 µm were obtained by using a confocal laser scanning microscope (Leica SP5). Scale bar = 30µm.

We subsequently stimulated the transiently transfected OK V5-NaPi-IIa cells with 0.1 µM PTH and fixed the cells 0, 120 and 270 min after stimulation prior to immunostaining (data not shown). Unfortunately we had to face the following problems: 1st only approximately 5% of the OK cells polarized at all. 2nd of the polarized cells, only few cells showed expression of NaPi-IIa, rendering the NaPi-IIa expression highly inhomogeneous. It was therefore impossible to differentiate between stimulated and non-expressing cells. Additionally, this reduced the probability of polarized cells positive for transient transfection of Mtm1 and Pdzk2. Western blot analysis of PTH stimulated OK V5-NaPi-IIa cells confirmed these observations. V5-NaPi-IIa was not detectable and degradation of NaPi-IIa upon PTH stimulation could not be observed (data not shown).
therefore concluded that the OK V5-NaPi-IIa cells were inappropriate for our purposes and stopped the experiments at this point.

7.2 Structural analysis of MTMRs and MTMR-complexes
Up to date no crystal complexes of MTMR proteins with PDZ domain containing proteins or with other MTMR family members have been reported. The knowledge of the structure on the atomic level, the position and conformation of the individual proteins to each other as well as the detailed information about the macromolecular interfaces would help to understand e.g. differences in localization and binding affinities towards phosphoinositides. To study the binding domain characteristics of Mtmr2 with the PDZ-domain containing protein Sap97 and the inactive phosphatase Sbf2, we expressed the protein complexes in insect cells for further analytical purification as well as crystallization.

7.2.1 Structural analysis of the Mtmr2/Sap97 complex
Synapse-associated protein 97 (Sap97), also known as Disc large 1 (Dlg1), is a PDZ-domain containing scaffolding molecule of the membrane-associated guanylate kinase-like (MAGUK) protein family. The structure of Sap97 is composed of a L27 domain important in the formation of large protein assemblies, 3 PDZ modules, a Src-homology 3 (SH3) domain and a guanylate kinase-like domain (GK). Previous work has shown that Mtmr2 associates with Sap97 in Schwann cells and its localization in Mtmr2-null mice was altered (Bolino et al, 2004). It was also shown that the C-terminal amino acids of Mtmr2 are essential for this interaction (Bolis et al, 2009). Interestingly, loss of Sbf2 did not affect the levels of its binding partner Mtmr2 and the Mtmr2-binding Dlg1/Sap97 in peripheral nerves (Tersar et al, 2007).

Our PDZ-array (Figure 7-3) and GST pulldown results (Figure 7-4) confirmed the interaction of Mtmr2 with Sap97. Here we further analyzed the binding specificity of Mtmr2 to the different PDZ modules of Sap97 and performed a domain mapping experiment. Therefore, we cloned the HA-tagged Sap97 PDZ domains PDZ1, PDZ2, PDZ3, PDZ1-2 or PDZ1-3, all lacking the L27, SH3 and GK
Results

domains, into the pre-existing pFBDMc His-Mtmr2 vector. His-Mtmr2 and HA-Sap97 PDZ proteins were produced in baculovirus infected Sf21 cells. Cell lysates were used for immunoprecipitation and analytical protein purification. All Sap97 PDZ motifs coprecipitated with His-Mtmr2. A particular strong interaction could be observed for the second PDZ domain (Figure 7-10, A), suggesting a higher affinity of Mtmr2 towards Sap97-PDZ2.

![Figure 7-10: Analysis of Mtmr2/Sap97-PDZ1-2 complex](image)

(A) Coimmunoprecipitation of His-Mtmr2 (75 KDa) and HA-Sap97-PDZ1 (14.0 KDa), -PDZ2 (13.7 KDa), -PDZ3 (13.2 KDa), -PDZ1-2 (24.0 KDa) or -PDZ1-3 (38.9 KDa) using anti-penta-His antibody. Immunoblot detection was performed with anti-HA antibody. Sap97-PDZ2, -PDZ1-2 and -PDZ1-3 clearly interacted stronger with Mtmr2 compared to Sap97-PDZ1 or -PDZ3. (B) His-Mtmr2/Sap97-PDZ1-2 complex was purified via NiNTA (data not shown) followed by size exclusion chromatography using a Superdex 200 16/60. Eluate (highlighted in green and orange) was analyzed by Coomassie stained SDS-PAGE. Mtmr2 and Sap97 eluted with an elution volume maximum of 67 ml. Compared with the BioRad gelfiltration standard this peak maximum corresponds to a molecular weight of 208 KDa.

Since Mtmr2 forms a homodimer in solution, we expected Mtmr2/Sap97-PDZ1-2 complex to appear as Mtmr2 dimer with one or two SAP97-PDZ1-2 attached on each Mtmr2, forming a tetramer (198 KDa). To reveal the complex structure of His-Mtmr2/HA-Sap97-PDZ1-2 we purified the proteins on a NiNTA column and analyzed the purified complex by size exclusion chromatography (Figure 7-10, B). Sap97-PDZ1-2 eluted together with Mtmr2 with an elution volume maximum
Results

of 67 ml, corresponding to a molecular mass of 208 KDa. Considering the expected size of a tetramer of 198 KDa, the tetrameric appearance of Mtmr2/Sap97-PDZ1-2 has to be suggested. In a further step we also pooled the fractions including the complex (Figure 7-10, B, highlighted in orange) and subjected the concentrated protein sample to SAXS analysis. However, the protein sample was damaged by X-ray radiation resulting in unfolded, aggregated protein and quality loss of the obtained scattering data, which could not be further processed (data not shown).

7.2.2 Purification and analysis of Mtmr2/Sbf2 complex
It has been shown that a homodimeric active Mtmr2 interacts with a homodimeric inactive Sbf2, thereby forming a tetrameric complex (Berger et al, 2006a). Of all myotubularin family members, only the crystal structure of Mtmr2 monomer has been solved (Begley et al, 2003; Begley et al, 2006). To get further insights into the structural aspects of active/inactive MTMR-complexes, we expressed and purified the full length His-Mtmr2/CBP-Sbf2 complex (Figure 7-11) using the baculovirus-based expression system. Since the flexible N- and C-terminal linkers of Sbf2 reduce the solubility of the protein, we also expressed two truncated versions of Sbf2: one lacking the N-terminus (DENN domain and a large undefined region) but composed of the PH-GRAM, phosphatase domain and C-terminal PH-domain (named MinTetra+PH or MT+PH) (Figure 7-12, A), the other lacking the N-terminus and the C-terminus (PH domain) and composed of the minimal part, the PH-GRAM and phosphatase domain (named MinTetra or MT) (Figure 7-13, A). All protein samples were initially purified with an IMAC followed by biochemical analysis using SEC. Purification and analysis of His-Mtmr2/CBP-Sbf2 is represented in Figure 7-11. Purification with IMAC clearly resulted in coelution of Sbf2 (B), supporting that Mtmr2 and Sbf2 form a complex. Excess of His-tagged Mtmr2 in the eluted fractions was observed after IMAC purification. Eluted fractions were pooled and subjected to SEC analysis (C). By experience purification of Mtmr2 alone using the same SEC 200 16/60 column (see Figure 7-16) usually resulted in the elution of Mtmr2 in the range of 64-75 ml with an elution maximum of ~69 ml (~175 KDa). Here, Mtmr2 eluted with an
Results

apparent molecular weight of 183 KDa, agreeing with the previous results and suggesting that Mtmr2 eluted as homodimer (expected: 150 KDa). Mtmr2 and Sbf2 eluted between 46-62 ml. The void peak seemed to overlap with a second UV-absorption detected peak visible as a shoulder. The eluted fractions ranging from 56 ml to 62 ml correlated to molecular masses of 616 KDa to 455 KDa, compared with a standard. Coomassie stained SDS-PAGE of these particular samples let us assume a composition of Sbf2 to Mtmr2 of 1:1. The expected size of a His-Mtmr2/CBP-Sbf2 tetramer is 570 KDa and would fit in this range quite well. Nonetheless, the purification quality as reported in (Berger et al, 2006a) could not be reproduced. Crystallization screens with SEC purified Mtmr2/Sbf2 complex did not yield any crystals.

Figure 7-11: Purification of His-Mtmr2/CBP-Sbf2 complex
(A) Scheme of the Mtmr2/Sbf2 tetramer. Mtmr2 and Sbf2 each form a homodimer and together a tetramer. (B) IMAC purification of harvested Mtmr2/Sbf2. Eluted fractions were analyzed with Coomassie stained SDS-PAGE. CBP-Sbf2 eluted together with His-Mtmr2. (C) SEC using a Superdex200 16/60 of the IMAC purified fractions (red box in B). Fractionates were analyzed with Coomassie stained SDS-PAGE. Mtmr2 homodimer eluted with an elution volume maximum of 68.2 ml (~183 KDa). Sbf2 was found in the void and eluted together with Mtmr2 from 56 ml to 62 ml, covering the range of molecular masses from 616 KDa to 455 KDa, respectively.

Purification and analysis of His-Mtmr2/CBP-Sbf2-MT+PH is represented in Figure 7-12. His-Mtmr2/Sbf2-MT+PH clearly eluted as complex after a single
Results

IMAC purification step (Figure 7-12, B). Analyzing the SEC fractions by Coomassie stained SDS-PAGE, Mtmr2/Sbf2-MT+PH complex eluted with a peak maximum of 65.4 ml (~243 KDa). The expected size of a tetramer however, is 376 KDa. Revising the Coomassie stained SDS-PAGE the elution maximum of Mtmr2 seemed to be slightly shifted to smaller mass. We therefore have to consider that the 243 KDa peak most probably reflected the composition of two overlapping peaks: the Mtmr2 homodimer (~150 KDa) and the Sbf2-MT+PH homodimer (~226 KDa). Although the formation of a heterodimeric complex of Mtmr2/Sbf2-MT+PH with an expected molecular weight of 188 KDa could explain the 243 KDa peak, the shifted protein maxima of Mtmr2 and Sbf2-MT+PH in the Coomassie stained gel do not support this possibility. Therefore, it has to be suggested that the complex was not stable during gel filtration. SAXS analysis of a concentrated sample (Figure 7-12, C, bordered in red) was unsuccessful. The sample was damaged by radiation and aggregation, reflecting the instability of the complex. Crystallization screens also did not yield any crystals, underscoring these negative results.

Figure 7-12: Purification of His-Mtmr2/CBP-Sbf2-MT+PH complex
(A) Scheme of the Mtmr2/Sbf2-MT+PH tetramer. Sbf2-MT+PH domain misses the N-terminal undefined region with the DENN domain. (B) IMAC of harvested His-Mtmr2/CBP-Sbf2-MT+PH. Eluted fractions were analyzed with Coomassie stained SDS-PAGE. CBP-Sbf2-MT+PH eluted
Results

Results together with His-Mtmr2. (C) SEC using a Superdex200 16/60 of the IMAC purified fractions (red box in B). Mtmr2 and Sbf2-MT+PH elute with an elution volume maximum of 65.4 ml (~243 KDa). SEC fraction bordered in red was used for SAXS analysis.

Figure 7-13 summarizes the purification and analysis of His-Mtmr2/CBP-Sbf2-MT. IMAC purification of harvested His-Mtmr2/CBP-Sbf2-MT lysate resulted in the elution of both His-Mtmr2 and CBP-Sbf2-MT protein (Figure 7-13, B), suggesting that both proteins interacted. Analyzing the SEC fractions by Coomassie stained SDS-PAGE, Mtmr2/Sbf2-MT complex eluted with a peak maximum of 65.5 ml (~240 KDa). The expected size of a tetramer however was 352 KDa. Also here it seemed that we have the situation of two overlapping elution maxima, the Mtmr2 homodimer (~150 KDa) and the Sbf2-MT homodimer (~226 KDa). Revising the Coomassie stained SDS-PAGE, the elution maxima of Mtmr2 and Sbf2-MT seemed to be slightly shifted, consistent with the SEC purification of Mtmr2/Sbf2-MT+PH. SAXS analysis of a concentrated sample was unsuccessful (Figure 7-13, C, bordered in red). The sample was damaged by radiation and scattering data was not further analyzed (data not shown). Crystallization screens, which have been set up did also not yield any hits.

Figure 7-13: Purification of His-Mtmr2/CBP-Sbf2-MT complex
(A) Scheme of the Mtmr2/Sbf2-MT tetramer. Sbf2-MT domain misses the N-terminal DENN domain with undefined region and the C-terminal PH domain. (B) IMAC of harvested
Results

His-Mtmr2/CBP-Sbf2-MT. Eluted fractions were analyzed with Coomassie stained SDS-PAGE. CBP-Sbf2-MT co-eluted together with His-Mtmr2. (C) SEC using a Superdex200 16/60 of the IMAC purified fractions (bordered in red in B). Mtmr2 and Sbf2-MT eluted with an elution volume maximum of 65.5 ml (~240 KDa). Expected MW of a tetramer was 352 KDa.

Summarizing our findings, the N-terminal DENN domain and the C-terminus including the PH domain of Sbf2 seemed to contribute to the stability of a heterotetrameric complex with Mtmr2. The truncated versions Mtmr2/Sbf2-MT+PH and Mtmr2/Sbf2-MT were not stable during a gelfiltration step. However, to achieve a pure sample for structural analysis such as crystallization and SAXS, a gelfiltration step would be absolutely required.

7.3 Structural and functional characterization of Mtmr2, 3-Pap and Mtmr2/3-Pap complex

7.3.1 Characterization of the Mtmr2/3-Pap interaction in HEK293 cells

3-Pap/Mtmr12 was identified as adaptor unit of Mtm1 and Mtmr2 in reciprocal coimmunoprecipitation studies of both endogenous and recombinant proteins expressed in K562 cells (Nandurkar et al, 2003). The Mtmr2/3-Pap interaction was additionally confirmed in mouse Schwann cells by immunoprecipitation and MS-based screening procedure (Tersar, 2008). Whether the interaction occurs also in HEK293 cells and whether it takes place over the coiled-coil motifs of one or both binding partners, has not yet been analyzed. To address this question, we transiently transfected HEK293 cells with plasmids coding for the full length proteins His-tagged Mtmr2 and V5-tagged 3-Pap as well as for the truncated versions His-Mtmr2 P589X and V5-3-Pap G654X lacking the C-terminal coiled-coil motif. Two days after transfection the cells were lysed and incubated for immunoprecipitation with anti-penta-His antibody or anti-V5 antibody. The protein expression was too low to detect the protein input in the crude cell lysate. Therefore, the immunoprecipitated protein was taken as loading control. The experiment has been repeated more than three times with the same outcome (data not shown). The results displayed in Figure 7-14 represent the merge of two independent experiments. As expected, full length proteins V5-3-Pap and His-Mtmr2 both coprecipitated each other. However, V5-3-Pap G654X could not
Results

precipitate His-Mtmr2 and vice versa His-Mtmr2 P589X did not coprecipitate V5-3-Pap. Thus, the coiled-coil domain of both Mtmr2 and 3-Pap is therefore essential for complex formation.

V5-3-Pap
V5-3-Pap G654X
His-Mtmr2
His-Mtmr2 P589X

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Figure 7-14: Mtmr2 and 3-Pap interact through the C-terminal tail including the coiled-coil

Immunoprecipitation of V5-3-Pap, V5-3-Pap G654X, His-Mtmr2 and His-Mtmr2 P589X using mouse anti-penta-His (upper panel) and mouse anti-V5 antibody (lower panel) in HEK293 cells. Precipitate and protein input was analyzed by immunoblotting using anti-penta-His and anti-V5 antibody. 3-Pap coprecipitated only with His-Mtmr2, but not with His-Mtmr2 P589X. His-Mtmr2 coprecipitated only with V5-3-Pap, but not with V5-3-Pap G654X. His-Mtmr2 P589X and V5-3-Pap G654X did also not coprecipitate each other. As control single V5-3-Pap G654X, His-Mtmr2 P589X and untransfected cells were used.

7.3.2 Characterization of the purified Mtmr2/3-Pap complex

The expression vector pFBDM His-Mtmr2/CBP-Sbf2 was successfully used for expression in Sf21 insect cells (Berger et al, 2006a). We therefore replaced CBP-Sbf2 in pFBDM His-Mtmr2/CBP-Sbf2 by Strep-tagged 3-Pap by PCR based subcloning. The protein yield of Strep-3-Pap however was extremely low (data not shown). We therefore cloned the expression vector pFL Strep-3-Pap for baculovirus-based expression and co-infected Sf21 cells with pFBDM His-Mtmr2 and pFL Strep-3-Pap baculovirus. The protein complex was purified in a first step
using a StrepTactin column and analysis of the eluate by Coomassie stained SDS-PAGE showed that His-Mtmr2 clearly co-eluted with Strep-3-Pap (Figure 7-15, A). His-Mtmr2/Strep-3-Pap complex can be produced and purified from Sf21 cells. To further characterize the complex formation between His-Mtmr2 and Strep-3-Pap, the StrepTactin eluate was separated by size exclusion chromatography (Figure 7-15, B). Strep-3-Pap eluted in excess with an elution maximum of 74.30 ml (~100 KDa). An excess of Strep-tagged protein was expected after StrepTactin purification. The expected molecular mass of Strep-3-Pap based on the amino acid sequence is 89 KDa. The elution peak of Strep-3-Pap with a molecular mass of 100 KDa points to a monomer in solution. His-Mtmr2 and Strep-3-Pap were found within a peak with a maximum of 67.62 ml (~195 KDa). This molecular mass could be explained by the formation of a His-Mtmr2/Strep-3-Pap heterodimer. In contrast, the Mtmr2 dimer eluted with a molecular mass of ~175 KDa (see Figure 7-16, B). To verify and confirm the heteromeric complex in a last purification step, the Mtmr2/3-Pap complex fractions of the SEC (bordered in red) were pooled and purified with a NiNTA column (Figure 7-15, C). The NiNTA eluate was separated by SDS-PAGE and analyzed by immunoblotting. Strep-3-Pap was detected with Strep-tagII antibody and His-Mtmr2 with penta-His antibody. Strep-3-Pap clearly coeluted with His-Mtmr2. The Mtmr2/3-Pap complex purification by StrepTactin and SEC was repeated three times, and in all three experiments Mtmr2 and 3-Pap coeluted with a corresponding mass of a heterodimer.
Results

Figure 7-15: Purification and analysis of His-Mtmr2/Strep-3-Pap complex
Sf21 cells were coinfected with baculovirus for the expression of His-Mtmr2 and Strep-3-Pap. Three to five days after infection the cells were harvested and proteins were purified from the lysate. (A) StrepTactin purification and Coomassie stained SDS-PAGE of eluted fractions. Mtmr2 eluted together with 3-Pap. (B) Size exclusion chromatography of pooled StrepTactin fractions using a gelfiltration column 200 16/60. Strep-3-Pap eluted alone with an elution maximum of 74.3 ml (~100 KDa) and together with Mtmr2 at 67.62 ml (~194 KDa). (C) SEC eluted fractions (framed in red, B) were purified in a third step by IMAC. Eluted samples were analyzed by SDS-PAGE and immunoblotting using anti-penta-His and anti-Strep-tagII antibodies. His-Mtmr2 co-eluted with Strep-3-Pap.

7.3.3 Protein purification and structural analysis using SAXS
Small-angle X-ray scattering (SAXS) is a powerful tool to study molecular structures in solutions such as proteins and protein complexes, to investigate conformational changes upon activation such as ligand binding and it enables the modeling of flexible structures like linkers, which are impossible to crystallize (Bernado et al, 2007; Svergun et al, 2003). Compared to crystallography, SAXS gives only access to low resolution structures ranging from 20-30 Å upwards. Prerequisites for successful data collection are highly pure protein in the range of 1-5 mg/ml and monodispersity. From the initial collected data, the scattered
Results

Intensity (I) as a function of the scattering vector (q/[Å⁻¹]), the following parameters can be easily extracted: the molecular weight (MW), radius of gyration (Rg), hydrated particle volume (Vp) and maximum particle diameter (Dmax) (Mertens et al, 2010). The Rg is calculated by the Guinier approximation from the initial collected data. Rg is the square root of the average squared distance of each scatterer from the particle center and can also be calculated from the distance distribution function (P(r)). P(r) function results from the Fourier-transformation of the scattering data and illustrates the distributions of intramolecular distances. The maximal intramolecular distance is described as Dmax. Ab-initio modeling programs as DAMMIN from the ATSAS package use Rg and P(r) to reconstruct the averaged shape.

7.3.3.1 Mtmr2 homodimer
Baculovirus derived from pFBDM His-Mtmr2 was already available in our lab. Sf21 insect cells were infected with the virus and the cells were harvested five days after infection. In a first step His-Mtmr2 protein was purified from the lysate with an IMAC column (Figure 7-16, A). In a further purification step, the IMAC fractions (framed in red, A) were pooled and purified by size exclusion chromatography. His-Mtmr2 eluted as dimer with an elution maximum of 68.7 ml, corresponding to a molecular mass of 176 KDa (Figure 7-16, B). Analysis of SEC purified His-Mtmr2 confirmed the homodimeric form of Mtmr2, which eluted with a MW of 160 KDa (Figure 7-16, C).
Results

Figure 7-16: Purification and analysis of His-Mtmr2 homodimer by SEC and MALS
Recombinant His-tagged Mtmr2 was expressed in Sf21 insect cells. Five days after infection the cells were harvested and purified. (A) Purification of His-Mtmr2 by IMAC and analysis of eluted fractions by Coomassie stained SDS-PAGE. (B) SEC using a Superdex200 16/60 gelfiltration column of the IMAC purified fractions. Mtmr2 protein eluted with an elution volume maximum of 68.7 ml (~176 KDa). Eluted fractions were analyzed by Coomassie stained SDS-PAGE. (C) Multi-angle light scattering of SEC purified Mtmr2 (bordered in red, B). Mtmr2 eluted with a molecular weight of 160 KDa.

The published crystal structure of Mtmr2 (PDB: 1LW3) lacks the first N-terminal 73 amino acids and the last C-terminal 57 amino acids, including the coiled-coil motif and the PDZ binding domain (Begley et al, 2003). A possible dimer found in the crystal packing has also been proposed (PDB: 1M7R) (summarized in Figure 7-18, A). Analysis of the macromolecular interfaces and assemblies of both mentioned PDB files with the interactive tool PISA (Krissinel et al, 2007) revealed that the interface of the Mtmr2 dimer (1M7R) with a Complexation Significance Score (CSS) of 0.000 had no biological significance and was unstable. A CSS ranges from 0 to 1, with 1 achieving the highest relevance of interfaces for complexation. In contrast, PISA uncovered in the crystal packing of 1LW3 a
Results

stable Mtmr2 dimer interface with a CSS of 0.201, rendering the newly discovered dimer interface as biologically relevant. This structure is from now on called PISA-dimer. Despite the biological relevance, the Mtmr2 structure lacks the coiled-coil motif, which was shown to be absolutely essential for homodimerization (Berger et al., 2003). Although the PISA-dimer model could be still an artefact of crystal packing, the biological relevance of the interface lets suggest a function in supporting the dimerization. To shed light on this issue, we decided to analyze the structure of Mtmr2 full length in solution using SAXS and to compare it with the calculated, biologically relevant crystal structure from PISA. After having successfully produced and purified His-Mtmr2 homodimer with high purity, the sample was subjected for SAXS analysis. We measured three different concentrations: 1.48 mg/ml, 3.03 mg/ml and 4.64 mg/ml. The initial collected data were plotted as intensity as a function of the scattering vector q (Figure 7-17, A) and were consistent for all three measured concentrations. The Kratky-plot confirmed that the protein was folded (Figure 7-17, B). The linear Guinier-region showed that no higher order aggregates were formed (Figure 7-17, C). The average Rg was 44 Å. The P(r) function showed an average maximum distance (Dmax) of 150 Å. The bell shaped curve indicated a spherical shape and the elongated tail to longer distances an appendix.
Figure 7-17: SAXS data analysis of purified His-Mtmr2 in solution
(A) Scattered intensity plotted against the scattering vector q, after background substraction.
(B) Kratky plot of the measured Mtmr2 concentrations. (C) By fitting the Guinier equation with the program PRIMUS (Konarev, 2003), the value of Rg was determined for the Mtmr2 concentrations 1.48 mg/ml, 3.03 mg/ml and 4.64 mg/ml to be 43.55 Å, 44.17 Å and 43.65 Å, respectively. The average Rg is 44 Å. (D) The P(r) function was calculated by the program AUTOGNOM (Svergun, 1992). Dmax ranges from 145 Å to 153 Å and is in average 150 Å.

The evaluated distance distribution functions were used as an input for the ab-initio shape reconstruction program DAMMIN. Twenty independent reconstructions were run for each concentration and resulted in globular shapes with a tail. Consistency between these individual runs allowed averaging with the program DAMAVER (Konarev et al, 2006). Superimposition of the PISA-dimer with the averaged SAXS model is depicted in (Figure 7-18, B). Howsoever the PISA-dimer was positioned and rotated it never fitted perfectly into the SAXS model. Normally, the positioning of the two monomers in a parallel manner into the SAXS shape resulted in a better fit (Figure 7-18, C).
Results

Figure 7-18: Ab-initio shape reconstruction of Mtmr2 homodimer
(A) Mtmr2 protein consists of a GRAM-PH domain (green), a phosphatase domain (blue), a coiled-coil (orange) and at the C-terminus a PDZ binding domain (grey). A crystal structure of the monomer has been published by Begley et al. (2003; 2006) and a complex of two monomers has been stored in the PDB database. The crystal structure lacks the first N-terminal 73 amino acids and the last C-terminal 57 amino acids, including the coiled-coil motif. (B) Ab-initio shape reconstruction of full length His-Mtmr2 homodimer (grey) with DAMMIN and averaged with DAMAVER (ATSAS 2.4), manually superimposed with the antiparallel Mtmr2 PISA-dimer structure. (C) Two Mtmr2 monomer structures manually fitted into the SAXS model, showing a parallel alignment.

7.3.3.2 3-Pap monomer
3-Pap belongs to the catalytically inactive members of the MTMR family and consists only of the characteristic domains: GRAM-PH, phosphatase domain and a coiled-coil motif (Figure 7-22, A). Based on the amino acid sequence retrieved from the UniProt database 3-Pap is 104 amino acids longer compared to Mtmr2 (73.4 KDa), resulting in a 12.7 KDa heavier protein (86.1 KDa in total). Based on the sequence alignment with Mtmr2 and its PDB file (PDB: 1LW3) a surface model of 3-Pap has been proposed (Begley et al, 2006) (Figure 7-22, B). Begley
et al. reported electrostatic polarization of the PH-GRAM and phosphatase domains to be likely for active myotubularins, as shown in Figure 7-22 (B) for Mtmr2 and Mtm1, but not the inactive myotubularins such as 3-Pap/Mtmr12. They propose that the two subgroups may have different affinities for PI-containing membranes. Since structural information of 3-Pap was missing, we performed structural analysis of 3-Pap by analytical purification, crystallization and SAXS.

Analysis of Mtmr2/3-Pap complex by size exclusion chromatography (see Figure 7-15, B) has shown that in addition to the Mtmr/3-Pap heterodimer, 3-Pap eluted as a monomer. To verify these findings, pFL His-3-Pap baculovirus was produced for infection of Sf21 insect cells and protein production. In a first step His-3-Pap was purified by its His-tag with an IMAC column (Figure 7-19, A). In a second purification step the protein was separated from impurities by size exclusion chromatography (Figure 7-19, B). Three peaks were observed, of which the first consisted of impurities with a high molecular weight. 3-Pap eluted within the last two peak maxima corresponding to 162 KDa and 91 KDa (compared with the gelfiltration BioRad standard). The second peak might reflect small amounts of homodimers (expected MW based on the amino acid sequence: 173 KDa). However, under the analyzed conditions most of the 3-Pap protein eluted with a peak maximum of 91 KDa (expected MW is 87.5 KDa), confirming the monomeric state. Peptide mass analysis of His-3-Pap by mass spectrometry (LC/ESI/MS) resulted in a molecular weight of 87.372 KDa, which was 104.9 Da smaller than expected. This discrepancy could neither be explained by an N-terminal methionine substitution (-131 Da) nor by a C-terminal valine substitution (-99 Da). (Figure 7-19, C). Also SEC coupled multi-angle light scattering confirmed the monomeric condition of His-3-Pap. Summarizing these results, we propose that under the measured conditions in vitro 3-Pap is a monomer.
Results

A IMAC

B SEC 200 16/60

C MS

D MALS

Figure 7-19: Protein purification and analysis of 3-Pap by SEC, MS and MALS

Recombinant His-tagged 3-Pap was expressed in Sf21 insect cells. Three days after baculovirus infection the cells were harvested and purified. (A) Purification of His-3-Pap by IMAC and analysis of eluted fractions by Coomassie stained SDS-PAGE. (B) SEC using a Superdex200 16/60 column of the IMAC purified fractions. His-3-Pap eluted with elution maxima of 72.07 ml (~162 KDa) and 79.97 ml (~91 KDa). Eluted fractions were analyzed by Coomassie stained SDS-PAGE. (C) MS analysis of gel filtration purified 3-Pap resulted in a MW of 87.371 KDa. The expected MW, based on the amino acid sequence, was 104.9 Da bigger. (D) Multi-angle light scattering (MALS) analysis of SEC purified 3-Pap protein. 3-Pap eluted with a molecular weight of 89 KDa, confirming the monomeric state.

Crystallization screens have been set up with purified His-3-Pap (see Table 6-8). These however, did not yield any positive hits. A common technique to improve crystallization is the removal of flexible linkers and domains of the protein. This can either be achieved on the transcriptional level, by expressing truncated versions of the protein or on the protein level by in situ proteolysis prior crystallization (Wernimont et al, 2009). The latter is technically the fastest to test. I therefore screened the two standard proteolytic enzymes trypsin and α-chymotrypsin in weight to weight (w:w) ratios of 1:10, 1:100, 1:1,000 and
Results

1:10'000. Evaluating the Coomassie stained SDS-PAGE gel, a dilution of 1:100 for both tested enzymes resulted in one stable fragment of ~55 KDa (Figure 7-20). His-3-Pap was therefore incubated with a 1:100 α-chymotrypsin dilution prior to crystallization (see Table 6-8). However, also the proteolytically treated protein samples did not yield any crystals and further refinement would be needed.

Figure 7-20: *In situ* proteolysis series of purified His-3-Pap for crystallization

4 μg purified His-3-Pap was incubated with trypsin or α-chymotrypsin for 30 min in w:w ratios of 1:10, 1:100, 1:1000 and 1:10000 trypsin or α-chymotrypsin. Reactions were stopped by addition of SDS sample buffer and boiling for 5 min at 95°C. Samples were analyzed by Coomassie stained SDS-PAGE. The first lane represents untreated purified His-3-Pap protein.

Since crystallization was unsuccessful, structural analysis of 3-Pap was continued by small-angle X-ray scattering analysis. Purified His-3-Pap originating from a SEC purification, as shown in the representative Figure 7-19 (framed in red), was used for SAXS analysis. Eluates corresponding to the molecular weight of a monomer were pooled and concentrated. Samples were measured in the presence of EDTA alone or together with DTT at three different concentrations: 1.50 mg/ml, 2.75 mg/ml and 4.38 mg/ml. The highest concentrated protein sample was damaged by radiation, which is why it was not further processed. Initial collected intensities after background subtraction are plotted in Figure 7-21 (A). The Kratky-plot confirmed that the protein was folded (Figure 7-21, B). Compared with the Kratky-plot of Mtmr2 (see Figure 7-17, B) 3-Pap lacked the typical features of two maxima, suggesting a monomeric structure. The linear Guinier-approximation was consistent for both measured samples and resulted in
Results

an average Rg of 41 Å (Figure 7-21, C). The P(r) function is characterized by an average maximum distance (Dmax) of 145 Å. Comparing the P(r) functions of 3-Pap and Mtmr2, 3-Pap is only 5 Å shorter in maximum distance and the distribution lets assume a thinner corpus (Figure 7-21, D).

Figure 7-21: SAXS data analysis of His-3-Pap monomer
(A) Scattered intensity plotted against the scattering vector q, after background substraction. (B) Kratky plot of the measured 3-Pap concentrations. (C) By fitting the Guinier equation with the program PRIMUS (Konarev, 2003), the value of Rg was determined for the 3-Pap concentrations 1.5 mg/ml and 2.75 mg/ml to be 41.96 Å and 40.79 Å, respectively. The average Rg is 41 Å. (D) Comparison of the calculated P(r) functions of 3-Pap (continuous line) and Mtmr2 (dashed line). The P(r) function was generated by the program AUTOGNOM (Svergun, 1992). The maximum diameter (Dmax) for 3-Pap ranges from 143 Å to 147 Å and is in average 145 Å.

The evaluated distance distribution functions were used as an input for the ab-initio shape reconstruction program DAMMIN. Twenty independent structures were generated and averaged with DAMAVER. Here we propose the first ab-inito SAXS model of a full length 3-Pap monomer (Figure 7-22, C). Superimposition
Results

with the Mtmr2 crystal structure was possible with two different orientations, with either the phosphatase domain or the GRAM-PH domain facing the head. Consistent with the interpretation of the $P(r)$ function, 3-Pap shape reconstruction had similar maximum dimensions as Mtmr2. Furthermore, superimposition of both Mtmr2 and 3-Pap SAXS shape reconstructions clearly showed that the corpus of 3-Pap (blue) was thinner (Figure 7-22).

Figure 7-22: Ab-initio shape reconstruction of 3-Pap monomer
(A) 3-Pap consists of a GRAM-PH domain (green), an inactive phosphatase domain (blue) and a coiled-coil motif (CC, orange). (B) Surface electrostatic potentials of human MTMR2, MTM1 and 3-PAP/MTMR12 (adapted from Begley et al., 2006). The surface is colored by its electrostatic potential showing saturating potential in blue and red, 10 and $-10\,\text{kT/e}$, respectively. (C) Ab-initio shape reconstruction of full length His-3-Pap monomer (blue) with DAMMIN and averaged with DAMAVER (ATSAS 2.4). MTMR2 structure (PDB: 1LW3) was manually superimposed. Two
orientations of MTMR2 are possible: phosphatase-to-head or GRAM-PH-to-head (in box).

(D) Superimposition of the Mtmr2 (grey) and 3-Pap (blue) SAXS models.

7.3.4 Localization of Mtmr2 and 3-Pap in HEK293 cells

Previous localization studies of Mtmr2 in COS cells showed that Mtmr2 is diffusely distributed in the cytosol with stronger staining in the perinuclear region (Berger et al, 2003; Kim et al, 2003; Berger et al, 2006a). 3-Pap was reported to localize to punctuate structures in the cytosol of COS-7 cells (Nandurkar et al, 2003). To analyze whether Mtmr2 and 3-Pap colocalize, HEK293 cells were transiently transfected with expression vectors for RGS-His-tagged Mtmr2 and V5-tagged 3-Pap. Mtmr2 staining was observed in punctuate structures in the cytosol and in membrane ruffles at the surface of the cell. 3-Pap colocalized with Mtmr2 in membrane ruffles, however no colocalization was observed in the cytosol (Figure 7-23).

Figure 7-23: Colocalization of Mtmr2 and 3-Pap at membrane ruffles

HEK293 cells were transiently cotransfected with RGS-His-tagged Mtmr2 and V5-tagged 3-Pap. Cells were probed with RGS-His and V5 specific primary antibodies. Two confocal sections of the same cell are shown. Merged images are shown on the panels on the left and right. Colocalization of Mtmr2 (red) with 3-Pap (green) is visible in the merged image as yellow in membrane ruffles (arrow) at the plasma membrane (upper row). No colocalization was observed in the same cell imaged in the center (lower row). White boxes indicate regions, represented as magnified views on the right. Scale bar = 10 µm.

Hypo-osmotic stress has been reported to activate PI-3,5-P\(_2\) synthesis and to lead to a transient increase of PI-3,5-P\(_2\) levels (Dove, 1997). Berger et al. (2003) reported that upon hypo-osmotic stress of transiently transfected COS cells
Results

Mtmr2 or Sbf2 localized at membranes of stress-induced vesicles. Furthermore, it was shown that in cotransfected cells expressing high levels of Sbf2, Mtmr2 staining was absent at these membranes (Figure 7-24, A). They therefore concluded that Sbf2 bound with higher affinity than Mtmr2 to membranes of these vacuoles, thereby competing for Mtmr2-binding sites. To analyze whether 3-Pap has a similar effect on Mtmr2 as Sbf2, HEK293 cells were transiently transfected with Mtmr2 and/or 3-Pap and incubated under hypo-osmotic conditions. HEK293 cells expressing 3-Pap or Mtmr2 alone showed staining at the plasma membrane and at membranes of the stress induced vesicles (Figure 7-24, B and C). Interestingly, in cells coexpressing Mtmr2 and high levels of 3-Pap (Figure 7-24, D), Mtmr2 still localized to the lipids of the vacuoles. Thus, 3-Pap did not compete for Mtmr2-binding sites as it was proposed for Sbf2. These findings let us assume that Mtmr2 and 3-Pap either have similar binding affinities or that they bind as a heterodimer to these membranes.
Figure 7-24: Colocalization of Mtmr2 and 3-Pap in HEK293 cells under hypo-osmotic conditions

(A) COS cells were transiently cotransfected with Sbf2 and Mtmr2 expression constructs and stained after hypo-osmotic shock. Sbf2 preferentially associated with the stress-induced vesicles, but not Mtmr2 (adapted from Berger et al., 2006). (B-D) HEK293 cells were transiently transfected with V5-tagged 3-Pap, RGS-His-tagged Mtmr2 or both expression constructs simultaneously. (B) V5-3-Pap staining was localized at the membranes of the stress induced vesicles, at the plasma membrane and partially in the cytosol. (C) RGS-His-Mtmr2 showed staining at the vesicles, at the plasma membrane and partially in the cytosol. (D) In Mtmr2/3-Pap cotransfected cells V5-3-Pap and RGS-His-Mtmr2 colocalized to membranes of stress induced vesicles and to the plasma membrane. Scale bar = 10 µm.

7.3.5 Influence of Mtmr2, 3-Pap and Mtmr2/3-Pap complex on EGFR trafficking in HEK293 cells

Based on the finding, that Mtmr2 overexpression in HEK293 cells blocks EGF stimulated EGFR degradation and that coexpression of Sbf2 abolished this effect (Berger et al, 2009), we wanted to study the localization of Mtmr2, 3-Pap, Mtmr2/3-Pap complex and EGF receptor in the context of EGF stimulated internalization and trafficking through endosomal compartments. Rab GTPases
were used before as subcellular markers for the localization of MTMRs (Cao et al, 2007; Cao et al, 2008; Franklin et al, 2011). The data are sometimes conflicting. This might be explained by the fact that these vesicles are often positive for several Rab GTPases (Ballmer-Hofer et al, 2011). We therefore analyzed colocalization with four different Rab GTPases to study trafficking through the endocytic compartment. Rab5-Rab4-Rab11 define the recycling axis, whereas Rab5-Rab4-Rab7 define the degradation axis. To monitor the localization throughout the different steps of internalization, HEK293 cells were transiently cotransfected with the expression constructs for the constitutive active endosomal markers YFP-Rab4 Q67L, GFP-Rab5A Q79L, EGFP-Rab7 Q67L or YFP-Rab11 Q70L. Prior to fixation, the Mtmr2 and 3-Pap transfected cells were stimulated with Alexa647 coupled EGF. By using the confocal microscope SP5, we were restricted to the use of three lasers covering the spectra of three different fluorescence markers. These were already occupied by Mtmr2 (Cy3), 3-Pap (Cy5) and the endosomal markers (yellow and green). HEK293 cells transiently cotransfected with MTMR, RabX and EGFR and positive for MTMR and RabX were in general also positive for EGFR (data not shown). We therefore stimulated Mtmr2/3-Pap cotransfected cells only with unstained EGF. Immunostainings of HEK293 cells transiently transfected with RGS-His-tagged Mtmr2, EGFR and GFP-tagged Rab GTPases and stimulated with Alexa647-EGF are represented in Figure 7-25. Based on the binding specificities of Mtmr2 for the phosphoinositides PI-3,5-P$_2$ and PI-5-P, localization of Mtmr2 would be expected at membranes harboring these lipids. Under steady state conditions (T=0) Mtmr2 localized on Rab4, Rab5, Rab7 and Rab11 vesicles, visible as yellow dots and marked with asterisks in the merged Figure 7-25 (A-D). Thirty minutes after EGF stimulation (T=30), Mtmr2 was still visible on Rab4, Rab5 and Rab7 vesicles, joined by internalized EGF (Figure 7-25, A-C, marked with arrows). After 180 min EGF stimulation (T=180), Mtmr2 was no longer found on Rab4 or Rab7 vesicles, but on Rab5 and colocalized with Rab11 vesicles. Interestingly, under steady state conditions and 30 min after EGF stimulation we found Mtmr2 not only at the lipid membranes of the Rab vesicles, but also inside
Results

Rab4, Rab5 and Rab7 vesicles (Figure 7-25, A-C, marked with an asterisk). One possible explanation could be the formation of multivesicular bodies inside these vesicles. In Rab11 transfected cells however, we could not make such observations. EGF staining was found in all four positive Rab endosomes. Considering the finding that after 180 min stimulation Mtmr2 colocalized strongly with Rab11, suggests that Mtmr2 got relocalized to Rab11 vesicles. Additionally, EGF was still observed in Rab11 vesicles, also after 180 min. Recently published localization study of EGFR with Rab GTPases in COS cells has shown that after 180 min EGF stimulation, phosphorylated EGFR was mainly found in Rab5, Rab4 and Rab7 positive vesicles but barely in Rab11 vesicles (Ballmer-Hofer et al, 2011). As mentioned above, it was also reported that Mtmr2 overexpression blocks EGFR degradation and leads to sustained Akt activation (Berger et al, 2009). We can thus hypothesize that Mtmr2 overexpression blocks phosphorylated EGF receptor in Rab11 vesicles, where it further activates downstream signaling leading to sustained Akt activation.
Results

A

T=0

RGS-His-Mtr2

Rab4

EGF

Merge

T=60

T=180

B

T=0

RGS-His-Mtr2

Rab5

EGF

Merge

T=60

T=180

105
Figure 7-25: Localization of Mtmr2 with endosomal markers in EGF stimulated HEK293 cells
HEK293 cells transiently transfected with the expression plasmids coding for RGS-His-tagged Mtmr2, human EGFR and the endosomal markers YFP-Rab4 Q67L (A), GFP-Rab5A Q79L (B), EGFP-Rab7 Q67L (C) or YFP-Rab11 Q70L (D). Prior fixation and immunostaining, the cells were starved overnight and then stimulated for 30 min and 180 min with Alexa647 conjugated EGF (blue). RGS-His-Mtmr2 (red) was detected and stained with RGS-His specific primary antibody.
Results

Vesicles with Mtmr2 inside are marked with an asterisk. EGF loaded vesicles positive for Mtmr2 inside or outside are labeled with an arrow. Rab vesicles only positive for EGF are marked with a filled arrow head and EGF vesicles only positive for Mtmr2 are marked with an empty arrow head. Mtmr2 localized inside Rab4 (A), Rab5 (B) and Rab7 (C) vesicles after 0 min and 30 min stimulation, but not after 180 min. Mtmr2 colocalized with Rab11 after all three time points (D). EGF was found on all four Rab positive endosomes. Scale bar = 10 µm.

Transiently transfected HEK293 cells with V5-tagged 3-Pap, EGFR and GFP-tagged Rab GTPases expression constructs, stimulated with Alexa647-EGF and immunostained are represented in Figure 7-26. At steady state conditions 3-Pap did not colocalize with any of the four expressed endosomal markers, nor was it observed inside the endosomes. Thirty minutes and 180 min after EGF stimulation, 3-Pap was observed at Rab4, Rab5 and Rab11 positive vesicles, pointing to the localization at vesicles important in the recycling pathway. EGF was observed in all four endosomal compartments after stimulation.
Figure 7-26: Localization of 3-Pap with endosomal markers in EGF stimulated HEK293 cells
HEK293 cells transiently transfected with the expression plasmids coding for V5-tagged 3-Pap, human EGFR and the endosomal markers YFP-Rab4 Q67L (A), GFP-Rab5A Q79L (B), EGFP-Rab7 Q67L (C) or YFP-Rab11 Q70L (D). Prior fixation and immunostaining, the cells were starved and stimulated for 30 min and 180 min with Alexa647 conjugated EGF (blue). V5-3-Pap (red) was detected and stained with V5 specific primary antibody. 30 min and 180 min after
Results

stimulation 3-Pap localized to Rab4, Rab5 and Rab11 vesicles, which were positive for EGF (arrow). Rab vesicles only positive for EGF are marked with an arrow head. EGF was found on all four Rab positive endosomes. Scale bar = 10 µm.

The first immunostaining experiments were performed in COS-7 cells, which have the advantage to express endogenous EGF receptor in higher amounts compared to HEK293 cells. Therefore, transient transfection of COS-7 with EGFR is unnecessary. We observed that in transiently cotransfected cells with 3-Pap and Mtmr2 expression constructs, the 3-Pap transfection efficiency was constantly higher compared to Mtmr2 (data not shown). Less than 5% of 3-Pap transfected cells were cotransfected with Mtmr2, making localization studies quantitatively difficult. However, we could collect a dataset of transiently transfected COS-7 cells with V5-tagged 3-Pap and GFP-tagged Rab GTPases expression constructs, stimulated with Alexa647-EGF (Figure 7-27), representing a valuable verification of the HEK293 results. In COS-7 cells, 3-Pap already colocalized at steady state conditions with all four endosomal markers. Thirty minutes after EGF stimulation, 3-Pap colocalized with EGF positive Rab4 and Rab5 vesicles (arrow in A and B), however not with Rab7 or Rab11 vesicles. After 180 min EGF stimulation, 3-Pap was still found at Rab4 and Rab5, but also at Rab11 vesicles. Internalized Alexa647-EGF was found in all four Rab positive vesicles (arrow head). Comparing and summarizing these results with the HEK293 immunostaining results (Table 7-2): in COS cells, at steady state conditions, 3-Pap could already be found at Rab4, Rab5 and Rab11 vesicles; in HEK293 cells however, this was not the case until 30 min and 180 min after EGF stimulation; in both tested cell lines, 3-Pap clearly did not localize to Rab7 vesicles. These findings all point to a localization of 3-Pap at early endosomes of the recycling axis.
Results

A

T=0

V5-3-Pap
Rab4
EGF
Merge

T=30

T=60

B

T=0

V5-3-Pap
Rab5
EGF
Merge

T=30

T=180
Figure 7-27: Localization of 3-Pap with endosomal markers in EGF stimulated COS-7 cells
COS-7 cells transiently transfected with the expression plasmids coding for V5-tagged 3-Pap and the endosomal markers YFP-Rab4 Q67L (A), GFP-Rab5A Q79L (B), EGFP-Rab7 Q67L (C) or YFP-Rab11 Q70L (D). Prior fixation and immunostaining, the cells were starved and stimulated for 30 min and 180 min with Alexa647 conjugated EGF (blue). V5-3-Pap (red) was V5 specific primary antibody. At steady state conditions (T=0min) 3-Pap colocalized with all four endosomal markers (arrows). After 30 min and 180 min stimulation 3-Pap localized on Rab4, Rab5 and
Rab11 vesicles, which were positive (arrow) or negative (asterisk) for EGF. Rab vesicles only positive for EGF are marked with an arrow head. EGF was located in all four Rab positive endosomes. Scale bar = 10 µm.

The immunostaining results of EGF stimulated HEK293 cells transiently transfected with RGS-His-tagged Mtmr2, V5-tagged 3-Pap, human EGFR and GFP-tagged Rab GTPases are represented in Figure 7-28. Prior to stimulation (T=0), Mtmr2 was found on Rab4, Rab7 and Rab11 vesicles (asterisk), but not on Rab5 vesicles. Triple positive vesicles were only found on Rab4 vesicles (marked with an arrow, A). Thirty minutes after EGF stimulation, Mtmr2 was found on all four Rab positive endosomes, after 180 min however, only on Rab4 and on Rab11 vesicles. A weak colocalization of 3-Pap with Rab5 was observed and marked with an arrow head (B). It is interesting that the only clear colocalization between Mtmr2, 3-Pap and endosomes 180 min after EGF stimulation occurred in Rab4 and Rab11 vesicles (A and D, marked with an arrow), which belong to the recycling axis.
Results
Figure 7-28: Localization of Mtmr2 and 3-Pap with endosomal markers in EGF stimulated HEK293 cells
HEK293 cells transiently transfected with the expression plasmids coding for RGS-His-tagged Mtmr2, V5-tagged 3-Pap and the endosomal markers YFP-Rab4 Q67L (A), GFP-Rab5A Q79L (B), EGFP-Rab7 Q67L (C) or YFP-Rab11 Q70L (D). Prior fixation and immunostaining, the cells were starved and stimulated for 30 min and 180 min with unconjugated EGF. Vesicles with Mtmr2 inside are marked with an asterisk. Vesicles positive for Mtmr2 inside or outside and 3-Pap are
labeled with an arrow. Colocalization between 3-Pap and Rab vesicles are marked with an arrow head and colocalizations between Mtmr2 and 3-Pap are marked with a V. Mtmr2 localized inside Rab4 (A), Rab7 (C) and Rab11 (D) vesicles after 30 min stimulation and after 180 min only in Rab4 and Rab11. Mtmr2 colocalized with Rab11 after all three timepoints (D). Scale bar = 10 µm.

The immunostainings are summarized in Table 7-2. Comparing the localization of Mtmr2 with 3-Pap alone, Mtmr2 did localize inside Rab7 vesicles, but 3-Pap did not localize at the membrane of these vesicles. The differences get even more clear 180 min after EGF stimulation. Mtmr2 was no longer present in Rab4 or Rab7 vesicles, however at Rab11 vesicles. 3-Pap in contrast, was found at Rab4 vesicles. Both, Mtmr2 and 3-Pap were found in or at Rab5 and Rab11 vesicles. In Mtmr2/3-Pap double transfected cells, Mtmr2 did not relocalize 3-Pap to Rab7 vesicles. Furthermore, Mtmr2 was no longer found in Rab5 vesicles, but in Rab4 vesicles. Considering the findings that in COS cells 3-Pap localized already at steady state conditions at Rab4 and Rab5 vesicles and remained there also after EGF stimulation (in HEK293 cells and COS cells), might support the hypothesis that the affinity of 3-Pap for Rab5 vesicles is stronger than of Mtmr2. Whether the presence of 3-Pap at Rab4 vesicles might retain Mtmr2 in these vesicles would have to be investigated. All together it can be concluded, that after stimulation the myotubularins Mtmr2 and 3-Pap localized independently or in complex along the recycling axis at Rab4 and Rab11 vesicles.

Table 7-2: Summary of Mtmr2 and 3-Pap localizations with Rab positive vesicles before and after EGF stimulation

<table>
<thead>
<tr>
<th>HEK293</th>
<th>T=</th>
<th>Rab4</th>
<th>Rab5</th>
<th>Rab7</th>
<th>Rab11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>180</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Mtmr2</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Mtmr2/3-Pap</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
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<td>√</td>
<td>-</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Mtmr2/3-Pap</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>COS-7</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3-Pap</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

Presence or absence in or at the respective Rab vesicles 0 min (0), 30 min (30) and 180 min (180) after EGF stimulation is symbolized with a tickmark (√) or a minus (-), respectively. In the case of Mtmr2/3-Pap cotransfected cells, the evaluated results refer to the underlined protein.
8 Discussion and Outlook

8.1 MTMRs interact with PDZ domain containing scaffolds
MTMRs are phosphatases which regulate the phosphoinositol pool at cellular membranes and influence trafficking and sorting of internalized membrane proteins. Loss of function of MTM1, MTMR2 and SBF2 has been linked to the human diseases XLMTM, CMT4B1, and CMT4B2, respectively, highlighting their essential role in human health. MTM1 and MTMR2 share high sequence similarity, same substrate specificity and are both expressed in the same tissues. However, the molecular mechanism leading to the distinct diseases XLMTM and CMT4B1 is poorly understood. One possibility to explain this differential outcome could be that the C-terminal PDZ-binding motif of MTM1 (-VQTHF) and MTMR2 (-VQTVV) interact with different PDZ domains of scaffolding proteins.

The goal of the first part of this thesis was to identify and characterize novel or known PDZ-interactors of MTMRs. First of all we screened GST-tagged C-termini of MTMRs against 96 different PDZ domains spotted on a membrane (Fam et al, 2005). Thereby we identified the first two PDZ domains of Sap97 to bind specifically to the GST-tagged C-termini of MTMR1 (-VHTSV), Mtmr1 (-VHTSV) and MTMR2, but not Mtm1. The finding that Mtmr2 interacts with Sap97 as such was nothing new. Initially, the Sap97-Mtmr2 interaction was identified in Schwann cells of mice, where Sap97 is enriched in the paranodal cytoplasm. Interestingly, Sap97 localization was altered in Schwann cells of conditional Mtmr2-null mice. Loss of the interaction between Sap97 and Mtmr2 results in myelin outfoldings at the paranodes, suggesting that this interaction regulates cellular junctions or membrane remodelling in Schwann cells (Bolino et al, 2004).

To specifically localize and characterize the Mtmr2-binding domain of Sap97 more precisely, we coexpressed and purified full length His-tagged Mtmr2 and HA-tagged PDZ domains of Sap97 (Sap97-PDZ1, Sap97-PDZ2, Sap97-PDZ3, Sap97-PDZ1-2 or Sap97-PDZ1-3) from Sf21 insect cells. Our coimmunoprecipitation experiments revealed that Mtmr2 preferentially binds to the second PDZ domain of Sap97 (Figure 7-10). Meanwhile, Bolis et al. (2009)
showed by GST-pulldown experiments from COS-7 cells expressing Myc-tagged Mtmr2 and GST-tagged Sap97 PDZ domains (PDZ1, PDZ1+2 or PDZ2+3) that Mtmr2 preferentially interacted with Sap97-PDZ1+2 and Sap97-PDZ2+3. Their results clearly support our findings, whereas we additionally could show a specific affinity of Mtmr2 towards the second PDZ domain of Sap97. Rescue experiments in \( Mtmr2 \)-null explants with Mtmr2 and Mtmr2 devoid of the PDZ-binding motif (Mtmr2ΔPDZ) showed that Mtmr2ΔPDZ rescued myelin outfoldings significantly less effectively than Mtmr2 (Bolis et al, 2009). Additionally, Bolis et al. identified in Schwann cells the plus-end directed motor protein kinesin 13B (kif13B) and the exocyst component Sec8 to interact with Sap97. They proposed a model in which Sap97-Sec8 interaction promotes membrane addition to sites of membrane remodelling and Mtmr2 negatively regulates membrane formation (Figure 8-1, A). Cotter et al. (2010) silenced Sap97 by injection of lentiviral vectors expressing shRNA in myelinating Schwann cells in mouse sciatic nerves at early stages of myelination (3 to 4 days postnatal). Two months after injection the thickness of myelinating Schwann cells was strikingly increased, whereby internodal length remained unaffected. Furthermore, they observed that some Sap97-silenced cells displayed myelin outfoldings, typical characteristics of CMT4B. Sap97 overexpression prevented Schwann cell myelination, indicating an inhibitory function. At last, they observed that axonal neuregulin-1 type III stimulated myelination was negatively regulated by Sap97 and Pten. Furthermore, Pten and PI3K regulate the turnover of PI-4,5-P\(_2\) and PI-3,4,5-P\(_3\), whereby loss of Pten was shown to result in increased levels of PI-3,4,5-P\(_3\) which have been shown to trigger myelination in PNS and CNS (Goebbels et al, 2010). Summerizing the above mentioned observations: Mtmr2, Pten and Sap97 influence membrane remodeling and myelination either alone or in complex with each other. Regarding our results and taking the above mentioned observations together, it would be interesting to see which effect a deletion of the second PDZ domain of Sap97 would have on myelin formation and on downstream signaling of Pten. Furthermore, it would be interesting to analyze whether weaker binding of Mtmr2 to the first or third PDZ domain of Sap97 would be enough for myelin
formation. Sap97 has been reported to bind via its PDZ domains to various C-termini of receptors and ion channels, thereby regulating function and localization of these membrane proteins (see Table 8-1). Additional potential PDZ-domain binding interactors of Sap97 predicted from the primary PDZ binding motif sequence were summerized in the Table S6 by Tonikian et al. (2008). In addition to the PDZ dependent interaction of Sap97 to the plus-end directed microtubule motor protein KIF1Bα, Sap97 has also been reported to interact through its N-terminus to the minus-end directed actin motor protein myosin VI and through its MAGUK domain with the plus-end directed microtubule motor protein Kif13B (Hanada et al, 2000; Wu et al, 2002; Mok et al, 2002). In Schwann cells KIF13B could transport Sap97 at sites of membrane remodeling, where interaction with Sec8 and Mtmr2 could regulate the rate of membrane addition (Bolis et al, 2009). The exact molecular mechanism of Mtmr2 on membrane remodeling during Schwann cell myelination is still unclear. The interaction of a PDZ scaffold with motor proteins has recently been shown to be important for the sequence-dependent recycling of receptors. Analysis of endosomal sorting of beta-2 adrenergic receptor (β2AR) revealed concentrated localization of β2AR in actin-stabilized recycling tubules positive for Rab4 and Rab11. Furthermore, they could show that the linkage of the receptor to the local actin cytoskeleton was mediated through PDZ-domain interactions (Puthenveedu et al, 2010). Similar results were found for the intracellular trafficking of internalized Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Stimulation with Neuropilin-1 (NRP1)-binding isoform VEGF-A_{165a} led to receptor recycling to the plasma membrane through Rab11, while an isoform unable to bind NRP-1, VEGF-A_{165b}, failed to do so (Ballmer-Hofer et al, 2011; Berger et al, 2011). They could show that the transition of internalized VEGFR-2/NRP-1/VEGF-A_{165a} complex from Rab4 to Rab11 vesicles was mediated by the PDZ binding of NRP-1 to GIPC. These results suggested a GIPC-mediated recycling of VEGFR-2/NRP-1/VEGF-A_{165a} complex. Since GIPC was previously reported to associate with myosin VI (Aschenbrenner et al, 2003; Naccache et al, 2006), they proposed that the attachment of a
Discussion and Outlook

NRP-1/GIPC/myosin VI complex to VEGFR-2 by VEGF A$_{165}$a lead to association with the actin cytoskeleton early after internalization. Myosin VI can therefore be seen as mediator for trafficking of internalized cargo to early and recycling endosomes. Recycling back to the plasma membrane however, would need a plus-end-directed myosin motor as for example myosin Vb. Interestingly, Rab11a has been reported to form a ternary complex with the myosin Vb and Rab11-family interacting protein 2 (FIP2), representing a possible sorting mechanism back to the plasma membrane along the actin cytoskeleton and/or by tubulation (Hales et al, 2001; Hales et al, 2002).

Up to now, the role of MTMR1 is poorly understood. During differentiation and development of skeletal muscle cells the MTMR1 gene is subject to alternative splicing and a muscle-specific transcript was reported. In patients with congenital myotonic dystrophy (cDM1) mRNA splicing of various genes is disturbed, including the muscle-specific isoform of MTMR1 (Buj-Bello et al, 2002a). The only so far known interaction of the PDZ binding motif of MTMR1 with a PDZ-domain containing protein has been identified in a yeast-two-hybrid screen using TIP-15 as bait (splice variant of PSD95/DLG4/SAP90 harboring the first two PDZ domains) (Fabre et al, 2000). The C-terminal PDZ-binding motif of MTMR1 with the sequence –VHTSV belongs to the class I PDZ-BD consensus sequence X-S/T-X-V/L. By the PDZ-domain array, as well as by GST pulldowns, this interaction was confirmed (Figure 7-4). In addition, I could show that the C-term of Mtmr1 interacted with the third PDZ domain of PSD95. Thereby we monitored a stronger signal for mouse Mtmr1 compared to human MTMR1. Interestingly, also the PDZ domains SAP97 PDZ1+2, SAP97 PDZ3 and PTPN13 PDZ4+5 precipitated stronger with Mtmr1 compared to MTMR1 (Figure 7-4). Although the C-termini of both Mtmr1 (-TSVHTSV) and MTMR1 (-TPVHTPV) are homologous in the last five amino acids, they differ at the sixth last position. The proline of MTMR1 might have introduced sterical changes into the peptide chain explaining the differential binding properties between MTMR1 and Mtmr1. PSD95 is an abundant excitatory postsynaptic scaffolding protein which has been implicated together with the MAGUK family members in the regulation of excitatory synapse
formation, maturation and plasticity (reviewed in (Zheng et al, 2011)). PSD95 interacts and scaffolds a subset of membrane receptors which are listed in Table 8-1. Recently, Mtmr2 was localized in excitatory synapses of rat central neurons and was identified to interact with the first two PDZ domains of PSD95 (Lee et al, 2010). The C-terminal PDZ-BD sequence –VQTVV of Mtmr2 is highly similar to Mtmr1 (-VHTSV). Furthermore they observed that knockdown of Mtmr2 in cultured neurons reduced excitatory synapse density and function and shifted the intensity of EEA1-positive signal on EEs from dendrites to the cell body region. This effect was rescued by wild-type Mtmr2 but not by Mtmr2ΔPDZ or Mtmr2 lacking the phosphatase activity. Suppression of Mtmr2 promoted endocytosis of the AMPA receptor subunit GluR2, which mainly localized to late endosomes/lysosomes in the cell body. Lee and coworkers therefore suggested that PSD95 promotes the synaptic localization of Mtmr2, and that synaptically targeted Mtmr2 maintains excitatory synapses by inhibiting excessive endosomal production and destructive trafficking to lysosomes. Based on the findings of Mtmr2 function on EGFR trafficking I would suggest, that Mtmr2 phosphatase activity alters the local phosphoinositide pool and thereby redirects the receptor complex back to the plasma membrane (Figure 8-1, B). The question which arises is, whether PSD95 knockdown or mutant lacking the first two PDZ domains would recapitulate the Mtmr2 knockdown phenotype in the excitatory synapses. Moreover, does Mtmr1 also play a role in excitatory synapses or does PSD95-Mtmr1 interaction have another cell specific area of action? As a next step Mtmr1-Sap97 interaction should be confirmed in vitro and in vivo with full length proteins. Assuming that Mtmr1 and Mtmr2 have overlapping PDZ-domain binding specificities, it would be interesting to analyze, whether Mtmr1 is able to compete with Mtmr2 for binding to the PDZ domain or whether Mtmr1 might even compensate Mtmr2 knockdown phenotype.

The PDZ array revealed that the third PDZ domain of PDZK2 specifically and only interacted with the C-term of Mtm1. We confirmed the Pdzk2-Mtm1 interaction in vitro and in vivo and could show that the interaction was dependent on the last 5 carboxy-terminal amino acids. mRNA based expression patterns of
Pdzk2 identified Pdzk2 mainly in intestine and kidney (Gisler et al, 2001; Scott et al, 2002). Interestingly, immunoprecipitation of Mtm1 from mouse tissue coprecipitated Pdzk2 not only from intestine and kidney tissue lysates, but also from cerebellum, muscle, stomach, heart, spleen and liver tissue lysates. To our best knowledge, Pdzk2 has yet not been correlated to XLMTM. Considering that mutations in Mtm1 gene cause the muscle specific phenotype CNM and regarding our finding that Pdzk2 is expressed in mouse muscle and does interact with Mtm1, strongly suggest to analyze the role of Mtm1-Pd zk2 interaction in muscle cell function more intensively. Assuming Pd zk2 has a scaffolding function in muscle cells at the plasma membrane or at endosomes strongly points to follow up with an analysis of Pd zk2-interacting proteins via pulldowns from muscle tissue. PDZK2 (also named NHERF4 or I KEPP) belongs to the Na⁺/H⁺ exchanger regulatory factor (NHERF) family of epithelial-enriched PDZ domain scaffolding proteins. Out of four family members NHERF1 and NHERF2 are characterized by two PDZ domains and a C-terminal MERM (Moesin, Ezrin, Radixin, Merlin) binding domain. PDZK1/NHERF3 and PDZK2/NHERF4 lack the MERM domain but consist of two additional PDZ domains. NHERFs play important roles for the regulation of intestinal ion transport and have been shown to bind through their PDZ domains to ion channels, receptors, transporters and signaling molecules, thereby regulating transport activity, subcellular localization and ion homeostasis (Gisler et al, 2001; Gisler et al, 2003; Shenolikar et al, 2004; Thelin et al, 2005; Lamprecht et al, 2006). Proteins interacting with the PDZ domains of Pd zk2 are listed in Table 8-1. OCTN2 is a sodium-dependent uptake transporter for L-carnitine, expressed in kidney, heart, skeletal muscle and placenta (Tamai et al, 1998). Interestingly, in HEK293 cells coexpressing PDZK2 and OCTN2, cell surface expression level and L-carnitine transport capacity of OCTN2 was increased (Watanabe et al, 2006). Whether this was due to a stabilizing effect of PDZK2 at the plasma membrane was not analyzed. It could also be suggested that internalized OCTN2 localized at EEs bound PDZK2 and then recycled back to the plasma membrane. Furthermore, it could be hypothesized that PDZK2 interacted with phosphatases e.g. Mtm1, which
promoted recycling of OCTN2/PDZK2 complexes from EEs. The recycling hypothesis is supported by the finding that the C-terminal PDZ-BD of CFTR is required for the recycling of CFTR from EEs/REs back to the plasma membrane (Moyer et al, 1999; Swiatecka-Urban et al, 2002; Lamprecht et al, 2006). Thereby, binding to members of the NHERF family has been suggested to promote the recycling of CFTR (Lamprecht et al, 2006). The calcium transporters transient receptor potential vanilloid member 5 (TRPV5) and TRPV6 tightly control transcellular Ca\(^{2+}\) (re)absorption in epithelial cells of intestine and kidney and thereby regulate calcium homeostasis (reviewed in (Renkema et al, 2011)). Gene ablation of TRPV5 and TRPV6 in mice leads to hypercalciuria, which forms the main risk factor for renal stone formation (Hoenderop et al, 2003; Coe et al, 2005). Long term survivors with XLMTM have been reported with clinical complications including gall stones, kidney stones, nephrocalcinosis and biochemical evidence of liver dysfunction (Herman et al, 1999). Considering the hypothesis that Mtm1 might influence ion homeostasis and absorption e.g. in the kidney via binding of Pdzk2 to TRPV5 and/or TRPV6 might explain the observed formation of kidney stones and nephrocalcinosis in long term XLMTM patients, as well as other accompanied complications. NaPi-IIa cotransporter plays an important role in renal phosphate reabsorption and homeostasis at the brush border membrane. Parathyroid hormone (PTH) regulates phosphate homeostasis by inducing internalization and degradation of NaPi-IIa (Pfister et al, 1998; Pfister et al, 1997). In transfected OK cells the transporter NaPi-IIa was reported to localize predominantly at the apical plasma membrane. Deletion of the last three C-terminal amino acids partially prevented apical expression of NaPi-IIa and increased its localization in intracellular compartments (Karim-Jimenez et al, 2001). NaPi-IIa was shown to bind with its C-terminal PDZ-BD to the third PDZ domain of both Pdzk1 and Pdzk2 and to the first PDZ domain of NEHRF1 (Gisler et al, 2001). Similar to CFTR, localization of NaPi-IIa has been suggested to be regulated by NHERF scaffolding proteins (reviewed in (Bacic et al, 2004)). Immunostaining of mouse proximal tubules showed that, Nherf1, Nherf2 and Pdzk1/Nherf3 colocalized with NaPi-IIa within the brush border
Discussion and Outlook

membrane (BBM) and Pdzk2 localized subapically (Gisler et al., 2001). Furthermore, it should be mentioned that overexpression of the third PDZ domain of Pdzk1, which is highly homologous to the third PDZ domain of Pdzk2, had a dominant negative effect on the apical expression of NaPi-IIa (Hernando et al., 2002; Thelin et al., 2005). Nherf1 and Pdzk1 could therefore stabilize NaPi-IIa at the apical plasma membrane or support recycling as described above for OCTN2. We therefore need functional assays which analyze the influence of Mtm1 and Pdzk2 on the trafficking and activity of Pdzk2-bound receptors such as TRPV5, TRPV6, NaPi-IIa or OCTN2. By immunostainings of transiently transfected COS-7 cells and HEK293 cells we observed that Mtm1 and Pdzk2 colocalized in vesicles, suggesting Mtm1 and Pdzk2 to form a complex at these structures (Figure 7-6 and Figure 7-7). Immunostainings of OK cells stably expressing NaPi-IIa and transiently transfected with Mtm1 and Pdzk2, NaPi-IIa clustered at the apical plasma membrane and in microvilli, consistent with previous localization studies (Blaine et al., 2009; Pfister et al., 1997; Pfister et al., 1998). As expected, Pdzk2 localized subapically of the plasma membrane in the cytoplasm, however we could not observe colocalization of Pdzk2 with Mtm1 in vesicles (Figure 7-9). Thus we hypothesized, that upon PTH stimulation Pdzk2 and/or Mtm1 might relocate e.g. to NaPi-IIa positive endosomal compartments. However, due to the highly inhomogeneous NaPi-IIa expression and the low rate of transiently transfected polarized cells positive for Mtm1 and Pdzk2, we were not able to assess NaPi-IIa, Pdzk2 and Mtm1 localization under PTH stimulated conditions in OK cells. The question whether Mtm1 overexpression influences NaPi-IIa localization and trafficking remains therefore still unanswered. The following experimental changes, improvements or alternatives should be considered: (1) Establish OK cell line stably expressing NaPi-IIa, Pdzk2 and Mtm1 simultaneously and repeat PTH-directed internalization and localization studies; analyze alternatively the influence of Mtm1 and Pdzk2 on (2) OCTN2 localization, transport activity and carnitine-uptake in HEK293 (Watanabe et al., 2006) or on (3) TRPV5/TRPV6 localization, transport activity and Ca\(^{2+}\)-uptake in HEK293 or HeLa cells (van de Graaf et al., 2006; van de Graaf et al., 2008).
### Table 8-1: List of interaction partners of SAP97, PSD95/SAP90/DLG4 and PDZK2/IKEPP and their PDZ domain binding interactors

<table>
<thead>
<tr>
<th>PDZ Protein</th>
<th>PDZ Domain</th>
<th>Interactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAP97/DLG1</strong></td>
<td>1</td>
<td>NrCAM</td>
<td>(Dirks et al, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR2B</td>
<td>(Wang et al, 2005)</td>
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<td></td>
<td></td>
<td>GluR6</td>
<td>(Mehta et al, 2001)</td>
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<td></td>
<td></td>
<td>KIFIBα</td>
<td>(Mok et al, 2002)</td>
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<td></td>
<td></td>
<td>SEC8</td>
<td>(Bolis et al, 2009)</td>
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<tr>
<td></td>
<td></td>
<td>Kir2.2</td>
<td>(Leonoudakis et al, 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KIFIBα</td>
<td>(Mok et al, 2002)</td>
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<tr>
<td></td>
<td></td>
<td>MTMR2</td>
<td>Figure 7-10 and (Bolis et al, 2009)</td>
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<tr>
<td></td>
<td></td>
<td>PTEN</td>
<td>(Valiente et al, 2005)</td>
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<tr>
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<td>SST1</td>
<td>(Cai et al, 2008)</td>
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<td></td>
<td>9ORF1</td>
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<td>3</td>
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<td>1-3</td>
<td>GluR1</td>
<td>(Leonard et al, 1998)</td>
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<td><strong>PSD95/SAP90/DLG4</strong></td>
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<td>KIFIBα</td>
<td>(Mok et al, 2002)</td>
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<td>KCNA4/Kv1.4</td>
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<td>(Garcia et al, 2000)</td>
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<td></td>
<td></td>
<td>MTMR1</td>
<td>(Fabre et al, 2000)</td>
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### PDZK2/IKEPP/NHERF4

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<th>PDZK2/IKEPP/NHERF4</th>
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In summary, the function of MTMRs in trafficking and recycling of transmembrane receptors and their influence on endosome maturation through their phosphatase activity has been reported many times. The identification and characterization of the interaction of MTMRs with PDZ-domain containing scaffolding proteins however, is still scarce and only few MTMR-PDZ protein interactions have been analyzed in detail. Although I have not discussed all single positive PDZ-array results here, the abundance of potential PDZ-interactors for each tested MTMR points to a significant role. Differential interaction of MTMRs with PDZ domain containing proteins, which themselves also interact with different membrane receptors can therefore result in differential function and specificity of MTMRs. Whether the different PDZ-dependent specific interactions between Mtm1 and Mtmr2 are the answer to the differential clinical features of XLMTM and CMT4B diseases, respectively, remains open.
Nevertheless, we have shown that Pdzk2 is bound by Mtm1 and not by Mtmr2 pointing to such a selectivity. Based on the findings (1) that overexpression of MTMRs can block the degradation of membrane receptors and can promote their recycling, (2) that overexpression of certain PDZ scaffolds enhances receptor activity and abundance at the plasma membrane and (3) MTMRs as well as receptors and channels bind to PDZ scaffolding proteins prompted us to merge these findings and propose an updated model. Figure 8-1 (A) represents a modified model proposed by Bolis et al. (2009). Mtmr2 interacts through its PDZ-BD with the second PDZ domain of Sap97. Sap97 interacts via its MAGUK domain with the plus-end directed microtubule motor protein Kif13B, which directs the whole protein complex in Schwann cells towards the plasma membrane and the site of membrane remodeling. In the modified and generalized model (Figure 8-1, B) a receptor binds via its PDZ-BD to a PDZ scaffold protein at EEs. The PDZ protein also interacts with an active member of the MTMR family. Thereby, the phosphatase activity of the MTMR alters the local pool of phosphoinositides. The reduction of PI-3-P and PI-3,5-P\(_2\) pool leads to a reduced recruitment of adaptors for degradation such as the ESCRT machinery including Vps24 or Hrs. The local increase of PI-5-P triggers the recruitment of recycling adaptors such as SNX5. The binding of MTMRs to receptor-PDZ-scaffold complexes therefore promotes and directs recycling of the whole complex to the plasma membrane. Dependent on the domain structure of the PDZ scaffold, the protein complex can be transported along the cytoskeleton by association with actin or microtubule binding motor proteins.
8.2 Interactions between active and inactive MTMR members

Specificity among the MTMRs can not only be gained by differential interaction with PDZ scaffolds, but also by differential interaction with other MTMR family members. The majority of MTMRs have been shown to homodimerize and/or heteromerize (see Table 5-1). Heteromerization between active and inactive MTMRs resulted in several changes including increased catalytic activity of the active member (Kim et al, 2003; Nandurkar et al, 2003; Mochizuki et al, 2003; Berger et al, 2006a; Zou et al, 2009), relocalization of the protein complex to specific subcellular sites (Nandurkar et al, 2003; Kim et al, 2003; Lorenzo et al, 2006) and modified substrate specificity, as shown for the Mtm1/3-Pap heterodimer purified from platelets which can dephosphorylate PI-3,4-P_2 (Nandurkar et al, 2001). Both Mtm1 and Mtmr2 interact with the inactive 3-Pap/Mtmr12 (Nandurkar et al, 2003), but only Mtmr2 was shown to heteromerize with Sbf1/Mtmr5 and Sbf2/Mtmr13 (Kim et al, 2003; Berger et al, 2006a).
8.2.1 The Mtmr2/Sbf2 protein complex
Previously, Mtmr2 and Sbf2 were both shown to homodimerize each through their coiled-coil domain and to form a heterotetramer (Berger et al, 2006a). To understand how Sbf2 increases the catalytic activity of Mtmr2 from the structural point of view we aimed to express and purify the Mtmr2/Sbf2 complex for structural analysis by crystallization and SAXS. We successfully expressed His-tagged Mtmr2 simultaneously with the full length CBP-tagged Sbf2 or with the Sbf2 truncated versions CBP-Sbf2-MT+PH and CBP-Sbf2-MT. Complex formation of His-Mtmr2/CBP-Sbf2, His-Mtmr2/CBP-Sbf2-MT+PH and His-Mtmr2/CBP-Sbf2-MT was confirmed after a single IMAC purification (Figure 7-11, Figure 7-12 and Figure 7-13). Separation of the His-Mtmr2/CBP-Sbf2 complex however was not optimal. Although Mtmr2 mainly eluted as homodimer and Sbf2 in an aggregated state, low amounts of Mtmr2/Sbf2 complex eluted with an apparent molecular size consistent with a tetrameric state. Against expectations, SEC purification of His-Mtmr2/CBP-Sbf2-MT+PH and His-Mtmr2/CBP-Sbf2-MT resulted not in the elution of a tetramer, but rather in the elution of a His-Mtmr2 dimer together with a CBP-Sbf2-MT dimer or a CBP-Sbf2-MT+PH dimer. This let suggest that the N-terminal region including the DENN domain and a large unidentified domain (~451 amino acids) might contribute to the stability of a heterotetrameric complex formation with Mtmr2. This hypothesis could be verified by coexpression and analytical purification of Mtmr2 together with a truncated Sbf2 version possessing the N-terminal domain but missing the C-terminal PH domain. This however, was not tested in this work. Furthermore, it was observed that CBP-Sbf2-MT and CBP-Sbf2-MT+PH expressed in similar amounts compared to His-Mtmr2, whereas CBP-Sbf2 expression was lower and during gelfiltration mainly eluted together with aggregates. Although the large N-terminal domain upstream of the PH-GRAM domain of Sbf2 seemed to contribute to the stability of a heterotetramer formation with Mtmr2, it also decreased together with the C-terminal domain the solubility of Sbf2. Since purification of a stable Mtmr2/Sbf2 tetramer devoid of any flexible domains for crystallization was unsuccessful this project was stopped at this
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point. Alternatively we analyzed the characterized Mtmr2 interaction with the inactive phosphatase 3-Pap/Mtmr12.

8.2.2 Structural characterization of Mtmr2 homodimer, 3-Pap monomer and Mtmr2/3-Pap heterodimer

Originally, 3-phosphatase adapter protein (3-Pap) was purified from rat brain in form of a heterodimer with an associated unidentified 65 KDa PI-3-P 3-phosphatase (Caldwell et al, 1991). Size exclusion chromatography revealed that this 65 KDa protein existed as homodimer and as heterodimer with 3-Pap, whereby the heterodimeric complex exhibited increased specificity and phosphatase activity towards its substrate PI-3-P. Ten years later, the cDNA of human 3-PAP was cloned and its coding protein characterized as catalytically inactive phosphatase of the MTMR family (Nandurkar et al, 2001). In 2003, MTM1 was identified as catalytic subunit associated with 3-PAP. Interaction of 3-PAP with MTM1 caused relocation of MTM1 from the plasma membrane to the cytosol and to punctuate vesicles and attenuated the MTM1-overexpressing cell phenotype in COS-7 cells. In addition, also MTMR2 was found to interact with 3-PAP (Nandurkar et al, 2003). The Mtmr2/3-Pap interaction was later confirmed in mouse Schwann cells (Tersar, 2008). In the second part of this thesis I described the successful expression, purification and characterization of the proteins Mtmr2 and 3-Pap and their protein complex Mtmr2/3-Pap. Furthermore I identified the relevant domains for Mtmr2/3-Pap complex formation and I determined the subcellular localization of Mtmr2, 3-Pap and EGFR in cells under steady state and EGF stimulated conditions.

As previously described, purification by gelfiltration of His-Mtmr2 clearly resulted in an apparent molecular size consistent with a homodimeric state (Berger et al, 2003) (Figure 7-16). Crystal structures of Mtmr2 monomer (PDB: 1LW3) was previously described, but lack the first N-terminal 73 amino acids and the last C-terminal 57 amino acids, including the coiled-coil motif and the PDZ binding domain (Begley et al, 2003; Begley et al, 2006). SAXS analysis of Mtmr2 homodimer enabled us to confirm the dimeric state of Mtmr2 and led us to propose a 3D shape reconstruction of the full length Mtmr2 homodimer. The
SAXS envelope of Mtmr2 exhibited an elongated tail and a major globular domain, which we proposed to be formed by the two Mtmr2 monomers. Furthermore, manual superimposition of two individual Mtmr2 monomers (PDB: 1LW3) with the SAXS shape matched if oriented in parallel (Figure 7-18). We therefore propose that the two Mtmr2 monomers orient in parallel to form a homodimer. This is supported by the finding that the coiled-coil domains of Mtmr2 form a parallel dimer (Berger et al, 2003).

In contrast to Mtmr2, no structural information on 3-Pap has been reported. We therefore analyzed the protein by analytical gelfiltration. To my best knowledge, this is the first report of recombinant 3-Pap protein expression and purification. Gelfiltration of His-3-Pap suggested an apparent molecular size consistent with a monomeric state (Figure 7-19). To a smaller extent, 3-Pap also eluted as homodimer. 3-Pap homodimers were previously described in yeast-two-hybrid screenings (Lorenzo et al, 2006) and we could confirm their existence by immunoprecipitation experiments from transiently transfected HEK293 lysates (data not shown). It remains to be investigated, whether the presence and stability of 3-Pap homodimers is increased in vivo under physiological conditions. To obtain structural information at the molecular level crystallization screens were performed, which did not yield any crystals. Thus, to obtain the overall structure of the full length His-3-Pap monomer in solution we performed SAXS analysis. The distance distribution functions of 3-Pap monomer compared to Mtmr2 dimer clearly deviated from each other, suggesting a thinner corpus for 3-Pap (Figure 7-21). The SAXS derived shape of 3-Pap compared to Mtmr2 was clearly thinner, supporting the monomeric state of 3-Pap and the dimeric state of Mtmr2 (Figure 7-22). Higher resolution data of the 3-Pap structure in future is absolutely required since the low resolution of the 3-Pap SAXS shape did not allow detailed conclusions regarding to structure and orientation of monomers.

The characteristics of Mtmr2/3-Pap complex formation were solved by analytical two step purification of differentially tagged His-Mtmr2 and Strep-3-Pap proteins (Figure 7-15). We could show that Mtmr2 formed a heterodimer with 3-Pap. It would be interesting to know, whether the conformation of an Mtmr2/3-Pap
heterodimer is similar to the Mtmr2 homodimer or not. The Mtmr2/3-Pap complex formation was shown to be dependent on the C-terminus including the coiled-coil motif (Figure 7-14). If the C-termini of Mtmr2 and 3-Pap would dimerize by their coiled-coil, it would be interesting to know whether the coiled-coil domain forms a parallel or an antiparallel dimer. In future experiments a SAXS analysis of the Mtmr2/3-Pap heterodimer should be envisaged. Similar to the complex formation with Mtm1 (Caldwell et al, 1991), 3-Pap heterodimerized with Mtmr2, assuming that 3-Pap generally occurs as a heterodimer with active MTMRs. Interestingly, gelfiltration analysis of Mtm1 in the absence of substrate resulted in a molecular weight representing a monomeric state (Schaletzky, 2002). Titration of Mtm1 with the catalytically dead mutant Mtm1 C375S in the presence of PI-3-P not only increased catalytic activity of the active Mtm1, but also promoted oligomerization giving rise to heptameric ring structures (Schaletzky et al, 2003). Interaction interfaces or domains of Mtm1 oligomers have not been analyzed. Furthermore, it is also unknown, whether Mtm1/3-Pap complexes interact through their C-terminus, as we observed for the Mtmr2/3-Pap complex. Considering the high sequence similarity among the MTMRs and the multiple reports of coiled-coil dependent MTMR-MTMR complex formations (see 5.1.4 for references), one could suggest that 3-Pap also heterodimerizes with Mtm1 in a coiled-coil dependent manner. In contrast to the other dead phosphatase Sbf2 or the binding partner Mtmr2, 3-Pap preferentially is a monomer. It would be interesting to investigate, whether the dead phosphatase Mtmr10, which is the closest relative of 3-Pap in the MTMR family, is also monomeric. Interestingly, yeast-two-hybrid screenings revealed that Mtmr10 interacts with Mtm1 and Mtmr2, similar to 3-Pap. Hence, it would also be interesting to characterize Mtmr10/Mtm1 or Mtmr10/Mtmr2 complex formation more closely. Since both Mtm1 and Mtmr2 were found in a heterodimeric complex with 3-Pap, this lets us to suggest that monomeric 3-Pap triggers heteromeric complex formation. Although the exact mechanism of heterodimer formation is not known yet, one possible scenario could be that monomeric 3-Pap interacts through its coil domain with the coiled-coil of Mtmr2 homodimer, thereby 3-Pap attacks and destroys the coiled-coil
dependent Mtmr2-Mtmr2 interaction and establishes an Mtmr2/3-Pap heterodimer. The dead phosphatases Sbf1/Mtmr5, Sbf2/Mtmr13 and 3-Pap/Mtmr12 have in common that complexing with an active MTMR resulted in increased catalytic activity of the active partner and relocalized the latter to other specific subcellular sites (Kim et al, 2003; Nandurkar et al, 2003; Berger et al, 2006a; Lorenzo et al, 2006). However, dependent on the proteins involved, this resulted in completely distinct subcellular localization. In the case of Mtmr2/Sbf2 complexes additional domains like the PI-3,4,5-P$_3$ binding PH domain of Sbf2 have been proposed to play a role in relocalization and function (Berger et al, 2006a). Furthermore, in contrast to 3-Pap, Sbf2 homodimerizes and has been shown to form heterotetramers with Mtmr2 dimers (Berger et al, 2006a). It has to be considered that not only binding of structurally different dead phosphatases, but also the resulting altered stoichiometry of the complex formation (heterodimer vs tetramer) might influence Mtmr2 function and localization. I therefore strongly suggest making a distinction of at least two different classes of dead phosphatases interacting with Mtmr2. A phylogenetic survey of myotubularin genes revealed that dead phosphatases appeared early in evolution and independently on three separate occasions in eukaryotic evolution: *Giardia lamblia*, *Dictyostelium discoideum* and *Entamoeba histolytica* (Laporte et al, 2003; Kerk et al, 2010). Based on the genome sequences, MTMRs were classified into six subgroups, three of which are represented by the dead phosphatases. From these the first subgroup comprises MTMR9, the second MTMR10, MTMR11 and MTMR12 and the third subgroup includes MTMR5/SBF1 and MTMR13/SBF2. Inactive MTMR9 has been shown to homodimerize and to heteromerize with active members of the subgroup comprising MTMR6, MTMR7 and MTMR8 (Mochizuki et al, 2003; Lorenzo et al, 2006; Zou et al, 2009). The stoichiometry of these complexes however, has not been solved yet. Up to date, MTMR2 is the only active MTMR reported to interact with two different types/subgroups of dead phosphatases, accentuating the specificity and importance of these interactions.
8.2.3 Subcellular localization of Mtmr2, 3-Pap and EGFR under resting and growth factor stimulated conditions

Finally, we determined the subcellular localization of Mtmr2 and 3-Pap by immunostaining of transiently transfected HEK293 cells. Under resting conditions, Mtmr2 staining was found in punctuate structures whereas 3-Pap staining was diffusely distributed in the cytosol. Mtmr2 and 3-Pap colocalized at the surface in membrane ruffles but not in the cytosol (Figure 7-23). Membrane ruffles are regions of increased PI-4,5-P$_2$ levels, where processes like endocytosis and macropinocytosis take place (McLaughlin et al, 2002; Doherty et al, 2009). Under resting conditions the level of PI-3-P is high within cells, whereas the level of PI-3,5-P$_2$ is low. Hypo-osmotic shock, UV radiation or EGF stimulation however, has been shown to lead to a transient increase of PI-3,5-P$_2$ levels (Dove, 1997; Jones et al, 1999; Tsujita et al, 2004; Cao et al, 2008). In COS cells expressing Mtmr2 or Sbf2, both Mtmr2 and Sbf2 were confined to membranes of stress induced vesicles under hypo-osmotic conditions (Berger et al, 2003; Berger et al, 2006a). In COS cells coexpressing Mtmr2 and Sbf2, Mtmr2 was no longer observed at these membranes, suggesting Sbf2 to compete for Mtmr2-binding sites due to binding with higher affinity (Berger et al, 2006a). Beside its function as adaptor for the correct localization of Mtmr2 and as regulator of Mtmr2 phosphatase activity, Sbf2 was also proposed to protect PI-3,5-P$_2$ from degradation (Cui et al, 1998). Here we report that under hypo-osmotic condition of transiently transfected HEK293 cells, both Mtmr2 and 3-Pap localized to membranes of stress induced vesicles (Figure 7-24). In HEK293 cells coexpressing Mtmr2 and 3-Pap, both proteins were clearly colocalized at these vesicles. In contrast to Sbf2, 3-Pap did therefore either not compete for Mtmr2 binding sites or had similar binding affinity compared to Mtmr2. In addition, colocalization of Mtmr2 and 3-Pap suggested a specific function of the Mtmr2/3-Pap complex at these membranes, supported by our finding that these proteins heterodimerize. Differential function, regulation and localization for Mtmr2/3-Pap homodimers and Mtmr2/Sbf2 tetramers, respectively, are therefore likely. Mtm1 and Mtmr2 were both reported to relocalize under conditions with elevated PI-3,5-P$_2$, e.g. under hypo-osmotic conditions or EGFR stimulation.
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(Berger et al, 2003; Tsujita et al, 2004). In COS-7 cells PI-3,5-P\(_2\) peaks 40 min after EGF stimulation and Mtm1 was shown to translocate to EGFR-positive late endosomal compartments. Furthermore, it was reported that Mtm1 negatively regulated EGFR trafficking from the late endosomes to lysosomes for degradation (Tsujita et al, 2004). Mtmr2 was also reported to negatively regulate EGFR degradation, however coexpression of Sbf2 counteracted this effect (Berger et al, 2009). How and where Sbf2 fulfills the counteracting effect, alone or in complex with Mtmr2, is not clear yet. Next, it would be highly interesting to study localization of Mtmr2, Sbf2 and 3-Pap in cotransfected cells under hypo-osmotic conditions. This experiment could address whether Sbf2 (or 3-Pap) compete for Mtmr2/3-Pap (or Mtmr2/Sbf2) complex formation as well as for localization at stress induced vesicles. Since in cells coexpressing Mtmr2/3-Pap under hypo-osmotic conditions both Mtmr2 and 3-Pap were found at these membranes, one could argue that 3-Pap or the Mtmr2/3-Pap complex do not fulfill a protecting function but rather induce PI-3,5-P\(_2\) degradation at these membranes and therefore stimulate recycling. Thus, we were interested whether 3-Pap or Mtmr2/3-Pap complex affect Mtmr2 in blocking EGFR degradation. As mentioned above, Mtm1 was shown to block EGFR trafficking from the late endosomes to the lysosomes for degradation (Tsujita et al, 2004). Whether Mtmr2 also blocks EGFR trafficking for degradation in specific endosomal compartments was not analyzed till now. Rab GTPases were used many times as subcellular markers for the localization of MTMRs as well as to track transport routes of membrane proteins such as RTKs (Cao et al, 2007; Cao et al, 2008; Zwang et al, 2009; Stenmark, 2009; Franklin et al, 2011). Recent published data showed that EGFR recycled through Rab11 vesicles under resting conditions. However 180 min after EGF stimulation, EGFR was routed along the degradation axis to Rab5, Rab4 and Rab7 vesicles but barely to Rab11 vesicles (Ballmer-Hofer et al, 2011). Thus, we used the endosomal markers Rab4, Rab5, Rab7 and Rab11 to monitor EGFR localization along the recycling (Rab5-Rab4-Rab11) and the degradation axis (Rab5-Rab4-Rab7). Mtmr2, 3-Pap and Mtmr2/3-Pap complex localization was analyzed before and after EGF stimulation in transiently
transfected cells. Prior to EGF stimulation, Mtmr2 was found throughout the whole cell in or at vesicles which are part of the recycling axis (Rab5-Rab4-Rab11) as well as the degradation axis (Rab5-Rab4-Rab7) (Figure 8-2, A). Thirty minutes after EGF stimulation, when the PI-3,5-P$_2$ level should nearly have reached its maximum (Tsujita et al, 2004), no relocalization of Mtmr2 was observed. However, 180 min after EGF stimulation, Mtmr2 was mostly observed in Rab5 and Rab11 recycling vesicles but no longer in Rab7 vesicles (Figure 8-2, B). Cao et al. (2008) reported that Mtmr2 colocalized with Rab7, but not with Rab5 or EEA1, in baby hamster kidney (BHK) and human epithelial carcinoma A431 cells. Whether colocalization of Mtmr2 with Rab5 vesicles is dependent on the cell type remains to be investigated. Subcellular localization of Mtmr2 after EGF stimulation was not reported. In HEK293 cells overexpressing Mtmr2 and stimulated 180 min, EGF was still detected in Rab5, Rab4 and Rab11 vesicles. Cao et al. analyzed EGFR trafficking in Mtm1 or Mtmr2 siRNA silenced A431 cells. In mock transfected cells, EGFR was mainly degraded and occasionally observed in Rab7 vesicles, consistent with other data (Ballmer-Hofer et al, 2011). In A431 cells transiently transfected with Mtm1 siRNA, EGFR accumulated in EEA1 positive vesicles 180 min after EGF stimulation. In Mtmr2 siRNA silenced cells however, EGFR was detected in Rab7 vesicles. Based on these findings, it was proposed that Mtm1 and Mtmr2 regulate distinct subcellular pools of PI-3-P and PI-3,5-P$_2$ through their specific membrane recruitment to early and/or late endosomes. Our results confirm Mtmr2 localization at Rab7 vesicles before and 30 min after stimulation, but in addition we observed loss of Mtmr2 from Rab7 vesicles 180 min after EGF stimulation. Furthermore, we could show that in Mtmr2 overexpressing cells, EGFR was still observed in Rab11 vesicles 180 min after EGF stimulation. This let us suggest that Mtmr2 keeps EGFR in the recycling axis, where it probably further activated downstream signaling. This coincides with the findings that Mtmr2 dependent blocking of EGFR degradation lead to sustained Akt activation (Berger et al, 2009). Localization of 3-Pap was determined in transiently transfected COS-7 and HEK293 cells. At steady state conditions 3-Pap localization clearly differed between these cell lines. In HEK293
cells, 3-Pap staining was more diffuse and no colocalization with any of the four tested endosomal markers was observed. In COS-7 cells however, 3-Pap colocalized with Rab4, Rab5 and Rab11. Differential protein content, protein expression level and/or signaling might explain these differences. Interestingly, in HEK293 cells 30 min after an EGF pulse, 3-Pap concentrated along the recycling axis of Rab5-Rab4-Rab11 vesicles. Consistent with the COS-7 cell stainings, 3-Pap was still present 180 min after stimulation. Although relocalization of 3-Pap was delayed, EGF stimulation resulted in the concentration of both Mtmr2 and 3-Pap along membranes of the recycling pathway. EGF was observed in all four Rab endosomes, especially in Rab5 vesicles, 180 min after stimulation. Whether 3-Pap overexpression also leads to blocking of EGFR degradation and sustained Akt activation has to be verified by immunoblotting and these experiments are under way. A clear difference we observed between Mtmr2 and 3-Pap localization was the presence of Mtmr2 and absence of 3-Pap in Rab7 positive late endosomes at steady state conditions and 30 min after EGF stimulation. The question is what exactly regulates Mtmr2 to leave Rab7 positive LEs? I suggest investigating the role of Sbf2 in this context, especially because localization of Sbf2 under hypo-osmotic conditions pointed to a high affinity of Sbf2 towards membranes of stress induced vesicles (Berger et al, 2006a). In HEK293 cells coexpressing Mtmr2 and 3-Pap, Mtmr2 was not able to attract 3-Pap to Rab7 vesicles nor did 3-Pap relocalize Mtmr2. We can therefore conclude that no Mtmr2/3-Pap complex is formed at membranes of late endosomes, but that it forms along the recycling axis under resting conditions and EGF stimulated conditions. The observation that 180 min after EGF stimulation, Mtmr2 and 3-Pap clearly colocalized with Rab4 and Rab11 vesicles, but not with Rab5 or Rab7 vesicles, further support a function along the recycling axis (Figure 8-2, B). As mentioned above, 3-Pap coexpression in COS cells altered MTM1 localization (Nandurkar et al, 2003). Coexpression of 3-Pap in HEK293 cells, however, did not alter Mtmr2 localization. We can therefore conclude that by heterodimerizing with both Mtm1 and Mtmr2, 3-Pap had a differential impact on the localization of its binding partners.
Figure 8-2: Subcellular localization of Mtmr2, 3-Pap and Mtmr2/3-Pap complex before and 180 min after EGF stimulation

Depicted are simplified graphs of Figure 5-2 showing the localization of Mtmr2 (red star), 3-Pap (blue cross) and Mtmr2/3-Pap complex (red star in yellow circle) as observed in the immunostainings (A) at resting conditions and (B) 180 min after EGF stimulation (see original data in Figure 7-27, 7-28 and 7-30). Membranes of early endosomes (EE), recycling endosomes (RE), late endosomes (LE) and lysosomes (Lys) are shown.
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(RE) and late endosomes (LE) are colored dependent on their Rab GTPase content in orange (Rab4), green (Rab5), light blue (Rab11) and violet (Rab7).

Another important difference we recognized between Mtmr2 and 3-Pap was that at resting conditions and 30 min after EGF stimulation Mtmr2 localized inside Rab4, Rab5 and Rab7 vesicles resembling “MVBs”. 3-Pap however, was never observed inside these vesicles, but on the outside of the membranes. Whether localization of Mtmr2 in MVBs reflects its degradative fate to the lysosomes is not known, but might represent a possible scenario.

The concentration of Mtmr2 and the Mtmr2/3-Pap complex along membranes of the recycling axis (Rab5-Rab4-Rab11) 180 min after EGF stimulation strongly suggests a function in the regulation of the local PI content at these membranes. As described above (see chapter 8.1) increased levels of Mtmr2 could decrease the local PI-3-P and PI-3,5-P$_2$ content and increase PI-5-P pool at these membranes. Reduction of PI-3,5-P$_2$ pool would result in less binding of effectors such as the ESCRT-II subunit Vps36 or ESCRT-III subunit Vps24, which have been shown to regulate sorting of ubiquitinated cargo and/or MVB formation, respectively (Whitley et al, 2003; Slagsvold et al, 2006). MVB formation at EEs and their maturation is highly dependent on the PI-3-P pool and on the production of PI-3,5-P$_2$ (Roth, 2004). Internalized EGFR destined for degradation is sorted to intraluminal vesicles (ILVs) of endosomes, which mature to LEs (Piper et al, 2007; Williams et al, 2007). Consequently, the reduction of PI-3-P and PI-3,5-P$_2$ at EEs by Mtmr2 might influence not only MVB formation but also explain the influence on EGFR trafficking at this level. Furthermore, EGFR was reported to directly interact with the BAR retromer component SNX1 (Kurten et al, 1996; Chin et al, 2001). SNX1 binds to PI-3-P, PI-3,5-P$_2$ and PI-3,4,5-P$_3$ and was shown to directly interact with SNX5 (Cozier et al, 2002; Zhong et al, 2002). SNX5 also binds to PI-4-P and PI-5-P, but not to PI-3-P (Liu et al, 2006). SNX1 and SNX5 are both BAR retromer components and are involved in retrograde transport of EGFR (Chin et al, 2001; Liu et al, 2006; van Weering et al, 2010; van Weering et al, 2011). SNX1 has also been shown to interact with the ESCRT-0 subunit Hrs, which controls receptor sorting and internalization within the MVBs
(Lloyd et al, 2002; Raiborg et al, 2002; Raiborg et al, 2001). Interestingly, independent overexpression of Hrs and SNX5 was shown to inhibit stimulated degradation of EGFR, whereas overexpression of SNX1 attenuated this effect (Kurten et al, 1996; Chin et al, 2001; Liu et al, 2006). The influence of MTMRs on the local PI pool, like the increase of PI-5-P, could therefore influence local binding of SNXs and subsequently regulate vesicle tubulation and promote recycling. Localization studies of Mtmr2 and Sbf2 in COS cells have shown that 20 min after EGF stimulation Mtmr2 and Sbf2 colocalized with SNX1, SNX2 and SNX5, supporting a function of MTMRs in SNX-BAR tubulation and retrograde transport (Berger et al, 2009). Recently, the DENN domains of SBF1 and SBF2 were reported to have GEF activity towards Rab28 (Yoshimura et al, 2010). Using RNA interference Rab28 was depleted in trypanosomes and was thereby found to mediate maintenance of the Golgi complex and to maintain expression levels and locations of the retromer subunit Vps26 and the ESCRT-I subunit Vps23. Based on localization studies Rab28 was suggested to localize to late endosomes and pre-lysosomes (Lumb et al, 2011). Binding of Sbf2 to PI-3,5-P₂ might regulate the function and localization of Rab28 to LEs. In the case that Mtmr2/Sbf2 complex would also bind to Rab28, Mtmr2 could influence the local PI-3,5-P₂ pool and thereby alter Rab28 function in retromer and ESCRT-I regulation. In summary, different combinations of active and inactive MTMR complexes might influence the PI pool in different endosomal membranes and thereby affect membrane and receptor trafficking from EEs to LEs.

To draw further conclusion, we first need to investigate whether 3-Pap overexpression has an impact on EGFR degradation and whether it blocks the effect of Mtmr2 on EGFR degradation (1). These experiments are of highest priority and already in preparation. (2) It would also be interesting to investigate EGFR degradation in an Mtmr2/Sbf2/3-Pap overexpressing cell system. (2a) One possible approach could be to stably transfect the existing stable HEK293 FlpIn His-Mtmr2/HA-Sbf2 cells with V5-3-Pap or (2b) to establish a new stable HEK293 cell line stably expressing Mtmr2, Sbf2 and 3-Pap by using a single MultiLabel plasmid for transfection. To complete the picture of localization and trafficking of
EGFR in an Mtmr2/3-Pap overexpressing background, the following experiments are also in preparation: (3) The cDNAs for Mtmr2, 3-Pap, one of the four endosomal markers (Rab4, Rab5, Rab7 or Rab11) and EGFR will be cloned into one single plasmid using MultiLabel technology (Kriz et al, 2010). The generated MultiLabel plasmids enable us: (3a) to monitor EGFR trafficking throughout the different Rab positive endosomes making sure that Mtmr2/3-Pap are overexpressed and (3b) to investigate EGFR degradation in Mtmr2/3-Pap expressing cells. The influence of Sbf2 on EGFR degradation in Mtmr2 overexpressing cells is known, however its localization in cells under resting versus EGF stimulated conditions at different timepoints was not investigated yet. (4) To understand this effect in more detail, we need to study the site of action more precisely, as shown in this work for Mtmr2 and 3-Pap. Our results strongly point to a differential effect in the regulation and localization of the two dead phosphatases Sbf2 and 3-Pap while binding the active phosphatase Mtmr2. Therefore, the spatiotemporal accumulation of both Mtmr2/Sbf2 and Mtmr2/3-Pap protein complexes needs to be investigated in more detail. Mtmr2, Sbf2, 3-Pap, and one of the endosomal Rab markers and/or EGFR should be inserted into MultiLabel plasmids to study their localization at steady state and after EGF stimulation.

8.2.4 Future pharmacological aspects in CMT disease
What are the future aspects and potential implications with regard to the peripheral neuropathy CMT? Currently there is no effective drug therapy for CMT patients. CMT4B is a severe demyelinating peripheral neuropathy with myelin outfoldings probably caused by increased synthesis or impaired membrane degradation in regions of active myelin turnover (Quattrone et al, 1996). By influencing the pool of PI-3-P and PI-3,5-P$_2$, MTMRs have been shown to alter the turnover of membrane receptors and subsequently cell signaling (Cao et al, 2008; Berger et al, 2009). In mammals, PIKfyve synthesizes PI-3,5-P$_2$ from PI-3-P and PI-5-P from PtdIns (Sbrissa et al, 1999; Sbrissa et al, 2002). Curcumin is a polyphenol derived from the curry spice turmeric and has been traditionally used for centuries for treating numerous diseases (Visioli et al,
Discussion and Outlook

Although curcumin was shown to inhibit a variety of kinases, in live cells at a very low concentration ($ID_{50} = 6 \mu M$), PIKfyve was reported to be a major target (Ikonomov et al, 2002). The *Trembler-J* mouse carries a missense mutation in the peripheral myelin protein 22 gene (*PMP22*) and is a validated CMT1 mouse model (Suter et al, 1992b; Suter et al, 1992a). Inhibition of PIKfyve by oral curcumin administration could mitigate the severe neuropathy phenotype of the *Trembler-J* mouse, supporting the hypothesis that PI-3-P and PI-3,5-P$_2$ pools play a general role in peripheral neuropathies (Khajavi et al, 2007). *PMP22, P0/MPZ, Cx32/GJB1* and periaxin, are all associated with CMT diseases, are structural components of compact myelin, and play a major role in membrane homeostasis (see also Figure 3 of (Niemann et al, 2006) and Figure 1 of (Berger et al, 2006b)). In transiently transfected COS-7 and Schwann cells, mutant PMP22 was shown to be retained in the ER or in the intermediate compartment (Naef et al, 1999). Curcumin treatment of HeLa cells expressing mutant PMP22 could partially release mutant PMP22 from the ER (Khajavi et al, 2007). Enzymes involved in the turnover of PI-3-P and PI-3,5-P$_2$, which include the kinase PIKfyve and the phosphatases MTM1, MTMR2, SBF2, 3-PAP and FIG4, represent therefore potential and promising pharmacological targets.
Appendix

9 Appendix

9.1 Remaining PDZ-domain array results

Supplementary Figure 9-1: MS analysis and PDZ-array overlay result of GST-Mtmr1 C-term

Recombinant GST-tagged Mtmr1 C-term was expressed in E. coli Acella cells and purified with a GST Trap column. (A) Mass spectrometry result of purified GST-Mtmr1 C-term shows a main signal for 31810 Da, corresponding to the full length protein (expected MW: 30894 Da) which was glutathionylated at three cysteines. Glutathionylation at one cysteine leads to an increase of 305 Da. Some glutathionylated protein fraction misses the methionine (orange), leading to a final mass of 31678 Da. (B) The PDZ array spotted with 96 class I PDZ domains (listed in C) was overlaid with 25 nM purified GST-Mtmr1 C-term. Positive interactions appear as dark spots. (C) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.
Supplementary Figure 9-2: MS analysis and PDZ-array overlay result of GST-MTMR1 C-term

Recombinant GST-tagged MTMR1 C-term was expressed in E. coli Acella cells and purified with a GST Trap column. (A) Mass spectrometry result of purified GST-MTMR1 C-term shows a main signal for 32048 Da, corresponding to the full length protein (expected MW: 30826 Da) which was glutathionylated at four cysteines. Glutathionylation at one cysteine leads to an increase of 305 Da. (B) The PDZ array spotted with 96 class I PDZ domains (listed in C) was overlaid with 25 nM purified GST-MTMR1 C-term. Positive interactions appear as dark spots. (C) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.

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Appendix

A  MS of GST-Mtmr2 C-term

B  PDZ-array overlaid with GST-Mtmr2 C-term

Supplementary Figure 9-3: MS analysis and PDZ-array overlay result of GST-Mtmr2 C-term

Recombinant GST-tagged Mtmr2 C-term was expressed in E. coli Acella cells and purified with a GST Trap column. (A) Mass spectrometry result of purified GST-Mtmr2 C-term shows a main signal for 32430 Da, corresponding to the full length protein (expected MW: 30903 Da) which was glutathionylated at five cysteines. Glutathionylation at one cysteine leads to an increase of 305 Da. (B) The PDZ array spotted with 96 class I PDZ domains (listed in C) was overlaid with 25 nM purified GST-Mtmr2 C-term. Positive interactions appear as dark spots. (C) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.
Supplementary Figure 9-4: MS analysis and PDZ-array overlay result of GST-Sbf2 C-term

Recombinant GST-tagged Sbf2 C-term was expressed in E. coli Acella cells and purified with a GST Trap column. (A) Mass spectrometry result of purified GST-Sbf2 C-term shows a signal for 32105 Da, corresponding to the full length protein (expected MW: 31190 Da) which was glutathionylated at three cysteines. Glutathionylation at one cysteine leads to an increase of 305 Da. (B) The PDZ array spotted with 96 class I PDZ domains (listed in C) was overlaid with 25 nM purified GST-Sbf2 C-term. Positive interactions appear as dark spots. (C) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.
Appendix

A  PDZ-array overlaid with GST-Sbf1 C-term

![Supplementary Figure 9-5: PDZ-array overlay result of GST-Sbf1 C-term](image)

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<td>C4</td>
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<td>MUPPI PDZ1</td>
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**Supplementary Figure 9-5: PDZ-array overlay result of GST-Sbf1 C-term**

Recombinant GST-tagged Sbf1 C-term was expressed in *E. coli* Acella cells and purified with a GST Trap column. (A) The PDZ array spotted with 96 class I PDZ domains (listed in B) was overlaid with 25 nM purified GST-Sbf2 C-term. Positive interactions appear as dark spots. (B) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.
Appendix

A  PDZ-array overlaid with GST-Pten C-term

Supplementary Figure 9-6: PDZ-array overlay result of GST-Pten C-term
Recombinant GST-tagged Pten C-term was expressed in E. coli Acella cells and purified with a GST Trap column. (A) The PDZ array spotted with 96 class I PDZ domains (listed in B) was overlaid with 25 nM purified GST-Pten C-term. Positive interactions appear as dark spots. (B) List with the 96 class I PDZ domains spotted on the membrane. Positive interactions are highlighted in black, negative results are kept in grey.
Supplementary Figure 9-7: Arbitrary rating of the PDZ array results by Philipp Berger
Depicted is a scheme representing the PDZ array with the 96 different spotted PDZ domains listed in Table 6-5. Positive signals for GST-Mtm1 C-term, GST-MTMR1 C-term, GST-Mtmr1 C-term, GST-Mtmr2 C-term and GST-Sbf2 C-term were weighted in an arbitrary manner considering the darkness of the spot and are illustrated as green dots: weak signal (one green dot) to strong signal (four green dots). Mtm1 and MTMR specific interactions are colored with blue or brown background, respectively. Signals which differed between human MTMR1 and mouse Mtmr1 are colored with a grey background.
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Ng EL, Ng JJ, Liang F, Tang BL (2009) Rab22B is expressed in the CNS astroglia lineage and plays a role in epidermal growth factor receptor trafficking in A431 cells. J Cell Physiol 221: 716-728


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dephosphorylation through PP2A inhibition. *Biochem Biophys Res Commun* 387: 127-131


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References


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11 Publications

11.1 List of publications


11.2 Poster presentations

Annual Symposium of the Neuroscience Center Zurich (ZNZ), 12th September 2008, ETH Zurich, Switzerland

Annual meeting of the Union of the Swiss Societies for Experimental Biology (USGEB): 29th - 30th January 2009, Interlaken, Switzerland

11th - 12th February 2010, Lugano, Switzerland
12 Curriculum Vitae

Katharina Gegenschatz-Schmid
Rathausplatz 1
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Date of Birth: 07. May 1980
Nationality: Swiss and German

Education

11/2007 – 03/2012 PhD student at the Paul Scherrer Institut (PSI, Villigen) and participant of the international PhD program in Neuroscience (ZNZ) of the ETH Zurich, Switzerland
Supervision by:
Dr. Philipp Berger
Prof. Kurt Ballmer-Hofer
Prof. Ueli Suter

Doctoral Thesis:
„Structural and Functional Characterization of Interactions of Myotubularin-Related Proteins“

08/2005 – 04/2007 Master of Science in Biology, Plant Sciences, University of Zurich, Switzerland (Total Mark: 5.6)

Master Thesis:
„Molecular Characterization of the MATE transporter TT12 using Site-directed Mutagenesis“ (Mark: 6)

08/2001 – 08/2005 Bachelor of Science in Biology, University of Zurich, Switzerland

08/2000 – 07/2001 Forensic Sciences, University of Lausanne, Switzerland
08/1996 – 07/2000 Kantonsschule Sargans (Matura Type E, Economy)
### Employment History

<table>
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<tr>
<td>2007</td>
<td>Private Lesson Teacher in Zurich, Switzerland</td>
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<tr>
<td></td>
<td>By giving lessons in Biology and designing specific exercises I prepared an autistic biology student for his intermediate degree in Biology (at the University of Zurich), which he passed successfully.</td>
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<tr>
<td>2003 – 2006</td>
<td>Temporary Laboratory Assistant at the ETH Zurich, Switzerland</td>
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<td>In the laboratory of the research group of Prof. Dr. E. Hafen in the Institute of Molecular Systems Biology ETH Zurich, I supervised and maintained mutant <em>Drosophila melanogaster</em> strains.</td>
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<tr>
<td>2003 – 2004</td>
<td>Freelance Garden Teacher at the Botanical Garden of the University of Zurich, Switzerland</td>
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<td>This activity included the organization, preparation and guidance of school classes of different ages through the botanical garden.</td>
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