Combining artificial Membrane Systems and Cell Biology Studies: New Insights on Membrane Coats and post-Golgi Carrier Formation

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Geboren am 25. November 1981 in Sebnitz

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Summary

In mammalian cells, homeostasis and fate during development relies on the proper transport of membrane-bound cargoes to their designated cellular locations. The hetero-tetrameric adaptor protein complexes (APs) are required for sorting and concentration of cargo at donor membranes, a crucial step during targeted transport. AP2, which functions at the plasma membrane during clathrin-mediated endocytosis, is well characterized. In contrast, AP1 a clathrin adaptor mediating the delivery of lysosomal hydrolases via mannose 6-phosphate receptors (MPRs) and AP3 an adaptor ensuring the proper targeting of lysosomal membrane protein are difficult to study by classic cell biology tools. To gain new insights on these APs, our lab has previously designed an \textit{in vitro} system. Reconstituted liposomes were modified with small peptides mimicking the cytosolic domains of \textit{bona fide} cargoes for AP1 and AP3 respectively and thereby enabling the selective recruitment of these APs and the identification of the interacting protein network.

In the study at hand we utilize above-described liposomes to generate supported lipid bilayers and Giant Unilamellar Vesicles (GUVs), large-scale membrane systems suited for analysis by fluorescence microscopy.

By using cytosol containing fluorescently-tagged subunits, we visualized clathrin coats on artificial membranes under near physiological conditions for the first time. Moreover, we demonstrated clathrin-independent recruitment of AP3 coats on respective GUVs. Presence of active ARF1 was sufficient for the selective assembly of AP1-dependent clathrin coats and AP3 coats on GUVs. By using dye-conjugated ARF1, we show that ARF1 colocalized with AP3 coats on GUVs and that increased association of ARF1 with GUVs coincided with AP1-dependent clathrin coats.
Our previous study identified members of the septin family together with AP3 coats on liposomes. Here we show on GUVs, that active ARF1 stimulated the assembly of septin7 filaments, which may constrain the size and mobility of AP3 coats on the surface. Subsequent cell biology studies in HeLa cells linked septins to actin fibers on which they may control mobility of AP3-coated endosomes and thus their maturation. An actin nucleation complex, based on CYFIP1 was identified together with AP1 on liposomes before. Here we show on GUVs, that CYFIP1 is recruited on the surface surrounding clathrin coats. Upon supply of ATP, sustained actin polymerization generated a thick shell of actin on the GUV surface. The force generated by actin assembly lead to formation of long tubular protrusions, which projected from the GUV surface and were decorated with clathrin coats. Thereby the GUV model illustrated a possible mechanism for tubular carriers formation. The importance of CYFIP1-reliant actin polymerization for the generation of MPR-positive tubules at the trans-Golgi network (TGN) of HeLa cells was subsequently demonstrated in our lab. The notion that tubulation of artificial membranes could be triggered by actin polymerization allowed us to perform a comparative mass spectrometry screen. By comparing the abundance of proteins on liposomes under conditions promoting or inhibiting actin polymerization, candidates possibly involved in stabilization, elongation or fission of membrane tubules could be identified. Among the proteins enriched under conditions promoting tubulation, we identified type I phosphatidylinositol-4-phosphate 5-kinases. Their presence suggested an involvement of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) in tubule formation. By cell biology studies in HeLa we show, that down regulation of these enzymes altered the dynamics of fluorescently-tagged MPRs, illustrating the importance of locally confined PI(4,5)P₂ synthesis during formation of coated carriers at the TGN.
Bin–Amphiphysin–Rvs (BAR) domains are known to sense membrane curvature and induce membrane tubulation. Among various BAR domain proteins, Arfaptin2 was enriched under conditions allowing tubulation of liposomes. By microscopy studies on HeLa cells we show, that Arfaptin2 as well as its close paralog Arfaptin1 were present on AP1-coated MPR tubules emerging from the TGN. We further show, that tubule fission occurred at regions were Arfaptin1 is concentrated and that simultaneous down regulation of both Arfaptins lead to increased number and length of MPR tubules. Since fission of coated transport intermediates at the TGN is poorly understood, our findings contribute a valuable component towards a model describing the entire biogenesis of coated post-Golgi carriers.

In conclusion, combining artificial membrane systems and cell biology studies allowed us to propose new models for formation as well as for fission of AP1-coated transport intermediates at the TGN. Further we gained new insights on AP3 coats and the possible involvement of septin filaments in AP3-dependent endosomal maturation.
Acknowledgements

First and foremost I would like to thank my supervisor Dr. Bernard Hoflack for the opportunity to work on an unique scientific project. I am especially grateful for Bernard’s patience in times when experiments failed or my motivation dropped. Bernard’s scientific instinct surprised me again and again. Often we passionately debated about experiments, results and the conclusion from those and as often Bernard turned out right at the end. Along the way he taught me the “soft-skills” of successful investigators: creativity, critical assessment and the ability to work under pressure. I further want to express my gratitude to the all past and current members of the Hoflack lab for creating an enjoyable and inspiring working atmosphere. In particular I would like to thank Mihaela Anitei. We productively collaborated on publications as well as on teaching. Mihaela’s experience in virtually all aspects of the scientific process saved me all too often. Moreover, I would like to thank Cornelia Czupalla, with whom I conducted and analyzed the mass spectrometry experiments, as well as Sofia Traikov and Thomas Wassmer for the cooperation on the septin project. I use this opportunity to acknowledge the great work of Luisa Irmscher. Without her organization talent, nobody in our lab would be able to work professionally. I would like to thank the members of the Schwille lab, in particular Grzegorz Chwastek, Salvatore Chiantia, Jonas Ries and Heiko Keller, for sharing their precious experience on artificial membrane systems.

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>APs</td>
<td>adaptor protein complexes</td>
</tr>
<tr>
<td>ARF1</td>
<td>ADP ribosylation factor protein 1</td>
</tr>
<tr>
<td>ARL1</td>
<td>ADP-ribosylation factor-like 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BAR domain</td>
<td>Bin–Amphiphysin–Rvs domain</td>
</tr>
<tr>
<td>BDPs</td>
<td>BAR domain proteins</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cd</td>
<td>cytoplasmic domain</td>
</tr>
<tr>
<td>CDC42</td>
<td>cell division control protein 42 homolog</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ciMPR</td>
<td>cation-independent MPR</td>
</tr>
<tr>
<td>CLC</td>
<td>clathrin light chain</td>
</tr>
<tr>
<td>COPI</td>
<td>coat protein I</td>
</tr>
<tr>
<td>COPII</td>
<td>coat protein II</td>
</tr>
<tr>
<td>CYFIP1</td>
<td>cytoplasmic FMR1-interacting protein 1</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindol</td>
</tr>
<tr>
<td>DiD</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzensulfonate</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complexes required for transport</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GGAs</td>
<td>Golgi localized -ear containing Arf binding proteins</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>GpI</td>
<td>glycoprotein I</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-O-[gamma-thio]triphosphate</td>
</tr>
<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPS</td>
<td>Hermansky-Pudlak syndrome</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish-peroxidase</td>
</tr>
</tbody>
</table>
LAMP1     Lysosomal-associated membrane protein 1
LIMP2     Lysosome membrane protein 2
M(6)P     mannose 6-phosphate
MPRs      mannose 6-phosphate receptors
mRFP      monomeric red fluorescent protein
mRNA      messenger RNA
MS         mass spectrometry
MVBs      multivesicular bodies
PA         phosphatidic acid
PACS-1     phosphofurin acidic cluster sorting protein 1
PBS       phosphate buffered saline
PC         phosphatidylcholine
PE         phosphatidylethanolamine
PH domain  pleckstrin homology domain
PI         phosphatidylinositol
PI(3)P     phosphatidylinositol 3-phosphate
PI(4)P     phosphatidylinositol 4-phosphate
PI(4,5)P2  phosphatidylinositol 4,5-bisphosphate
PIP5KI     type I phosphatidylinositol-4-phosphate 5-kinase
PIPs      phosphatidylinositol-phosphates; phosphoinositides
PLA        phospholipase A
PLC        phospholipase C
PLD        phospholipase D
PS         phosphatidylserine
RAC1       ras-related C3 botulinum toxin substrate 1
RNA        ribonucleic acid
RNAi       RNA interference
SH3 domain  Src homology 3 domain
siRNA      small interfering RNA
SNAREs     soluble N-ethylmaleimide sensitive factor attachment protein receptors
TGN        trans-Golgi network
TIRF       total internal reflection fluorescence
TMD        trans membrane domain
YFP        yellow fluorescent protein
wt         wild type
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1. INTRODUCTION:

MECHANISM OF INTRACELLULAR MEMBRANE TRAFFIC AND ITS REGULATION

1.1 Preface

When the first high resolution electron microscopy pictures of intact mammalian cells were published in the late 1940s scientists were astonished, yet perplexed by the manifesting complexity of cellular architecture (Porter et al., 1945). Negative staining with heavy metal salts revealed an unprecedented pandemonium of internal membranes filling the cytosol as well as a heterogeneously featured outer cellular membrane. Critical peers even claimed that these structures reflected artifacts of the novel fixation and staining methods. More than 60 years of cell biology research proved the skeptics wrong and although recent advances in prokaryotic cell biology have refuted the perception of bacteria being “bags filled with enzymes”, still the sheer complexity of membrane organization is a hallmark of eukaryotic cells.

This sophisticated organization imposed two major questions on cell biologists:

Why do eukaryotic cells, in contrast to prokaryotes, exhibit this complex internal membrane system? Or in other words: What are the fundamental functions of these compartments? This matter becomes more intriguing in light of emerging data, that even evolutionary most distinct protists share homologue internal compartments as well as the same corresponding protein machineries (Dacks et al., 2009).
How do cells maintain this elaborated organization? This question certainly baffled scientists since the early years of cell biology, but became even more fascinating in view of the enormous dynamics of membranes, revealed by utilizing radioactive and fluorescent probes. The rapid reappearing of up taken plasma membrane components suggested a complex network of uptake, recycling and secretion processes (Thilo and Vogel, 1980). An overview on current models approaching these questions is given in the following chapters.

1.2 Cellular membrane systems are interconnected by transport processes

In order to comprehend compartmentalization and membrane transport of mammalian cells, a preface on the general architecture of biological membranes is obligatory. Even though being refined and updated over the last 40 years, the fluid mosaic model, introduced in the early 1970s, still is the basis of our understanding (Engelman, 2005; Singer and Nicolson, 1972). Its essential statements are summarized below. Although the model is applicable on mitochondrial membranes as well, those are excluded from the further discussion, due to different evolutionary background.

In mammalian cells, biological membranes are three to four nanometer thick, sheet-like structures that compartmentalize cell space (Mitra et al., 2004). They are made up of proteins and lipids. Membrane lipids comprise glycerophospholipids, sphingolipids and cholesterol, with relative abundance of distinct lipid species varying among subcellular compartments (Pomorski et al., 2001).
The more hydrophobic moieties of individual lipid molecules are facing each other. This cooperative hydrophobic interaction together with the hydrophilicity of the surrounding milieu is the foundation for the characteristic bilayer organization of membranes as well as for its barrier function towards polar molecules. Since membranes are not held together by covalent interactions, but by weak cooperative interactions, lipids as well as proteins can diffuse quickly in the plane of the membrane (Webb et al., 1981). Proteins are partially or penetrating the bilayer with acylations and hydrophobic peptide stretches or, in case of integral membrane proteins, completely traversing the bilayer with transmembrane domains. Bilayers are asymmetric. Transmembrane proteins display a discrete orientation, determined during the process of incorporation into the bilayer. Membrane asymmetry also affects lipids, creating two compositionally distinct leaflets of an individual bilayer (Rothman and Lenard, 1977; van Meer et al., 1980). Lipid asymmetry is maintained by flippases, as well as by specific lipid transfer proteins and lipid-modifying enzymes present in respective membrane separated environments (Menon, 1995; Rogers and Bankaitis, 2000). Generalized for all membranes in mammalian cells, one leaflet is facing the cytosol while the other leaflet faces the lumen of membrane bound compartments or, in case of the plasma membrane, the extracellular space. The maintenance of this separation between “inside” and “outside” - in other words: cytosol and exoplasmic milieu - is the key aspect of cellular homeostasis.

Homeostasis also involves the maintenance of protein distribution within the cell. Soluble as well as membrane bound enzymes have to be transported to and maintained in their designated compartments. Cell type dependent, soluble proteins have to be secreted into the environment at distinct sites and in a regulated fashion.
Cell surface proteins have to reach selectively their destinations. Depending on the cell type these comprise specialized membrane domains such as: cilia, the cleavage furrow during cell division, apical or basolateral membranes in polarized cells, dendritic spines and neurite growth cones of neurons. Furthermore surface proteins also have to be down regulated in a controlled manner. These tasks are fulfilled utilizing two major transport pathways: The secretory pathway delivers newly synthesized proteins to the cell surface, while proteins are internalized from the cell surface and subsequently degraded or recycled back in the endocytic pathway.

1.2.1 The secretory pathway

The cargoes of the secretory pathway, also termed biosynthetic pathway, are newly synthesized proteins. After nuclear export of mRNA, protein biosynthesis on ribosomes is taking place in the cytosol. There are two modes of secretion: unconventional secretion is the direct translocation of folded cytosolic proteins into the extracellular environment by various processes (Nickel, 2005). Yet only a small subset of secreted proteins exploits the unconventional path. In contrast all conventionally secreted proteins are translocated from the cytosol in the course of translation at the rough ER. These proteins display an universal N-terminal signal sequence which ensures co-translational injection of the nascent polypeptide into the ER lumen, where protein folding takes place (Johnson and van Waes, 1999). At the ER membrane the same machinery facilitates the membrane insertion of integral membrane proteins. Also the majority of membrane lipids is synthesized at the cytosolic leaflet of ER membrane (Rogers and Bankaitis, 2000). Soluble proteins and the luminal domains of integral membrane proteins are modified with disulfide bridges as well as glycosylations by ER resident enzymes (Helenius and Aebl, 2001).
From specialized ER exit sites, protein cargo is transported to the cis-face of the perinuclear-situated Golgi apparatus. The pathway from ER to Golgi is traversing the tubular ER-Golgi intermediate compartment (ERGIC). ER resident proteins are transported back to the ER from Golgi and ERGIC by a specific retrieval pathway (Lee et al., 2004). Protein cargo being not retrieved is successively conveyed through the adjacent cisternae of the Golgi. During maturation within the Golgi, glycosylations on protein cargo are sequentially modified by enzymes being active in respective cisterna (Helenius and Aebi, 2001; Miller and Ungar, 2012). Also lipid glycosylations takes place at this location. Eventually cargo proteins arrive in the trans-Golgi network (TGN), a complex system of tubules and vesicles representing the main sorting station of the pathway (Traub and Kornfeld, 1997). From the TGN, cargo is either directly sent to the cell surface via constitutive secretion or is packaged in secretory granules, which dock close to the plasma membrane for regulated secretion (Anitei and Hoflack, 2011; Polishchuk et al., 2003). Lysosomal targeting of cargo, the third possible pathway diverging from the TGN, will be discussed below.

1.2.2 The endocytic pathway

The cargoes of the endocytic pathway are extracellular proteins, often bound to specific cell surface receptors, as well as cell surface transmembrane proteins. In contrast to the biosynthetic pathway, which is - apart from the sorting at the TGN - quite linear, the endocytic pathway is multifaceted. Depending on the protein machinery involved, numerous initial uptake pathways can be discriminated, including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolar-type endocytosis, flotillin-dependent endocytosis and ARF6-dependent endocytosis (Conner and Schmid, 2003; Doherty and McMahon, 2009).
All pathways lead to generation of differently sized endocytic carriers, that at some point fuse with an endosomal compartment. Phagosomes, which result from phagocytosis, fuse with lysosomes to ensure degradation of their content. Yet for most uptake routes the first station is the peripheral early endosome. Early endosomes are comprised of a vesicular-tubular membrane network. Here cargo delivered from the cell surface, is sorted on different routes (Gruenberg and Stenmark, 2004): upon arrival in the early endosome, ligands often dissociate from their respective receptors due to the lower pH in the lumen. Soluble cargo is maintained in the vesicular part of the endosome, whereas receptors and other membrane bound proteins are concentrated in the tubular part, from where they are either rapidly recycled towards the plasma membrane or are sent on a direct retrograde pathway to the TGN. Cargo can alternatively be transported to recycling endosomes. These specialized compartments are characterized by their localization in the perinuclear region, a mainly tubular appearance as well as by concentration of recycled cargo. From recycling endosomes membrane bound cargo is sent back to the plasma membrane in a slow recycling pathway. Retrograde transport towards the TGN takes place on the level of the recycling endosome as well. Whether membrane proteins are recycled slowly or fast and whether retrograde transport occurs on a direct or indirect route depends on the individual cargo and cell type. Polarized cell types harbor specialized recycling endosomes, distributing cargo derived from distinct membrane surfaces as well as common recycling endosomes (Ang et al., 2004; Futter et al., 1998). Cargo being not recycled is eventually sent for degradation. Down regulated receptors and soluble cargo are concentrated during maturation of early towards late endosomes. The maturation process is accompanied by translocation towards the cell centre, increasing acidification as well as accumulation of lysosomal membrane proteins.
Separation of membrane proteins of different fate is a crucial step during endosomal maturation. Transmembrane proteins, destined for degradation by ubiquitination, are concentrated in internal membranes. These accumulate within the lumen of endosomes, a process regarded as multivesicular body (MVB) formation (Katzmann et al., 2003). Maintenance of lysosomal membrane proteins on the limiting membrane of the late endosome as well as retrograde transport of cargo to the TGN is mediated by distinct sorting machineries (Pfeffer, 2009). It is still a matter of debate whether endosomal maturation occurs progressively or via transport intermediates fusing with preexisting late endosomes (Huotari and Helenius, 2011; Rink et al., 2005). Late endosomes eventually fuse with mature lysosomes. These compartments display the lowest pH and the highest activity of hydrolytic enzymes and thus represent the end station of the endocytic pathway. Lysosomes are mainly characterized by their strong intrinsic electron density on micrographs. However their discrimination from mature late endosomes according to marker proteins is delicate (Gruenberg, 2001).

1.2.3 Delivery to the lysosomal system –

connecting endocytic and secretory pathways

Integrity and degradation function of the lysosomal compartment relies on its continuous replenishment with newly synthesized components. Impaired delivery of lysosomal proteins leads to severe lysosomal storage disorders (Eskelinen et al., 2003). The lysosomal delivery routes integrate biosynthetic and endosomal pathways. Newly synthesized proteins are delivered into endosomes, where they ensure proper degradation or, in case of lysosome-related organelles, performance of particular functions. Two types of protein cargo are delivered by particular pathways: hydrolytic enzymes and lysosomal membrane proteins (Le Borgne and Hoflack, 1998b).
Soluble hydrolases are synthesized in the ER as inactive precursors. During Golgi passage specific modifications on their N-linked high mannose oligosaccharides take place. GlcNAc-phosphate is attached to a terminal mannose residue, present on N-linked glycosylations. In a subsequent step the GlcNAc moiety is removed, exposing a mannose-residue phosphorylated at position 6. This mannose 6-phosphate (M6P) is the signal destining hydrolases to the endosomal system (Ludwig et al., 1991). At the neutral pH of the TGN, M6P binds with high affinity to its respective receptors, the cation dependent M6P receptor (cdMPR) (Hoflack and Kornfeld, 1985) and the cation independent M6P receptor (ciMPR) (Dahms, 1996). From the level of the TGN, hydrolases bound to respective MPRs are transported to early endosomes. The lower pH in the lumen of early endosomes leads to dissociation of hydrolases from the MPRs. In the course of endosomal maturation hydrolases are activated by proteolytic cleavage of the precursors. This cleavage is mediated by membrane bound proteases, which reach endosomes by the same pathway as their substrates (Rohn et al., 2000). While the enzymes are maintained in the lumen of endosomes, vacant MPRs are transported back to the TGN by various retrograde pathways (Pfeffer, 2009; Seaman, 2004). MPRs are also retrieved from the cell surface as well as from maturing secretory granules, ensuring integrity of those (Kuliawat et al., 1997).

The heavily glycosylated lysosomal membrane proteins shield the limiting membrane of lysosomes and late endosomes from hydrolysis, thus maintaining organelle integrity. An additional receptor function for lysosomal membrane proteins has been exemplified (Reczek et al., 2007). Newly synthesized proteins can be transported to lysosomes via a direct or an indirect pathway (Carlsson and Fukuda, 1992).
The transport of lysosomal membrane proteins from the TGN to maturing late endosomes via specialized transport intermediates is regarded as direct pathway. The indirect pathway is initiated by cargo release to the cell surface via constitutive secretion and subsequent endocytosis. The cargo is then sorted in early endosomes, where lysosomal membrane proteins are largely excluded from both, internalization during MVB-formation and recycling to plasma membrane or TGN. Subsequently the lysosomal membrane proteins are transported to lysosomes in the course of endosomal maturation. It is still a matter of debate, whether the indirect pathway reflects a bypass mechanism, since it seems to be the predominant route for some cargo (Cook et al., 2004; Harter and Mellman, 1992). Either way, in the course of endosomal maturation, lysosomal membrane proteins become increasingly enriched on the limiting membrane of the respective compartments and display their highest concentration in lysosomes (Eskelinen et al., 2003), while a subset also accumulates in internalized membranes (Escola et al., 1998).

1.2.4 The paradigm of vesicular traffic

The above described membrane pathways coalesce in an equilibrated network of membrane flow. This dynamic system is fed with membranes at the level of the ER, whereas the lysosomes can be regarded as sink. Given this plethora of interconnected pathways, it may be surprising that all transport processes follow the same sequence of events, employing analogous protein machineries (Behnia and Munro, 2005; Bonifacino and Glick, 2004). The vesicular transport model, illustrated in Fig.1.1, can be exemplified as follows: Transport cargoes comprises membrane bound proteins, receptor associated soluble molecules as well as lipids. In the initial sorting step, cargo is concentrated and clustered in microdomains at the donor compartment.
Cargo clustering may be aided by assembly of coat proteins on the cytosolic face if the donor compartment (Bremser et al., 1999). The coat recruitment leads to formation of a initial membrane bud. While cargo further accumulates into that nascent carrier, it is stabilized on the cytosolic site by membrane binding proteins as well as cytoskeleton elements (Kaksonen et al., 2005). Eventually the connection to the donor compartment is sieved, releasing a membrane bound carrier. This fission step is mediated by curvature specific assembly of cytosolic proteins on the small membranous connection between emerging transport intermediate and donor, as well as by local action of lipid-modifying enzymes (Campelo and Malhotra, 2012).

Transport intermediates range from spherical vesicles with diameters smaller than 100 nm to structures of vesicular-tubular shape, referred to as large pleomorphic carriers (Luini et al., 2005; Waguri et al., 2003). The detached carrier is transported along microtubules or actin tracks employing respective motor proteins. Compartment specific protein tethers support the recognition of the designated acceptor compartment (Whyte and Munro, 2002). When carrier and acceptor membrane reach close distance, interaction of pairs of Soluble NSF Attachment Protein Receptor proteins (SNAREs), present on both membranes, facilitate membrane fusion (Chen and Scheller, 2001). In contrast to tethering factors and motors, which are recruited from the cytosol, selected v-SNAREs have to be sorted into the carrier during initial steps of its formation. During fusion, the carrier membrane completely coalesces with the acceptor membrane, distributing its membrane cargo into the destined compartment. Alternatively, the transport intermediate can release its soluble cargo in a “kiss and run” mechanism. By sieving the membrane continuum between transport intermediate and acceptor shortly after fusion, large scale mixing of membranes is prevented and the non collapsed carrier can be “reused” (Rizzoli and Jahn, 2007).
Fig. 1.1. The concept of vesicular membrane traffic. Clustering of membrane cargo and receptor bound soluble molecules at the donor membrane is reinforced by assembly of cytosolic coat proteins. **Budding:** coat polymerization mediates formation of a membrane bud, eventually pinching off, generating vesicular or tubular carrier. **Movement:** coats dissociate from transport intermediate. Motor proteins attach to the carrier, allowing locomotion on cytoskeleton tracks. **Tethering:** interaction of tethering proteins immobilize the carrier nearby the respective acceptor membrane. **Fusion:** at close distance, interaction of v-SNAREs and t-SNAREs leads to membrane fusion. Membrane cargo diffuses into acceptor membrane and soluble molecules are released into the lumen of the acceptor compartment.

Although the principle mechanisms are similar, selectivity of the above reviewed transport process is maintained at each step: cargo clustering at the donor compartment accompanied by membrane recruitment of specific coat proteins, the subsequent association of the transport intermediate with distinct motors and tethering proteins as well as the presence of matching pairs of SNARE proteins on the membranes of carrier and acceptor compartment (McNew et al., 2000). Since for successful cargo delivery the first step - formation of a cargo-enriched membrane microdomain reinforced by coat recruitment - is of outmost importance, this step will be discussed in more detail.
1.3 Cargo clustering in microdomains is sustained by coats proteins and lipid segregation

Clustering of cargo in microdomains within the donor compartment membrane is induced by three mechanisms acting either on the exoplasmic site, in the plane of the bilayer or on the cytosolic site. In distinct trafficking pathways, several of these mechanisms may act cooperatively or alone.

1.3.1 Cargo clustering by lectin molecules

The “crosslinking” of the glycosylated exoplasmic domains is one way of clustering transmembrane proteins. The carbohydrate binding protein family of lectins can interact with glycans on proteins and lipids. Lectins are known to induce certain uptake events, most notably phagocytosis in cells of the immune system (Sharon, 1984). Lectins are also involved in cargo clustering and quality control during ER to Golgi traffic (Nonaka et al., 2007; Schrag et al., 2003). They can stabilize microdomains formed by protein-lipid interactions by linking glycosylated proteins and glycolipids (Lajoie et al., 2009). Recent findings in polarized cells emphasize a role for lectins in lipid raft-independent clustering of cargo for apical secretion at the level of the TGN (Delacour et al., 2007).

1.3.2 Implication of lipids in microdomain formation

The notion that the lipid bilayer itself influences the sorting of embedded integral membrane proteins arose, when studies revealed that variations among the length of transmembrane domains (TMD) affected subcellular localization of membrane proteins (Munro, 1995; Sharpe et al., 2010).
This hydrophobic matching constrains between TMD and lipid environment seem to be the major mechanism for retaining ER-resident membrane proteins and for segregating them from secreted cargo (Dumas et al., 1999). There are also more specific interaction between peptide stretches within the TMD and the surrounding lipid species, that thus display selectivity towards specific cargoes (Radhakrishnan et al., 2008). Another way of cargo segregation is the inclusion of membrane proteins in so called lipid rafts. These represent transiently stabilized nano-scale assemblies, rich in sphingolipids, cholesterol, and glycoproteins. Lipid rafts are responsible for the clustering of apical secreted cargo at the TGN as well as for receptor clustering at the plasma membrane with implication for various endocytic events (Lingwood and Simons, 2010; Schuck and Simons, 2004). The role of lipid rafts in sorting on the level of early endosomes is a matter of debate (Gruenberg, 2001).

The selective presence of lipids on the cytosolic leaflet of donor compartments controls the recruitment of coat components and coat accessory proteins. The phosphatidylinositol-phosphates (PIPs), also termed phosphoinositides, are the best studied examples. Through reversible phosphorylation on the 3, 4, and 5 positions of the inositol ring, seven individual PIPs can be generated. Each of these lipid species displays a distinctive distribution in cells: PI(4,5)P$_2$ and PI(3,4,5)P$_3$ are preferentially concentrated on certain regions of the plasma membrane, PI(3)P and PI(3,5)P$_2$ are predominantly localized to endosomes, PI(4)P is enriched at the Golgi and PI is found at the ER. The levels of PIPs on individual compartments are maintained by specialized kinases and phosphatases (Di Paolo and De Camilli, 2006; Wieffer et al., 2012). Many membrane associated proteins harbor specialized domains or sequences facilitating their recruitment to compartments enriched for these lipids (Roth, 2004).
1.3.3 Protein cargoes sustain coat formation

Cargo sorting by assembly of cytosolic coat proteins is the best studied mechanisms of microdomain formation. In eukaryotic cells the distinct coats follow a common mechanistic principle. They represent higher order molecular assemblies of cytosolic building blocks (Bonifacino and Lippincott-Schwartz, 2003; Hughson, 2010). The schematic architecture of assembled coats is depicted in Fig. 1.2. The process of coat polymerization provides some of the free energy necessary to bend the donor membrane. Cargo accumulation and membrane deformation are concomitant during coat assembly. Transmembrane protein cargo is distinguished on the basis of sorting motifs within its cytosolic domain. Coat subunits or coat interacting adaptors mediate cargo recognition (Le Borgne and Hoflack, 1998a). After the detachment of the coated transport intermediate, uncoating mechanisms redistribute coat building blocks to the cytosol, allowing subsequent fusion of the carrier (Bonifacino and Lippincott-Schwartz, 2003). The architecture and mediated trafficking steps of the three best characterized coats are reviewed below.

Fig. 1.2. Schematic architecture of COPI, COPII & Clathrin coats. COPII and COPI coat subunits form rod-like assembly units. Assembled into cage structures, three or four of these units orient themselves around a vertex. The vertex represents the point where lattice edges intersect in the cage. In case of clathrin the cytosolic building blocks (triskelions) already exhibit a vertex structure, similar to that of COPI coats. Therefore the COPI cages resemble clathrin cages. COPII again, with its cross-like vertex structure, assembles into remarkably different cages. Depicted cages are idealized models. Coated vesicles can diverge a lot from the isotropic shape due to the mechanic flexibility within coats.
On the ER, COPII coats are comprised of four individual proteins. Sec13 and Sec31, that form a heterotetramer, representing the actual cage assembly. Sec23 and Sec24 form a heterodimer, implicated in recognition of sorting motifs present in cargo proteins (Barlowe, 2003a; Stagg et al., 2008). Together with the ER membrane protein Sec12 and the small GTPase Sar1 the cargo-associated Sec23/24 acts as an adaptor for Sec13/31 assembly. The function of COPII is clearly defined: at ER exit sites these coats mediate formation of cis-Golgi destined carries, that incorporate non ER-resident transmembrane proteins, harboring the respective export signals (Antonny and Schekman, 2001; Barlowe et al., 1994). Selective incorporation of soluble cargo into COPII coated structures is less understood, albeit lectin-related cargo receptors may be involved as described above (Barlowe, 2003b).

On Golgi membranes, COPI coats are comprised of seven subunits termed alpha-, beta-, beta', gamma-, delta-, epsilon- and zeta-COP. While the other subunits have structural functions, beta-, gamma, delta and zeta comprise a sub-complex, which is involved in recognition of cargo sorting motifs and is structurally homologous to the heterotetrameric clathrin adaptor complexes (Edeling et al., 2006; Hirst et al., 2012). COPI coats mediate the formation of ER-destined retrograde carriers at the cis- and medial-Golgi as well as on the ERGIC. A less defined role is the transport within the cisternae of the Golgi stack responsible for Golgi maintenance (Pelham, 2001; Yang et al., 2011). The main cargo of this coat is the KDEL receptor and receptor-bound ER resident enzymes (Orci et al., 1997). Also p23 and p24, putative ER resident cargo receptors, are retrieved via COPI coated carriers (Fiedler et al., 1996). COPI subunits have been also found associated to endosomes, where they have been shown to contribute to endosomal maturation by an unknown mechanism (Daro et al., 1997).
Clathrin coated membrane buds were found on the plasma membranes, at the TGN, on tubular parts of endosomes (Heuser, 1980; Pesacreta and Lucas, 1984). The intriguing profiles of membrane decorating clathrin cages, detectable on electron micrographs, have been fascinating cell biologists since the 1960s (Kanaseki and Kadota, 1969). In addition flat clathrin lattices were detected on the vesicular part of endosomes and on the plasma membrane (Raiborg et al., 2006). These distinct localizations indicate the involvement of clathrin in several transport pathways. The cytosolic building blocks of clathrin coats are termed triskelions. They represent hetero-hexamers with a bended, three-legged shape, comprised of clathrin light chain and clathrin heavy chain with an equimolar distribution (Ungewickell and Branton, 1981). Triskelions can polymerize into cage-like structures displaying hexagonal and pentagonal lattices (Fig.1.2), or into flat assembles displaying mainly hexagonal lattice features (Heuser, 1980). The clathrin light chains are dispensable for triskelion stabilization and coat assembly, but are important for the dynamics of clathrin coats (Brodsky et al., 2001; Poupon et al., 2008; Wilbur et al., 2008). In contrast to COPI & COPII, clathrin coats do not directly interact with cargo sorting motifs, but rely on adaptor protein complexes or monomeric adaptor proteins respectively. The terminal domain of clathrin heavy chain projects inward toward the membrane where it directs incorporation of specific cargo by facilitating its interactions with adaptors (Kirchhausen, 2000). Thus only at the edges of the assembled coat terminal domains are accessible for interaction with clathrin accessory proteins. These are therefore preferentially involved in the initiation of coat assembly as well as in uncoating.

The architecture, cargo specificity and the transport pathways mediated by clathrin adaptors and related protein complexes will be discussed in the following chapter.
1.4 The Adaptor Protein complexes

Initially the adaptor protein complexes 1 and 2 (AP1 and AP2) have been purified from isolated clathrin coated vesicles (Pearse and Robinson, 1984). Electron micrographs and structural biology approaches showed, that AP1 as well as AP2 are situated underneath the assembled clathrin coat, thus physically linking clathrin and cargo. The complexes AP3, AP4 and - most recently - AP5 have later been identified by homology search in protein sequence databases. These adaptors are not detected in clathrin coated vesicles and may function independent of clathrin (Hirst et al., 2012). It remains ambiguous whether coats comprising these adaptors rely on a higher order assembly of structural components, analogous to clathrin.

The adaptor protein complexes, also referred to as adaptors, exhibit a heterotetrameric architecture, consisting of analogous subunits: one small subunit ($\sigma$1 - 4), one medium subunit ($\mu$1 - 4) and two big subunits ($\beta$1 - 4 as well as $\alpha$, $\gamma$, $\delta$, $\varepsilon$ respectively) (Robinson, 2004). Several tissue specific isoforms of individual subunits have been identified in the case of AP1 and AP3, giving rise to distinct adaptor protein complexes with tissue specific function (Mattera et al., 2011). Both big subunits are comprised of a trunk region, mediating interaction with the other subunits, a flexible hinge region and a protruding appendage domain, allowing interaction with coat accessory proteins. Specific sequences in the hinge region of $\beta$1 and $\beta$2 as well as $\gamma$ and $\alpha$ subunits facilitate interactions with the terminal domains of clathrin heavy chain. The $\sigma$ subunit may stabilize the complexes. In all complexes $\beta$ and $\mu$ subunits are involved in cargo sorting motif recognition. Some sorting motifs are recognized by all four adaptors, whereas others are specific for one complex.
The binding to sorting motifs triggers conformational changes within the complexes, reinforcing their membrane association (Owen et al., 2004). In general recruitment of adaptor protein complexes, and thus subsequent coat assembly on membrane microdomains, relies on a mosaic of interactions: combined interactions with cargo sorting motifs, specific phosphatidylinositol-phosphates, regulative small GTPases and coat accessory proteins stabilize adaptors on donor membranes (Robinson, 2004).

1.4.1 AP2 dependent sorting

AP2 is the best studied adaptor complex. AP2 subunits colocalize with all clathrin coated structures at the plasma membrane. These comprise bended clathrin coated pits as well as flat clathrin plaques. Pits and plaques employ different machineries for carrier formation and are presumably required for uptake of distinct cargo (Saffarian et al., 2009). The sole function of AP2 is the facilitation of clathrin-dependent endocytosis, the major mechanism for cellular uptake of nutrient receptors as well as for cell surface receptor down regulation during receptor-mediated signaling. The initial membrane targeting of AP2 is initiated by interaction of α and μ2 with PI(4,5)P2 (phosphatidylinositol 4,5-phosphate), concentrated on the cytosolic leaflet of the plasma membrane (Honing et al., 2005; Padron et al., 2003). The subsequent interaction of AP2 with cargo sorting motifs ensures cargo selectivity and stabilizes its membrane association. AP2 interacts with motifs in the cytosolic domains of a broad spectrum of transmembrane proteins: Subunit μ2 recognizes the prevalent tyrosine-based sorting motif (YXXΦ; Φ representing a bulky hydrophobic residue) in a tyrosine phosphorylation dependent manner. Alternatively α together with ο2 recognize a dileucine-based motif (DE]xxL[LI]), which is present in a subset of cargoes (Collins et al., 2002).
The general view on how AP2 interacts with clathrin and cargo during the events leading to the formation mature clathrin coated vesicles, has changed in the past ten years. It was believed that polymerization of clathrin on the plasma membrane is initiated by its recruitment via PI(4,5)P$_2$ binding accessory proteins, like AP180 or epsin1. Subsequently cargo would incorporate into the emerging clathrin coat via interaction with AP2 (Ehrlich et al., 2004; Ford et al., 2001). However recent studies suggest a distinct mechanism. The membrane binding proteins FCHo1/2 prime sites of cargo clustering by AP2, which - in conjunction with accessory proteins - recruit clathrin coats at this sites (Henne et al., 2010; McMahon and Boucrot, 2011). Yet a very recent study on zebrafish embryos challenges the primacy of FCHo1/2 in coat initiation (Umasankar et al., 2012). Either way, the notion prevails that presence of membrane stabilized AP2 is strictly required for efficient clathrin-mediated endocytosis (Boucrot et al., 2010).

### 1.4.2 AP4 dependent sorting

Our understanding of AP4 function is very limited. AP4 subunits can be localized to the TGN and to endosomal structures (Hirst et al., 1999). The μ4 subunit has been shown to recognize the tyrosine-based sorting motif, although only few distinctive cargo proteins and only one accessory protein have been identified so far for this adaptor (Aguilar et al., 2001; Borner et al., 2012). AP4 mediates endosomal delivery of newly synthesized cargo, like the amyloid precursor protein, to endosomes (Burgos et al., 2010). In polarized cells it is important for proper targeting of selective basolateral cargo, possibly by mediating transport from TGN to recycling endosomes (Simmen et al., 2002). The loss of functional AP4 is associated with congenital neuronal disorders of severe cognitive dysfunction (Moreno-De-Luca et al., 2011).
1.4.3 AP1 dependent sorting

AP1 subunits are detected mainly on the TGN as well as on tubular profiles of endosomes, in both cases remarkably colocalizing with clathrin. It should be noted, that endosomes display also separate, AP1-independent clathrin coats (Raiborg et al., 2006). Analogous to AP2, AP1 recognizes tyrosine-based and dileucine-based sorting motifs in cytosolic tails of cargo (Ohno et al., 1998; Rapoport et al., 1998). In addition an acidic cluster sorting motif, often in close proximity to the above described motifs, mediates cargo recognition by AP1 (Chen et al., 1997; Stockli et al., 2004; Tortorella et al., 2007). Moreover, AP1 was shown to recognize more complex sorting motifs hidden in the tertiary structure of cytosolic domains (Ma et al., 2011). In addition to cargo binding, membrane association of AP1 is heavily reinforced by the presence of PI(4)P (Wang et al., 2003). The major function of AP1 in non polarized cells, facilitating the delivery of ciMPR and cdMPR, has been identified early (Le Borgne et al., 1996; Meyer et al., 2000; Teuchert et al., 1999). However there is an ongoing debate about the directionality of AP1 dependent transport and about the role of other clathrin adaptors in MPR sorting (Hinners and Tooze, 2003). The family of Golgi-localized, gamma adaptin ear-containing, ARF-binding (GGA) proteins represents monomeric adaptors displaying partial homology to the γ subunit of AP1. At the TGN GGAs interact with MPRs by recognition of distinct dileucine sorting motifs (DxxLL). GGAs and AP-1 can facilitate clathrin recruitment independent of each other (Puertollano et al., 2003). Yet the conception emerges, that effective TGN to endosome transport of MPRs is facilitated by GGAs and AP1 in conjunction (Doray et al., 2002; Hirst et al., 2009; Zhu et al., 2001). However in a recent study, rapid depletion of AP1 mainly affected the retrograde transport of ciMPR, illustrating the indispensable function of AP1 in this trafficking step (Robinson et al., 2010).
On endosomes, the AP1 accessory protein phosphofurin acidic cluster sorting protein (PACS-1) binds the acidic cluster motif of ciMPR, thereby sustaining its interaction with AP1 and promoting retrograde transport (Crump et al., 2001). The retrograde transport of MPRs from early endosomes presumably requires cooperation of AP1 with the retromer complex (Johannes and Wunder, 2011; Seaman, 2004). Another important function of AP1 is directing the transport of the pro-protein convertase furin from the TGN to endosomes. In this case PACS-1, but not GGAs, are also involved in cargo recognition (Teuchert et al., 1999). Recent studies suggest two novel implications of AP1, both possibly linked to its function in furin delivery and MPR retrieval: the maturation of specialized secretory granules (Burgess et al., 2011; Metcalf et al., 2008) and the regulation of Notch- and Wnt-mediated signaling during embryonic development (Kametaka et al., 2012). Moreover in fibroblasts, AP1 in complex with Gadkin is involved in an unconventional secretory route, the calcium-induced endo-lysosome secretion (Laulagnier et al., 2011). As other cellular processes, AP1 dependent sorting is exploited by pathogens. Envelop glycoproteins of the human herpes virus 3 are recognized by AP1 at the TGN and subsequently delivered to the plasma membrane for virus particle assembly (Alconada et al., 1996).

Polarized cells contain two populations of AP1: the ubiquitous expressed AP1A and the tissue specific AP1B, containing the subunit isoforms μ1A and μ1B respectively (Folsch et al., 1999). Whereas AP1A is mainly present at the TGN, AP1B localizes to the common recycling endosome, a cell type specific, juxtanuclear compartment receiving recycled cargo from apical and basolateral endosomes (Folsch et al., 2001). AP1B was shown to be indispensable for maintenance of cell polarity (Folsch et al., 2003; Gravotta et al., 2007).
A recent study, integrating AP1A function, proposes following model for AP1 in polarized cells (Fig. 1.3): At the TGN newly synthesized cargo is transported to the apical membrane by a lipid raft-mediated mechanism. Cargo containing basolateral sorting motifs is sorted by AP1A into clathrin coated carriers, taking a direct route to the basolateral membrane (Gravotta et al., 2012). MPRs are sorted by AP1A in conjunction with GGAs in distinct clathrin coated transport intermediates towards endosomes (Doray et al., 2002), while it remains unclear, whether AP1A or AP1B are required for retrograde traffic of MPRs from endosomes. At the common recycling endosome cargo, recycled from the basolateral membrane, as well as newly synthesized basolateral cargo from the TGN is sorted by AP1B in cell surface destined carriers (Ang et al., 2004; Gravotta et al., 2007).

Additional functions of tissue specific AP1 complexes are emerging (Mattera et al., 2011). A neuron specific pool of AP1, containing the subunit isoform σ1B, has recently been linked to synaptic vesicle recycling in hippocampal synapses (Glyvuk et al., 2010).

1.4.4 AP3 dependent sorting

Upon its discovery, the AP3 complex was described as a new clathrin adaptor (Dell'Angelica et al., 1997). While early colocalization- and in vitro binding-studies suggested a direct interaction with clathrin, the notion prevails that AP3 functions independent of clathrin (Peden et al., 2002; Vowels and Payne, 1998). Concatenated phylogenetic analysis indicates, that AP3 is the evolutionary most ancient adaptor protein complex, whereas the clathrin interacting adaptors AP1 and AP2 as well as presumably clathrin itself emerged later in the evolution of eukaryotic cells (Hirst et al., 2012; Klute et al., 2011).
The majority of AP3 is found on endosomes, mostly on tubular features devoid of recycled cargo but partially colocalizing with clathrin (Peden et al., 2004). A pool of AP3 can be found on features of the TGN as well (Dell'Angelica et al., 1998). In yeast AP3 was initially shown to mediate cargo-selective protein transport from the Golgi to the vacuole, a lysosome-related organelle (Cowles et al., 1997). Subsequently AP3 was linked to the delivery of lysosomal membrane proteins in mammalian cells. It binds to tyrosine- or dileucine-based sorting motifs, present in the cytosolic tails of lysosomal-associated membrane protein 1 (LAMP1) or lysosomal integral membrane protein 2 (LIMP2) respectively (Le Borgne et al., 1998). Interference with AP3 leads to increased cell surface accumulation of lysosomal membrane proteins, suggesting a role in the direct transport of lysosome-destined cargo from the TGN to endosomes (Chapuy et al., 2008; Rous et al., 2002; Yang et al., 2000). Yet AP3 seems to be implicated in an indirect pathway as well. Lysosomal cargo, possibly harboring weak AP3 sorting motifs, is transported to the cell surface. After AP2-mediated uptake, the cargo is delivered to early endosomes, where AP3 segregates it to the limiting membrane during maturation toward late endosome (Ihrke et al., 2004). AP3 is also involved in cargo delivery to cell type specific lysosome-related organelles, like melanosomes or platelet dense granules, and is thus important for biogenesis of these specialized compartments (Bonifacino, 2004; Chapuy et al., 2008; Starcevic et al., 2002). To mediate these specialized traffic routes, AP3 directly interacts with subunits of biogenesis of lysosome-related organelles complex 1 (BLOC-1) (Di Pietro et al., 2006). Further the neuronal specific isoform AP3B, containing β3B and σ3B, has shown to mediate a distinct pathway of synaptic vesicle recycling (Danglot and Galli, 2007; Nakatsu et al., 2004).
Many insights on adaptors were derived from mutant animal models. Mice homozygous for deletion of AP1 or AP2 subunits display embryonic lethality (Ohno, 2006). In contrast, mice harboring mutations in AP3 subunits that lead to depletion of the entire complex, show only a mild phenotype. These mice are models for Hermansky-Pudlak syndrome (HPS), a group of human disorders caused by different mutations in subunits of AP3, BLOC-1 or BLOC-2 (Dell'Angelica et al., 2000). *Pearl* mice harbor a disruption of σ3A, depleting the ubiquitous complex but leaving the neuronal AP3B intact. These mice display altered pigmentation and prolonged bleeding (Feng et al., 1999). The observed symptoms are in conjunction with an AP3 function in the genesis of melanosomes and platelet granules. In *mocha* mice, the δ subunit is affected and as a result the AP3 complex is depleted in all cell types. These mice show the *pearl* phenotype and additional neurological disorders, thus highlighting the brain specific function of AP3 (Kantheti et al., 1998). In these mutant animals or HPS patients the integrity and performance of lysosomes is not drastically compromised, indicating the presence of AP3 independent pathways for the delivery of lysosomal enzymes (Dell'Angelica et al., 1999).

An increasing number of studies emphasize novel AP3 functions in transport of non-lysosomal cargo. In mouse neurons AP3 mediates the long distance transport of phosphatidylinositol-4-kinase type II alpha (Larimore et al., 2011; Salazar et al., 2005) and the sorting of cargoes like zinc transporter 3 and certain v-SNARES (Martinez-Arca et al., 2003; Salazar et al., 2004). An unexpected role for AP3 in regulated secretion was discovered in a screen using *Drosophila* cells. Yet also in mammalian neuroendocrine cells, loss of AP-3 dysregulates exocytosis due to a primary defect in large dense core vesicle formation (Asensio et al., 2010).
Fig. 1.3. Scheme of adaptor protein complex directed traffic routes. Depicted is a polarized epithelial cell. Yellow: cell surface cargo is clustered and up-taken by AP2. Red: MPRs and furin are transported from TGN to early endosomes (EE) by AP1A, possibly traversing the common recycling endosomes (CRE). Dashed lines: retrieval to the TGN is mediated by the retromer complex and AP1A or AP1B. Purple: newly synthesized lysosomal membrane proteins are transported to EE and possibly segregated there by AP3. Green: apical cargo is secreted from TGN and CRE by a lipid raft-mediated mechanism. Blue: newly synthesized basolateral cargo is secreted from the TGN by AP1A and possibly delivered to CRE by AP4. On the level of the CRE recycled as well as TGN-derived cargo is secreted to the basolateral membrane by AP1B dependent clathrin coated carriers.

On the donor compartment, coats cluster cargo molecules in microdomains to permit efficient transport. Individual transmembrane proteins are recognized by coat subunits or coat associated adaptors via short sorting motifs in their cytosolic domains. In cells the coat formation is regulated in space and time, yet regulatory elements are missing in the molecular details reviewed above. As shown for various cellular processes, small GTPases, in particular members of the Rab and ARF families, act as molecular switches during steps of intracellular trafficking. Their role in coat formation will be discussed in the following chapter.
1.5 Small GTPases regulate various aspects of vesicular traffic

The function of all small GTPases relies on the same mechanism. They exist in two distinct states displaying different conformation. In general the GTP bound conformation represents the active state, since it allows high affinity interactions with effector proteins (Wennerberg et al., 2005). The intrinsic nucleotide exchange activity of GDP-bound GTPases is remarkably increased by interaction with designated Guanine nucleotide exchange factors (GEFs). Membrane associated GTPases have a low membrane specificity and are recruited onto distinct compartments by resident GEFs, since their membrane interaction is highly reinforced in the GTP-bound state (ten Klooster and Hordijk, 2007). After GTP hydrolysis and subsequent conformational changes, the effectors dissociate and membrane associated GTPases redistribute to the cytosol. The intrinsic GTP hydrolysis rate is highly accelerated by GTPase-Activating Proteins (GAPs). Like other effector proteins, GAPs are recruited from the cytosol by active GTPases. Thus the activity of a GTPase at a distinct location depends on the interplay of GEFs and GAPs at this site. Fidelity of both GEFs and GAPs is fine tuned by phosphorylation and interactions with regulatory proteins (Bernards and Settleman, 2004).

Rab GTPases are referred to as membrane organizers and compartment identifiers (Zerial and McBride, 2001). By mediating membrane association of their multifaceted effectors, these GTPases regulate several steps of vesicular transport. Rabs enable interaction of carriers with motor proteins and thus long distance movement (Jordens et al., 2005). They permit the tethering of carriers to their acceptor compartments and are also involved in membrane fusion (Grosshans et al., 2006).
Specific Rabs are enriched on the transport intermediate already during budding and a recent study emphasizes a role in carrier fission (Miserey-Lenkei et al., 2010). However, Rabs are generally not required for the initial steps of carrier formation.

Members of the family of ADP ribosylation factor (ARF) GTPases are involved in membrane traffic by regulating cytoskeleton dynamics and coat recruitment (Gillingham and Munro, 2007). Activation of Sar1 initiates COPII assembly on ER exit sites. Membrane association of COPI, AP1, GGAs, AP3 as well as AP4 is regulated by ARF1 (Bui et al., 2009; Nie and Randazzo, 2006). In contrast, the plasma membrane recruitment of AP2 may be to the independent of small GTPases. ARF6 clearly is involved in clathrin-mediated endocytosis but its precise role in AP2 assembly remains a matter of debate (Krauss et al., 2003; Paleotti et al., 2005).

The multifaceted regulator ARF1 illustrates, how one GTPase can direct coat assembly on various compartments, depending on local GEFs, GAPs and lipids. The N-terminus of ARF1 harbors a myristoylation as well as an amphiphatic helix, which sits in a hydrophobic pocket in the GDP-bound state (Goldberg, 2000). By insertion of its myristoyl-tail ARF1 transiently interacts with membranes. Upon GEF aided nucleotide exchange, the amphiphatic helix flips out of its pocket and inserts into the bilayer. This event reinforces membrane interaction, renders interaction with effector proteins and also induces membrane deformation (Behnia and Munro, 2005; Krauss et al., 2008). Site directed mutations were generated to study ARF1 function. The dominant active version of ARF1, termed Q71L, displays drastically reduced GTP hydrolysis, while the dominant negative version – T31N – binds GDP nearly irreversibly (Dascher and Balch, 1994; Zhang et al., 1994).
Another useful tool to address ARF1 function is Brefeldin A (BFA), an inhibitor for a subset of ARF-GEFs, that thereby prevents ARF1 activation and subsequent recruitment of COPI or AP-1 to cis-Golgi or TGN membranes respectively (Donaldson et al., 1992), as well as recruitment of AP3 on endosomes (Peyroche et al., 1999). As a consequence, retrograde tubules emanate from the Golgi and fuse with the ER, while anterograde tubules appear at the TGN and fuse with endosomes (Lippincott-Schwartz et al., 1990; Wood et al., 1991). However ARF1 activity is not perturbed at sites, where its regulation relies on BFA-insensitive GEFs. Endocytic pathways involving ARF1 are thus not affected by BFA treatment (Kumari and Mayor, 2008). On the other hand, various members of the ARF family of GTPases can be influenced by BFA, when their activity is regulated by BFA-sensitive ARF-GEFs.

At the cis-Golgi nucleotide exchange of ARF1 is promoted by GBF1. The recruitment of this GEF to Golgi membranes is barely understood, yet it seems to depend on Rab1b and Golgi tethers (Garcia-Mata and Sztul, 2003; Monetta et al., 2007). Moreover, the cis-Golgi association of ARF1 itself is reliant on its interaction with cargo molecules like p23/24 and certain SNARES (Gommel et al., 2001). Once activated, ARF1 recruits stepwise COPI subunits, thus facilitating coat assembly (Sun et al., 2007). In addition, the ARF1 effectors ARFGAP2 and ARFGAP3 are crucial for coat polymerization, while it is controversial, whether the curvature sensitive ARFGAP1 is only implicated in coat disassembly or participates in coat formation as well (Bigay et al., 2003; Hsu, 2011; Kartberg et al., 2010). By recruiting phospholipase D2 to COPI coated membranes, ARF1 is also involved in carrier fission (Yang et al., 2008).
At the TGN, the established ARF-GEFs are BIG1 and BIG2. The differential roles of these proteins are a matter of debate. A recent model suggests, that BIG1 mainly acts at the TGN, while BIG2 activity is restricted to endosomal compartments (Boal and Stephens, 2010; Ishizaki et al., 2008). Both GEFs are not sufficient to promote COPI assembly (Manolea et al., 2008). Most recently it could be shown, that ARL1, an ARF family GTPase involved in the maintenance of the Golgi structure, is necessary for Golgi recruitment of BIG1 and BIG2 but not GBF1 (Christis and Munro, 2012). The molecular interactions of ARF1 during membrane stabilization of AP1 and GGA have been analyzed in detail (Austin et al., 2002; Collins et al., 2003), yet these interactions seem to be transient, since ARF1 is generally not detected in purified coated vesicles (Zhu et al., 1998). ARF1 additionally recruits phosphatidylinositol 4-kinase β (Haynes et al., 2005) and thereby possibly promotes local PI(4)P synthesis which reinforces AP1 binding (Wang et al., 2003). Apart from stabilizing AP1/GGA coats, ARF1 is involved in the recruitment of various factors that are essential for later stages of carrier formation (Donaldson et al., 2005), which will be reviewed in the following chapters. At the TGN, the implication of GAPs in ARF1 regulation is ambiguous. Liposome-based studies suggest that ARFGAP1 might facilitate AP1 coat disassembly (Meyer et al., 2005). Cell biology studies emphasize that the TGN localized AGAP2, also termed centaurin γ1, regulates ARF1 in the context of AP1 coats, yet it might not be the only relevant GAP (Nie et al., 2005; Nie and Randazzo, 2006). Especially the GIT family of ARF-GAPs may be involved in regulation of ARF1 at the TGN as well (Premont et al., 2000).

ARF1 regulates the formation of the clathrin independent coats AP3 and AP4 (Boehm et al., 2001; Ooi et al., 1998). Yet its molecular interactions as well as relevant GEFs and GAPs remain a field of intensive research.
When activated by respective GEFs, small GTPases of the ARF family initiate coat formation by stabilizing coat subunits or coat adaptors on membranes. Yet distinct effector proteins, recruited by active GTPases, are further involved in shaping and scission of the coated carrier as well as in disassembly of the coat.

Carrier formation requires mechanical deformation of the donor membrane. The assembly of cytosolic proteins on microdomains may generate only a portion of the free energy required for this process. Additional mechanisms of regulated membrane bending are described in the following chapter.
1.6 Membrane curvature and its impact on carrier formation

Cellular membrane systems display different types of curvature. By convention, positively-curved membranes are convex in respect to the cytosol, whereas negative curvatures are concave (Antonny, 2011). Consequently intracellular compartments display mainly positive curvatures with local negatively curved features (Fig. 1.4). The curvature of a membrane is considered to be small, when the corresponding radius of curvature is much larger than the thickness of the membrane. In case the curvature radius is close to membrane thickness, the membrane curvature is regarded being very high (Zimmerberg and Kozlov, 2006). The smallest stable membranous structures have radii around 10 nm, yet in the course of fission and fusion events membranes transiently exhibit higher curvatures (Burger, 2000).

![Diagram of membrane curvatures](modified from Suetsugu (2010))

**Fig. 1.4. Positive and negative curvatures on cellular membranes.**

(A): plasma membrane invaginations as well as protrusions from internal compartments display positive curvature on the tip and alongside the tubule. A small region at the neck exhibits negative curvature. (B): intracellular vesicles display uniformly positive curvature. (C): exosomes as well as intraluminal vesicles of MVBs exhibit uniformly negative curvature. (D): plasma membrane protrusions as well as invaginations in endosomes or MVBs display negative curvature in the tip and on the inner surface of the tubule. A small region at the neck exhibits positive curvature.
Homeostasis of cellular membranes does not only involve lipid and protein composition as described above, but also their curvature. Cells have developed various ways to maintain membrane curvature and thus the characteristic shape of internal compartments and plasma membrane structures (McMahon and Gallop, 2005). The general mechanisms are summarized in Fig.1.5. Shaping of different regions of the ER sets a descriptive example. The reticular tubular regions, exhibiting a high positive curvature are, at least partially, maintained by reticulons. The unusual hairpin topology of these abundant transmembrane proteins acts like a wedge in the ER membrane. The nuclear envelope, on the other hand, is devoid of reticulons. Thus it displays a sheet-like shape of very low curvature - apart from nuclear pores, locally displaying high positive curvature, being stabilized by the pore complex machinery (Zurek et al., 2011).

On the other hand, many cellular processes require regulated and spatially confined changes of membrane curvature. During transport carrier formation the membrane of the donor compartment is deformed, thus locally more positive membrane curvature is generated. While intracellular transport intermediates exhibit a high positive curvature in general, the extent of membrane deformation during their formation varies. Clathrin coated endocytic vesicles and COPI carriers have a small spherical surface, thus their generation individually requires little change in curvature. In contrast extensive tubulation occurs during the formation of large pleomorphic carriers at the TGN and during sorting on endosomes (Maxfield and McGraw, 2004; Polishchuk et al., 2003; Waguri et al., 2003). The molecular machineries involved in the regulated induction and the stabilization of high membrane curvature during carrier biogenesis are reviewed in the following three chapters.
Fig. 1.5. Mechanisms of membrane curvature induction and maintenance.
The lipid bilayer can be deformed leading to negative or positive membrane curvature. In order to induce or maintain curvature on cellular membranes, several of following mechanisms act in concert:
(A): insertion of an amphiphatic helix into one leaflet of the bilayer. (B): asymmetric changes in lipid composition. (C): influence of transmembrane proteins harboring intrinsic curvature or displaying curvature upon oligomerization. (D): polymerization of cytoskeleton towards the membrane and pulling of tubules by motor proteins. (E): direct scaffolding of the bilayer by membrane binding proteins, like BAR domain proteins., or indirect scaffolding by higher order assemblies, like clathrin coats.

1.6.1 Lipid modifications affect membrane curvature
The shape of lipid molecules, reliant on the sizes of their head groups and acyl chains, defines the effective spontaneous curvature of an individual lipid species. These can differ a lot: the spontaneous curvature of the cone shaped lipids like phosphatidic acid (PA) and diacylglycerol (DAG) is negative, while it is generally positive for the inverted cone-shaped lysophospholipids (Kooijman et al., 2003; Zimmerberg and Kozlov, 2006). Therefore changes in membrane curvature rely on the presence of distinct lipids in one leaflet. Thus local bilayer asymmetry, stabilized by diffusion barriers like integral membrane proteins or lipid interacting cytoskeleton, may trigger membrane deformation. On the other hand, lipids may respond to external curvature changes by accumulating in domains of curvature, that they prefer (Roux et al., 2005).
Fig. 1.6. Influence of lipid shape on membrane curvature. The bilayer is mainly composed of cylindrical phospholipids (PL). Enrichment of inverted conical PLs in the cytosolic leaflet generates positive curvature. This curvature is stabilized by presence of conical PLs in the exoplasmic leaflet. Conical PLs are also required in the cytosolic leaflet to maintain the negative curvature at the neck of the protrusion. Note the differences in lipid packing between cytosolic and exoplasmic leaflet. While identical in the flat part, the cytosolic leaflet in the positively curved part contains more lipid molecules, due to the larger surface in respect to the exoplasmic leaflet.

During the formation of transport carriers and corresponding generation of high positive curvature, bilayer asymmetry has to be introduced. Flippases maintain segregation of lipids among leaflets. In yeast interfering with certain flippases affected Golgi export and endocytosis, probably due to impeded carrier generation (Hua et al., 2002). However more relevant during the process of carrier formation may be the local synthesis of conical as well as inverted cone-shaped phospholipids by lipid-modifying enzymes recruited from the cytosol.

As shown in Fig. 1.6 conical lipids favor negative curvature. Therefore they have the potential to reduce the energetic barrier to initiate membrane fission and fusion (Roth, 2008). The importance of conical lipids in membrane fusion of viruses and liposomes has been exemplified (Chernomordik et al., 1997; Vicogne et al., 2006). The conical lipid DAG can be generated from PI(4,5)P2 by Phospholipase C (PLC) at the plasma membrane, where it is relevant for signal transduction (Carrasco and Merida, 2007). At other locations DAG may also be generated by dephosphorylation of PA.
Lowering of DAG levels at the TGN results in attenuated constitutive secretion due to impaired fission. Yet the role of DAG in fission may rely rather on its function as signaling platform than on promotion of curvature (Baron and Malhotra, 2002; Litvak et al., 2005). The unconventional conical lipid lysobisphosphatidic acid (Matsuo et al., 2004) as well as the cone-shaped ceramides (Trajkovic et al., 2008) are required in formation of intralumenal vesicles during MVB formation, a process relying on generation of negative curvature (Woodman and Futter, 2008). The abundant conical lipid PA is produced from phosphatidylcholine (PC) by phospholipase D (PLD). At the TGN, levels of PA regulate the budding of secretory vesicles (Siddhanta and Shields, 1998). At the cis- and medial-Golgi the isozyme PLD2 interacts with ARF1. It locally generates PA during late stages of formation of COPI carrier, permitting their fission (Stamnes et al., 1998; Yang et al., 2008). The same enzyme is also required for transferrin receptor recycling on endosomes (Padron et al., 2006). While several studies connected PA to fusion and fission, it also has the potential to induce positive curvature when converted to lysophosphatidic acid (LPA).

The inverted cone-shaped lysophospholipids are generated via deacylation of lipids by phospholipase A (PLA). The isozyme PLA2 catalyzes LPA formation from PA. Similar to DAG, LPA is a potent mediator of intracellular signaling (Moolenaar et al., 2004). In general, production of LPA seems to promote positive membrane curvature as shown during formation of COPI coated tubules from purified Golgi membranes (Yang et al., 2011). A recent model suggests that at the Golgi, production of LPA by PLA2 triggers the generation of coated membrane tubules. LPA is subsequently converted to conical lipids by lysophospholipid acyltransferase, promoting fission and thereby fragmentation of these tubules into detached vesicles (Ha et al., 2012).
All transport processes may involve lipid modifications as described above. The initiation of carrier formation may coincide with formation of lysophospholipids, generating local positive curvature. Fission and fragmentation of the tubular transport intermediate may be promoted by acylation of lysophospholipids as well as by PLD or PLC catalyzed cleavage of PC or PI(4,5)P₂, producing local negative curvatures (Bossard et al., 2007; Haucke and Di Paolo, 2007).

1.6.2 Cytoskeleton aided membrane deformation

The majority of studies linking cytoskeleton dynamics and membrane trafficking are restrained to filamentous actin, microtubules and respective motor proteins. However implications of intermediate filaments and septin filaments are emerging (Spiliotis et al., 2008; Styers et al., 2005)

Polymerization of actin monomers into filamentous actin (F-actin) is facilitated by different mechanisms. The seven subunit ARP2/3 complex nucleates a branched network of actin filaments, while formins nucleate and sustain the elongation of non-branched actin filaments and are also involved in actin bundling (Pollard, 2007). The nucleator activity of ARP2/3 is locally increased by nucleation promoting factors, present at different sites. These comprise: WASH, acting in conjunction with the retromer complex on endosomes, WHAMM, connecting cytoskeleton and membrane dynamics at the cis-Golgi, the poorly characterized JMY as well as the nucleation promoting factors of the WASP family (Rottner et al., 2010). Last-mentioned act mainly at the plasma membrane, where they are linked to dorsal ruffles (WAVE1), lamellipodia (WAVE2), as well as filopodia, podosomes and certain uptake pathways (WASP, N-WASP) (Takenawa and Suetsugu, 2007).
Recent studies suggest additional functions for WASP or N-WASP at the Golgi and on endosomes (Matas et al., 2004; Taunton et al., 2000; Vicinanza et al., 2011). Actin polymerization is temporally regulated by GTPase of the Rho family, most notably RAC1 (ras-related C3 botulinum toxin substrate 1) and CDC42 (cell division control protein 42 homolog) (Tapon and Hall, 1997). In turn activity of these GTPases is controlled at distinct locations by respective GEFs and GAPs, as reviewed above.

Bursts of ARP2/3 nucleated F-actin generate force, that is utilized to move organelles or pathogens within the cytosol (Cossart, 2000; Taunton et al., 2000). Depending on the direction of polymerization and the rigidity of adjacent membrane, local F-actin foci provide force to induce both, membrane protrusions and invaginations (Takenawa and Suetsugu, 2007). Several molecular links between AP2-dependent clathrin coats and nucleation promoting factors are known (Brady et al., 2010; Hussain et al., 2001; Wilbur et al., 2008). In yeast actin polymerization is tightly linked to clathrin coat formation and is indispensable for generation of clathrin coated vesicles (Kaksonen et al., 2005). In mammalian cells actin polymerization is not strictly necessary for clathrin-mediated endocytosis (Fujimoto et al., 2000). Yet the force provided by actin polymerization is required for endocytosis of clathrin plaques, huge cargo loads like viruses as well as under conditions of high membrane tension (Boulant et al., 2011; Cureton et al., 2010; Saffarian et al., 2009). The role of ARP2/3 nucleated actin assembly in these cases is to constrict and elongate the neck of the clathrin coated intermediate and push the endocytosed vesicles away from the plasma membrane (Collins et al., 2011). Analogous mechanisms could be discussed for carrier formation at the Golgi and on endosomes, since interactions of coats and nucleation promoting factors at this location become more evident.
ARP2/3-independent nucleation is relevant on early endosomes. Interaction of annexin A2 and the actin nucleator spir-1 is required for assembly of short actin filaments. These F-actin patches are necessary for maturation towards late endosomes, possibly by aiding membrane deformation processes (Morel et al., 2009). Likewise formin nucleated F-actin as well as its crosslinking with microtubules was shown to be involved in endosome positioning. (Fernandez-Borja et al., 2005; Gasman et al., 2003).

Newly synthesized actin filaments act as supports for myosins. These F-actin-associated motor proteins can bind to emerging tubules via coat interacting proteins or via direct lipid interaction (Dippold et al., 2009; Spudich et al., 2007). By sliding along F-actin filaments, myosins may mechanically deform linked membranes by pulling them. On another hand some myosins promote actin polymerization (Lechler et al., 2000). Specific myosins can generate force, that controls the local ARP2/3 dependent assembly of F-actin foci and thereby promote the elongation of tubules, as recently shown for TGN derived (Valderrama et al., 2001) and endocytic carriers (Cheng et al., 2012). The contribution of myosins aided membrane deformation comprises also late stages of carrier formation and fission (Valente et al., 2010).

Microtubules organize the interior of the cell. Emanating from the perinuclear organizing center, microtubules reach to the cell cortex. Internal compartments are positioned on microtubules or utilize them as tracks for anterograde and retrograde locomotion by the use of microtubule associated motors (Caviston and Holzbaur, 2006; Hirokawa et al., 2009). Plus- or minus-end directed motor proteins may also be implicated in carrier formation.
Motors can directly attach to coats, as shown for AP1, which interacts with the kinesin KIF13A during delivery of MPRs (Nakagawa et al., 2000). Direct binding of motors to PIPs on the cytosolic leaflet of donor membranes via lipid interacting domains has been illustrated (Hoepfner et al., 2005; Klopfenstein et al., 2002). Rab GTPases present on emerging carriers can mediate interaction with motors via their effectors, as shown for the Rab11 effector Rip11/FIP5 and kinesin II, which is implicated in receptor recycling on endosomes (Schonteich et al., 2008). By sliding along microtubular tracks, membrane-attached motors apply force on the donor compartment. By doing so, motors overcome membrane resistance, thus sustaining the formation of positive curvature and therefore the generation of tubular carriers. Tubule pulling along microtubular tracks could be illustrated on artificial vesicles (Koster et al., 2003). Nonetheless it is difficult to untangle the precise function of microtubular motors in carrier elongation, fission and subsequent transport.

1.6.3 BAR domain containing proteins as curvature sensors

The members of the BAR (Bin-Amphiphysin-RVS) domain protein super family, further referred to as BDPs, display a characteristic molecular architecture. The BAR domains assemble to curved helical-bundle dimers, exhibiting a concave surface on which clusters of positive charges are positioned to interact with negatively charged phospholipid head groups (Casal et al., 2006; Peter et al., 2004; Suetsugu et al., 2010). In vitro these dimers are able to congregate into filaments decorating membrane tubules in a helical fashion (Fig. 1.7-B) (Shimada et al., 2007). Depending on the mode of assembly, reflected in the slope of the helix, various tubule diameters can be coated (Mim et al., 2012).
BDPs can be classified into three groups according to the curvature of the dimer surface (Fig. 1.7-A): N-BAR, also termed “classic” BAR domain proteins generate the highest curvature and tubulate liposomes to narrow tubules. The F-BAR proteins harbor a smaller intrinsic curvature, thus induce larger tubules in vitro (Qualmann et al., 2011). Contrary I-BAR domains display a negative curvature and therefore stimulate invaginations into liposomes (Mattila et al., 2007). Most BDPs additionally contain specialized domains for recognition of PIPs, aiding membrane selectivity, and for protein-protein interactions. Thereby BDPs act as versatile modular binding platforms in various cellular processes. Likewise some GAPs and GEF acontain BAR domains (Peters et al., 2002).

In cells BDPs interact with membranes, thus sensing and likely also stabilizing their curvature. In case of numerous BDPs over expression leads to induction of stable intracellular membrane tubules. As observed in vitro on liposomes, F-BAR proteins induce tubules with larger diameter than N-BAR BDPs in vivo (Frost et al., 2008). To which extent BDPs alter membrane curvature under physiological conditions is a matter of debate. The F-BAR proteins FCHO1/2 generate small plasma membrane deformations that initiate clathrin-mediated endocytosis (Henne et al., 2010). In the course of clathrin coated vesicle formation, additional N-BAR BDPs are successively recruited. By stabilizing the neck of the coated intermediate they act as binding platforms for factors implicated in fission and uncoating: Amphiphysin can recruit dynamin to the nascent carriers and regulate its activity (Ferguson et al., 2009; Smaczynska-de et al., 2012). Endophilin, assembled on the narrow neck of the endocytic intermediate, recruits synaptojanin1, a lipid phosphatase which promotes membrane fission by local PI(4,5)P₂ hydrolysis (Chang-Ileto et al., 2011).
Membrane associated BDPs can recruit actin nucleation promoting factors via their interaction domains. At the plasma membrane Toca-1 as well as FBP17 recruit N-WASP in a CDC42 dependent manner to sites of endocytosis (Ho et al., 2004; Takano et al., 2008). On the other hand CDC42 is activated by the BAR domain containing Rho-GEF tuba (Cestra et al., 2005). On early endosomes BDPs of the sorting nexin family recruit WASH to tubular projections during cargo recycling to plasma membrane or TGN (Gomez and Billadeau, 2009; Temkin et al., 2011). At these sites BDPs can activate and sustain F-actin nucleation via ARP2/3. Thereby BDPs can support elongation of tubules, initially formed by action of coat-associated actin nucleation promoting factors, as described above. A number of SH3 domain containing BDPs can interact with the membrane constricting GTPase dynamin, as described above for clathrin-dependent endocytosis (Cestra et al., 2005; Ferguson et al., 2009; Modregger et al., 2000). Thus those BDPs may control membrane tubule scission, either by dynamin alone or by dynamin/cortactin-mediated actin reorganization (Cao et al., 2005). A recent study suggests a novel mechanism for BDPs in stabilization of membrane tubules: by covering the tubule membrane, BDPs restrict its accessibility for factors mediating fission (Boucrot et al., 2012).

Although ER and Golgi display features of high membrane curvature, only few BDPs have been localized to these compartments so far. Paralogs of endophilin and amphiphysin have been described at the TGN, yet their function is ambiguous (Farsad et al., 2001; Sarret et al., 2004). ICA69, a Rab2 effector is found at the cis-Golgi and is involved in secretory granule formation (Buffa et al., 2008; Spitzenberger et al., 2003).
Arfaptin2 has been shown to bind both ARF1 and RAC1 \textit{in vitro}, thus potentially linking activity of both GTPases at the level of the Golgi (Tarricone et al., 2001). However, a recent study in HeLa cells shows that Golgi localization of Arfaptin2 and its paralog Arfaptin1 is independent of ARF1, but relies on the ARF family GTPase ARL1 (Man et al., 2011).

Other membrane-associated proteins can alter membrane curvature by acting like a molecular wedge on the cytosolic leaflet. Upon GTP loading, ARF family GTPases stabilize or further facilitate changes in membrane curvature through the bilayer insertion of an amphiphatic helix (Krauss et al., 2008; Lundmark et al., 2008). Epsin N-terminal homology (ENTH) domain-containing proteins display a similar mechanism. At the plasma membrane, binding of epsin1 to PI(4,5)P$_2$ induces the membrane penetration of the N-terminal $\alpha$-helix, aiding membrane deformation in context of clathrin-mediated endocytosis (Ford et al., 2002). Its paralog epsinR possibly conducts a similar role at the TGN or on endosomes (Legendre-Guillemin et al., 2004; Saint-Pol et al., 2004). A recent model suggests an interplay between tubule stabilization by BDPs and curvature induction by above described molecular wedges in regulation of fission (Haucke, 2012).
Fig. 1.7. Architecture and assembly of BAR domains. (A): Ribbon diagrams depicting dimers of the F-BAR domain of FBP17, the N-BAR domain of amphiphysin and the I-BAR domain of IRSp53. The individual molecules comprising the dimers are colored respectively. Note the differences in curvature. (B): Model for assembly of FBP17 BAR domain dimers (individually colored) around the tubular portion of a membrane bud. Note the helical arrangement.

A mosaic of mechanisms is involved in membrane curvature induction during carrier formation: local lipid modification, bilayer penetration by amphiphatic amino acid stretches, assembly of BAR domain containing proteins, regulated F-actin polymerization as well as the force of motor proteins. These mechanisms may act successively, cooperatively or competitively in the individual steps: initial membrane deformation, tubule stabilization, tubule elongation as well as in subsequent fission and fragmentation of the carrier. This sequence of events and the involved protein networks are fairly well characterized in the case of clathrin-mediated endocytosis. It is likely that similar mechanisms and analogous proteins may be involved in the formation of clathrin coated transport intermediates at the level of the TGN (McNiven and Thompson, 2006).
2. AIM OF THE THESIS

The adaptor protein complex mediated trafficking pathways are essential for the regulated delivery of membrane-bound protein cargo and are thereby crucial for cellular homeostasis and cell fate during development. In non-polarized mammalian cells, AP1 mediates the delivery of lysosomal hydrolases via MPRs, AP2 is required for clathrin-mediated endocytosis and AP3 ensures proper targeting of lysosomal membrane proteins (Ohno, 2006; Robinson, 2004). While the molecular details of AP2-dependent endocytic uptake are well characterized, cellular functions and the molecular interaction networks of AP1 and AP3 are not fully understood. By employing total internal reflection fluorescence (TIRF) microscopy, the sequence of events taking place at the plasma membrane during clathrin-mediated endocytosis has been dissected in great detail (Ehrlich et al., 2004; Taylor et al., 2011; Yarar et al., 2005). In contrast, AP1 and AP3 localize to the TGN and to endosomes, compartments where numerous trafficking routes converge. This diversity together with the complex shape and the high dynamics of these organelles as well as their lacking proximity to the plasma membrane hamper studies by TIRF microscopy.

Reconstituted systems using purified or artificial membranes have effectually been employed to shed light on various aspects of membrane traffic, difficult to investigate in living cells (Mellman and Warren, 2000). Our group addressed AP1 and AP3 coat formation in vitro by the use of proteo-liposomes, harboring sorting peptides specific for individual adaptor protein complexes. This approach allowed the selective recruitment of AP1 and AP3 from a cytosolic pool onto liposomes and thus enabled the identification of known as well as novel interaction partners of the individual adaptor protein complexes (Baust et al., 2008; Baust et al., 2006).
The intriguing presence of an ARP2/3-dependent actin nucleation complex in case of AP1 as well as the discovery of septin cytoskeleton elements together with AP3 raised new questions to be addressed in further *in vitro* experiments and cell biology studies: What is the spatial relationship between the actin nucleation complex and AP1-dependent clathrin coats? Is actin dynamics affecting for the formation of clathrin coats? May F-actin assembly be involved in membrane deformation and thus in biogenesis of transport carriers, as shown for endocytosis (Kaksonen et al., 2006)? Unlike endocytic carriers, TGN derived AP1-coated transport intermediates are tubular (Waguri et al., 2003). Can we utilize our liposome system to identify proteins and lipids involved in elongation, stabilization and fission of those tubular carriers? Organization and interaction network of AP3 coats remain elusive (Dell'Angelica, 2009). Can we use our *in vitro* system to illustrate the assembly of AP3 coats in comparison to clathrin-associated AP1 coats? How is the spatial relationship of AP3 coats and septin filaments on artificial membranes as well as in living cells? The membrane recruitment of AP1 and AP3 is dependent on GTP-bound ARF1 (Ooi et al., 1998; Zhu et al., 1998). Is active ARF1 sufficient to co-recruit AP1 and the actin nucleation complex as well as AP3 and septin filaments? How is ARF1 distributed on artificial membranes in respect to the coats?

To address these questions, we want to exploit our proteo-liposome system, which can be used to generate supported bilayers or Giant Unilamellar Vesicles. These large-scale artificial membrane systems are suitable for examining the spatial organization and dynamics of coats and other membrane interacting proteins by various imaging methods. Hence artificial membranes can act as a simplified model reproducing certain steps of AP1 or AP3-mediated sorting and carrier formation in space and time, and could be considered a model for an intracellular compartment.
3. RESULTS

3.1 Visualization of AP1-dependent clathrin coat assembly on artificial membranes

Liposomes covalently modified with a small peptides mimicking the cytosolic domain of the varicella-zoster virus glycoprotein I (Gpi cd), can recruit AP1 coats from the cytosol (Baust et al., 2006). A tyrosine-based sorting motif next to an acidic cluster within the peptide is responsible for the interaction with AP1, mediating coat clathrin assembly (Alconada et al., 1996). This specific recruitment from the cytosol allowed the identification of a protein network associated with AP1 coats. It consisted of coat core components and accessory proteins like γ-synergin (Page et al., 1999). Small GTPases like ARF1 as well as its GEF BIG2, RAC1 as well as its GEF β-PIX, and several Rabs were detected as well. Further identified was an actin nucleation machinery consisting of the ARP2/3 complex and a homologue of the WAVE/SCAR complex based on CYFIP, NAP-1, ABI-1 and WAVE (Innocenti et al., 2004).

In order to visualize coat components as well as coat-interacting proteins, cytosol of cells stably expressing fluorescently tagged proteins has been utilized. Although thousands of proteins were present in the respective cytosolic extracts, the localization and dynamic behavior of the fluorescently tagged protein could be selectively studied. GFP-CLC, for example, is incorporated into productive clathrin coated pits together with AP2 and clathrin heavy chain (Ehrlich et al., 2004). Thus GFP-CLC was used as a probe to label clathrin coats on artificial membrane systems.
3.1.1 Clathrin is assembled in patches on supported lipid bilayers

The first attempt to visualize AP1-dependent clathrin coats was carried out using supported lipid bilayers. The bilayers were produced on flat mica supports using liposomes modified with the GpI cd peptide (Baust et al., 2006). Integrity of the supported lipid bilayer was examined after formation by taking advantage of the red fluorescent lipid dye DiI (Korlach et al., 1999). The bilayer appeared homogeneous, the lipid dye was evenly distributed. Lateral mobility within the membrane could be demonstrated by fluorescence recovery after bleaching, utilizing the lipid dye (data not shown). It is worth mentioning that in the case of control bilayers, which were generated using glycine-modified liposomes, large areas (several square millimeters) were evenly covered by DiI, which did not form any specific domains. In contrast when GpI cd peptide-containing liposomes were utilized, the evenly covered areas were smaller, with mica surface remaining uncovered. This could be explained by the presence of charged peptides on the liposomes surface interfering to some degree with the fusion to the mica surface and thus with proper bilayer formation. In both cases, e.g. glycine and GpI cd modified liposomes, upon addition of cytosol, circular areas (0.5 – 2 μm in diameter) devoid of DiI could be observed on the bilayer within seconds. By applying atomic force microscopy (AFM) it could be shown, that these features were not simple “holes” in the membrane, exposing uncovered mica surface, but were actually about 15 nm higher than the underlying membrane (Fig. 3.3-C). These features may represent rapidly forming aggregates of cytosolic proteins on the mica surface, that replace the bilayer. Lipid phase separations, induced by cytosolic proteins, could be ruled out since the height difference of about 15 nm was much larger than the 1 - 2 nm detected by AFM on lipid bilayers, separated into liquid ordered and liquid disordered phases (Mingeot-Leclercq et al., 2008).
Fig. 3.1 Specificity of clathrin recruitment onto supported lipid bilayers. Bilayers were formed using liposomes, which contain either the GpI cd peptide (gpI) or glycine (gly) and include 1% Phosphatidylinositol-4-phosphate (PI(4)P) as indicated. After 20 min incubation at 37°C in presence of cytosol containing GFP-CLC (green) and GTPγS, bilayers were imaged. Lipid dye DiI is labeled in red. Bars = 10 μm.

Next clathrin recruitment on bilayers was analyzed. After inspection bilayers were overlaid with cytosol from BSC-1 cells stably expressing GFP-CLC. After 20 min incubation at 37°C in presence of GTPγS, the bilayers were examined. Strikingly, we observed green fluorescent structures on the bilayers in the presence of the GpI cd peptide, but not of PI(4)P alone (Fig. 3.1). The GFP-CLC positive structures were uniformly scattered on the bilayer and had a mean diameter of about 1 μm, but also smaller, diffraction limited structures could be detected (Fig. 3.3-B). The formation of the clathrin structures could be followed by incubating the bilayer with cytosol at 37°C directly on the microscope. First structures started to appear at about 9 min of incubation. It cannot be ruled out that structures were present earlier, but their fluorescence was too low to allow their detection. Subsequently these patches grew and reached their final size and fluorescence intensity after about 18 min (Fig. 3.2). It is also noteworthy that the CLC-GFP structures appeared stable and confined to the place where they had initially formed, with no visible movement in the plane of the membrane. This could indicate that these structures have a high molecular mass and thus display a reduced lateral diffusion.
With a sophisticated setup, using a temperature-controlled AFM device mounted on a confocal fluorescence microscope, the clathrin patches, indicated by GFP-CLC fluorescence, could be correlated with height features visible in AFM. AFM was carried out with the kind assistance of Salvatore Chiantia (Stony Brook University, New York, USA). Apart from the DiI devoid “holes”, which had a height of about 15 nm and a flat surface, the majority of the patches detected by AFM were about 20 nm higher than the flat bilayer and correlated well with the GFP-CLC signal (Fig. 3.3-A). The GFP-CLC structures visible in AFM slightly differed in shape from structures visible in fluorescence microscopy. This could indicate, that clathrin was not the only component of these patches and other structures, possibly cytoskeleton elements, are covering or surrounding the clathrin coats. Another reason may be the better x-y resolution of AFM in comparison to light microscopy (Muller and Dufrene, 2011).
Fig. 3.3. Atomic force microscopy on bilayers displaying clathrin patches.
An area, containing clathrin patches, was imaged by fluorescence microscopy (B) and was subsequently probed by atomic force microscopy (AFM) (A): Bright features represent high objects, the dark background represents the flat bilayer (height image). Numbers represent dimensions of the imaged areas in μm. Note the correlation of GFP-CLC patches with elevated features on the AFM image. (C): A cross section obtained from the AFM image (white line in A, B). The profile on the left represents a clathrin structure (asterisk in A, B). The other profile represents an area devoid of DiI. (D): Examples of high resolution AFM profiles, depicting regular structures present on supported bilayer (deflection image). Note the resemblance to clathrin coats in spacing and angularity. Bars = 100 nm.
The height of the clathrin structures was about 20 nm (Fig. 3.3-C) above the bilayer. This in conjunction with the thickness of about 20 nm for clathrin coats observed on purified coated vesicles by electron microscopy studies (Vigers et al., 1986). The large lateral dimension and the rather flat appearance resemble a type of flat clathrin coats found at the plasma membrane, so called clathrin plaques (Saffarian et al., 2009). In consecutive AFM experiments with kind assistance of Grzegorz Chwastek (Schwille Lab, BIOTEC, Dresden) we tried to resolve the characteristic lattice structure of the clathrin coat, and analyze if it was similar to that described using electron microscopy (Heuser, 1980). Regular features could be detected on some of the profiles (Fig. 3.3-D). Nonetheless, the acquired images were not completely convincing, since the characteristic hexagonal lattice could not be evidently resolved. This might be due to the fact that the used imaging circumstances resemble native conditions. In contrast to the case of the fixed samples used for electron microscopy, the clathrin coats may be either hidden underneath a layer of interacting proteins or may be not rigid enough to be probed by AFM. Indeed, published high resolution AFM images of native clathrin structures also failed to resolve the lattice structure known from electron micrographs (Jin et al., 2006; Wagner et al., 1994).

Here it could be shown that GFP-tagged clathrin light chain can be recruited onto supported bilayers. The recruitment depends on the presence of the AP1 sorting motif harboring Gpl cd peptide, similar to AP1 and clathrin heavy chain recruitment on liposomes (Baust et al., 2006). This specificity as well as the dimensions of the GFP-CLC containing structures suggests, that flat AP1-dependent clathrin coats did assemble onto the supported lipid bilayer. However future experiments will have to prove if these structures are organized as clathrin lattices.
3.1.2 Clathrin assembles in large domains on GUVs

The Giant Unilamellar Vesicle (GUV) system was chosen since it represents a free standing and deformable artificial membrane system. Therefore GUVs may represent a more physiological model for studying the assembly of clathrin coats.

GUVs were generated using the above described liposomes. As in case of supported bilayers, GUV formation was more efficient, when no peptide was present on the liposomes. Nonetheless each GUV preparation contained several hundred GUVs with diameters from 5 – 50 μm. In the big majority of GUVs no large scale phase separation, indicated by segregation of the lipid dye DiI (Korlach et al., 1999), could be detected. The presence of cytosol did not affect GUV integrity, indicating that on supported bilayers the DiI segregation was indeed caused by interactions with the mica support. It should be noted that in every preparation there was some heterogeneity among the GUVs. Albeit being unilamellar, some GUVs contained higher concentrations of lipid dye. Frequently GUVs engulfing smaller GUVs as well as onion-like GUVs could be observed. First the specificity of GFP-CLC recruitment was assessed. GUVs were formed from liposomes containing either GpI cd or glycine as well as with or without PI(4)P. As an additional control, PI(4)P containing liposomes modified with GpI cd trunc, a peptide missing the acidic cluster sorting motif, have been utilized. GFP-CLC structures were recruited onto the GUV surface from the cytosol after incubation with GTPγS at 37°C. As exemplified by the equatorial sections in Fig. 3.4-A, the recruitment was dependent on presence of the intact GpI cd peptide. Quantification of GFP fluorescence showed that the effect of the intact peptide was significant (p-value < 10⁻²⁰; n = 43), whereas the PI(4)P content did not affect the clathrin assembly significantly (p-value > 0.05; n = 10) (Fig. 3.4-B).
To rule out that clathrin just aggregates on the GUV by the presence of an arbitrary peptide, following setup was chosen: two populations of GUVs, color-coded with distinct lipid dyes, and containing either the intact GpI cd peptide or the trunc version were incubated in GFP-CLC containing cytosol (Fig. 3.4-C). Strikingly, clathrin structures assembled only on GUVs harboring peptides with the intact sorting motif. Moreover, GUVs did not fuse with each other, even when the membranes were in close contact. Clathrin patches could only be observed on areas of GUV surface, which are accessible by the cytosol (Fig. 3.4-C, lower panel).

Fig. 3.4. Specificity of clathrin recruitment onto GUVs. (A): GUVs were generated using denoted liposomes which were modified with either GpI cd peptide, GpI cd trunc peptide or with glycine and include 1 % (PI(4)P) as indicated. After 20 min incubation at 37ºC in cytosol containing GFP-CLC (green) and in presence of GTPγS, GUVs were imaged. Equatorial sections are depicted. The lipid dye DiI is represented in red. (B): Quantification of GFP-CLC fluorescence on the GUV surface. (C): GUVs containing GpI cd, labeled with DiI (red), and GUVs containing GpI cd trunc, labeled with DiD (blue) were incubated together as described above. Note the selective assembly of GFP-CLC (green). Bars = 10 μm.
Whereas the analysis of equatorial sections through GUVs was adequate to quantitatively compare recruitment, the actual shape of the clathrin structures was studied on polar sections and projections of z-stacks. As noticeable in Fig. 3.5-A there is a high variation among GUVs concerning the size and shape of the clathrin coats. Some structures appear as big uniform assemblies, others seem to be clusters of small patches (Fig. 3.5-B). This diversity in surface coverage is also reflected in the big variance obtained in the quantification of GFP-CLC fluorescence on GUV surface (Fig. 3.4-B). The clathrin structures were flat and did not protrude visibly from the GUV surface. With a diameter of up to 5 μm, the clathrin patches were bigger than those observed on supported bilayers (Fig. 3.3). Thus they were much larger than clathrin coats detected at the TGN with a few hundred nanometers and also bigger than clathrin plaques at the plasma membrane (Puertollano et al., 2003; Saffarian et al., 2009).

**Fig. 3.5. Various shapes of clathrin coats on GUVs.** GUVs containing GpI cd peptide and PI(4)P were incubated with GFP-CLC containing cytosol. (A): A z-stack with slices of 1 μm was acquired. 3D projection of the stack. The lipid dye is represented in red and GFP-CLC is represented in green. Bar = 15 μm. (B): Examples of clathrin structures on polar sections of GUVs. Bars = 5 μm.
In order to address the question whether the GFP-CLC-containing clathrin coats were AP1-dependent, the above described experiment was repeated with cytosol from cells stably expressing GFP-AP1-σ. The specificity of recruitment was similar to that of GFP-CLC. GFP-AP1-σ assembled in patches on the GUV surface and its recruitment was prominently dependent on the presence of the GpI cd peptide (Fig. 3.6-A).

**Fig. 3.6. Co-recruitment of AP1 and clathrin on GUVs.** (A): GFP-AP1σ containing cytosol was incubated with GUVs (red) containing GpI cd peptide and PI(4)P as indicated. (B): GUVs containing PI(4)P and GpI cd, labeled with DiD (blue), were incubated in cytosol containing both GFP-AP1-σ (green) and tomato-CLC (red). Z-stack with slices of 1 μm was acquired. 3D projection and one single slice are depicted. Note the coalescence of red and green fluorescence signal. Bars = 10 μm.
To ensure, that the GFP-AP1\(\sigma\) patches represent the same structures as the GFP-CLC patches, GUVs were incubated with a mixture of cytosol obtained from cells expressing GFP-AP1-\(\sigma\) and cells expressing tomato-CLC. In this case the GUVs were labeled with the far-red dye DiD-C16. As expected GFP-AP1\(\sigma\) colocalized with clathrin patches containing tomato-CLC (Fig. 3.6-B). Using the above-mentioned setup no clathrin structures devoid of GFP-AP1\(\sigma\) could be detected.

In conclusion, clathrin coats, visualized by fluorescently-tagged clathrin light chain, assemble specifically on GUVs harboring peptides with AP1 sorting motif. The large flat clathrin structures on the GUV surface contain AP1, as visualized using GFP-AP1-\(\sigma\). These two findings imply that visualized structures are indeed similar in composition and sorting function with AP1-dependent clathrin coats detected in vivo.
3.1.3 Clathrin assembly is ARF1 dependent

In order to investigate the implication of ARF1 in AP1-dependent clathrin coat formation, we purified two versions of myristoylated recombinant ARF1. ARF1-cys harbors an additional C-terminal cysteine. It has been shown, that the introduced aminoacid can be utilized for labeling of the protein with maleimide-coupled fluorescent dyes (Manneville et al., 2008). ARF1Q71L is a version of ARF1, which hydrolyze GTP very slowly. Once loaded with GTP, ARF1Q71L is constitutively active (Dascher and Balch, 1994; Zhang et al., 1994).

First, the importance of ARF1 for coat recruitment on Gpl cd and PI(4)P containing liposomes was studied. All experiments described above were carried out the presence of GTPγS, a non-hydrolyzable nucleotide which keeps GTPases, that are present in the cytosol, in an active GTP-bound state. To assess the individual role of ARF1 GTPase, we used ARF1Q71L instead of relying on activation of endogenous ARF GTPases by GTPγS. After incubation with brain cytosol supplemented with ARF1Q71L or not, liposomes were purified by equilibrium density centrifugation and bound proteins were analyzed by SDS-PAGE. First the Coomassie stained gel provided an indication of the ARF1Q7L-reliant effect on recruitment. In comparison to liposomes just incubated with GTP, in the presence of ARF1Q71L and GTP additional protein bands were visible, corresponding to proteins recruited by ARF1Q71L. In the case of GTPγS these bands were present as well, but additional bands showed up (Fig. 3.7-B). It can be assumed, that the complementary proteins are recruited due to the activation of other Rab- or ARF-family GTPases activated by GTPγS. Next the recruitment of members of the AP1-dependent protein network (Baust et al., 2006) was assessed by Western blotting (Fig. 3.7-A).
For AP1 and Clathrin, the same protein amounts were recruited onto liposomes incubated with either ARF1Q71L or GTPγS. Addition of 0.5 mM GTP only slightly improved the recruitment in the case of ARF1Q71L, indicating that intrinsic GTP levels in the cytosol were sufficient to load ARF1Q71L with GTP. Although added in a 10-fold excess, the ARF-GEF inhibitor brefeldin A only slightly decreased the recruitment of AP1 and clathrin. This suggests that ARFQ71L may interact with brefeldin A insensitive GEFs present in the cytosol. Although ARF1 has shown to be necessary for membrane association of AP3 (Ooi et al., 1998), no recruitment could be observed when liposomes were incubated in presence of ARF1Q71L or GTPγS. This was expected, since GpI cd does not contain AP3 sorting motifs. The recruitment of members of the actin nucleation machinery, co-recruited with AP1 coats on liposomes, was only slightly improved by addition of ARFQ71L. Compared to GTPγS about 7 % of CYFIP1 and about 20 % of Beta-PIX (RhoGEF7) were recruited respectively. Apparently the activation of additional GTPases by GTPγS is necessary to recruit CYFIP1 and Beta-PIX more efficiently. Indeed, the CYFIP containing WAVE/SCAR complex was shown to be stabilized by GTPγS (Innocenti et al., 2004).

Subsequently, the effect of ARF1Q71L on coat recruitment was studied using GUVs containing GpI cd and PI(4)P. After incubation with GFP-CLC cytosol in presence of GTPγS, patchy clathrin structures were detected. In consistance with the Western blot, incubation with ARF1Q71L and GTP lead to the appearance of GFP-CLC structures with comparable fluorescence intensity. These coats displayed a similar variety of shapes as observed in the case of incubation with GTPγS (Fig. 3.8-B). Surprisingly the addition of GTP alone also lead to formation of clathrin coats on a subset GUVs, where coats covered a smaller area of the GUV surface, compared to addition of ARF1Q71L or GTPγS (Fig. 3.8-A).
Fig. 3.7. ARF1Q71L facilitates the recruitment of clathrin coat components on liposomes. GpI cd and PI(4)P containing liposomes were incubated with brain cytosol. As indicated, the reaction was supplemented with final concentrations of 1.6 μM ARF1Q71L, 500 μM GTP, 100 μg/ml Brefeldin A and 150 μM GTPγS. Liposomes were harvested by floatation and bound proteins were applied on gel. (A): The recruitment of indicated proteins was assessed by immunodetection with specific antibodies respectively. B): Coomassie stained gel.

Fig. 3.8. Assembly of clathrin coats onto GUVs by ARF1Q71L. (A):GpI cd and PI(4)P containing GUVs were incubated with GFP-CLC cytosol, supplemented with 1.6 μM ARF1Q71L, 500 μM GTP, or 150 μM GTPγS as indicated. (B): Polar sections displaying clathrin structures induced by ARF1Q71L  Bars = 10 μm.
To visualize ARF1 on GUVs, myristoylated ARF1-cys was fluorescently labeled at its C-terminus with Cy5-maleimide and is further referred to as ARF1-Cy5. In order to test the activity of ARF1-Cy5, control GUVs were incubated with cytosol containing or lacking GTPγS. Fluorescence detection revealed a strong background of soluble ARF1-Cy5 in both cases. Nevertheless, the addition of GTPγS alone led to membrane binding of ARF1-Cy5 (Fig. 3.9-A). This selective membrane binding upon activation of the GTPase by GTPγS indicated that, at least, a subset of ARF1-Cy5 molecules were active. Subsequently the association of ARF1-Cy5 with clathrin coats was examined. When control GUVs were incubated with cytosol containing GFP-CLC and ARF1-Cy5 in the presence of GTPγS, no clathrin coats were detectable. However ARF1-Cy5 was still recruited onto the GUV surface and appeared to be enriched in patches. When GUVs containing GpI cd were utilized, the ARF1-Cy5 signal was stronger and evenly distributed over the surface. As expected, GFP-CLC was recruited patches. Even though no local enrichment of ARF1-Cy5 in clathrin-coated areas could be detected, we observed a correlation between the recruitment of both: noticeably more ARF1-Cy5 was recruited on GUVs displaying a stronger GFP-CLC signal (Fig. 3.9-B). It could be discussed, whether a larger pool of ARF1 leads to reinforced clathrin assembly or whether AP1-dependent clathrin coats on GUVs could reinforce the membrane association of additional ARF1-Cy5, possibly by recruiting ARF-GEFs to the membrane.
By utilizing recombinant, constitutive active ARF1Q71L it could be shown, that ARF1 activation is sufficient to recruit AP1 dependent clathrin coats on liposomes as well as on GUVs. Visualization using ARF1-Cy5 indicates that ARF1 displays a uniform distribution on the surface of clathrin coated GUVs. The intensity of ARF1-Cy5 signal and clathrin recruitment correlate on GUV surface.
3.2 Visualization of AP3 coats on artificial membranes

The above described experiments demonstrated the recruitment of AP1 dependent clathrin coats on specific proteo-liposomes. A similar approach has previously been chosen to specifically assemble AP3 coats in vitro. For this purpose liposomes were modified with short peptides corresponding to the cytosolic domains of LAMP1 and LIMP2, lysosomal membrane proteins transported along the AP3 dependent pathway (Le Borgne et al., 1998). By analyzing the proteins selectively recruited from the cytosol onto these liposomes, new interactors of AP3 dependent coats could be identified. Among these were members of the septin family of cytoskeleton elements as well as the septin assembly regulator BORG4 (Baust et al., 2008). The spatial organization of AP3 coats on membranes as well as their link to septin filaments remained ambiguous. Since the GUV system has been established as a suitable system for imaging of coats and coat interacting proteins, we used a similar approach to shed light on the assembly of AP3 coat, especially in respect to polymerization of septin filaments.

3.2.1 AP3 specifically assembles on GUV surface

To generate proteo-liposomes specific for AP3, a new coupling chemistry, developed in our lab (Pocha et al., 2011), was employed. A maleimide-functionalized lipid anchor, introduced into liposomes, was coupled to polypeptides containing one unique cysteine at the N-terminus. To allow the selective recruitment of AP3, peptides, representing the cytosolic tails of human LAMP1 and LIMP2 and including an additional N-terminal cysteine, were synthesized.
Peptides with substituted AP3 sorting motifs were referred to as LAMP1 Y/A and LIMP2 LL/AA, to distinguish them from the LAMP1 wt and LIMP2 wt for peptides with intact sorting motifs. In order to test the applicability of the new coupling method for the specific recruitment of AP1, a GpI cd peptide harboring a N-terminal cysteine was synthesized as well.

Proteo-liposomes modified with these peptide were used for recruitment of protein from brain cytosol. First the specificity of the new peptides was tested: Liposomes containing 1% PI(3)P as well as either peptides with intact sorting motif, peptides with substituted sorting motif or cysteine (as negative control) were incubated with brain cytosol in the presence of GTPγS. After liposomes were purified by floatation, bound proteins were analyzed by Western blot. Indeed, only liposomes containing LAMP1 or LIMP2 peptides with intact sorting motifs recruited AP3, as shown with antibodies against the AP-3 sigma-subunit (Fig. 3.10-A).

Fig. 3.10. AP3 was recruited selectively using proteo-liposomes. Liposomes containing indicated peptides or cysteine were incubated with brain cytosol in presence of GTPγS. After purification by floatation bound proteins were analyzed by Western blot using indicated specific antibodies. (A): PI(3)P containing liposomes modified with indicated peptides were used. (B): effect of PI(3)P on coat recruitment was tested. Utilized liposomes contained 1% PI(3)P as indicated. (C): LIMP2 wt, cysteine or new GpI cd peptide harboring a N-terminal cysteine was coupled to liposomes lacking PI(3)P. The differential recruitment of AP1 and AP3 was assessed.
In an analogous experimental setup, the implication of PI(3)P on AP3 recruitment on liposomes was assessed. The Western blot revealed that the presence of 1% PI(3)P increased the AP3 recruitment by 50% in the case of liposomes containing the LIMP2 wt peptide (Fig. 3.10-B), which is in conjunction with the previous study (Baust et al., 2008). As shown by detection with antibodies against the AP-1 gamma-subunit, AP1 was co-recruited with AP3 to some extent in these conditions. Yet in contrast to AP3, AP1 was not as much enriched on liposomes compared to the input (Fig. 3.10-A,B). Subsequently the levels of proteins recruited onto PIP-lacking liposomes containing either GpI cd peptide, LIMP2 wt peptide or cysteine as a negative control were compared. Only the GpI cd peptide was sufficient to enrich AP1 and clathrin heavy chain on liposomes compared to the input. Similarly, only LIMP2 wt was sufficient to recruit AP3 efficiently on liposomes (Fig. 3.10-C).

After the fidelity of coupling and the specificity of the recruitment was evaluated, visualization of AP3 on GUVs was attempted: GUVs were prepared from liposomes containing 1% PI(3)P as denoted and either LIMP2 wt or cysteine. LIMP2 was chosen since it recruited less AP1 together with AP3, than LAMP1 (compare Fig. 3.10-A). Moreover, a similar LIMP2 peptide was used for the initial screen (Baust et al., 2008). GUVs were incubated in presence of GTPγS with cytosol of BSC-1 cells stably expressing GFP-AP3-αA. It should be noted, that instead of using brain cytosol spiked with 10-20% (v/v) GFP containing cytosol, as done in case of GpI cd before, undiluted cell cytosol was used. As predicted, only in case of LIMP2 peptide GFP-AP3-αA signal could be detected on the GUV surface, while the presence of PI(3)P did not have a noticeable influence (Fig. 3.11-A).
Fig. 3.11. GFP-AP3-σA could be specifically recruited on GUV surface.

(A): GUVs containing LIMP2 peptide or cysteine and PI(3)P as indicated were incubated with GFP-AP3-σA (green) cytosol in presence of GTPγS. GUVs were labeled with DiI (red). Bars = 50 μm. (B): GUVs harboring indicated peptides were incubated with a mixture of cytosol containing both, GFP-AP3-σA and tomato-AP1-σ (red), in presence of GTPγS. GUVs were labeled with DiI D (blue). Bars = 20 μm.

To further demonstrate the specificity of GFP-AP3-σA recruitment, PI(3)P-lacking GUVs containing either GpI cd or LIMP2 were incubated with a mixture of cytosol, from cells stably expressing GFP-AP3-σA or tomato-AP1-σ. Fluorescence signal of GFP-AP3-σA was detectable only on the surface of GUVs containing LIMP2. On the other hand, the assembly of tomato-AP1-σ in large domains could be detected only in presence of GpI cd (Fig. 3.11-B). This also exemplifies the difference in the organization of respective coats on the GUV surface. Whereas AP1 coats form large clusters (compare Fig.3.6), AP3 is detectable in smaller, scattered patches that occasionally form clusters (Fig. 3.12-A,D).
Compared to the GFP-AP1σ, GFP-AP3σA was more difficult to detect on GUVs, due to a lower ratio between cytosolic and membrane associated fluorescence. LIMP2 containing GUVs did not recruit tomato-AP1σ and, accordingly, they also failed to assemble clathrin structures on their surface when incubated with cytosol from cells expressing the GFP-CLC (data not shown).

3.2.2 ARF1 is responsible for the recruitment of AP3 coats on GUV surface

AP3 coat assembly on membranes depends on active ARF1 (Ooi et al., 1998). To test this in vitro, we used ARF1Q71, which was shown to facilitate the assembly of AP1-dependent clathrin coats on GUVs. GUVs harboring LIMP2 peptide and 1% PI(3)P were incubated with GFP-AP3-σA containing cytosol. GFP-AP3-σA was only detectable on the GUV surface, when either 150 μM GTPγS or 1.6 μM ARF1Q71L were added to the reaction. The shape and fluorescence intensity of these GFP-AP3-σA structures promoted by GTPγS or ARF1Q71L were similar (Fig. 3.12-A). No addition of GTP to the cytosol was necessary to promote the effect of ARF1Q71L, indicating that endogenous levels of these nucleotides might be sufficient to activate this GTPase, as shown earlier (compare Fig. 3.7-A). Since ARF1 activation is sufficient to mediate AP3 assembly on GUVs, the question arises, how active ARF1 and AP3 coats are spatially distributed on the GUV surface. In order to visualize ARF1, the above described ARF1-Cy5 was utilized.
Fig. 3.12 ARF1 recruits AP3 coats and colocalizes with AP3 on GUVs. (A): LIMP2 and PI(3)P containing GUVs were incubated in GFP-AP3-σA cytosol in presence of 150 μM GTPγS or 1.6 μM ARF1Q71L as indicated. Lower panels display polar sections. Bars = 20 μm. (B): Above described GUVs were incubated with GFP-AP3-σA cytosol containing ARF1-Cy5. GTPγS was added to reaction as indicated. Bars = 10 μm. (C): GUVs containing either cysteine or LIMP2 were incubated with ARF1-Cy5 containing cytosol in presence of GTPγS. Cy5-signal is depicted. Bars = 10 μm. (D): GUVs were incubated in GFP-AP3-σA cytosol containing ARF1-Cy5 (red) in presence of GTPγS. Lower panels display polar sections. Bars = 10 μm

Control GUVs were incubated with GFP-AP3-σA cytosol containing ARF1-Cy5. As expected, ARF1-Cy5 only bound to the GUV surface when its activation was permitted by the presence of GTPγS. When recruitment was performed using GUVs containing the LIMP2 peptide in presence of GTPγS, both ARF1-Cy5 and GFP-AP3-σA were recruited on the GUV surface (Fig. 3.12-B). In this case the ARF1-Cy5 appeared enriched in AP3 patches and was less abundant in areas outside the patches (Fig. 3.12-D). This observation was in contrast to the case of Gpl cd, where ARF1-Cy5 appeared evenly distributed on the GUV surface (compare Fig. 3.9-B).
On control GUVs containing cysteine, ARF1-Cy5 assembled in patches (Fig. 3.12-B,C) indicating that ARF1-Cy5 recruitment was dependent on GTP\textsubscript{S}, but not on the presence of a sorting peptide. These ARF1-Cy5 patches were comparable in fluorescence intensity and size to those observed in case of LIMP2 peptide (Fig. 3.12-C). This suggested that on patch-like areas enriched in ARF1, AP3 assembly may be promoted when the sorting peptides are present. It remains ambiguous which factors constrain membrane bound ARF1 to such patch-like organization in the absence of coats. The membrane bound ARF1 remains associated with AP3 coated membrane areas and does not evenly cover the entire GUV surface, like it was the case for clathrin coats (Fig. 3.12-D, compare Fig. 3.9-B). Apparently, AP3 coats do not recruit factors that further sustain ARF1 recruitment on the GUV surface.

3.2.3 Septin7 assembles on GUVs and spatially restricts AP3 coats

Various septins as well as the septin regulator BORG4 were identified among the proteins assembled together with AP3 on liposomes (Baust et al., 2008). Septins are GTP-binding proteins that assemble into hetero-oligomeric complexes, which give rise to higher-order structures such as filaments and rings (Oh and Bi, 2011). They comprise a conserved family of proteins that are found primarily in fungi and animals. Unlike for filamenous fungi and yeast, the physiological significance of mammalian septin complexes remains ambiguous (Weirich et al., 2008). The 13 humans septins can be divided in several groups of homologous and functionally redundant proteins. Septin7 is the only unique member, since its loss can not be substituted by other septin family members in its hetero-trimeric complex with septin6 and septin2 (Zent et al., 2011). Yet septin7 may be part of other septin complexes as well (Nagata et al., 2004).
To visualize septin filaments on GUVs, HEK cell lines stably expressing YFP-septin7 or mCherry-septin7 were generated. Septin7 was chosen for the visualization of septin filaments since it represents the best characterized member among the septins identified in the initial screen: In fixed HEK cells, YFP-septin7 was detectable as loose meshwork of bended filaments, scattered throughout the cell (Fig. 3.13-A).

Fig. 3.13. Septin7 assembly on GUV surface. (A): HEK cells stably expressing YFP-septin7 were generated. A characteristic cell is depicted. GUVs were incubated with cytosol, obtained from these cells. (B): GUVS containing either LIMP2 peptide or cysteine were incubated in presence of GTPγS. (C): GUVs containing LIMP2 were incubated with 150 μM GTPγS or 1.6 μM ARF1Q71L as denoted. (D): 3D-projections of z-stacks displaying YFP-septin7 assembly on GUVs. Bars = 10 μm.
First we tested which conditions promote the assembly of YFP-septin7 filaments on artificial membranes: LIMP2 containing GUVs were incubated with cytosol of HEK cells stably expressing YFP-septin7 under different conditions. Without supplements, no fluorescence signal could be detected on GUVs, whereas the addition of 150 μM GTPγS facilitated YFP-septin7 recruitment. Surprisingly, the supplement of 1.6 μM ARF1Q71L was sufficient to facilitate septin7 assembly as well (Fig. 3.13-C). Subsequently the dependence of septin7 assembly on the LIMP2 sorting motif was assessed: GUVs containing either LIMP2 or cysteine were incubated with YFP-septin7 cytosol in the presence of GTPγS. YFP-septin7 fluorescence signals of similar intensity could be detected on the surface of GUVs in both cases (Fig. 3.13-B). Regardless of the presence of a sorting peptide, a big variation in surface bound septin7 was observed among GUVs. In equatorial sections the YFP-septin7 signal on GUVs appeared patchy and, as described above, heterogeneous. However polar sections and 3D projections of z-stacks revealed unique and highly organized structures. The YFP-septin7 signal was reminiscent of tape wrapped around the GUV, sometimes displaying loops and circles (Fig. 3.13-D). It could be speculated that these structures were a result of self-organization of septin-filaments on the GUV surface. It should be noted that these filaments might also contain other members of the septin family or other cytoskeleton elements, but only septin7 was visualized via the YFP-tag. Astoundingly, the YFP-septin7 structures on GUVs were reminiscent of the filaments detected in YFP-septin7 expressing HEK cells (compare Fig. 3.13-A). The observation that septin7 filaments assemble uncoupled from AP3 coats on GUVs did not rule out a possible interaction of AP3 with septin filaments.
Fig. 3.14. Spatial relationships of AP3 coats and septin7 structures. (A) LIMP2 GUVs were incubated with cytosol containing both, GFP-AP3-σA (green) and mCherry-septin7 (red) in presence of GTPγS. 3D- projections of the polar region are depicted. Note the spatial confinement of AP3 coats by septin7. (B): LIMP2 GUVs were incubated in cytosol containing both, YFP-septin7 (green) and as ARF1-Cy5 (red) in presence of GTPγS. Equatorial sections and polar sections are depicted. Note confinement of ARF1 surface occupation by septin7. Bars = 10 μm.

To visualize the interplay between septin7 structures and AP3 coats a mixture of cytosol from cells stably expressing GFP-AP3-σA and mCherry-septin7 was prepared. LIMP2 containing GUVs were incubated in this mixture in presence of GTPγS. Only a subset of GUVs recruited both AP3 coats and septin7. When present on the same GUV, AP3 coats are detectable in areas devoid of septin7 filaments.
Septin assembly on GUVs appeared to spatially confine the AP3 coats and to limit their size. Occasionally AP3 coats were detected close to septin structures (Fig. 3.14-A). When the experiment was repeated with YFP-septin7 cytosol, spiked with ARF1-Cy5, the distribution of ARF1-Cy5 on GUV surface seemed to be confined by septin7 structures as well (Fig. 3.14-B). These findings point towards the possibility that the patchy appearance of ARF1-Cy5, recruited on GUVs by GTP\(\gamma\)S, may be caused by septin filaments that co-assemble during ARF1 recruitment and constrain ARF1 diffusion in the plane of the membrane. The spatially limited pool of ARF1 would further limit the distribution of AP3 coats. This would go along the line with the suggested septin function as diffusion barriers and membrane organizers (Hu et al., 2010; Schmidt and Nichols, 2004).

### 3.2.4 Interaction of septin7 and AP3 positive carriers in HeLa cells

The above described GUV experiments shed light on an interesting property of septin filaments, yet their association with AP3 coats remained ambiguous. An initial cell biology approach was chosen to assess how AP3 and septins interact in vivo. Therefore, we expressed tagged versions of septin7 and septin6 in HeLa cells. The ectopic expression of tagged septins was delicate. Higher expression levels lead to occurrence of spherical aggregates in case of septin6 and fiber-like aggregates in case of septin7. Filamentous structures were detected only at very low expression levels. Septin filaments were aligned to actin cables, partially covering those (Fig. 3.15-A, B). GFP-septin6 completely colocalized with endogenous septin7, but showed only little overlap with microtubules (Fig. 3.15-C). However, AP3 structures only occasionally were aligned to septin6 or septin7 filaments in fixed cells (Fig. 3.15-A,B,D).
Fig. 3.15. Localization of septin structures in HeLa cells. (A): HeLa cells transfected with GFP-septin6 were stained for AP3 (red) and actin (blue). (B): HeLa cells transfected with GFP-septin7 and were stained for AP3 (red) and actin (blue). (C): HeLa cells transfected with GFP-septin6 were stained for endogenous septin7 (red) and tubulin (blue). (D): HeLa cells stable expressing GFP-AP3-δ were stained for endogenous septin7 (red) and tubulin (blue) (E): HeLa cells were co-transfected with GFP-AP3-δ and mCherry-septin7. Low expressing cells were imaged by video microscopy. A montage of a 32 s time interval from the boxed area (right) is depicted. Note how AP3-positive structures and jump between septin filaments. Bars = 20 μm.
In order to investigate the dynamic interactions between AP3-coated structures and septin7, HeLa cells were double transfected with plasmids encoding mCherry tagged septin7 and the GFP-tagged δ subunit of AP3. Transfected cells were studied by dual color video microscopy. Only at very low expression level, the fluorescence signals appeared vesicular in case of GFP-AP3δ and filamentous in case of mCherry-septin7. In these cases a subset of AP3 positive carriers was aligned to septin7 filaments at each time point, as suggested by staining of fixed cells (Fig. 3.15-D). When AP3 carriers traveled through the cell, movement was briefly interrupted at septin filaments. It appeared like AP3 structures jumped between filaments (Fig. 3.15-E). Moreover events of fission of vesicles located on septin filaments as well as fusion with incoming carriers could be observed. These findings indicate an implication of septin filaments in the movement and positioning of AP3-positive endosomal carriers.

In conclusion, we utilized the GUV system to visualize AP3 coats and demonstrate the specificity of its recruitment as well as its dependency on ARF1 activation. Moreover, the assembly of septin7 in intriguing shapes could be visualized on the GUV surface. Septin assembly on artificial membranes may in general constrain the lateral diffusion of membrane interacting proteins, and thus limit the size and the lateral mobility of recruited coats. The actual cell biological implication of septin7 in the context of AP3 dependent trafficking remains elusive, yet it may be linked to its interaction with cytoskeleton elements, as indicated by the initial live cell imaging experiments.
3.3 Coordinated actin polymerization on GUVs gives an indication for a mechanism of carrier formation at the TGN

An actin nucleation machinery has been identified in the proteomic analysis of proteins recruited together with AP1-dependent clathrin coats onto liposomes (Baust et al., 2006). This protein network was comprised of members of the WAVE-SCAR complex, CYFIP1, CYFIP2, ABI1 and NAP1 as well as the actin nucleation promoting factors WAVE1 and WAVE3 together with the actin nucleator ARP2/3. The presence of a molecular link between coats and the described actin nucleation machinery as well as the physiological relevance of the newly identified interaction remained ambiguous. Therefore we used the GUV system to reconstitute and visualize clathrin together with CYFIP1 and actin. This may help to explain the mode of interaction and the function of these molecules.

3.3.1 Actin polymerization on GUVs is ATP dependent

To visualize CYFIP1, as a part of the actin nucleation complex, GpI cd and PI(4)P containing GUVs were incubated with a mixture of cytosols from cells stably expressing GFP-CYFIP1 and tomato-CLC respectively. After incubation in presence of GTPγS, GFP-CYFIP1 could be detected on the GUV surface. The fluorescence signal did not overlap with, but rather surrounded clathrin patches (Fig. 3.16-A). In order to test whether the recruited actin polymerization machinery was functional, GUVs were incubated in cytosol containing GFP-actin. No significant GFP-actin signal could be detected on GUV surface after incubation with GTPγS (data not shown). Actin polymerization could be observed when ATP, provided by an ATP regeneration system, was supplied.
In this case GUVs were surrounded by actin shells of various thickness, as indicated by a strong GFP-actin signal. Supplementing the cytosol with Latrunculin B to a final concentration of 50 μM completely inhibited actin assembly (Fig. 3.16-B).

As previously observed for clathrin recruitment, there was a high variation among GUVs in amount of actin assembly, indicating heterogeneity generated during GUV formation. The actin shells appeared uniform and had a thickness ranging from 1 to 10 micrometer. Actin “comets”, described as a result of anisotropic actin polymerization on GUVs (Heuvingh et al., 2007), could only rarely be detected.

**Fig. 3.16. Coordinated Actin assembly on GUVs.** (A): GpI cd containing GUVs were incubated with cytosol containing tomato-CLC (red) and GFP-CYFIP1 (green). Equatorial and polar sections are depicted. (B): GUVs were incubated with GFP-actin (green) containing cytosol in presence of ATP regeneration system and either DMSO or 50 μM Latrunculin B. The lipid dye is represented in red. (C): GUVs were incubated with mRFP-actin (red) containing cytosol in presence of ATP regeneration for 15 min. Then GFP-actin (green) was added and GUVs were incubated for 10 more min before imaging. Bars = 10 μm.
To identify the location of actin assembly, a pulse experiment has been conducted. mRFP-actin was allowed to assemble on GUVs for 15 min, followed by addition of GFP-actin and 10 more min of incubation. Newly-assembled, GFP-positive actin filaments were detected close to the GUV surface and pushed “older” mRFP-actin away from the membrane (Fig. 3.16-C). This is in conjunction with the notion, that the actin polymerization promoting complex, as exemplified using GFP-CYFIP1, is enriched on the GUV surface and may promote actin polymerization at this site.

3.3.2 Clathrin decorated membrane protrusions are induced by actin polymerization on GUVs

Since actin assembly on GUVs was promoted by supplying ATP, it could consecutively be tested, how actin polymerization affected clathrin coats. GUVs containing GpI cd and PI(4)P were incubated in cytosol containing CLC-GFP in presence of GTPγS and an ATP regeneration system. Instead of large domains or clusters, under conditions promoting actin polymerization the appearance of clathrin coats changed. In this case we detected clathrin patches smaller than 1 μm (Fig. 3.17). The size of these clathrin patches was comparable to those observed on bilayers.

More strikingly in a number of cases clathrin patches that projected from the GUV surface could be detected. (Fig. 3.17, arrows). These protrusions corresponded to thin membrane tubules. Those had not been noticed in previous experiments, since they were barely detectable due to low amount of lipid dye. The formation of these projections was strictly linked to actin polymerization and was completely abolished by the actin destabilizing drug Latrunculin B (Fig. 3.17).
The formation of these membrane tubules was studied in further experiments by using cytosol containing GFP-actin or GFP-CLC. As expected, the distance of the membrane protrusion from the GUV surface corresponded to the observed thickness of the GFP-actin shell, which ranges from 1 to 10 μm (Fig. 3.18-A). Actin assembly was an ongoing process: the actin shells were becoming more evident after 30 min incubation at 37°C and were continuing to grow during observation on the microscope, leading to GUV deformation in the end. Several steps could be observed during sustained actin polymerization on GUVs (Fig. 3.18-C, from left to right): formation of a thin (few micrometers) actin shell led to straight membrane tubules emerging from the GUV surface in a perpendicular fashion (compare Fig. 3.18-B). Those tubules were barely detectable by lipid dye but could be seen by observing clathrin coats, which were predominantly localized at the very tips of these tubules.

Fig. 3.17. Effect of actin polymerization on coat formation on GUV surface. GpI cd and PI(4)P containing GUVs were incubated with cytosol containing GFP-CLC (green) under conditions allowing (DMSO) or preventing (Latrunculin B) actin polymerization. Equatorial sections and projections of image z-stacks are depicted. Arrows indicate coats projected from GUV surface. Bars = 5 μm.
As the actin shells grew, the tubules became longer and occasionally appeared bended or branched, whereas the GUV lumen seemed to be compressed. In the case of longer tubules, in addition to clathrin being present at the tip, discrete patches decorating the tubules could be observed. At the final stage, the GUV was completely collapsed, with no detectable lumen and long tubular projections. Due to the obvious heterogeneity of the GUVs, all these stages could be concomitantly observed in the same preparation. Nevertheless, after a long observation time (more than 30 min), mainly collapsed GUVs remained, next to GUVs not displaying any tubules at all.

Fig. 3.18. Tubular projections from GUV surface are generated by actin polymerization. (A): GUVs are incubated with cytosol containing GFP-actin (green) under conditions that promote actin polymerization. The signal of the lipid dye DiI (red) was detected with high laser power. (B): 3D-projection of GUV, labeled with DiI, displaying tubular protrusions. (C): GUVs are incubated with cytosol containing GFP-clathrin (green) under conditions that promote actin polymerization as indicated. Stages of increasing GUV deformation are depicted from left to right. Bars = 10 μm.
As shown above, CYFIP1 was present on the GUV surface, but excluded from clathrin-coated areas (Fig. 3.16-A). Accordingly, the CYFIP1 containing actin nucleation machinery promoted actin assembly on membrane areas surrounding coats, as seen in Fig. 3.16-C. Polymerization of actin in the vicinity of coats, but not on top of them, generated force towards GUV surface eventually forming tubular protrusions with coats being projected away from the membrane. This would be in conjunction with clathrin coats being present on the tips of emerging tubes.

### 3.3.3 ARF1 activation is not sufficient to promote actin assembly on GUVs

Active ARF1 has the potential to recruit factors that in turn could recruit or activate Rho GTPases necessary to promote actin assembly (Dubois et al., 2005; Koronakis et al., 2011; Shin and Exton, 2005). Thus ARF1 could coordinate coat formation and actin dynamics. Indeed, as shown by Western blot, ARF1Q71L was capable of recruiting the RAC1-GEF beta-PIX (Rho guanine nucleotide exchange factor 7) on liposomes, although not as efficiently as GTPγS (compare Fig. 3.7-A).

We next asked, whether ARF1 activation and its crosstalk with Rho-GTPases can also promote actin polymerization on membranes. A noticeable GFP-actin shell was formed only in the presence of GTPγS, but not ARF1Q71L, on GUVs incubated with cytosol containing GFP-actin and ATP supply (Fig. 3.19). Thus no long tubular projections and no thick actin shells are detectable on GUVs incubated with ARF1Q71L. This is consistent with the finding that a much smaller amount of the actin nucleation complex member CYFIP1 (7 %) and Beta-PIX (20 %) were recruited in case of ARF1Q71L compared to GTPγS, as seen by western blot (Fig. 3.7-B).
Nevertheless, ARF1Q71L was sufficient to assemble clathrin coats, as indicated by tomato-CLC. In the presence of ATP supply however, the GUV membrane underneath these coats was bended. These deformations were clearly different from the micrometer long tubular projections seen in the case of GTPγS and were not detectable without ATP supply. Thus, ARF1 activation may mediate local actin assembly at clathrin coats, leading to tiny protrusions. Obviously it may not recruit enough nucleation promoting factors and/or may not activate enough Rho GTPases to activate the nucleation promoting factors for prolonged actin polymerization. The massive actin assembly, observed in case GTPγS, might be sustained by factors being activated and recruited onto the membrane in a GTPγS-dependent manner.

Fig. 3.19. ARF1Q71L is not sufficient to promote actin polymerization on GUV surface. DiD labeled GpI cd containing GUVs (blue) were incubated with cytosol containing both GFP-actin (green) and tomato-CLC (red). Cytosol was supplemented with 150 μM GTPγS or 1.6 μM ARF1Q71L and ATP regeneration system as indicated. Bars = 10 μm.
3.3.4 Actin mediated GUV tubulation is dependent on CYFIP1 & RAC1

The GpI cd peptide mediated the recruitment of AP1 dependent clathrin coats, together with described actin nucleation complex onto liposomes. In mammalian cells GpI utilizes an AP1 dependent transport pathway (Alconada et al., 1996). Therefore GpI can be regarded as an AP1 model cargo. Thus the idea suggested itself to test the implication of actin nucleation on intracellular transport of *bona fide* AP1 cargo like MPRs. Cell biology studies by Mihaela Anitei indeed indicated, that interference with CYFIP1 and RAC, both had been found associated with clathrin coats on liposomes, reduced the formation of tubular ciMPR carriers at the TGN (Anitei et al., 2010). This implied that spatially confined and coordinated actin assembly was required for generation of tubular post-Golgi carriers *in vivo*.

To investigate whether these proteins, shown to affect ciMPR tubule generation at the TGN *in vivo*, are involved in tubule formation on GUVs following experiments had been conducted with assistance of Mihaela Anitei: cytosols were generated from HEK cells stably expressing GFP-actin. Theses cells were treated for 72 h with siRNAs against CYFIP1 and RAC1 as well as with control siRNAs. Cytosols of untreated cells were used as control and the RAC1 inhibitor NSC2376 was added to 200 μM final concentration when indicated. GUVs were studied by confocal microscopy after 20 min incubation with cytosol. Visualization of GFP-actin aided the identification of tubulated GUVs. Indeed interference with CYFIP1 and RAC1, which is likely the GTPase required for the activation of the CYFIP1 containing actin nucleation complex, significantly reduced the percentage of tubulated GUVs from about 60 % to 25-30 % (Fig. 3.20-B). This is in conjunction with above-mentioned *in vivo* data.
It should be mentioned that down regulation of CYFIP1 and RAC1 as well as drug-mediated inhibition of RAC1 activity did not completely abolish but only slowed down the dynamics of actin-mediated GUV tubulation, (Fig. 3.20-A). This may be due to alternative mechanisms that contribute to some extent to actin polymerization on GUVs, likely due to activation of other GTPases like CDC42 by GTPγS.

**Fig. 3.20. Effect on RAC1 and CYFIP1 inhibition on actin mediated GUV tubulation.** GpI cd containing GUVs were incubated with cytosol of GFP-actin HEK cells, either transfected with indicated siRNAs or treated with 200 μM NSC23766 (RAC1 inhibitor) as denoted. GUVs tubulated by actin were counted after 20 min incubation with cytosol in presence of ATP regeneration system and GTPγS. (A): examples for siNon and siCYFIP1. Bars = 20 μm. (B): quantification of the occurrence of tubulated GUVs.
In conclusion, we have shown that a CYFIP1 containing actin nucleation machinery was recruited onto GUVs. When ATP was supplied, actin polymerization took place on the GUV surface. GTPγS, but not ARF1 activation alone, lead to sustained actin polymerization. Continuous actin assembly on GUV membrane generated tubular projections decorated with clathrin coats. The generation of those membrane tubules could be reduced by interfering with CYFIP1 and RAC1, however could be completely inhibited by destabilizing actin with Latrunculin B. A very similar mechanism, although tightly regulated, might act during the early steps of carrier formation in vivo. Therefore it can be hypnotized, that the tubule formation on GUVs could act as an in vitro model to understand steps of tubular carrier generation at the TGN.
3.4 Screening for proteins enriched on tubulated membranes reveals new candidates implicated in post-Golgi carrier formation

Our previous in vitro studies showing that we can manipulate the actin-dependent formation of tubular carriers, gave us the possibility to identify by quantitative mass spectrometry the protein networks leading to the stabilization, elongation and fission of these tubules. Candidates could subsequently be tested in cell biological assays for involvement in the formation and fate of tubular carriers in vivo and in vitro.

3.4.1 Proteomic screen to identify proteins enriched or depleted on liposomes tubulated by actin polymerization.

The small liposomes, used for recruitment, had been extruded to an average size of 400 nm. This reflects a relatively low membrane curvature, comparable to endosomes or features of the TGN. Indeed clathrin-coated protrusions with a diameter of 25 μm, reflecting a very high membrane curvature, had occasionally been detected on EM pictures of liposomes (Baust et al., 2006). It should be possible to promote liposome tubulation, increasing membrane curvature, and thus promote assembly of membrane curvature-dependent proteins accordingly.

We used different experimental conditions to conduct the identification of the protein network controlling tubule formation. We used liposomes containing GpI cd peptide and PI(4)P, incubated in the absence and presence of ATP regeneration system to control actin polymerization. On the other hand, we used 20 μM Latrunculin B as an inhibitor of actin polymerization.
When the conditions favored actin polymerization, liposomes increased in density, possibly due to the surrounding actin shell. Therefore conditions for floatation had to be optimized to separate denser membranes from the cytosol by increasing the sucrose concentration and thus the density of the floatation medium.

**Fig. 3.21. Quantitative mass spectrometry approach.** (A): flow scheme of the experiments: liposomes were incubated with cytosol under indicated conditions and purified by floatation. Bound proteins were applied on gel, bands of same height were cut out and in-gel trypsin digested in either heavy or light water. Peptides of corresponding gel slices were extracted, pooled and analyzed by LC-MS/MS. (B): a selection of candidate proteins being enriched on liposomes tubulated by actin polymerization.

After gel separation, proteins bound to liposomes were identified by mass spectrometry with kind assistance of Cornelia Czupalla. For quantification corresponding gel pieces were trypsin digested in the presence of H$_2$O$^{18}$ or H$_2$O$^{16}$ respectively and paired samples were combined immediately before nano-LC-MS/MS (Lange et al., 2010). A large number of proteins has been identified in both experiments, including all those identified in the initial study (Baust et al., 2006).
The more sensitive mass spectrometry set-up as well as updated protein databases can explain the newly identified proteins. These include among others previously not identified coat accessory proteins like aftiphilin, HIP1R, GGA3 and synaptojanin-1. Yet the main focus of the experiment was on those proteins that changed in abundance upon sustained actin polymerization and membrane tubulation. A distinct protein was defined as changed, when in between conditions the normalized MS intensities differed at least twofold for the majority of corresponding its tryptic peptides.

As expected, many members of the nucleation promoting complex as well as members of the ARP2/3 complex were increased, when actin assembly was permitted. Several myosin motors, in contrast, were displaced from liposomes under conditions promoting F-actin assembly. Abundance of clathrin heavy chain and AP1 subunits were decreased under these conditions as well. This is in conjunction with observations on GUVs, where coats occupied a smaller fraction of the GUV surface when actin-mediated tubules are formed (compare Fig. 3.17 and Fig. 3.18-C). The enrichment of N-BAR domain containing proteins amphiphysin, endophilin-A1, Arfaptin2 and PRKCA-binding protein gave further indication that the chosen experimental set-up was successful, since N-BAR domains preferentially bind to highly curved membranes (Gallop et al., 2006). Yet apart from Arfaptin2, the N-BAR domain proteins identified have been shown so far to function in endocytosis (Hanley and Henley, 2005; Simpson et al., 1999). Moreover the abundance of several lipid-modifying enzymes has been altered: The phosphatidylinositol 4-kinase beta, an enzyme generating PI(4)P from PI, as well as the Inositol polyphosphate 5-phosphatase J (INPP5J), an enzyme converting PI(4,5)P_2 to PI(4)P, were depleted.
On the other hand, all three members of the type I phosphatidylinositol-4-phosphate 5-kinase family were enriched. The presence of these enzymes might imply that generation of PI(4,5)P$_2$ from PI(4)P was promoted on tubulated liposomes. This went along the line with several characteristic PI(4,5)P$_2$ binding proteins being enriched as well. Among those was dynamin-1, a GTPase with known affinity for tubular membranes (Takei et al., 1995), and was already shown to assembles on tubules, mechanically pulled from GUVs (Roux et al., 2010). In addition, several proteins involved in intracellular calcium-signaling, binding inositol 1,4,5-trisphosphate have been identified as enriched. The generation of this secondary messenger depends on the presence of PI(4,5)P$_2$ as well.
3.4.2 A role for PI(4,5)P₂ synthesis in post-Golgi carrier formation.

The members of the type I phosphatidylinositol-4-phosphate 5-kinase (PIP5KI) family were preferentially recruited from the cytosol on PI(4)P containing liposomes, when actin polymerization and thus tubulation of liposomes was promoted. This suggests that the enzymatic activity of these kinases, synthesis of PI(4,5)P₂ from PI(4)P, may take place during formation or elongation of those tubules. As discussed above, the Gpi cd liposome system could act as a model for AP1 dependent carrier formation at the TGN. Regulated and spatially confined synthesis of distinct phosphoinositides has been linked to membrane transport processes in various cases (Cullen et al., 2001; De Camilli et al., 1996). Therefore, these findings prompted the investigation of type I phosphatidylinositol-4-phosphate 5-kinases function in formation of coated carriers at the TGN. To study the dynamics of AP1 sorted cargo, HeLa cells stably expressing a chimera formed of GFP fused to the transmembrane and cytosolic domain if the ciMPR (GFP-MPR) were utilized (Waguri et al., 2003).

In contrast to PIP5KIGamma which localizes to the plasma membrane, activity of PIP5KIAlpha and PIP5KIBeta has been linked to the Golgi and endosomal structures (Doughman et al., 2003b; Jones et al., 2000). Therefore, they were chosen for subsequent studies and targeted with corresponding siRNAs. Using specific siRNAs PIP5KIAlpha levels were decreased by about 85 % (Fig. 3.22-B) and were not affected by siRNA targeting the Beta isozyme (data not shown). Unfortunately the assessment of PIP5KIBeta levels by Western blot and RT-PCR was not successful, thus knock-down efficiency and overall expression of this protein in HeLa cells remained ambiguous. First, the effect of down regulation of phosphatidylinositol-4-phosphate 5-kinases on GFP-MPR steady state distribution had been addressed.
In fixed control cells the majority of GFP-MPR signal was condensed in the perinuclear region, with numerous small polymorphic endosomal structures being detectable throughout the cytosol (Waguri et al., 2003). In the case of PIP5K1Alpha knock-down, the overall GFP-MPR signal was reduced compared to control siRNA. The endosomal signal was barely detectable and the perinuclear signal appeared scattered (Fig. 3.22-A). Staining with the cis-Golgi marker GM130 revealed a less condensed, often fragmented overall Golgi morphology.

Fig. 3.22. Effect of PIP5KI down regulation on GFP-MPR distribution. HeLa cells stably expressing GFP-MPR were treated for 72 h with 10 nM of indicated siRNAs. (A): antibody staining for GM130 (red) highlighting Golgi integrity. Note the scattering of the Golgi in both knock-downs. (B): effect of down regulation on PIP5KIAlpha protein level has been quantified by Western blot. (C): GFP-MPR signal of cells that were treated with denoted siRNAs for 60 h and were subsequently incubated for additional 12 h with 100 μM Chloroquine as indicated. Bars = 20 μm.
When cells, incubated with PIP5KIA Alpha specific siRNA, were treated with 100 μM Chloroquine for 12 h, the GFP-MPR fluorescence signal recovered to levels of control. In addition a more condensed perinuclear localization of GFP-MPR was observed, resembling its morphology in untreated cells (Fig. 3.22-C). Chloroquine interferes with the function of lysosomal hydrolases (Gonzalez-Noriega et al., 1980; Waguri et al., 2006). The rescue of GFP-MPR signal intensity and distribution by this drug indicates, that in PIP5KIA Alpha knock-down cells GFP-MPR levels were decreased due to degradation in lysosomes. Increased lysosomal degradation could indicate mis-routing of MPRs.

In comparison to the knock-down of PIP5KIA Alpha, treatment with siRNAs targeting PIP5KIBeta did not lead to a noticeable change of the overall fluorescence. However down regulation of PIP5KIBeta lead to an enlarged Golgi, yet the scattering was not as pronounced as in case of PIP5KIA Alpha down regulation. Characteristic was the occurrence of enlarged GFP-MPR structures in the perinuclear region, not colocalizing with GM130 (Fig. 3.22-A).

The alterations observed in fixed cells strongly pointed towards changes in the dynamics of GFP-MPR. Thus the formation of MPR tubules at the Golgi was examined by live cell imaging. GFP-MPR tubules emerged at the edges of the perinuclear cluster reflecting the Golgi, further elongated and eventually detached. Tubular structures were also detectable in the cell periphery. Some of these originated from the Golgi derived tubules, and are further referred to as tubular endosomal structures. The number of tubules forming at the TGN within 2 min as well as the maximal length of individual tubules has been quantified.
Cells treated with control siRNA displayed on average 4 tubules per cell, which is in accordance with published data (Anitei et al., 2010; Waguri et al., 2003), while 16% of cells did not display any tubules under this conditions (Fig. 3.23-A). In case of PIP5KIAlpha down regulation, no Golgi-derived tubules at all could be detected in 68% of cells. Therefore, the average number of tubules per cells was reduced by a factor of six (Fig. 3.23-C). The fragmented Golgi may partially account for this strong reduction of tubule formation, although cells with drastically perturbed Golgi structure were excluded from analysis. It is worth mentioning, that no tubular endosomal structures in the cell periphery could be observed (Fig. 3.23-B). In the few cells, still displaying tubules, possibly the knock-down efficiency not sufficient. Accordingly, the mean length of Golgi derived tubules did not change significantly upon down regulation (Fig. 3.23-C).

Cells treated with siRNAs targeting PIP5KIBeta displayed a reduced number of emerging tubules, compared to the control as well. However, the difference was not as striking and tubular endosomal structures were still present. That interference with PIP5KIBeta had a less pronounced effect on GFP-MPR dynamics, could be explained by its lower expression in HeLa cells and the fact that PIP5KIAlpha activity could largely compensate the loss of its isozyme, as shown for PIP5KIBeta knock out mice (Volpicelli-Daley et al., 2010). On the other hand, double knock-down of both kinases generated the same phenotype as PIP5KIAlpha silencing alone (data not shown). This suggests that in HeLa cells predominantly the activity of PIP5KIAlpha is involved in GFP-MPR dynamics.
Fig. 3.23. Effect of PIP5KI down regulation on GFP-MPR dynamics. HeLa cells stably expressing GFP-MPR were treated with indicated siRNAs. (A & B): after 72 h live cell imaging was performed for 2 min at 500 ms per frame. Representative montage of 20 s intervals are depicted. Bars = 8 μm. (C): Quantification of Golgi derived tubules per cell and of mean length of observed tubules. (D): after 72 h of treatment with siRNAs the cells were incubated with anti-GFP antibody for 15 min at 4°C. Subsequently cells were directly fixed or allowed to uptake the bound antibody for 15 min at 37°C. Bars = 20 μm. (E): Cells treated with for 72 h with indicated siRNAs were stained for AP1 with specific antibody. Bars = 10 μm.
The fact that the overall tubule formation was blocked, points towards a role of PI(4,5)P₂ in the early steps of GFP-MPR carrier formation. These steps are coat recruitment, followed by actin driven initial membrane deformation and membrane tubule stabilization (Anitei et al., 2011). The elongation of the nascent tubule to a several micrometer long carrier is a subsequent step, possibly requiring different protein machineries. If PI(4,5)P₂ was required for tubule elongation, shorter but not necessarily less numerous tubules would have been expected.

To test whether the transport of GFP-MPR was completely blocked by interference with PIP5KIAlpha, an antibody uptake assay was performed. In down regulated cells the luminal GFP-moiety of GFP-MPR could be detected on the cell surface, when intact cells were allowed to bind anti-GFP antibody. Probably GFP-MPR followed an alternative secretion pathway, not dependent on long tubular carriers. Its endocytosis was not blocked either, as revealed by allowing cells to uptake bound antibody for 15 min. The GFP antibody-signal shifted from the cell periphery towards the cell center, as it was the case in siNon2 treated cells (Fig. 3.23-D). Yet in control cells the GFP-antibody signal was stronger, which was likely due to the higher overall GFP-MPR signal in comparison to the PIP5KIAlpha knock-down. Endocytic uptake of fluorescently-labeled transferrin was not noticeable influenced either (data not shown). Also interference with coat recruitment was not evident, since colocalization of AP1 with GFP-MPR in the TGN region was not drastically perturbed by interference with PIP5KIAlpha. However the AP1 signal in the perinuclear region appeared more scattered (Fig. 3.23-E). The reduction of GFP-MPR signal, which could be reversed by Chloroquine, and the absence of tubular endosomal GFP-MPR structures, indicates that the retrograde transport was affected at some stage as well.
We showed that interfering with PI(4,5)P$_2$ synthesis by down regulating PIP5KIAlpha and – to a less extent – PIP5KIBeta affected the distribution and dynamics of AP1 dependent cargo. Knockdown of the alpha isozyme drastically reduced formation of tubular, endosomal destined GFP-MPR carriers at the Golgi. Altered transport processes lead to a depletion of the GFP-MPR signal possibly due to lysosomal degradation, as well as to the fragmentation of the overall Golgi complex. This highlights the importance of confined and regulated PI(4,5)P$_2$ production at the level of the TGN, though the molecular link between this lipid modification and transport carrier formation and stabilization remains ambiguous.
3.4.3 The BAR domain proteins Arfaptin1 and Arfaptin2 are involved in post-Golgi carrier formation

Arfaptin2 was detected among the proteins being enriched on liposomes, when actin assisted tubulation was promoted. Moreover Arfaptin2 has been identified in the initial screen among proteins recruited on liposomes together with AP1 dependent clathrin coats (Baust et al., 2006). Arfaptin2 presented itself as an interesting candidate for cell biology studies in the context of post-Golgi carrier formation. It was one of the first BAR domain containing proteins whose crystal structure had been resolved, displaying a prime example of the banana-shaped structure characteristic for the N-BAR fold (Tarricone et al., 2001). Like other BAR domain proteins, recombinant Arfaptin2 was shown to tubulate small liposomes (Casal et al., 2006; Peter et al., 2004). Additionally, its localization at the TGN and its proposed interaction with ARF1 suggested that Arfaptin2 is implicated in post-Golgi traffic (Man et al., 2011; Shin and Exton, 2001).

We used the GUV system to visualize Arfaptin2 on membrane tubules generated by actin polymerization. For this purpose we amplified the coding sequence of Arfaptin2, from human cDNA, cloned it into a GFP expression plasmid and generated a HEK cell line stable expressing GFP-Arfaptin2. GUVs were incubated with cytosol of these cells under conditions promoting actin polymerization. As predicted by the comparative proteomic screen, GFP-Arfaptin2 assembled on the tubular protrusion projected from GUVs by actin polymerization (Fig. 3.24). Noticeably less GFP-Arfaptin2 signal could be detected on the flat “rim” of tubulated GUVs. In contrast to clathrin, which was mainly present on the very tip (compare Fig. 3.18-C), Arfaptin2 coated the entire tubule, yet leaving small stretches of membrane uncoated.
In the comparative screen mentioned above as well as in the initial screen (Baust et al., 2006) only few peptides of Arfaptin1, the other member of the Arfaptin family of BAR domain containing proteins, were detected. This might reflect the low expression of Arfaptin1 in brain tissue (Kanoh et al., 1997), since mouse brain cytosol was the source of recruited proteins. However, in HeLa cells both Arfaptin paralogs are expressed (Man et al., 2011). Arfaptin1 and Arfaptin2 display a high degree of sequence similarity, suggesting their redundancy in function and interchangeability. For this reason, both proteins were analyzed in further cell biology studies, investigating their implications in the formation of post-Golgi carriers.
The coding sequence of Arfaptin1, amplified from human cDNA, was cloned into GFP- and mCherry- expression plasmids. Due to alternative splicing, HeLa cells express two isoforms of Arfaptin1. Both isoforms (39 kDa and 42 kDa) were cloned and the specific antibody recognized both as well. Because no differences in their localization and dynamics could be detected (data not shown), plasmids encoding the larger isoform have been employed for all further experiments.

**Fig. 3.25. Arfaptin1 & Arfaptin2 colocalize in HeLa cells.** (A): cells were transfected with plasmids encoding GFP-Arfaptin1 and stained for endogenous Arfaptin2 with specific antibodies. (B): cells were transfected with plasmids encoding GFP-Arfaptin2 and stained for endogenous Arfaptin1 with specific antibodies. (C): cells were double transfected with plasmids encoding GFP-Arfaptin2 and mCherry-Arfaptin1. Boxes depict enlarged details. Bars = 10 μm.
In order to address the possible differences between the cellular functions of the Arfaptin paralogs, the subcellular localization of both proteins has been investigated. Since the individual antibodies were not compatible for co-immunostaining, the localization of endogenous Arfaptin1 was compared with that of GFP-Arfaptin1 and vice versa (Fig. 3.25-A,B). In both cases, the two proteins co-localized in the perinuclear region, as well as on peripheral structures, which were scattered throughout the cytosol. Moreover, co-expression of GFP-Arfaptin2 and mCherry-Arfaptin1 revealed a complete overlap in the Golgi region and on the peripheral structures (Fig. 3.25-C). The latter also moved together, as observed by live cell imaging (data not shown). In all cases, Arfaptin1/2-positive tubular structures emerging from Golgi region could be observed (Fig. 3.25, boxes). In contrast to fluorescently tagged Arfaptin1, GFP-Arfaptin2 displayed a high cytosolic background. This may be due the N-terminal GFP-tag in case of Arfaptin2, since Arfaptin1 constructs harbored the tag at their C-terminus. Further microscopy studies were conducted to assess the association of Arfaptins with AP1-dependent clathrin coats and their respective cargo, the ciMPR. GFP tagged Arfaptin paralogs and mRFP tagged ciMPR displayed a strong colocalization in the Golgi area (Fig. 3.26-A). The colocalization of Arfaptin1/2 with endogenous ciMPR was verified by staining with specific antibodies (data not shown). Less signal overlap could be observed on endosomal structures. MPR tubules could occasionally be observed, partially overlapping with Arfaptin1/2 (Fig. 3.26-A, boxes). However, tubular GFP-MPR carriers are generally not well preserved in fixed cells (Waguri et al., 2003). Staining with antibody against endogenous AP1 revealed a strong colocalization with GFP-Arfaptin1 and GFP-Arfaptin2, especially on endosomal structures.
Fig. 3.26. Association of Arfaptin1 & Arfaptin2 with TGN markers. (A): HeLa cells were double transfected with mRFP-ciMPR (red) and GFP-Arfaptin1 or GFP-Arfaptin2 as indicated. (B): HeLa cells were transfected with GFP-Arfaptin1 or GFP-Arfaptin2 as indicated and stained for endogenous AP1 with specific antibodies (red). Boxes depict enlarged detail. Bars = 10 μm.
Golgi originated tubular structures labeled by GFP-tagged Arfaptins were decorated with patches of AP1 (Fig. 3.26-B, boxes). The evident colocalization of Arfaptin1 and Arfaptin2 with ciMPR and AP1 coats at the level of the TGN and Golgi-derived tubules prompted further studies utilizing live cell imaging.

The biogenesis of a post-Golgi carrier can be described as a sequence of steps, leading from a flat TGN membrane domain to a detached tubular carrier, enriched in respective cargo (Anitei and Hoflack, 2011; Luini et al., 2008). To investigate at which of those steps Arfaptins were implicated, dual color live cell imaging was performed: HeLa cells stably expressing GFP-MPR were transfected with mCherry-tagged Arfaptin1 and analyzed by TIRF- and epi-fluorescence-videomicroscopy. Cells expressing low levels of mCherry-Arfaptin1 were imaged, since high expression levels affected GFP-MPR distribution. Live cell imaging confirmed the strong colocalization in the Golgi region as also observed in fixed cells (Fig. 3.27-A, asterisk). In addition GFP-MPR and mCherry-Arfaptin1 were detected on smaller vesicular and tubular features, scattered throughout the cytosol. These highly mobile structures showed little overlap of fluorescence signal though. Overall, mCherry-Arfaptin1 displayed a stronger Golgi accumulation compared to GFP-MPR. As denoted above, only few GFP-MPR positive tubules emerged from the Golgi area within 1 - 2 min. In case of shorter tubules, MPR and arfaptin signals overlapped alongside the tubule early during tubule formation. Yet often both proteins appeared exclusively separated in distinct domains on the same tubular structure (Fig. 3.27-A, arrows). On longer GFP-MPR tubules Arfaptin1 appeared at a later time point during the process of elongation. Thus Arfaptin1 was rather detectable in stretches along the tubule or at the basis, but rarely at the tip (Fig. 3.27-B, upper panel).
Fig. 3.27. Arfaptin1 is present at sites of post-Golgi carriers fission. HeLa GFP-MPR were transfected with mCherry-Arfaptin1. Low expressing cells were observed by video microscopy. (A): a single frame of a movie illustrating colocalization at Golgi (asterisk) and on emerging tubules (arrows). Bars = 12 μm (B): montages depict representative 37 s sequences showing fission of carriers emerging from Golgi (upper right corner in picture). Note the partial segregation of both fluorescence signals along the tubule. Bars = 4 μm. (C): BSC-1 cells stably expressing GFP-AP1α were transfected with mCherry-Arfaptin1. Low expressing cells were observed by video microscopy. The montage depicts a representative 14 s sequence showing growth and fragmentation of a carrier. Note the regular decoration with AP1 coats and the persistence of individual coated vesicles after tubule fission. Bar = 4 μm.
Noticeably, fission occurred in regions where long GFP-MPR tubules displayed a local concentration of Arfaptin1 (Fig. 3.27-B, upper panel). Also, the fragmentation of the detached carrier coincided with presence of Arfaptin1 and its segregation from GFP-MPR signal (Fig. 3.27-B, lower panel). Shorter tubules tend to detach at the basis of the tubule near the TGN donor membrane, thus the localization of Arfaptin1, which is highly concentrated on the TGN, was difficult to assess. To further study the association of Arfaptin1 with coats during fission, BSC-1 cells stably expressing GFP-AP1σ were utilized. Transfection and imaging were carried out as described above. As observed in fixed cells, Arfaptin1 and AP1 colocalized at the Golgi and on mobile endosomal structures. Moreover, emerging mCherry-Arfaptin1 tubules were decorated with GFP-AP1σ in a regular fashion. The above described experiments with GFP-MPR cells emphasize that these Arfaptin1 positive tubules are enriched in ciMPR. Fission of the tubules occurred inbetween the AP1 coated stretches, leading to the formation of small vesicles coated with both Arfaptin and AP1 (Fig. 3.27-C). Therefore it could be speculated that domain segregation of AP1 coats and/or Arfaptins is important for fission of coated post-Golgi carriers.

In order to test the involvement of Arfaptin1 and Arfaptin2 in GFP-MPR tubule fission and fragmentation, respective genes were down regulated in the GFP-MPR cells by using specific siRNAs. Knock-down efficiencies were about 80% for Arfaptin1, while the protein levels of both isoforms were reduced similarly, and close to 100% for Arfaptin2, as shown by Western blot (Fig. 3.28-D,E). Interestingly, treatment with siRNAs targeting Arfaptin1 lead to about 40% increase in protein levels of Arfaptin2 and vice versa. This could represent a compensatory effect and probably indicated the functional interchangeability of Arfaptin1 and Arfaptin2.
Fig. 3.28. Effect of Arfaptin double knock-down on GFP-MPR dynamics. GFP-MPR cells were treated for 72 h with 10 nM final concentration of denoted siRNAs. Subsequently live cell imaging was performed for 2 min. (A): montages depict representative 20 s sequences of mentioned movies. (B): number of tubules per cell was quantified. (C): Occurrence of tubules representing indicated length classes was quantified. (D,E): Knock-down efficiency of individual targeting siRNAs was quantified by Western blot. (D): effect of indicated siRNAs on Arfaptin1 protein levels. (E): effect of indicated siRNAs on protein Arfaptin2 levels. (F): montages depict representative 33 s sequences focusing on peripheral tubular structures. Note the ongoing fragmentation of the tubule in case of siNon2. Bars = 5 μm.
Treatment of cells with siRNAs targeting one of the paralogs did not show any noticeable effect on GFP-MPR distribution after fixation. However, when both proteins were down regulated, GFP-MPR tubules were detected more frequently in comparison to cells treated with control siRNA. Since the integrity of membrane tubules was largely compromised by fixation methods, live cell imaging was employed to investigate the effect of Arfaptin double knock-down on MPR dynamics. The behavior of GFP-MPR structures was monitored by epi-fluorescence time-lapse video-microscopy 72 h after transfection with siRNAs. The main focus was on tubular carriers emerging from the Golgi region towards cell periphery. Indeed, the average tubule number per cell increased by 50% in the Arfaptin double knock-down compared to the control (Fig. 3.28-B). As the obtained movies indicated, these tubules were not only more frequent, but also longer (Fig. 3.28-A). The statistical analysis revealed, that in the double knock-down tubules shorter than 2.5 μm were less abundant, whereas as tubules longer than 7.5 μm were more common (Fig. 3.28-C). Moreover, in the double knock-down the frequency and length of tubular endosomal structures in the cell periphery was also increased compared to the control (Fig. 3.28-F).

These findings refutet the assumption that, presumably by stabilizing membrane tubules by their assembly, Arfaptins might be required for the formation of post-Golgi carrier. The presence of longer and more abundant GFP-MPR tubules in case of their down regulation rather suggested a role for Arfaptins in constriction and fission of tubules, probably by acting as docking platforms for key components required for this process. Thus Arfaptins may be implicated in the termination of post-Golgi carrier formation as well as in fragmentation of detached tubular carriers.
In conclusion, using the GUV system we could demonstrate that Arfaptin2, a protein enriched on tubulated liposomes, preferentially assembles on tubular membranes. Cell biology studies of Arfaptin2 and its closely related paralog Arfaptin1 showed that these N-BAR-domain containing proteins strictly colocalize and may be redundant in function. Arfaptin1 and Arfaptin2 are present on emerging AP1-coated post-Golgi tubules. The enrichment of Arfaptins on stretches along those tubular carriers may coincide with a phase separation process, eventually leading to the breakage of the tubule at this site. Longer GFP-MPR tubules were detected in case of Arfaptin double knock-down while tubules were less abundant in cells highly over expressing Arfaptin1 or Arfaptin2. By assembly of their N-BAR domains, Arfaptins may sense or induce stretches of high curvature on nascent tubular carriers (Peter et al., 2004). It can be hypothesized, that at these sites Arfaptins may successively recruit factors that permit membrane fission, as it has been shown for N-BAR proteins during release of endocytic vesicles (Chang-Ileto et al., 2011; Simpson et al., 1999).

We tried to enrich Arfaptin1/2 interacting proteins by immuno-precipitation with specific antibodies as well as by pull-down experiments using recombinant proteins. Yet the subsequent mass spectrometry-based analysis did not show conclusive results, since even known interactors like ARL1 and RAC1 were not enriched compared to the respective controls (data not shown). Thus the molecular link between Arfaptin1/2 and the process of membrane fission remains elusive and a matter of future research.
4. DISCUSSION

In this work we employed artificial membrane systems to visualize coat assembly as well as coat-associated membrane deformation. We further used artificial membranes to identify proteins involved in membrane remodeling and subsequently illustrated their function in vivo. Thereby we characterized new molecules and mechanisms controlling membrane deformation during transport carrier biogenesis at the TGN.

4.1 Visualization of coats on artificial membranes

Large-scale artificial membrane systems, GUVs in particular, allow the visualization and manipulation of complex systems which were difficult to illustrate otherwise. Thus they have been successfully applied to shed light on the function of coats and other membrane interacting proteins: purified fluorescently labeled COPI subunits assembled on GUVs in presence of recombinant ARF1 and covered large domains on the GUV surface, analogous to clathrin in our study (Manneville et al., 2008). These unusually large COPI assemblies probably result from lacking membrane organization, an aspect being discussed below. Further the function of the endosomal sorting complex required for transport (ESCRT) could be nicely illustrated using GUVs (Wollert and Hurley, 2010; Wollert et al., 2009). Four different fluorescently-labeled ESCRT subunits selectively promoted cargo clustering, membrane invagination and membrane constrictions thus recapitulating the sequential steps of MVB formation in vitro.
The membrane curvature dependent localization and activity of fluorescently labeled ARFGEF1 (Ambroggio et al., 2010) and dynamin (Roux et al., 2010) were illustrated on tubules mechanically pulled from GUVs. The membrane constricting activity of dynamin was illustrated on fluid supported bilayers with excess membrane reservoir (Pucadyil and Schmid, 2008). Lee and colleagues recapitulated the generation of filopodia-like structures on supported bilayers. Similar to the study at hand, they supplemented cytosolic extracts with individual fluorescently labeled components to study their localization and dynamic behavior during the filopodia formation (Lee et al., 2010). A sophisticated set-up employing free standing planar bilayers allowed the visualization of cargo recruitment into forming COPII vesicles as well as subsequent vesicle release (Tabata et al., 2009). In conclusion, our approach joins a growing number of studies exploiting the benefit of large-scale artificial membrane systems: the dynamic behavior of individual proteins can be followed by fluorescent detection at near physiological conditions.

Reconstituted clathrin coats have been visualized using various in vitro set-ups. Many of these approaches utilized clathrin and adaptors extracted from purified coated vesicles. Assembly of triskelions into cage structures in the absence of membranes has been shown in numerous studies (Engqvist-Goldstein et al., 2001; Ungewickell and Ungewickell, 1991). By scanning electron microscopy, the McMahon group depicted clathrin coats on flat PI(4,5)P₂-containing lipid monolayers. In presence of recombinant AP180, purified triskelions assembled into discrete flat lattices of about 70 nm diameter (Ford et al., 2001). After recruitment from a cytosolic pool, clathrin profiles on artificial liposomes could be visualized in several studies (Baust et al., 2006; Takei et al., 1998; Zhu et al., 1999).
Yet all these approaches relied on fixation, staining and subsequent visualization via electron microscopy. The work at hand is the first study visualizing clathrin coats on artificial bilayers under near physiological conditions. We depicted clathrin coats specifically recruited from a cytosolic pool, utilizing two different artificial membrane systems: supported lipid bilayers and GUVs. Clathrin coats were visualized indirectly by detecting the fluorescence signal of GFP-tagged clathrin light chain (GFP-CLC). Fluorescently-tagged CLC has been extensively used in prior studies and is an accepted tool to determine localization and dynamics of clathrin coats in living cells (Perrais and Merrifield, 2005), although a recent study suggests an inhibiting effect of the ectopic expression of CLC on clathrin dynamics (Doyon et al., 2011). On supported bilayers, clathrin patches with diameters of 0.5 to 1 \( \mu \text{m} \) and a height of about 20 nm above the bilayer could be observed. On the surface of GUVs, clathrin was detectable in much larger flat domains of variable shape. In both cases it remained unclear, whether these structures represent continuous clathrin lattices or clusters of smaller structures. The largest clathrin lattices observed in cells, representing clathrin plaques on the substrate adherent plasma membrane of fibroblasts, exhibit a diameter 0.3 to 1.0 \( \mu \text{m} \) (Heuser, 1980; Heuser, 1989). AP1/GGA dependent clathrin coats detectable at the TGN are even smaller (Polishchuk et al., 2006; Puertollano et al., 2003). In general, biological membranes exhibit a complex lateral organization mediated by transmembrane proteins, an underlying membrane-cytoskeleton, local differences in curvature and lipid-induced microdomains (Charrin et al., 2009; Lenne et al., 2006; Mukherjee and Maxfield, 2000). The artificial membranes utilized by us, on the other hand, were uniformly flat and did neither contain transmembrane proteins nor a scaffolding cytoskeleton. Therefore the lipid coupled AP1 sorting peptide was probably able to diffuse freely within the bilayer.
This lack of spatial organization may have lead to the assembly of unusual big clathrin lattices or to a large-scale clustering of coats respectively. When actin assembly was promoted, smaller clathrin structures could be detected on the GUV surface. This may reflect the organizing effect of actin assembly on coat formation, since a role for F-actin in the spatial organization and lateral movement of clathrin-coated endocytic sites has been discussed (Kaksonen et al., 2006). Another factor possibly promoting the increased size of coats was their stabilization by the non-hydrolyzable nucleotide GTPγS or the constitutive active ARF1Q71L, whereas in cells growth and disassembly of clathrin coats is tightly regulated (Traub, 2011). In case of the supported bilayer electrostatic interactions of membrane lipids with the charged mica surface possibly constrained clathrin coat size in comparison to GUVs.

In contrast to AP1, the nature of AP3 coats remains elusive. Immuno-gold labeling in fixed cells suggests the coexistence of clathrin-interacting and clathrin-independent AP3 coats on endosomes and the TGN (Dell'Angelica et al., 1998; Peden et al., 2002). However, so far there is no study in living cells, elucidating the dynamic association of AP3 coats with clathrin. In vitro experiments are inconsistent as well: Purified AP3 complexes co-assemble with purified clathrin on isolated Golgi membranes and liposomes (Drake et al., 2000). Yet studies in our lab show that, in comparison to AP1, AP3 specifically recruited from cytosol onto liposomes is accompanied by minor clathrin recruitment (Baust et al., 2008; Baust et al., 2006). In the work at hand, we visualize selective AP3 assembly on the GUV surface. Using GUVs modified with sorting motifs specific for AP3 and cytosol of cells expressing GFP-AP3σA, we could detect amorphous patches, smaller than 1 μm, that aggregated to larger clusters on a subset of GUVs. The membrane bound GFP-AP3σA signal was comparably weak.
In case of GFP-AP1 specifically assembled on GUVs, several micrometer-sized domains with intensive fluorescence were detected in contrast. The difference in morphology and signal intensity may be due to concentration and immobilization of membrane bound AP1 by clathrin scaffolds. Hence we assume that the detected GFP-AP3σA features were devoid of clathrin, since neither GFP-CLC nor tomato-AP1σ were detectable on respective GUVs. It can be speculated, whether the function of clathrin-independent coats (AP3, AP4 and presumably also AP5) relies on scaffolding supra-molecular assemblies, analogous to clathrin triskelions. While a recent study proposes alternative molecules (Hirst et al., 2012), such scaffolds might not be essential, since studies have shown that in the absence of clathrin, AP1 and AP2 still bind to membranes and form microdomains (Motley et al., 2003; Zhao et al., 2001).

Totally reconstituted in vitro system allow studies on the precise function of individual components. In a set-up combining seven purified core components, the in vitro reconstitution of AP2-dependent clathrin coated vesicle formation was recently demonstrated (Dannhauser and Ungewickell, 2012). The complexity of the revealed protein network involved in shaping AP1 or AP3 dependent carriers hampers establishing an in vitro system of defined purified proteins. Consequently, our model system was only partially reconstituted: artificial membranes were utilized together with complex proteins mixtures. This approach may be more physiological relevant in some aspects than reconstitution using minimal components and to some extent our system can be manipulated as well: certain components (as shown for CYFIP1 and RAC1) can be down regulated in the cells from which the cytosol is obtained. The function of individual components can be targeted with specific drugs. The role of added purified proteins can be studied, as done for ARF1Q71L and ARF1-Cy5.
Still, to gain new insights on coat formation the artificial membrane system itself could be augmented: the parallel introduction of several sorting peptides specific for distinct coats, for example AP1 and AP3 or AP3 and ESCRT, would allow the visualization of coat segregation on the GUV surface. This process is difficult to observe on TGN and endosomes. The successive modification of liposomes with distinct peptides using the maleimide- and hydrazone-based coupling chemistry may accomplish this task. Further the insertion of luminal-tagged transmembrane protein cargo into GUVs could enable us to directly visualize cargo sorting and clustering. Similarly the incorporation of v-SNAREs into coats could be studied. In conclusion, our proteo-liposomes based set-up is suited to visualize and manipulate coat formation and has the potential to illustrate additional aspects of transport intermediate formation.

### 4.2 ARF1 activation is required of coat formation

The promoting effect of ARF1 activation on membrane recruitment of AP1 (Zhu et al., 1998) and AP3 (Ooi et al., 1998) has previously been shown *in vitro*. In accordance, by using recombinant ARF1Q71L, a constitutive active version of ARF1 (Zhang et al., 1994), we showed that the AP1-dependent recruitment of GFP-CLC as well as recruitment of GFP-AP3σA on respective GUVs was strongly reinforced by ARF1 activation. Interestingly when ARF1Q71L or the non-hydrolyzable nucleotide GTPγS were used, signal intensity and morphology of respective coats were comparable This illustrates, that the promoting effect of GTPγS on membrane assembly of AP1 and AP3 largely relies on the activation of ARF1.
When ATP was supplied, the presence of GTPγS was sufficient to promote the assembly of a thick shell of GFP-actin on the GUV surface. GTPγS was previously shown to stabilize the CYFIP1-containing actin nucleation complex in vitro (Innocenti et al., 2004). A recent study showed that this complex promotes ARP2/3-nucleated F-actin assembly on supported bilayers on beads, when both ARF1 and RAC1 were active (Koronakis et al., 2011). Moreover, in our recent paper we propose a model for the crosstalk of ARF1 and RAC1 in triggering local actin polymerization at the TGN (Anitei et al., 2010). Via the interaction of its effectors GIT1 and GIT2 with the RAC1-GEF beta-PIX, active ARF1 may locally activate RAC1. However in our in vitro system, ARF1Q71L recruited beta-PIX insufficiently in contrast to GTPγS. Thus in presence of ATP supply, ARF1Q71L was not sufficient to promote the massive actin polymerization on GUVs. However, although no GFP-actin assembly was detectable, the GUV membrane was visibly deformed at clathrin coated regions, possibly due to small amounts of locally confined F-actin assembly.

By adding recombinant, far-red fluorescent ARF1-Cy5 to GUV assays, the behaviour of membrane bound ARF1 can be monitored (Manneville et al., 2008). Since we used cytosol containing hydrolytic enzymes, not GTP but the non-hydrolyzable GTPγS was sufficient to mediate membrane recruitment of ARF1-Cy5. In absence of sorting motifs, it was distributed in micrometer-sized patches on the GUV surface. In presence of respective sorting peptides, GFP-AP3σA colocalized with ARF1-Cy5, whose signal and distribution was not altered. In contrast, sorting peptides promoting AP1-dependent clathrin assembly increased the membrane-bound Arf1-Cy5 signal, which appeared uniform and was therefore not enriched in GFP-CLC coated areas. The distinct behaviour of ARF1 in case of AP1 and AP3 coats may reflect the presence of distinct effectors (GEFs/GAPs) recruited together with individual coats.
4.3 Septin filaments are involved in endosomal maturation

While architecture, dynamics and functional implications of actin filaments and microtubules have been studied in great detail, the function of a further class of cytoskeleton elements of mammalian cells, the septins, remains elusive. The septin family of filament-forming, GTP binding proteins had been first characterized in fungi. In budding yeast, septin filaments are required for cytokinesis by stabilizing the mother-bud neck and directing interactors to this site (Longtine and Bi, 2003) as well as by regulating the diffusion of plasma membrane components into the daughter cell (Gladfelter et al., 2001). The universal function of septins as scaffolds and diffusion barriers may apply to mammalian cells as well. An increasing number of studies, link mammalian septins to various cell-type dependent processes like cytokinesis (Schmidt and Nichols, 2004; Surka et al., 2002), ciliogenesis (Hu et al., 2010), axon branching (Hu et al., 2012), dendrite branching (Xie et al., 2007) and spermatogenesis (Ihara et al., 2005). A possible involvement of septin filaments in vesicular traffic is discussed in several reviews (Kartmann and Roth, 2001; Spiliotis and Nelson, 2006), yet the indications are emerging slowly: in rodent neurons, septins interact with the exocyst complex, suggesting their implication in exocytosis and thereby in neurite outgrowth (Hsu et al., 1998; Vega and Hsu, 2003). The Nelson lab could show that in epithelial cells, septin2 is required for post-Golgi transport of constitutively secreted cargo and maintenance of cell polarity (Spiliotis et al., 2008). Eventually, our lab identified septins assembled together with AP3 coats on proteo-liposomes, suggesting their involvement in AP3-dependent transport processes (Baust et al., 2008). Yet so far, the spatial relationship of septin filaments and AP3 coats on liposomes as well as within living cells remains elusive.
We addressed this issue employing the GUV system. In contrast to AP1 and AP3, the recruitment of fluorescently tagged septin7 was not dependent on the presence of peptides containing cargo sorting motifs and was thus uncoupled from coat assembly. Although a affinity for certain phosphoinositides has been described for some septins (Casamayor and Snyder, 2003; Zhang et al., 1999), YFP-septin7 bound similarly to GUVs containing PI(3)P or lacking phosphoinositides. However, YFP-septin7 assembly on the GUV surface was dependent on the presence of the non-hydrolyzable nucleotide GTPγS. Albeit septins contain a conserved GTPase domain, the importance of GTP binding/hydrolysis for their polymerization is not entirely clear (Mitchison and Field, 2002). GTP binding is necessary for septin assembly during bud emergence in yeast (Versele and Thorner, 2004). Moreover, purified vertebrate septin2 requires GTP binding, but not GTP hydrolysis to assemble into filaments in vitro (Mendoza et al., 2002), which is consistent with previous in vivo results (Kinoshita et al., 1997). Thus the observed GTPγS dependency of YFP-septin7 assembly on GUVs is in line those studies. However, the YFP-septin7 labeled filaments on GUVs possibly do not solely contain septin7, since biochemical studies suggest that septin7 is constituent of higher order assemblies of heterotrimers, also containing septin2 and septin6 (Kinoshita et al., 2002) or septin9b and septin11 (Nagata et al., 2004). While no YFP-septin7 assembly was observed in the absence of GTPγS, the addition of recombinant ARF1Q71L promoted septin filament formation as well. This finding may suggest that active ARF1 recruits effectors onto the GUV surface which promote the assembly of septin7 containing filaments. However, the molecular links remain completely elusive, since so far no interaction between septins and ARF family GTPases has been described.
Although our findings indicate that assembly of septin filaments and recruitment of AP3 coats are not directly linked, co-assembly of both on LIMP2 peptide containing GUVs was observed. Intriguingly, GFP-AP3σA was exclusively detectably on surface areas devoid of mCherry-septin7 filaments. Similarly, septin filaments restricted the surface occupancy of recombinant ARF1-Cy5 on GUVs. It can be speculated that membrane interacting septin filaments act as diffusion barriers for membrane bound ARF1 as well as for the cargo-peptide bound AP3. Septin filaments might constrain the size of AP1-dependent clathrin coats on GpI cd peptide containing GUVs as well. The concept of septins as barriers limiting lateral diffusion arose from studies on the mother-bud neck in yeast (Gladfelter et al., 2001; Takizawa et al., 2000) and recent studies exemplify the importance of mammalian septins in lateral compartmentalization of membranes (Caudron and Barral, 2009). In light of our observations on GUVs, it might be tempting to assign septin7 a role as diffusion barrier on endosomes. However, in such function septins have been described exclusively at plasma membrane features so far. No indication for an endosome-associated pool of septins could be derived from live cell imaging in HeLa cells using ectopic expressed mCherry-septin7 or mCherry-septin6. In contrast, fluorescent septins display long straight filaments, resembling actin bundles as well as a meshwork at the edges of cells, resembling cortical actin. The association of these septins with actin filaments has been described earlier (Kremer et al., 2007). When septin6 or septin7 were co-expressed with GFP-AP3δ, AP3 positive endosomes seemed to be immobilized in proximity of septin filaments. GFP-AP3δ structures traveling longer distances repetitively stopped at septin structures. Therefore it can be speculated that septin6 and septin7 are part of scaffolds, linking AP3-coated endosomal structures to cytoskeleton elements, actin filaments in particular.
A similar role for intermediate filaments in AP3 dependent traffic has been emphasized (Styers et al., 2004). Ongoing cell biology studies in our lab show that in HeLa cells, RNAi-mediated interference with septin6 and septin7 reproduces the phenotype of the AP3 knock down. Depletion of those molecules reduced the release of HIV-Gag particles and attenuated the down-regulation of activated epidermal growth factor receptor (unpublished data). Both processes rely on endosomal maturation, in particular on MVB formation (Dong et al., 2005; Futter et al., 1996). Moreover, live cell imaging revealed, that down regulation of septins altered the dynamics of GFP-AP3δ positive carriers. Therefore, the septin reliant positioning of AP3-coated endosomes may contribute the proper maturation of these by aiding endosomal sorting and MVB formation.

4.4 Actin polymerization induced deformation of membranes as a mechanism for tubular carrier generation

The generation of tubular projections from GUVs has been reported in various cases. At low membrane tension, COPI and ARF1 covered GUVs displayed short tubules (Manneville et al., 2008). Formation of long and straight tubules, could be observed when GUVs were incubated with purified yeast COPII components, but also by sole activity of Sar1p (Bacia et al., 2011; Long et al., 2010). In contrast, incubation with Shiga toxin or I-BAR domain-containing proteins induced the generation of tubular invaginations in GUVs (Romer et al., 2007; Saarikangas et al., 2009). Formation of membrane tubules could also be detected utilizing substrate adherent plasma membrane patches as a model (Wu et al., 2010). The authors choose an approach similar to ours, utilizing cytosol containing fluorescently tagged proteins.
The generated tubules were decorated with clathrin, and stabilized by F-actin and BAR domain proteins, which is in line with our findings. Actin polymerization on GUVs has been exemplified in a handful of studies as well. Assembly of F-actin from a cytosolic pool onto GUVs modified with recombinant ARF1 or N-WASP generated actin shells as well as actin comets (Delatour et al., 2008; Heuvingh et al., 2007). By utilizing the binding of recombinant N-WASP to PI(4,5)P₂ containing GUVs researches showed, that a dynamic, membrane-bound actin networks alone can control the formation of macroscopic membrane domains (Liu and Fletcher, 2006). Using a similar setup, the same group reported the appearance of bundled actin filament protrusions from branched actin networks, protruding into the lumen of GUVs (Liu et al., 2008). Yet our study is the first one utilizing GUVs to link coats to actin polymerization and membrane deformation.

Our GUV study suggest that factors promoting actin polymerization bind to membranes surrounding clathrin coats. Under conditions promoting F-actin assembly, actin polymerization takes place near the GUV surface, likely in an ARP2/3 dependent manner, forming a branched actin network. The force of the actin polymerizations towards the membrane leads to tubular membrane protrusions projected from the GUV surface, displaying clathrin coats on the tip. Sustained actin polymerization eventually leads to complete tubulation and collapse of GUVs.

While the importance of actin dynamics in clathrin-mediated endocytosis is well established, the notion of its involvement in post-Golgi carrier formation is not apparent. In contrast to the plasma membrane, F-actin, actin nucleators and promoting factors are difficult to detect at the Golgi region by microscopy (Schafer et al., 1998).
However, already the first paper illustrating GFP-MPR dynamics emphasized the importance of actin polymerization in formation of tubular carriers at the TGN (Waguri et al., 2003). In addition, drug-mediated interference with actin dynamics showed its involvement in secretion of both apical- and basolateral-targeted proteins in polarized cells (Fucini et al., 2002; Lazaro-Dieguez et al., 2007) as well as in COPI-mediated retrograde transport (Valderrama et al., 2001). Distinct pools of ARP2/3-nucleated F-actin were detected on the cis-Golgi and the TGN and were linked to ARF1 activation (Dubois et al., 2005; Fucini et al., 2000; Matas et al., 2004). Most recently the implication of myosin1b in directing ARP2/3-mediated actin assembly at the TGN was described. Interference with myosin1 affected formation of ciMPR-enriched post-Golgi carriers (Almeida et al., 2011).

The first direct molecular between F-actin and the formation of ciMPR-containing carriers at the TGN was the clathrin accessory protein Huntingtin-interacting protein 1-related protein (HIP1R) (Carreno et al., 2004). HIP1R had been shown to organize F-actin during clathrin-mediated endocytosis (Engqvist-Goldstein et al., 2001). Further studies revealed, that HIP1R, unlike most accessory proteins, interacts with clathrin light chain (Wilbur et al., 2008). Thereby, HIP1R may inhibit ARP2/3-mediated actin polymerization on top of the clathrin lattice, thus restricting it to the edges of clathrin cages during endocytosis as well as at the TGN (Boulant et al., 2011; Poupon et al., 2008).

Cell biology studies in our lab using GFP-MPR expressing HeLa cells emphasized a novel link between clathrin coats and actin polymerization promoting factors, which is important for formation of coated tubular carriers at the TGN (Anitei et al., 2010).
There were several lines of evidence for a direct interaction of clathrin with the WAVE/SCAR homolog actin nucleation machinery through CYFIP1: clathrin heavy chain could be co-immunoprecipitated with CYFIP1. A recent study confirmed this molecular link (Gautier et al., 2011). Yeast-2-hybrid studies showed an interaction of the CYFIP1 paralog CYFIP2 with the terminal domain of clathrin heavy chain. Immunostaining revealed colocalization of clathrin and CYFIP1 at the TGN. Moreover, CYFIP1 was redistributed to the cytosol when clathrin was down regulated. It was further illustrated that formation of GFP-MPR carriers at the TGN was impaired in HeLa cells, in which CYFIP1 was down regulated. Consequently lysosomal enzyme delivery was delayed in this case.

In the above described paper we could corroborate our in vitro system with in vivo data. While the GUV system illustrated a possible mechanism of coated carrier formation, subsequent cell biology studies revealed the functional link between clathrin coats and actin polymerization during post-Golgi transport. Figure 4.1 depicts and summarizes the current model for the initial steps of clathrin coated carrier formation at the TGN (Anitei et al., 2010).

The stabilization and elongation of the short initial membrane tubule to a micrometer long carrier may require additional protein machineries, including myosins, microtubule motors, BAR domain proteins as well as lipid-modifying enzymes, as exemplified in case of clathrin-mediated endocytosis (McMahon and Boucrot, 2011; McNiven and Thompson, 2006). In order to identify proteins involved in late stages of carrier formation, we chose a quantitative mass spectrometry approach.
Liposomes were incubated with cytosol under conditions either promoting or preventing F-actin assembly and therefore tubule generation. We identified the proteins bound to purified liposomes and compared their abundance under both conditions. Proteins enriched on tubulated liposomes represent candidates for *in vivo* studies, addressing their role in stabilization, elongation or fragmentation of coated carriers at the TGN.

**Fig. 4.1. Involvement of actin polymerization in clathrin coated carrier formation at the TGN.** Cargo recruits AP-1 to TGN membranes. Active ARF1 stabilizes the sorting motif-based interaction of AP1 with its designated cargo. AP1-mediated clathrin polymerization accompanies cargo clustering. At the edges of the assembled coat, the terminal domains of clathrin heavy chain mediate an interaction with the actin nucleation machinery via CYFIP1. RAC1 is activated by the Rho-GEF β-PIX and permits actin nucleation. At the edges of the coat polymerization of a branched actin network is locally promoted by N-WASP, while the interaction of clathrin light chain with HIP1R inhibits actin nucleation above the clathrin lattice. The force of F-actin assembly deforms the membrane, generating a clathrin covered membrane protrusion, representing the initial stage of a tubular post-Golgi carrier.
The above described approach was straightforward, but it had two major restraints: Since many membrane interacting proteins bind actin as well, some proteins enriched under conditions promoting actin polymerization might be more abundant solely due to their interaction with F-actin. On the other hand, usage of brain cytosol as protein source for the screen provokes the identification of neuron- or glia-specific proteins. In non-polarized models used for cell biology studies, distinctive proteins with analogous function may be implicated. For example, PIP5KIBeta was identified as enriched in the screen, however its expression was not detectable in HeLa cells. On the other hand, due to its higher expression in brain tissue (Kanoh et al., 1997), Arfaptin2 but not its close paralog Arfaptin1 was identified in the screen, albeit both are expressed in HeLa cells (Man et al., 2011).

Among the manifold proteins enriched on tubulated liposomes, two protein classes especially called our attention: Several N-BAR domain containing proteins were enriched. Amphiphysin and endophilin A1 have an established functions in endocytic carrier formation the plasma membrane (Qualmann et al., 2011), yet both have less characterized paralogs, that localize to the TGN (Farsad et al., 2001; Sarret et al., 2004). In contrast, Arfaptin2 localizes to the TGN exclusively (Man et al., 2011). By interaction via its N-BAR domain, Arfaptin2 could potentially stabilize narrow membrane tubules in the context of post-Golgi traffic (Peter et al., 2004). Our in vivo results on the role of Arfaptin2 and its paralog Arfaptin1 are discussed in the following chapter. The identification of the type I phosphatidylinositol-4-phosphate 5-kinases was not surprising, since their substrate PI(4)P was abundant in the liposomes. Yet the recruitment of the three isozymes Alpha, Beta and Gamma was strongly reinforced under conditions promoting tubulation.
This finding, together with the increased abundance of PI(4,5)P$_2$-binding proteins, could indicate that tubulation of liposomes is accompanied by conversion of PI(4)P to PI(4,5)P$_2$. This prompted us to address the importance of PI(4,5)P$_2$ synthesis for ciMPR carrier formation at the Golgi in cell biology studies, which are discussed below. Several known interaction partners of phospholipase C and phospholipase D, like inositol-triphosphate 3-kinase A and PIP5KIAlpha were enriched under conditions promoting tubule formation, yet the lipases themselves were not identified in the screen. Hence it is very likely that lipid modifications accompany tubulation of liposomes, in order to accommodate the changes in membrane curvature (Kooijman et al., 2003; Roux et al., 2005). However, only a lipidomic approach, identifying modifications of liposomal lipids by mass spectrometry, may address this issue satisfactorily.

4.5 Production of phosphatidylinositol 4,5-bisphosphate by type I PIP5 kinases is required for post-Golgi carrier formation

The lipid phosphatidylinositol 4,5-bisphosphate, also referred to as PI(4,5)P$_2$, is a signaling molecule involved a puzzling multitude of cellular processes (Czech, 2000). It acts as a binding platform for various membrane interacting proteins and modulates the activity of membrane associated enzymes (McLaughlin et al., 2002; Roth, 2004). Moreover, PI(4,5)P$_2$ represents a substrate for the generation of other phosphoinositides and it can give rise to the secondary messengers DAG and inositol-1,4,5-trisphosphat (Pizarro-Cerda and Cossart, 2004).
PI(4,5)P₂ is generated by phosphorylation of PI(4)P by type I phosphatidylinositol-4-phosphate 5-kinases (PIP5KI) (van den Bout and Divecha, 2009). In human cells three PIP5KI isoforms, termed Alpha, Beta and Gamma, are expressed, whereas PIP5KIGamma exists in three tissue specific splice variants (Ishihara et al., 1998). PI(4,5)P₂ may also be generated from PI(5)P by type II PIP kinases (Rameh et al., 1997), yet the physiological significance of this pathway is poorly understood (Doughman et al., 2003a). PI(4,5)P₂ is concentrated in the cytosolic leaflet of the plasma membrane as suggested by staining with specific antibodies (Gascard et al., 1991) as well as by live cell imaging employing GFP-tagged pleckstrin-homology (PH) domains (Stauffer et al., 1998; Varnai and Balla, 1998). Moreover, many reported implications of PI(4,5)P₂, like clathrin-mediated endocytosis (Honing et al., 2005; Rohde et al., 2002), phagocytosis (Scott et al., 2005), regulated exocytosis (Aikawa and Martin, 2003) and cortical actin dynamics (Raucher et al., 2000) rely on its presence on the plasma membrane. As it is the case for lipids in general (Kuershner et al., 2005), the determination of subcellular localization of PI(4,5)P₂ is delicate. The outcome of immunostaining is highly sensitive to applied fixation and permeabilization methods (Hammond et al., 2009) and the commonly employed GFP-PH domain constructs have a bias for plasma-membrane PI(4,5)P₂ (Lemmon, 2004). Indeed, electron microscopic studies suggest PI(4,5)P₂ localization on intracellular membranes, including Golgi endosomes and endoplasmic reticulum (Watt et al., 2002), as well as within the nucleus (Mazzotti et al., 1995). The presence of PI(4,5)P₂ on tubular endosomes is described in several studies and is important for proper cargo recycling, possibly by modulating ARP2/3-dependent assembly of endosomal F-actin (Brown et al., 2001; Kanzaki et al., 2004; Rozelle et al., 2000).
At the level of the Golgi, PI(4)P is generated from PI by various phosphatidylinositol 4-kinases (PI4 kinases) and thus represents the predominant phosphoinositide (De Matteis et al., 2005). In contrast, the role of PI(4,5)P2 at this compartment remains elusive. Yet there are a number of indications linking Golgi function and PI(4,5)P2. Upon incubation with cytosol in presence of active ARF1, PI(4,5)P2 generation could be detected on purified Golgi membranes (Jones et al., 2000), where its synthesis is catalyzed by PIP5KIAIpha in a phosphatidic acid dependent manner (Siddhanta et al., 2000). Requirement of PI(4,5)P2 synthesis for the cell surface delivery of raft-enriched apical cargo was shown, yet the authors emphasize a role for PI(4,5)P2 in actin-mediated propulsion of post-Golgi carriers (Guerriero et al., 2006). The inositol polyphosphate 5-phosphatase OCRL-1 localizes to the TGN and to endosomes, where it regulates the level of PI(4,5)P2 by its conversion to PI(4)P (Zhang et al., 1998). Both, down regulation and over expression of OCRL-1 results in miss-sorting of ciMPR (Choudhury et al., 2005). On early endosomes, OCRL-1 activity decreases local PI(4,5)P2 levels, thus presumably limiting PI(4,5)P2–dependent F-actin assembly and thereby enabling the successful retrograde transport of cargoes like ciMPR (Vicinanza et al., 2011). Nonetheless, the TGN-resident pool of OCRL-1 suggests the presence and physiological relevance of PI(4,5)P2 at this compartment as well. The reported subcellular localizations of the type I phosphatidylinositol-4-phosphate 5-kinases does not contradict their activity at the Golgi: besides being concentrated at the plasma membrane, PIP5KIAIpha is present on punctuate structures within the cytosol and PIP5KIBeta is found in the perinuclear region (Doughman et al., 2003b; Honda et al., 1999).
Interestingly, the *in vitro* activity of the type I PIP5 kinases is promoted by the presence of active ARF1 and phosphatidic acid (Honda et al., 1999; Ishihara et al., 1998; Jones et al., 2000), both being enriched at the Golgi. In conclusion, it may be possible that during transport carrier formation at the TGN, a spatial confined pool of PI(4,5)P₂ is synthesized from PI(4)P in an ARF-controlled manner, while the overall level of PI(4,5)P₂ in Golgi membranes is controlled by TGN-resident phosphatases. Last-mentioned task may be fulfilled by OCRL-1 and by Inositol polyphosphate 5-phosphatase J, an enzyme which has been identified in our comparative screen.

In this study we show that interference with local synthesis of PI(4,5)P₂ alters the dynamics of GFP-tagged ciMPR in HeLa cells. SiRNA-mediated down regulation of PIP5KIAlpha resulted in impaired formation of anterograde GFP-MPR tubules at the TGN, as shown by live cell imaging. The changed GFP-MPR dynamics resulted in a fragmented Golgi morphology, the absence of tubular GFP-MPR positive endosomes as well as in an overall reduced GFP-MPR signal, likely due to lysosomal degradation of miss-sorted GFP-MPR. However the PIP5KIAlpha down regulation did not perturb coat assembly, since colocalization between AP1 and GFP-MPR in the TGN region was not compromised. An effect on the retrieval of GFP-MPR from endosomes can not be ruled out. Yet the phenotype is not consistent with the accumulation of ciMPR in endosomes observed by directly interfering with retrograde transport by down regulation of OCRL-1 (Choudhury et al., 2005) or retromer components (Wassmer et al., 2009). Treatment with siRNAs specific for PIP5KIBeta resulted in a reduced number of Golgi derived GFP-MPR tubules and a altered Golgi morphology as well. However, no expression of PIP5KIBeta could be detected in HeLa cells.
The inconsistent reciprocal nomenclature for PIP5KI isoforms Alpha and Beta in the sequence databases for human and mouse complicates deriving PIP5KIBeta expression data from literature (Roth, 2004). It would be interesting to see, whether over expression of type I PIP5 kinases promotes GFP-MPR tubule formation. By utilizing a GFP-PH domain construct, Doughman and colleagues observed PI(4,5)P2-rich tubules in the perinuclear region upon PIP5KIAlpha over expression (Doughman et al., 2003b). Yet, they interpreted those structures as tubulated recycling endosomes.

As the cellular functions of PI(4,5)P2 are so multifarious, the role of PIP5KI activity in GFP-MPR carrier formation is difficult to interpret. Changing the levels of PI(4,5)P2 on TGN membranes may result in miscellaneous and superimposed effects, direct and indirect ones. Over expression of PIP5KIAlpha increased the ARP2/3-mediated F-actin assembly on endosomes in an N-WASP- and RAC1-dependent manner (Kanzaki et al., 2004; Rozelle et al., 2000; Shibasaki et al., 1997). Likewise at sites of carrier formation at the TGN, ARP2/3-mediated actin polymerization might be sustained by the presence of PI(4,5)P2. We could previously show that RAC1 dependent actin dynamics promotes force, required for initiation of GFP-MPR carriers (Anitei et al., 2010). Similar to the PIP5KIAlpha knock-down, interference with the actin nucleation machinery resulted in impaired GFP-MPR tubule formation and a scattering of the TGN.

Alternatively, the presence of PI(4,5)P2 on nascent tubules may be required for recruitment of membrane curvature stabilizing proteins. Indeed in COS-7 cells, over expression of GFP-tagged version of all three PIP5K isozymes results in accumulation of endosome-derived membrane tubules, resulting from reinforced assembly of the BAR domain protein ACAP1 (Shinozaki-Narikawa et al., 2006).
Among the proteins, enriched under conditions promoting tubulation in the comparative proteomic screen, we identified several PI(4,5)P\(_2\) interacting proteins, involved in membrane curvature generation: epsin1, amphiphysin and endophilinA1, which have no published function at the Golgi, as well as dynamin-1, which was shown to be required for transport carrier formation at the TGN (Jones et al., 1998). Although dynamin has affinity for PI(4,5)P\(_2\) (Achiriloaie et al., 1999), its recruitment to the TGN may not solely dependent on PI(4,5)P\(_2\), but relies on a mosaic of factors (Cao et al., 2005).

PI(4,5)P\(_2\) synthesis could affect coated carrier formation at the TGN in a more indirect manner as well. Since the presence of PI(4,5)P\(_2\) increases the activity of phospholipase D (Liscovitch et al., 1994), it may modulate levels of phosphatidic acid required for carrier formation at TGN (Siddhanta and Shields, 1998) and cis-Golgi (Stamnes et al., 1998). Alternatively, PI(4,5)P\(_2\) may be converted by phospholipase C to DAG which is required to stabilize protein kinase C\(\eta\) and subsequently recruit protein kinase D (PKD) to the TGN membrane (Ghanekar and Lowe, 2005; Malhotra and Campelo, 2011). PKD interacts with type I PIP5 kinases, replenishing PI(4,5)P\(_2\) at this site thus reinforcing its own recruitment (Nishikawa et al., 1998). PKD signaling is essential for carrier formation in constitutive secretion (Bossard et al., 2007), but an analogous mechanism may be relevant for lysosomal destined carriers as well.

Figure 4.2 summarizes the discussed hypothetical roles of PI(4,5)P\(_2\) in formation of coated post-Golgi carriers.
Fig. 4.2. Hypothetical functions for PI(4,5)P₂ in formation and stabilization of clathrin coated carriers at the TGN. At TGN membranes, PI is converted to PI(4)P by PI4 kinases. Our data suggests, that during the formation of coated tubular carriers, type I PIP5 kinases locally convert P(4)P to PI(4,5)P₂. The presence of this lipid may sustain tubule formation by promoting RAC1-dependent F-actin assembly towards the membrane. Moreover, PI(4,5)P₂ may stabilize the nascent tubular carrier by allowing recruitment of PI(4,5)P₂ interacting proteins. Tubule formation might be promoted by lipid modifications. At the rim of the tubule, negative membrane curvature may be reinforced by conversion of PI(4,5)P₂ to DAG by phospholipase C, as well as the conversion of PC to PA by phospholipase D. PA may locally promote the activity of type I PIP5 kinases at this sites, while the activity of inositol polyphosphate 5'-phosphatases prevent the accumulation of PI(4,5)P₂ at the TGN.

4.6 Arfaptin1/2 regulate post-Golgi carrier formation by aiding fission

BAR domain proteins (BDPs) are referred to as membrane curvature sensors (Galic et al., 2012; Madsen et al., 2010) and are have been associated with tubular membrane structures on endosomes, Golgi and plasma membrane (Carlton et al., 2004; Shinozaki-Narikawa et al., 2006; Spitzenberger et al., 2003; Wu et al., 2010).
Several BDPs were enriched on tubulated liposomes in our comparative proteomic screen, yet only Arfaptin2 (Peter et al., 2004) as well as its close paralog Arfaptin1 (Kanoh et al., 1997) have been linked to Golgi membranes in earlier studies. The interactions of Arfaptin2 (Shin and Exton, 2001; Shin and Exton, 2005; Tarricone et al., 2001) and Arfaptin1 (Ho et al., 2003; Williger et al., 1999) with ARF1 has been elaborated extensively by *in vitro* studies. However recent cell biology studies challenge this notion: in HeLa cells, membrane interaction of Arfaptin2 as well as Arfaptin1 does not depend on ARF1, but on ARL1 (Man et al., 2011; Nakamura et al., 2012). The study by Man et al. illustrates the formation of Arfaptin1/2 positive tubular carriers at the TGN, but fails to identify the nature of these transport intermediates. An initial study suggests a function of Arfaptin1 in the early secretory pathway, since it inhibited ARF1-mediated activation of phospholipase D *in vitro* and its over expression in fibroblasts attenuated secretion (Tsai et al., 1998; Williger et al., 1999). In consideration of the recent study (Man et al., 2011), roles of Arfaptin1 and Arfaptin2 in membrane traffic need to be readdressed.

In this study we show that in HeLa cells Arfaptin1 and Arfaptin2 strictly colocalize with each other. Both are concentrated at the TGN, colocalizing substantially with endogenous ciMPR as well as with ectopic expressed GFP-MPR. Arfaptin1/2 are also present on tubular-vesicular structures within the cytosol, partially colocalizing with GFP-MPR and AP1. Live cell imaging revealed the presence of Arfaptin1 on tubular GFP-MPR carriers emerging from the TGN. Arfaptin1 appeared separated from GFP-MPR in distinct domains along the tubule. Fission occurred frequently at interfaces between domain. The Arfaptin1 positive tubules were decorated with AP1 coats, identifying them as anterograde, endosome-destined carriers (Waguri et al., 2003).
The double knock-down of Arfaptin1/2 increased the frequency and length of GFP-MPR post-Golgi carriers and GFP-MPR-positive tubular endosomal structures. This suggests a role of Arfaptin1/2 in the release tubular transport intermediates from the TGN and in their further fragmentation, likely by promoting membrane fission.

Since over expression of various BDPs induces the formation of membrane tubules (Frost et al., 2008), their involvement in tubule fission may appear counterintuitive. However in case of clathrin-mediated endocytosis, various BDPs are linked to membrane fission. Via its N-BAR domain endophilin binds to the tubular neck of nascent clathrin coated vesicles (Gallop et al., 2006) and recruits synaptojanin via its SRC homolog 3 (SH3) domain (Schuske et al., 2003). The inositol polyphosphate 5’-phosphatases synaptojanin locally converts PI(4,5)P₂, to PI(4)P and this conversion is required for successful fission (Chang-Ileto et al., 2011). The F-BAR domain containing protein FBP17 is required for endocytosis by locally promoting ARP2/3-mediated F-actin assembly (Tsujita et al., 2006). It coordinates actin polymerization toward the tubulated neck of the nascent carrier, possibly creating force necessary for membrane fission (Suetsugu, 2009). The SH3-doamin containing BDPs sorting nexin 9 (Soulet et al., 2005) and amphiphysin (Takei et al., 1998) regulate the activity of dynamin on the neck of the nascent carrier. Constriction of membrane bound dynamin is believed to be the major driving force in endocytic fission (Sever et al., 2000). However, a recent study (Boucrot et al., 2012) as well as the above described alternative mechanisms challenge the universal role of dynamin in membrane fission (Callan-Jones and Bassereau, 2012).
Yet the role of Arfaptin1/2 as binding platform for factors, promoting fission – analogous to endophilin - is difficult to interpret. In contrast to the majority of BDPs (Suetsugu, 2010), Arfaptin1/2 are small molecules lacking conserved protein/protein or protein/lipid interaction domains apart from the N-BAR domain. This might be one reason, why our pull-down-based approaches to identify novel interaction partners representing a hypothetical link to membrane fission, were not successful. By its interaction with RAC1 (Tarricone et al., 2001), Arfaptin2 may locally regulate ARP2/3-mediated F-actin assembly, promoting fission in a similar fashion as discussed for FBP17. However, recent structure biological work suggests that Arfaptin2 binding to RAC1 interferes with its membrane association (Nakamura et al., 2012). An alternative model may illustrate how N-BAR domains promote fission of membrane tubules without the need for direct protein-protein interactions (Boucrot et al., 2012). Stretches of tubular membrane are covered by BDPs, thus the accessible membrane surface is restricted. At the interfaces between coated and accessible bilayer, insertion of amphipathic helices may locally change curvature, producing line tension. Alternatively lipid-modifying enzymes may change the lipid composition at such interfaces, generating lipid phase separation. Both processes may be sufficient for spontaneous fission of the tubule (Roux et al., 2005). This model is in conjunction with the observation, that GFP-MPR tubules often fragment at the interface between Arfaptin1-covered and uncovered stretches. Alternatively, by its N-BAR domain-driven assembly on tubular membranes, Arfaptin1/2 may generate a tubule diameter, adequate for subsequent fission by dynamin, since dynamin polymerization on membrane tubules was shown to be curvature dependent (Roux et al., 2010). Figure 4.3 illustrates schematically how Arfaptin1/2 might be involved in fission of coated post-Golgi carriers.
Fig. 4.3. Involvement of Arfaptin1/2 in late stages of clathrin coated carrier formation at the TGN. Arfaptin1/2 bind to TGN membranes via interaction with active ARL1. Our findings suggest that Arfaptin1/2 are recruited on pre-existing coated carriers, which are enriched in ciMPR. Arfaptin1/2 cover stretches of the membrane tubule and thus separate ciMPR into adjacent segments. Assembly of Arfaptin1/2 may also narrow the diameter of the tubule. At the edges of the Arfaptin1/2 coated membrane stretches, fission may be promoted either by the activity of lipid-modifying enzymes, phospholipases or inositol polyphosphate 5'-phosphatases, or by allowing the assembly of dynamin. It remains unclear whether Arfaptin1/2 directly interact with these fission promoting factors. Alternatively, Arfaptin1/2 may maintain membrane curvature required for activity of those factors. Moreover, by promoting lipid phase separation along the tubule, Arfaptin1/2 may generate line tension required for fission.
5. MATERIALS AND METHODS

5.1 Reagents

All general reagents were purchased from Cart Roth (Karlsruhe, Germany), if not stated otherwise. The media kitchen of the BIOTEC supplied bacterial growth medium, agar plates and standard buffer stock solutions.

Biochemicals

GTPγS, ATP, GDP, GTP, creatine kinase, creatine phosphate as well as complete protease inhibitor were from Roche Diagnostics (Mannheim, Germany). Latrunculin B as well as RAC inhibitor NSC23766 were from Calbiochem/Merck (Darmstadt, Germany). Brefeldin A as well as water free DMSO were from Sigma-Aldrich (Hamburg, Germany).

Antibodies

Goat anti-mouse Alexa488-conjugated, goat anti-mouse Alexa546-conjugated, goat anti-mouse Alexa633-conjugated, goat anti-rabbit Alexa488-conjugated, goat anti-mouse Alexa546-conjugated and donkey anti-goat Alexa546-conjugated secondary antibodies as well as DAPI, TexasRed-conjugated phalloidin and Alexa633-conjugated phalloidin were from Molecular Probes/Life Technologies (Paisley, UK). Goat anti-mouse HRP-conjugated, goat anti-rabbit HRP-conjugated and mouse anti-goat HRP-conjugated secondary antibodies were from Dianova (Hamburg, Germany). Mouse anti-AP1-γ (100/3), mouse anti-beta-tubulin (T-4026), rabbit anti-septin7 (HPA02309) and mouse anti-Arfaptin2 (clone 2B5) primary antibodies were from Sigma-Aldrich (Hamburg, Germany). Goat anti-Arfaptin1 (I-19), mouse anti-LAMP1 (H4A3) primary antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-PIP5K1A primary antibody was from GeneTex (Irvine, California, US).
Mouse anti-EEA1, mouse anti-AP1-γ (clone 88), mouse anti-clathrin-heavy-chain, mouse anti-GM130 primary antibodies were from Transduction Lab/BD Biosciences (Heidelberg, Germany). Rabbit anti-PIR121-1/Sra1/CYFIP1 primary as well as anti-beta-PIX SH3 domain primary antibodies was from Upstate/Millipore (Schwalbach, Germany). Mouse anti-AP3-δ (SA4) primary antibody was from Developmental Studies Hybridoma Bank (Iowa City, Iowa, US). Monoclonal mouse anti-GFP primary antibody was from Roche Diagnostics (Mannheim, Germany). The production of rabbit polyclonal antibodies against AP3-σ-A/B and ciMPR have been described earlier (Le Borgne et al., 1998; Ludwig et al., 1991)

*Lipids*

Extracted phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and cholesterol as well as synthesized maleimide anchor (18:1-PE-MCC), phosphatidylinositol-3-phosphate (PI(3)P) and phosphatidylinositol-4-phosphate (PI(4)P) were from Avanti Polar Lipids Inc (Alabaster, Alabama, US). The synthesis of Di-O-hexadecyl-rac-glyceraldehyde lipid anchor has been described earlier (Bourel-Bonnet et al., 2005). Lipids were solved in chloroform (for PC, PE, PS and cholesterol) or in a 1:1 mixture of chloroform and methanol (for lipid anchors and phosphatidylinositol-phosphates) and stored at -80°C. The lipid dyes DiI-C16 and DiD-C16 were purchased from Molecular Probes/Life Technologies (Paisley, UK). Dyes were solved in methanol and stored as 1 mM stocks at -80°C.
5.2 DNA constructs & molecular biology

The mammalian expression vectors pGFP-N3, pGFP-C1 and pmCherry-C1 were supplied by Takara Bio Europe/Clontech (Saint-Germain-en-Laye, France). pGFP-beta-actin and pmRFP-beta-actin were supplied from BD Biosciences (Heidelberg, Germany). pGFP-CYFIP1 was a gift from Annette Schenck (Nijmegen Centre for Molecular Life Sciences, Nijmegen). pYFP-septin7 and pGFP-septin6 have been designed by Thomas Wassmer (Aston University, Birmingham). Generation of pGFP-ciMPR, pmRFP-ciMPR and pGFP-AP3δ have been described earlier (Waguri et al., 2003).

All cloning procedures as well as plasmid preparation was carried out using electro-competent *E.coli DH5α*. All enzymes used for molecular biology tasks were purchased from New England Biolabs (Frankfurt am Main, Germany). The inserts of pYFP-septin7 and pGFP-septin6 were subcloned into pmCherry-C1 using BamH1/XhoI. The coding sequence of Arfaptin1 was amplified from HEK cell cDNA using primers harboring BamH1 & XhoI sites. Two splice variants, Arfaptin1A encoding the 373 aa isoform and Arfaptin1B encoding the 341 aa isoform, were cloned into pGFP-C1 as well as into pmCherry-C1 using BamH1/XhoI. The coding sequence of Arfaptin2 was amplified from HEK cell cDNA using primers harboring Kpn1 & NheI sites and cloned into pGFP-N3 using Kpn1/NheI. The bacterial expression vector pREF-duet-1 encoding both human ARF1 and human N-myristoyl-transferase was a gift from Volker Haucke (Freie Universität Berlin, Berlin). To generate a plasmid for production of dominant active myristoylated ARF1Q71L, the codon encoding glutamic acid at position 71 was changed from “CAG” to “TTG” by site directed mutagenesis.
To generate a plasmid for production of myristoylated ARF1-cys, harboring a C-terminal cysteine, the three nucleotides “TGC” were inserted between the codon encoding the C-terminal lysine “AAG” and the stop codon “TGA” by site directed mutagenesis. Success of cloning and mutagenesis was verified by sequencing (a service of the MPI-CBG, Dresden).

5.3 Gel electrophoresis and Western blot

Gels for SDS-PAGE were cast, run and subsequently transferred using equipment of the Mini-PROTEAN system purchased from Bio-Rad Laboratories GmbH (München, Germany). In all cases a discontinuous Tris/glycine system was employed. Depending on demands, gels with acrylamid concentrations varying from 7 - 15 % were used. Before loading on gel all protein samples were mixed with respective volumes of 4 x SDS sample buffer (200 mM Tris pH 6.8, 8% (w/v) SDS, 0.04% (w/v) bromphenole blue, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol) and were incubated for 5 min at 95°C. ColorPlus prestained protein marker, broad range (from New England Biolabs, Frankfurt am Main, Germany) was applied as standard on every gel. The transfer onto nitrocellulose membrane with pore size of 0.2 μm (from Peqlab, Erlangen, Germany) was carried out in presence of 20% (v/v) methanol and 0.01% (w/v) SDS using tank-blot procedure. Membranes were blocked with 3% (w/v) milk powder in PBS. Incubation with primary antibody in 3% (w/v) milk powder in PBS according to indicated dilution was carried out over night at 4°C. Incubation with 1:5000 HRP-conjugated secondary antibody in 3% (w/v) milk powder in PBS was done for 30 min at room temperature. Blots were developed using enhanced chemiluminescence reagent Luminata forte (from Millipore, Schwalbach, Germany) in the LAS-3000 CCD-Imaging System (from Fujifilm, Tokyo, Japan).
When no Western blotting was desired, gels were stained with Coomassie G-250 in 10% (v/v) acetic acid, 40% (v/v) methanol followed by destaining with 10% (v/v) acetic acid. Pictures of the destained gels were acquired using the LAS-3000 system.

**5.4 Preparation of cytosolic extracts**

Cytosolic extracts of mouse and pig brain tissue have been prepared as follows: All steps were carried out at 4ºC. Fresh brain tissue was supplied from local slaughterhouse in case of pig, or were extracted from sacrificed mice (provided from Biomedical Service Unit of the MPI-CBG, Dresden). After cerebellum and meninges were removed, brain tissue was cut in small cubes and was homogenized using a properly sized dounce tissue grinder (from Wheaton Science Products, Millville, New Jersey, US) in about 3 volumes of homogenization buffer (25 mM HEPES pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, supplemented with twofold excess of complete protease inhibitor). The homogenate was centrifuged at 10,000 g for 30 min, supernatant was recovered and centrifuged at 150,000 g for 1 h to pellet insoluble components. After ultra centrifugation, supernatant was recovered and snap frozen in liquid Nitrogen and stored at -80ºC. In case of cultured cells cytosolic extracts were produced as follows: All steps were carried out at 4ºC. Plates with confluent grown cells were washed with cold PBS and cells were gently detached using a cell lifter. Cells were pelleted by 5 min centrifugation at 450 g and resuspended in about 5 volumes of homogenization buffer. Cells were homogenized with a syringe by repeatedly passing through a 22 Gauge needle followed by repeatedly passing through a 27 Gauge needle. The homogenate was centrifuged at 10,000 g for 15 min, the supernatant was recovered and centrifuged at 150,000 g for 45 min to pellet insoluble components.
After ultra centrifugation the supernatant was recovered and snap frozen in liquid Nitrogen and stored at -80°C. Concentration of cytosol has been 10 - 20 mg/ml for brain tissue derived extracts and 4 - 5 mg/ml for cell derived extracts, as determined using microplate based colorimetric DC protein assay (from Bio-Rad Laboratories GmbH, München, Germany).

5.5 Recombinant protein production

The FPLC system, the Typhoon image reader, all chromatography columns as well as the fluorescent dye were purchased from GE Healthcare (München, Germany). Recombinant myristoylated ARF1-cys and ARF1Q71L were produced as follows: single colonies of E.coli BL21 DE3, transformed with respective bacterial expression plasmids, were used to inoculate 200 ml 2YT medium containing 30 µg/ml kanamycin and this starter culture was grown over night at 37°C. On the following day, each 30 ml of starter culture each were used to inoculate six times 1000 ml 2YT. This production culture was incubated at 37°C until OD₆₀₀nm reached a value of 0.6. At this point 100 x myristate solution (6 mM sodium myristate incubated with 3 % (w/v) fatty acid free BSA in water) was added to the culture. After the temperature was reduced to 27°C, culture was incubated for 20 more min in order to feed the cells with myristate. Next the expression of genes was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.3 mM. After 5 more hours at 27°C, cells were harvested by 15 min centrifugation at 3000 g and cell pellet was resuspended in 80 ml extraction buffer (50 mM Tris/HCl pH 8.0, 1 mM MgCl₂, 1 mM DTT, 200 μM GDP, containing complete protease inhibitor). From this point on all steps were carried out at 4°C. Cells were disintegrated using EmulsiFlex-C5 (from AVESTIN, Ottawa, Canada).
Cell debris was removed by 45 min centrifugation at 100,000 g. Supernatant was recovered and diluted with extraction buffer to 200 ml final volume. Ammonium sulfate was slowly added to the extract until a saturation of 35% has been reached. Precipitated proteins were pelleted by 20 min centrifugation at 8000 g. The pellet was solved in 7.5 ml resuspension buffer (20 mM Tris/HCl pH 8.0, 1 mM MgCl$_2$, 1 mM DTT, 20 μM GDP, complete protease inhibitor) and then desalted using PD-10 columns equilibrated with loading buffer (10 mM Tris/HCl pH 8.0, 1 mM MgCl$_2$, 1 mM DTT). Proteins that had aggregated during this process were removed by subsequent centrifugation at 100,000 g for 20 min. The supernatant was applied at 0.75 ml/min on a 20 ml HiTRAP DEAE FF column equilibrated with loading buffer. Ion exchange chromatography was carried out using ÄKTA Explorer 10s FPLC system equipped with 50 ml SuperLoop and Frac950 fraction collector. After washing with 3 column volumes of loading buffer, proteins were eluted from the DEAE column with a linear gradient from 0 to 100% (v/v) elution buffer (1M KCl, 10 mM Tris/HCl pH 8.0, 1 mM MgCl$_2$, 1 mM DTT) in 5 column volumes. Fractions of 0.75 ml were collected and analyzed on SDS-PAGE. Fractions containing ARF1-cys or ARF1Q71L respectively, eluting from the column at about 15% (v/v) elution buffer, were pooled, snap frozen in liquid nitrogen and stored at -80°C. Concentrations were 1.5 mg/ml for ARF1-cys and 0.75 mg/ml for ARF1Q71L, as determined using colorimetric protein assay. According to the shift in gel migration, visualized by Coomassie staining, about 80% of the purified ARF1 was myristoylated. ARF1-cys was subsequently fluorescence-labeled as follows: 0.5 ml ARF1-cys was dialyzed against 500 ml labeling buffer (20 mM HEPES ph7.2, 100 mM KCl, 1 mM MgCl$_2$) for 4 h using a Spectra/Por membrane with a molecular weight cutoff of 12,000-14,000 (from Spectrum Laboratories, Rancho Dominguez, California, US).
After dialysis TCEP was added to a final concentration of 3.6 mM. After 30 min incubation on ice, Cy5-maleimide mono-reactive dye in dimethylformamide was added to 360 µM final concentration and incubated for 2 more hours on ice. Subsequently the unbound dye was removed by gelfiltration at room temperature using a PD-10 column, equilibrated with labeling buffer. Elution fractions of 200 µl were collected and analyzed by SDS-PAGE with subsequent fluorescence analysis using the Typhoon 9410 imager, equipped with filters proper for Cy5 detection. Afterwards the gel was stained with Coomassie to estimate labeling efficiency. Fractions containing labeled ARF1 were snap frozen in liquid nitrogen and stored at -80°C.

5.6 Liposomes preparation and floatation assay

Proteo-liposomes have been produced using two different approaches: Either using reactive α-hydrazino acetyl peptides and an aldehyde lipid anchor, or alternatively using a reactive maleimide lipid anchor and peptides containing N-terminal cysteine.

The first method was applied for producing liposomes, used in the majority of supported bilayer and GUV experiments addressing clathrin and AP1. Details of the method as well as the synthesis of the GpI cd peptides has been described earlier (Baust et al., 2006; Bourel-Bonnet et al., 2005): PC, PE, PS, Cholesterol and aldehyde lipid anchor were mixed in a molar ratio of 41.5% : 31.5% : 10.5% : 10.5% : 5% in a low binding 1.5 ml tube (from Biozym, Wien, Austria). DiI-C16 or DiD-C16 were added at a molar ratio of 0.02% and when indicated phosphatidylinositol-phosphates were added at a molar ratio of 1%.
The solvents were evaporated to under a steam of nitrogen, allowing formation of a thin lipid film in the tube, followed by 15 min of drying in the vacuum Concentrator 503 (from Eppendorf, Hamburg, Germany). Coupling buffer A (15.4 mM citric acid, 69.2 mM dibasic sodium phosphate, pH 6.4) was added and lipids were suspended by vigorous vortexing. Unilamellar liposomes were formed from the lipid emulsion by ten cycles of freezing in liquid nitrogen & thawing, followed by eleven cycles of extrusion using a LiposoFast handheld extruder, equipped with a polycarbonate membrane (pore size 400 nm, from Avestin, Ottawa, Canada). For coupling to peptides, 250 µl of liposome suspension containing about 1.3 µmol of total lipids and thus about 65 nmol of anchor were mixed with 250 µl coupling buffer A containing either 1.8 mg/ml GpI cd peptide, 1.4 mg/ml GpI cd trunc peptide or, for control experiments, 10 mM glycine. The coupling mixture was incubated for 16 h at 20 °C in the dark. Subsequently unbound peptides as well as salts were removed by desalting over Sephadex G-25 NAP-5 columns (from GE Healthcare, München, Germany) equilibrated with water. The desalted liposomes, corresponding to a final lipid concentration of about 1.3 mM, were aliquot and used immediately for floatation assays or were stored at -80°C for later use in GUV or supported bilayer experiments.

The second method was applied to produce those liposomes, used for all experiments dealing with AP3 and more recent experiments concerning AP1. Following peptides had been synthesized by GenScript USA (Piscataway, New Jersey, US):

- LAMP1 wt  \((\text{NH}_2-\text{CGRKRSHAGYTQTI-COOH})\);
- LIMP2 wt  \((\text{NH}_2-\text{CRGQGSMDEGTADERAPLIRT-COOH})\);
- LAMP1 Y/A  \((\text{NH}_2-\text{CGRKRSHAGAQTI-COOH})\);
- LIMP2 LL/AA  \((\text{NH}_2-\text{CRGQGSMDEGTADERAPAART-COOH})\).
The peptides had following production specifications: HPLC-purity > 80%, Quantity 1 – 4 mg, no modifications.

A GpI cd peptidewas synthesized by EZBiolab (Carmel, Indiana, US) and had following production specifications: HPLC-purity > 70%, Quantity 10 mg, no modifications: (NH₂-CGKRMVRVKAYRVDKSPYNQSMYYAGLPVDDFEDSES TDTEE-COOH)

PC, PE, PS, Cholesterol and maleimide lipid anchor were mixed in a molar ratio of 42.5% : 32.5% : 11% : 11% : 2% in a safe lock 1.5 ml tube (from Eppendorf, Hamburg, Germany). DiI-C16 or DiD-C16 was added at a molar ratio of 0.02% and when indicated phosphatidylinositol-phosphates were added at a molar ratio of 1%. The solvents were evaporated to under a steam of nitrogen, allowing formation of a thin lipid film in the tube, followed by 15 min of drying in the vacuum Concentrator 503. Coupling buffer B (20 mM HEPES pH 7.2, 125 mM potassium acetate, 1 mM EDTA) was added and lipids were suspended by vigorous vortexing. Since the maleimide anchor hydrolys quickly in the presence of water, the homogenization protocol has been shortened. Unilamellar liposomes were formed from the lipid emulsion by six cycles of freezing in liquid nitrogen & thawing. For coupling 150 µl liposome suspension, containing about 1 µmol of total lipids and thus about 20 nmol of anchor was supplemented with TCEP to a final concentration of 1 mM. In parallel peptides were dissolved in 150 µl of coupling buffer B containing 1mM TCEP to a final concentrations of either 2.5 mg/ml for GpI cd or 1,25 mg/ml for LAMP1 wt, LAMP1 Y/A, LIMP2 wt and LIMP2 LL/AA. Peptide solutions were mixed with liposome suspension, while for control experiments the liposome suspension was mixed with 150 µl 2 mM cysteine in coupling buffer B.
After 60 min incubation at 20°C, β-mercaptoethanol was added to a final concentration of 1 mM to saturate vacant maleimide anchor molecules. To remove unbound peptides as well as salts, liposomes were pelleted by 15 min centrifugation at 20,000 g at 20°C. The supernatant was carefully removed and liposomes were resuspended in 400 µl water by pipetting up and down. The centrifugation was repeated and the liposome pellet was finally suspended in 300 µl water. The liposomes, corresponding to a final lipid concentration of about 3 mM, were aliquot and used immediately for floatation assays or were stored at -80°C for later use in GUV experiments.

The floatation assay was carried out as follows: All steps were carried out at 4°C, if not indicated otherwise. Brain cytosolic extract was defrosted and denatured proteins were precipitated by 60 min centrifugation at 150,000 g. Cleared cytosol was diluted to a protein concentration of 10 mg/ml with recruitment buffer (25 mM HEPES pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate). 500 µl of cytosol were transferred in a 1.5 ml tube on ice and supplements were added as indicated. Finally 50 µl of respective liposome suspension was added, tubes were mixed and transferred to 37°C. After 20 min incubation tubes were put on ice. The reaction mixture was mixed with 1.1 ml cold 60% (w/v) sucrose in recruitment buffer and the mixture was transferred into a 4.5 ml ultracentrifugation tube. The mixture was then carefully over layered with 2 ml 30% (w/v) sucrose in recruitment buffer and finally with 0.6 ml of recruitment buffer. To facilitate floatation of liposomes, the tube was centrifuged for 2 h at 60,000 RPM in the free swing rotor SW-60 using an Optima LE80 ultracentrifuge (from Beckman Coulter, Krefeld, Germany). The interface between 30% sucrose and top layer, containing the floated liposomes, was recovered, was transferred in a new 4.5 ml tube and was mixed with 4 ml cold recruitment buffer.
Liposomes were finally pelleted by 30 min centrifugation at 60,000 RPM in the SW-60 rotor. Supernatant was removed and pellet was dissolved in 40 μl 1x SDS sample buffer by pipetting up and down. Equal sample volumes were applied on SDS-PAGE. To prepare samples for mass spectrometry analysis, the protocol was modified as follows: 1000 μl mouse brain cytosol were incubated with 100 μl liposomes and indicated supplements for 25 min at 37ºC. Reaction mixture was mixed with 4.5 ml 65% (w/v) sucrose in recruitment buffer and transferred in a 14 ml ultracentrifugation tube. Mixture was over layered with 5.5 ml 40% (w/v) sucrose in recruitment buffer and 2 ml recruitment buffer. Centrifugation was carried out for 12 h at 35,000 RPM in the free swing rotor SW-40 using an Optima LE80 ultracentrifuge (from Beckman Coulter, Krefeld, Germany). The interphase containing liposomes was recovered and liposomes were harvested as described above. When indicated a ATP—regeneration system was added to recruitment reaction as follows: ATP was added to final concentration of 1 mM, creatine phosphate was added to final concentration of 10 mM and creatine kinase was added to final concentration of 50 μg/ml.

### 5.7 GUV and supported bilayer preparation

Supported lipid bilayers have been prepared as follows: A freshly cleaved sheet of mica was glued onto a #1.5 microscopy slide using UV-curable glue (from DYMAX, Torrington, Connecticut, US). 50 μl of liposomes were mixed with equal volume of 2x recruitment buffer (50 mM HEPES pH 7.2, 250 mM potassium acetate, 5 mM magnesium acetate) and were incubated for 10 min at 37ºC. Liposomes were applied on prewarmed mica substrate, CaCl₂ was added to a final concentration of 3 mM and bilayer was allowed to form for 30 min at 37ºC.
Non fused liposomes were removed by rinsing the bilayer several times with prewarmed recruitment buffer. The integrity of the bilayer was checked by microscopy and the bilayer was used for recruitment experiments immediately. For recruitment experiments pig brain cytosol was spiked with 10% (v/v) of indicated cytosol from cell expressing GFP-tagged proteins. The cytosolic mixture was centrifuged at 150,000 g for 30 min. When indicated, supplements were added to the cleared cytosol and the mixture was placed directly onto the bilayer after the overlaying buffer had been removed. After 20 min incubation at 37°C, bilayer was analyzed by confocal microscopy.

GUVs were prepared from liposomes by the electro-swelling method (Bacia et al., 2004) as follows: 10–20 μl of liposomes was dried onto two Indium tin oxide-slides (Präzisions Glas & Optik, Iserlohn, Germany) by 20 min desiccation under vacuum. Separated by a rubber ring spacer, the slides were assembled together, and 600 μl 330 mM sucrose was added in between the slides. GUVs were formed by applying alternating current (10 Hz, 1.8 V) for 2 h using a Voltcraft 8202 1-channel-function generator (from Conrad Electronic, Hirschau, Germany). Subsequently, the GUV containing solution was removed and diluted in 2 volumes of recruitment buffer. GUVs were allowed to settle down for 30 min and 200 μl from the bottom of the tube were recovered as GUV suspension. For recruitment experiments pig brain cytosol was spiked with 10% (v/v) of indicated cytosol from cell expressing GFP-tagged proteins. In indicated cases undiluted cell cytosol was used. The cytosolic mixture was centrifuged at 150,000 g for 30 min. When indicated, supplements were added to the cleared cytosol and the mixture was placed into the wells of an 8-well Lab-Tek glass bottom chamber (from Nunc, Langenselbold, Germany).
Finally 20 µl GUV suspension was added to 180 µl cytosol and was mixed by pipetting. After 20 min incubation at 37°C, the GUVs in the chamber were analyzed by confocal microscopy.

5.8 Cell culture, transfection and immunostaining

All cell culture reagents were purchased from Gibco/Life Technologies (Paisley, UK) and all cell culture plates were purchased from TPP/MIDSCI (St. Louis, Missouri US), if not stated otherwise. Cells were grown at 37°C and 5 % CO₂ in DMEM + GlutaMAX (4.5 mg/ml Glucose, containing pyruvate), supplemented with penicillin/streptomycin, 2 mM L-glutamine and 10 % (v/v) Fetal Bovine Serum Superior (from Biochrome, Berlin, Germany). The American Type Culture Collection (LGC Standards GmbH, Wesel, Germany) provided HeLa and HEK-293T cell lines. BSC-1 cells stably expressing GFP-clathrin-light-chain, tomato-clathrin-light-chain, GFP-AP1-σ, GFP-AP3-σA as well as HeLa cells stably expressing tomato-AP1-σ were kind gifts from Tomas Kirchhausen (Harvard Medical School, Boston). The generation of HeLa cells stably expressing GFP-tagged cytosolic domain of ciMPR (HeLa GFP-MPR) has been described earlier (Waguri et al., 2003).

For live cell imaging 60,000 – 120,000 cells were seeded in #1.5 3.5 cm live cell imaging dishes (from MaTek Cooperation, Ashland, Massachusetts, US). At the day of imaging, cells were washed with PBS and medium lacking pH indicator dye was added. For microscopy of fixed samples, cells were seeded on 11 mm #1.5 glass coversilps in individual wells of 24-well plates. At the day of staining, cells were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature. After fixation cells were washed twice with PBS and put on ice.
Permeabilization was carried out by adding ice cold 0.1% (v/v) Triton X-100 in PBS and incubation for 5 min. Subsequently cells were washed three times with PBS and blocked for 30 min in 3% (w/v) BSA in PBS at room temperature. Primary antibody solutions were prepared in 3% (w/v) BSA in PBS, using indicated dilutions. Incubation with primary antibody was carried out for 1 hour. Afterwards cells were washed three times with PBS, incubating 5 min each. Secondary antibodies as well as DAPI were diluted 1:450 in 3% (w/v) BSA in PBS, and when indicated labeled phalloidin was diluted 1:250 in parallel. Cells were stained for 30 min. Subsequently cells were washed three times in similar fashion and mounted on microscopy slides using MOWIOL (from Calbiochem/Merck, Darmstadt, Germany).

For anti-GFP antibody uptake, GFP-MPR cells in 24-well plates were washed with cold PBS and put on ice. Per well 150 µl serum free DEMEM containing 1 µg/ml anti-GFP antibody was added. After 15 min incubation on ice, cells were washed with cold PB, cells were fixed directly or alternatively, 1 ml warm serum free DEMEM was added. Cells were fixed after 15 min incubation at 37°C. After permaebilization cells were stained with secondary antibody as described above.

Cells were transfected with mammalian expression plasmids 24 h after seeding using JetPEI reagent (from Polyplus transfection, Illkirch, France) according to supplier’s protocol. Plasmid DNA was purified from transformed *E.coli DH5α* using phenol/chloroform extraction and subsequent isopropanol precipitation.

In case of 24-well plates, each well was treated for 24 h by addition of 1 µg plasmid DNA mixed with 2 µl JetPEI in 100 µl 150 mM NaCl.
In case of live cell imaging dish, cells were treated 24 or 48 hours by addition of 1.5 μg plasmid DNA mixed with 3 μl JetPEI in 200 μl 150 mM NaCl. HEK cells stably expressing GFP-CYFIP1, GFP-beta-actin, mRFP-beta-actin, YFP-septin7, mCherry-septin7 and GFP-Arfaptin2 as well as HeLa cells stably expressing GFP-AP3-δ were generated as follows: Cells grown in on well of a 24-well plate were transfected with 1 μg of respective plasmid as described above. Two days after transfection cells were detached with trypsin and seeded into 10 cm dishes containing medium supplemented with 0.5 mg/ml (for HeLa) or 0.8 mg/ml (for HEK) geneticin. After 2-3 weeks single clone-derived colonies were picked and transferred into wells of a 24-well plate. Clones were individually analyzed for expression by fluorescence microscopy and suitable clones were cultivated further in the presence of 0.3 mg/ml geneticin.

5.9 RNA interference

All small interfering RNA molecules (siRNAs) were supplied as annealed Silencer Select Pre-Designed siRNAs from Ambion/Life Technologies (Paisley, UK) and were stored as 20 μM stock solutions in water at -20°C. Following siRNA sequences were used:

- siArfaptin1a (Entrez GeneID: 27236; 5’-GAAUUCCAGUGACUAGUAtt-3’)
- siArfaptin1b (Entrez GeneID: 27236; 5’-GGGUGUAUUGAAGCAGGAtt-3’)
- siArfaptin2a (Entrez GeneID: 23647; 5’-GGACCCAACCUCAUGAAAtt-3’)
- siArfaptin2b (Entrez GeneID: 23647; 5’-CAACUGUUAUCAGAACGAUtt-3’)
- siPip5kIalpha (Entrez GeneID: 8394; 5’-CAAGAUCGGUAAAAAUGCtt -3’)
- siPip5kIbeta (Entrez GeneID: 8395; 5’-GAUCAUGGUAUAGCCUUt -3’
siNon2 represents Silencer Select Negative Control #2 (Ambion/Life Technologies, Paisley, UK). siRNA sequences used to target CYFIP1 and RAC1 have been published (Anitei et al., 2010).

Delivery of siRNAs into cells was mediated using INTERFERIN reagent (from Polyplus transfection, Illkirch, France) according to supplier’s protocol. The final concentration of siRNAs in medium was 10 nM, if not stated otherwise. In every case cells were incubated in presence of siRNAs for 72 h.

In case of 24-well plates 15,000 cells were seeded in each well 24 h beforehand. For one well 2.5 μl INTERFERIN and respective amount of siRNA stock were mixed in 100 μl OPTIMEM and after 15 min incubation this mixture was applied on cells in 500 μl of fresh medium. In case of individual wells of 6-well plates and in case of live cell imaging dishes, 75,000 cells were seeded 24 h beforehand. 10 μl INTERFERIN and respective amount of siRNA stock were mixed in 200 μl OPTIMEM and after 15 min incubation this mixture was applied on cells in 2000 μl of fresh medium. In case of HEK cells grown in 10 cm dishes, final siRNA concentration was 50 μM.

The efficiency of down-regulation was assessed using 6-well plates: All steps were carried out at 4°C. 72 h after individual wells had been transfected with siRNAs, cells were washed with cold PBS, were scraped in 150 μl cell lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % (v/v) TritonX-100, 2 mM MgCl₂, 1 mM EGTA, 0.5 mM NaF, 0.5 mM Na₃VO₄) and were collected in individual tubes. Cells were homogenized with a syringe by repeatedly passing through a 27 Gauge needle. The cell homogenate was cleared by 20 min centrifugation at 20,000 g.
The concentration of the cleared lysate was determined using colorimetric protein assay. Equal amounts of total protein (15 - 40 µg) were applied on SDS-PAGE. After Western blotting membranes were probed with primary antibodies against down-regulated proteins.

5.10 Microscopy

Fixed cells were imaged using either an inverted LSM 510 META microscope equipped with a 63x 1.4 numerical aperture Plan-Apochromat objective or a LSM 780 microscope equipped with a 100x 1.45 numerical aperture Plan-Apochromat objective (from Carl Zeiss Microimaging, Oberkochen, Germany) Supported lipid bilayers and GUVs were analyzed by confocal microscopy with an inverted LSM 510 META microscope equipped with a 40x 1.2 numerical aperture water-immersion objective.

High-speed time-lapse microscopy was performed with either epifluorescence or Total Internal Reflection Fluorescence using an AFLX6000 equipped with an EMCCD detector, temperature, CO2 and humidity control and a 100x 1.4 numerical aperture oil-immersion objective (Leica Microsystems, Mannheim, Germany). Epifluorescence video microscopy was alternatively performed with an Axiovert 200 M with temperature, CO2 and humidity control and a 63x 1.4 numerical aperture Plan-Apochromat objective (Carl Zeiss Microimaging, Oberkochen, Germany).

Atomic Force Microscopy on supported bilayers was performed by Salvatore Chiantia (Stony Brook University, New York, USA) and Grzegorz Chwastek (BIOTEC, Dresden) using a NanoWizard system (JPK Instruments, Berlin, Germany) mounted on an inverted LSM 510 META.
5.11 Mass Spectrometry

All steps were carried out by Cornelia Czupalla (BIOTEC, Dresden). Control and treated samples were separated by SDS-PAGE side-by-side. Gel lanes were cut into 30 slices in parallel fashion. Protein digestion and in-gel $^{16}$O/$^{18}$O-labeling was performed as described (Lange et al., 2010). In brief, gel pieces were incubated with 100 ng trypsin (Promega, Madison, WI) in the presence of $\text{H}_2^{18}\text{O}$ (97% $^{18}$O, Campro Scientific GmbH, Berlin, Germany) or $\text{H}_2^{16}\text{O}$ as indicated, and paired samples were combined immediately before nano-LC-MS/MS analysis. Label-free sample preparation for mass spectrometry was done as described previously (Czupalla et al., 2006). Peptides were separated on an EASY-nLC nano-HPLC system (Proxeon, Odense, Denmark) equipped with a fused silica microcapillary C18 analytical column (3 $\mu$m, 100 Å, 10 cm x 75 $\mu$m i.d.) directly coupled to the nanoelectrospray source of a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were eluted with a 90 min gradient of 5-50% acetonitrile in 0.1% formic acid at 300 nL/min. Mass spectra were acquired in a data-dependent mode with one MS survey scan (resolution of 60,000) in the Orbitrap and MS/MS scans of the five most intense precursor ions in the LTQ. MS/MS spectra of $^{16}$O/$^{18}$O-labeling experiments were processed and searched against the UniProtKB/SwissProt database (release 56.9, 412,525 sequences, 16,091 Mus musculus sequences) using a Mascot server (version 2.2, Matrix Sciences Ltd., London, UK).

Search criteria were: taxonomy, mouse; mass tolerance of precursor and sequence ions, 10 ppm and 0.35 Da, respectively; modifications, cysteine carbamidomethylation, methionine oxidation, serine/ threonine/ tyrosine phosphorylation, and C-terminal $^{18}$O1- and $^{18}$O2-isotope labeling; maximum two missed cleavages.
A protein was accepted as identified if the total Mascot score was greater than the significance threshold (p < 0.05) and if at least two unique peptides were detected. Based on decoy database searches, the false discovery rate was estimated to be <1%.

Quantification was carried out using the Mascot Distiller Quantification Toolbox (version 2.2.1.2, Matrix Sciences) and was based on calculations of at least two unique tryptic peptides. Relative protein ratios were calculated from the intensity-weighted average of all peptide ratios. Data analysis of label-free experiments was done using MaxQuant version 1.2.2.5 (Cox and Mann, 2008). Peak lists were searched against a database containing 20,253 entries from the UniProt-KB/Swiss-Prot human database (release 2011_02) and 255 frequently observed contaminants as well as reversed sequences of all entries and the search criteria listed above with the following exceptions: mass accuracy, 6 ppm and 0.5 Da for precursor ion and fragment ion mass tolerance, respectively; fixed and variable modifications, cysteine carbamidomethylation and methionine oxidation, respectively. Peptide identifications were accepted based on their posterior error probability until less than 1% reverse hits were retained while protein false discovery rates were < 1%. Proteins were considered if at least two peptides were identified.
6.REFERENCES


Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.


Desweiteren erkenne ich hiermit die Promotionsordnung der Fakultät Mathematik und Naturwissenschaften der Technischen Universität Dresden vom 23.02.2011 an.

Dresden,

Christoph Stange

Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified, notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from January 2007 to August 2012 under the supervision of Prof. Dr. Bernard Hoflack at the proteomics research group of the Biotechnological Centre of the Technical University of Dresden, Germany.

Dresden,

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