Proteomic analysis of the sorting machineries involved in vesicular traffic between the biosynthetic and endosomal compartments

Dissertation

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2006

Proteomic analysis of the sorting machineries involved in vesicular traffic between the biosynthetic and endosomal compartments

A dissertation submitted to the

Technical University of Dresden

For the degree of

Doctor rerum naturalium

In Biology

Presented by

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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 July *2002* to *April 2006* 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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Thorsten Gerhard Baust

Acknowledgements

I would like to thank my supervisor Bernard Hoflack that he gave me the opportunity to conduct my PhD thesis in his laboratory. I am grateful for his support and for the scientific discussions we had, providing me the required research environment to perform my studies throughout the time I was a PhD student in his lab. In addition, I would like to thank my PhD committee Prof. Dr. Marino Zerial and Prof. Dr. Marcos González-Gaitán for the strategic advices and time they spend on our meetings. In addition, I would like to express my gratitude to the members of our joint group meetings, especially Christiane Walch-Solimena, Kai Simons and Christoph Thiele for the scientific discussions on my project.

I would also like to thank my colleagues, especially Dorothee Thiel, Coralie Apfeldorfer and Cornelia Czupalla, who were the first members of our lab besides Bernard and me starting in 2002. In respect to the proteomic analysis, I am particular grateful to Cornelia Czupalla and Theresia Pursche who introduced me into the field of mass spectrometry. The other members of the lab, Tobias Geyer, Katrin Grosser, Tobias Heckel, Hannu Mansukoski, Iryna Parshyna, Thilo Riedl, and Arantxa Sanchez-Fernandez I would like to thank for making scientific research much more enjoyable by their comments, ideas and scientific discussions.

I am deeply grateful to my friends and family, especially to my parents for their love and support at everytime throughout my life. Thanks a lot! Furthermore, I would like to thank my future wife Beate for balancing my life since the first day we met.

Abstract

Vesicular traffic along the biosynthetic and endocytic pathways is essential for homeostasis of eukaryotic cells. However, it raised the question of how the proteins characteristic for each compartment are transported to their destination (Bonifacino and Glick, 2004). This study is especially focusing on the connection between the Golgi apparatus and the endosomal compartment, mediated by two parallel trafficking pathways regulated by the clathrin adaptors AP-1A and AP-3 (Owen et al., 2004). Typical cargo molecules sorted along the AP-1A regulated pathway are mannose 6-phosphate receptors (MPRs) (Ghosh et al., 2003) or the gpI envelop glycoprotein of the *Vesicular Zoster virus* (Alconada et al., 1996), while sorting of lysosomal membrane proteins like Lamp-1 and LimpII is AP-3 regulated (Eskelinen et al., 2003).

To study how AP-1A and AP-3 coats are stabilized on membranes and to identify the protein networks involved, a liposome based *in vitro* assay that recapitulates the fidelity of protein sorting *in vivo* was developed and combined with proteomic screens. Therefore, liposomes carrying cytoplasmic domains of gpI or Lamp-1/LimpII were used as affinity matrix to recruit selectively AP-1A or AP-3 and associated protein machineries. The coated liposomes were then analyzed by mass spectrometry.

Using the *in vitro* recruitment assay, it was possible to demonstrate that efficient and selective recruitment of AP-1A and AP-3 coats depends on the presence of several low affinity binding sites on membranes. Thus, AP-1A and AP-3 recognize their target membranes by activated Arf1 GTPases, organelle specific phosphoinositides, PI-4P and PI-3P respectively, and distinct cargo molecules carrying intact signals in their cytoplasmic domains. The implication of PI-3P in AP-3 recruitment was further supported by *in vivo* experiments. During the biochemical characterization of the assay, several lines of evidence indicated that cargo tails containing intact sorting signals stabilize not only AP-1A and AP-3 coats on membranes but also influence the membrane recruitment of Arf1. It is possible that cargo molecules indirectly drive an Arf1 amplification loop, thereby ensuring efficient AP coat assembly.

The proteomic screens identified protein networks of ≈40 proteins selectively recruited on AP-1A coated structures. The most appealing result of the analysis was the presence of two additional protein machineries, one involved in actin nucleation the other involved membrane fusion. More precisely, the AP-1A analysis identified the selective recruitment of the AP-1A subunits and interacting molecules (clathrin, γ-synergin), Arf1 and Arf1 effectors (Big2, Git1), Rac1 including Rac1 effectors (β-PIX, RhoGEF7) and a Rac1 dependent actin nucleation machinery (Wave/Scar complex, Arp2/3 complex, associated effectors) as well as members of a Rab machinery (Rab11, Rab14). This finding was further supported by *in vivo* colocalization studies of the AP-1A cargo CI-MPR with CYFIP2, a protein of the Wave/Scar complex, and the localization of Big2 and Git1 on Rab11 positive membranes (Matafora et

al., 2001; Shin et al., 2004). The biochemical characterization revealed that the stabilization of AP-1A coats, most probably driven by cargo molecules that stabilize AP-1A and Arf1 on membranes, leads as well to the stabilization of the two other machineries. Thus, the results support the notion that cargo sorting, vesicular movement and membrane fusion are coordinated during early steps of vesicular traffic.

In analogy, the proteomic screens on AP-3 coated structures identified as well ≈40 selectively recruited proteins, which constituted a similar supramolecular network of protein machineries involved in coat formation, action nucleation and membrane fusion via Rab proteins. Thus, beside the AP-3 coat including the AP-3 subunits, Arf1 and Arf effectors (Big1, ARAP1, AGAP1), members of the septin family involved in actin rearrangements and most of the already described effectors of Rab5 microdomains (EEA1, Rabaptin-5, Rabex-5, Vps45) involved in early endosomal dynamics were selectively recruited together with Rab5 and Rab7. Thus, the proteomic analysis of AP-1A and AP-3 coated structures suggest that both AP coats use similar principles - coats, actin nucleation devices and Rab fusion machineries - to assemble supramolecular structures needed for membrane traffic. Although we do not have the ultimate proves yet, it seems as AP-1A and AP-3 use different members of subcomplexes, hence different GTPase effectors, different actin nucleation machineries and different Rab GTPases, to regulate their specific transport pathways and to link the different protein machineries. The proteomic analysis revealed for example that they probably use different Arf and Rho GTPase effectors to link the coat with actin nucleation. However, this has to be proven experimentally.

In order to understand the networks of protein interactions, bioinformatic tools were used as a first approach. Even though some clues about the overall organization of the supramolecular protein complexes were provided, the direct links to the Rab machinery are still elusive. Maybe the proteins with thus far unknown functions could be involved.

The biochemical analysis, especially the role of PIPs, and the Rab GTPases identified in the context of AP-1A and AP-3, provide indications about AP-1A and AP-3 function *in vivo*. The results could be interpreted in a way that AP-1A functions either in traffic from PI-4P positive membranes towards Rab11/Rab14 positive membranes or AP-1A coats assemble on PI-4P and Rab11 or Rab14 positive membranes, hence, TGN to endosomes traffic. The same holds true for AP-3, the results either suggest AP-3 mediates traffic from PI-3P positive towards Rab5/Rab7 positive membranes or they could be interpreted in a way that AP-3 assembles on PI-3P and Rab5 positive membranes for subsequent transport to Rab7 positive membranes, thus traffic from early to late endosomes.

Overall, the results of this thesis research provided important insight into the formation of AP-1A and AP-3 coated structures and the potential interconnection between AP coats, actin nucleation and membrane fusion machineries.

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Preface

One of the central problems of modern cell biology is to understand the three-dimensional organization of cells. The extensive network of intracellular membrane bound organelles allows the eukaryotic cell to carry out a variety of specialized tasks, and greatly increases its surface-to-volume ratio. The existence of this network, however, raised the question of how the proteins, characteristic for each compartment are transported to their destination. This intracellular transport is absolutely necessary for the biogenesis of plasma membranes, lysosomes and endosomes, the secretion of proteins and other molecules from the cell, and the uptake of external molecules by endocytosis. Moreover, its specificity is essential to generate the distinct apical and basolateral surfaces needed for the polarized function of cells in most tissues. Any model of intracellular transport must include mechanisms to guarantee temporal and spatial specificity, because without such regulators, vesicular traffic would result in the rapid homogenization of all cellular compartments. How selective transport between membrane-enclosed organelles occurs and how each organelle maintains its characteristic set of resident macromolecules, are questions that have fascinated biologist for decades.

I. Introduction

I.1: The vesicular transport hypothesis

Based on the findings on protein secretion more than 30 years ago by George Palade and colleagues, the vesicular transport hypothesis was developed (Palade, 1975). This work established that newly synthesized secretory proteins are delivered to the lumen of the endoplasmic reticulum (ER), pass the Golgi complex for post-translational modifications, and are stored in secretory granules which will fuse with the plasma membrane to release their content into the extracellular space. Electron microscopy (EM) studies showed that secretory proteins are often found within small, membrane-enclosed vesicles interspersed among the major organelles. Several studies extended this concept to other membrane systems. This has led to the vesicular transport hypothesis, which states that the transfer of cargo molecules between organelles is mediated by shuttling transport intermediates. Such transport intermediates, which are enclosed by a membrane, bud from a donor compartment by a process that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment ('protein sorting'). Then, the transport carriers are subsequently targeted to a specific acceptor compartment ('targeting'), into which they release their cargo upon fusion of their limiting membranes ('fusion') (Bonifacino and Glick, 2004; Bonifacino and Lippincott-Schwartz, 2003; Pfeffer, 2003; Rothman, 1994).

I.2: Intracellular trafficking routes

The elaborated intracellular trafficking routes in eukaryotic cells are highly dynamic and involve forward (anterograde) and backward (retrograde) movement of transport intermediates in order to maintain the integrity of all organelles. In general, intracellular traffic can be divided into a biosynthetic and an endocytic pathway and is mainly sorting signal dependent. Within the biosynthetic pathway, the ER and the Golgi as well as the intra-Golgi compartments are connected by anterograde and retrograde traffic (Lee *et al.*, 2004). From the trans-Golgi network (TGN) the pathways are more diverse, especially in polarized cells. Cargo can either be transported to the plasma membrane or to the endocytic compartments or both. Even though the precise transport steps are not clear, it is believed that apical cargo can be transported via "lipid rafts" to the apical membrane (Schuck and Simons, 2004), while basolateral sorting is mediated by sorting signals probably from recycling endosomes (Folsch, 2005). Traffic between the TGN and the endocytic pathway can be mediated either directly via transport intermediates that cycle between both compartments in anterograde and retrograde manner or indirectly via transport to the plasma membrane and subsequent endocytosis. The endocytic compartment seems actually to be more complex than

the biosynthetic compartment. First, there are several mechanisms by which cells internalize material into transport intermediates at the plasma membrane including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, as well as clathrin- and caveolin-independent endocytosis (Conner and Schmid, 2003). Second, within the endocytic compartment, which resembles early-, late-, recycling-endosomes, as well as lysosomes, there exist a plethora of transport pathways that either recycle endocytosed cargo to the surface, lead to their degradation in lysosomes or deliver them to the TGN (Maxfield and McGraw, 2004).

Budding and cargo selection of transport carriers at different sites of the biosynthetic and endocytic pathway is mediated by different types of coats. Generally, transport intermediates are classified by the identity of the protein coat used during their formation as well as by the cargo they contain. At least 10 different coats have been identified, each of which mediates a different pathway between two or more organelles. The best studied coats and traffic pathways are those using the coatomer COP-I, COP-II, or clathrin and its adaptors (Kirchhausen, 2000b; Lee *et al.*, 2004; Owen *et al.*, 2004; Robinson, 2004; Traub, 2005). COP-I and COP-II vesicles traffic between the ER and the Golgi complex. COP-I mediates primarily retrograde traffic from the Golgi to the ER and between Golgi stacks, whereas COP-II functions in anterograde traffic from the ER to the Golgi. The clathrin-mediated pathways are responsible for a large fraction of vesicular traffic, e.g. between the Golgi and the endosomal compartment as well as traffic from the plasma membrane to early endosomes (Figure 1).

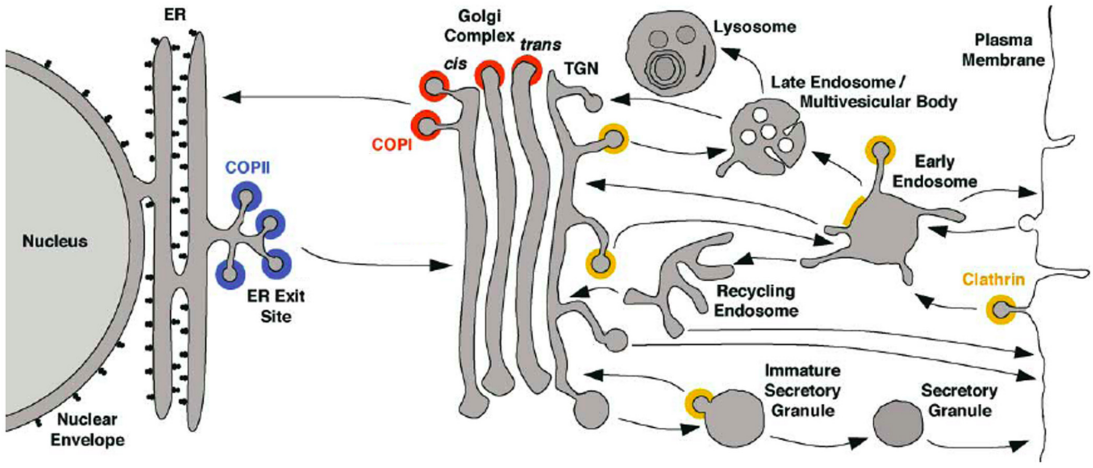

Figure 1: Intracellular trafficking pathways.

Trafficking pathways within the biosynthetic and endocytic compartments, colors indicate the locations of COP-I (red), COP-II (blue), and clathrin (orange). Clathrin coats are heterogeneous and contain different adaptors at different membranes. Additional coat or coat-like structures exist but are not represented in this scheme. Modified from (Bonifacino and Glick, 2004).

I.3: Coated transport intermediates

The budding of transport intermediates and the selective incorporation of cargo into the forming carrier are both mediated by the coats (Bonifacino and Glick, 2004; Kirchhausen, 2000b). Several coat machineries have been developed by eukaryotes that mediate budding from a donor membrane, however all follow a similar principle. Supramolecular assemblies of proteins are recruited from the cytosol to the membrane of the donor compartment. Patches of the flat membrane are deformed by the polymerizing proteins into spherical buds, eventually leading to the release of a protein-coated transport intermediate. The coat proteins dissociate from the membrane of the transport intermediate and recycle back to the cytosol. Finally, the uncoated carrier is free to fuse with the membrane of the acceptor organelle (Figure 2).

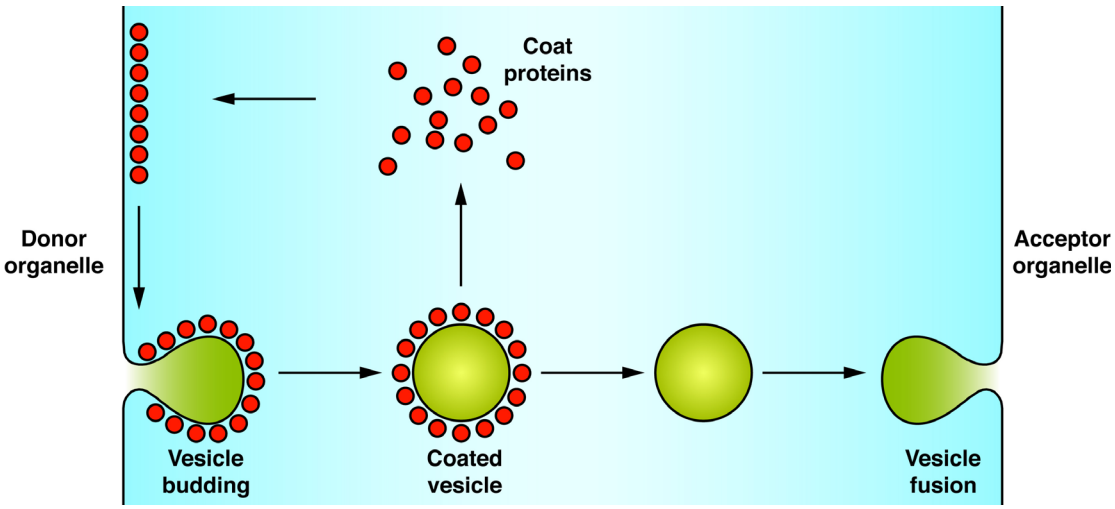

Figure 2: Generation of coated vesicles.

Coat proteins are recruited from the cytosol to the donor organelle. The coated membrane domain bends to form a coated bud. A spherical coated vesicle pinches off, after which the coat proteins dissociate back into the cytosol. The uncoated vesicle fuses with an acceptor organelle. Modified from (Bonifacino and Lippincott-Schwartz, 2003).

Imaging of transport carriers has shown that beside round and small coated vesicles with a diameter of <100nm, there are also large pleiomorphic carriers (LPCs) which are similarly involved in moving secretory traffic between distant compartment. Such LPCs are much larger and more variable in shape than vesicles, and they have interconnected tubular and cisternal components that range in size from 100-200nm or tubules that are several microns long as well as vesicular-tubular structures of various sizes and shapes. Such carriers are highly dynamic, often changing shapes or divide during transport (Bonifacino and Lippincott-Schwartz, 2003; Luini *et al.*, 2005).

I.4: Clathrin-coated vesicles

Since the first isolation and biochemical analysis of clathrin-coated vesicles (CCVs) by Barbara Pearse during the 1970s, much has been learned about their architecture, function, lifecycle as well as the proteins they are composed of and their implication in physiology and human diseases (Brodsky et al., 2001; Edeling et al., 2006; Owen et al., 2004; Traub, 2005). It became evident that the core machinery of CCVs is build by clathrin and adaptor complexes.

I.4.1: Clathrin

Clathrin-coated vesicles showing spike-like structures as well as regular pentagons and hexagons with sides of equal length have been discovered by EM studies roughly 40 years ago (Kanaseki and Kadota, 1969; Roth and Porter, 1964). Some years later, Barbara Pearse purified the coated vesicles and analyzed them biochemically. Pearse found that the major protein component is an approximately 180 - 190kDa protein that she suggested to call ‘clathrin’ in reference to the cagelike structure that it forms (Pearse, 1975). The clathrin assembly unit is a trimer of three extended subunits, which radiate from a central hub. Each triskelia is made of three 190kDa clathrin heavy chains and three 25kDa clathrin light chains, and has a approximately three-fold rotational symmetry. Typical electron micrographs of clathrin coated structures and triskelions are shown in Figure 3.

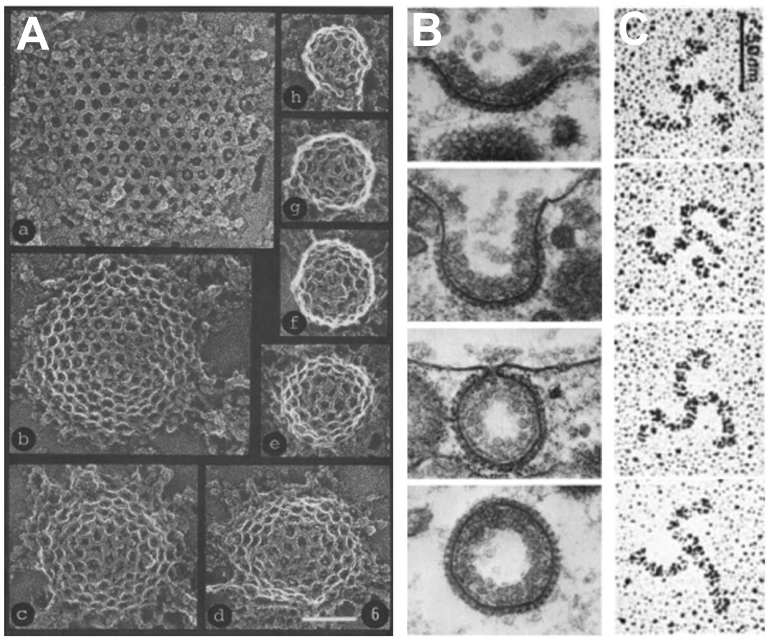

Figure 3: EM pictures of clathrin triskelia and clathrin-coated structures.

Panel A: Rapid-freeze, deepetch electron micrograph of clathrin coated pits and vesicles on the inner surface of the plasma membrane (Heuser, 1980); Panel B: Formation of CCVs from clathrin-coated pits at the plasma membrane (Perry and Gilbert, 1979); Panel C: Platinum shadowed electron micrographs of clathrin triskelions (Ungewickell and Branton, 1981).

I.4.1.1 Ultra structure of clathrin cages

Clathrin coats are three-dimensional arrays of triskelia. When triskelions assemble into a coat, the legs interdigitate to create a lattice of open hexagonal and pentagonal faces as shown on Figure 4. Recently, using electron cryomicroscopy, the structure of *in vitro* assembled clathrin lattices has been obtained at subnanometer resolution (Fotin *et al.*, 2004), for review see (Edeling et al., 2006; Kirchhausen, 2000a).

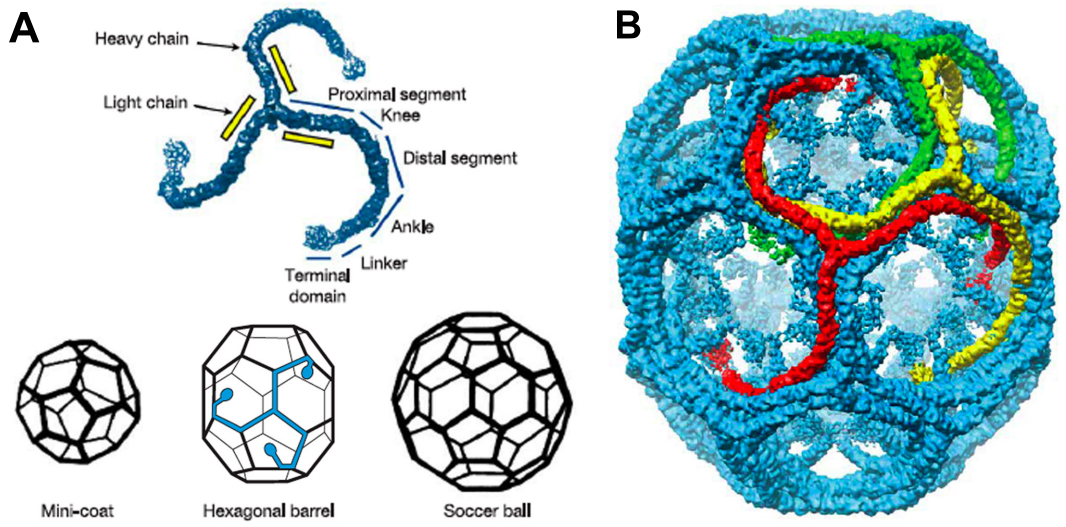

Figure 4: Clathrin triskelion and the structure of clathrin cages.

Panel A: Clathrin triskelion labeled with names for the segments of the heavy chain. The N-terminus of the chain is the terminal domain, and the C-terminus is at the vertex. Three structures that form when clathrin assembles into coats in vitro, schematic representation of one triskelion within the hexagonal barrel shown in blue. Panel B: Image reconstruction of a clathrin hexagonal barrel (heavy chains only) at 7.9Å resolution. Taken from (Fotin et al., 2004).

I.4.2: Adaptor complexes

Beside clathrin, CCVs were shown to contain some other major coat-protein components, which promote clathrin assembly *in vitro* (Keen *et al.*, 1979). The first ones described during the 1980s are the heterotetrameric adaptor protein (AP) complexes AP-1 and AP-2. Both were identified to be highly enriched in CCVs that are derived from the TGN or the plasma membrane, respectively (Pearse and Robinson, 1990). Until today, there have been at least 20 different adaptors for clathrin identified. Most of them are involved at individual sites of clathrin-mediated vesicular traffic inside the cell. The best-characterized adaptors for clathrin are the family of AP complexes. Four basic AP complexes have been described: AP-1, AP-2, AP-3, and AP-4. Each of these complexes is a heterotetramer composed of two large subunits (one each of γ/α/δ/ε and β1-4, respectively, 90 – 130kDa), one medium subunit (μ1-4,

~50kDa), and one small subunit (σ1-4, ~20kDa) (Owen et al., 2004; Robinson, 2004; Robinson and Bonifacino, 2001). The analogous subunits of the four AP complexes are homologous, but in general, the subunits of different AP complexes are not interchangeable. The corresponding β, μ, and σ subunits are showing the highest, whereas the γ, α, δ, and ε the lowest homologies on amino acid level to one another. Some of the APs occur as two or more closely related tissue-specific isoforms encoded by different genes or due to alternative splicing on the mRNA level. AP-1, AP-2, and AP-3 are expressed in all eukaryotes tested. On the other hand, AP-4 is ubiquitously expressed only in mammals and birds (Boehm and Bonifacino, 2001). AP-1 is found mainly on the TGN but also on endosomes, whereas AP-2 is found exclusively at the plasma membrane. AP-3 and AP-4 are both found on TGN/endosomal membranes, while AP-3 localizes more to endosomes and AP-4 more to the TGN (Owen *et al.*, 2004; Robinson, 2004) (Figure 5).

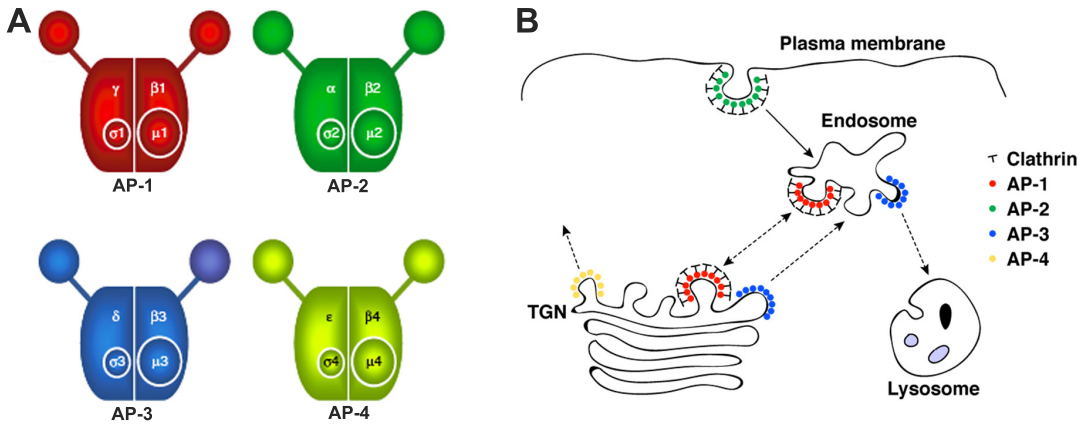

Figure 5: Adaptor protein complexes and pathways they mediate.

Panel A: Schematic diagrams of the four AP complexes, taken from (Robinson and Bonifacino, 2001); Panel B: Trafficking pathways mediated by the four AP complexes. Dotted lines represent pathways where the direction and/or the coat are still a matter of debate. Modified from (Robinson, 2004).

I.5: AP-mediated trafficking pathways

Transport of newly synthesized proteins between the biosynthetic and the endocytic compartments of eukaryotic cells is mediated by vesicular transport to maintain endosomal and lysosomal integrity (Luzio *et al.*, 2003). On the biosynthetic compartment, two transport systems are responsible for cargo delivery to the endocytic system. They are characterized by the AP-coat components they use. AP-1-coated structures are responsible for the transport of mannose 6-phosphate receptors (MPRs). MPRs are major AP-1 cargos that cycle between the TGN, endosomes and the plasma membrane, thereby mediating transport of soluble

hydrolases to the lysosome (Ghosh *et al.*, 2003). Furin, a prohormone processing endoprotease involved in several cellular processes, is a second major cargo transported in AP-1-coated structures (Thomas, 2002). AP-3-coated structures on the other hand, are responsible for transport of integral glycoproteins like Lamp-1 or LimpII that are destined for the limiting membrane of lysosomes where they protect the membrane from unwanted degradation (Eskelinen et al., 2003; Le Borgne and Hoflack, 1998).

I.5.1: AP-1 mediated traffic between the TGN and endosomes

Genetic and biochemical analysis revealed that there are two variants of AP-1: the already known ubiquitously expressed AP-1, now called AP-1A, and the epithelial-specific variant AP-1B (Ohno *et al.*, 1999). Both have an identical architecture, except for the medium subunits μ1A and μ1B, respectively. Despite their close homology, AP-1A and AP-1B form distinct vesicular populations and have separate functions (Folsch *et al.*, 2003). AP-1B is probably involved in cargo traffic to the basolateral surface of polarized cells and localizes primarily to recycling endosomes (Folsch *et al.*, 1999), for review see (Folsch, 2005).

AP-1A mediates transport between the TGN and endosomes, however, the direction is still a matter of debate (Hinners and Tooze, 2003). For MPRs, which were supposed to leave the TGN in AP-1A-coated structures, it was demonstrated that they accumulate in endosomes of μ1A-deficient fibroblasts (Meyer *et al.*, 2000). This result suggested that AP-1A is physiologically involved in the sorting of MPRs from endosomes back to the TGN. The involvement of AP-1A in retrograde traffic is further reinforced by the identification of the phosphofurin acidic cluster sorting protein 1 (PACS-1) (Wan *et al.*, 1998). PACS-1 is an AP-1A interacting protein, shown to be necessary for TGN localization of AP-1A cargos like MPRs and furin. The debate about AP-1A-dependent traffic was further enhanced by the identification of a novel clathrin adaptor family, named Golgi-localized, γ-ear-containing, Arf-binding family of proteins (GGAs) (Bonifacino, 2004). GGAs were shown to be involved in anterograde transport of MPRs from the TGN to endosomes (Puertollano *et al.*, 2001; Zhu *et al.*, 2001). This has led to two proposals concerning the function of AP-1A and GGAs in anterograde MPR transport from the TGN to endosomes. First, two types of coats function in parallel to pack MPRs into different vesicle carriers, or second, the GGAs could bind MPRs and facilitate their entry into AP-1A-CCVs. While the first proposal has not been excluded, there are several lines of evidence supporting the latter possibility (Ghosh and Kornfeld, 2004). The most convincing data beside EM and GGA-AP-1A interaction studies, are video microscopy data showing that GGA1 and AP-1A exit the TGN within the same tubulo-vesicular structures (Puertollano *et al.*, 2003). Thus, there are evidences that AP-1A is mediating both, anterograde and retrograde transport between the TGN and endosomes.

Noteworthy, the originally in yeast discovered and recently in mammals described retromer complex seems to have as well a role in the retrieval of MPRs from endosomes to the Golgi (Seaman, 2005). The mammalian retromer complex consists of probably 5 proteins and can be dissected into two subcomplexes that have either cargo selective roles (mVPS35, mVPS29, mVPS26) or structural functions (sorting nexin-1 and probably sorting nexin-2). Its early endosomal localization is mediated by the PX domains of the sorting nexin family members that recognize the endosomal specific lipid PI-3P. RNAi experiments indicated that the retromer complex is cargo-selective, thus performing retrograde traffic of MPRs but not of furin (Arighi *et al.*, 2004; Seaman, 2004). In addition to the already mentioned machineries involved in the retrieval of MPRs from endosomes to the Golgi, there are reports from Pfeffer and colleagues that Rab9 (Riederer *et al.*, 1994) and TIP47 (Diaz and Pfeffer, 1998) have a role in this process. They showed that TIP47 binds to Rab9 and that this interaction increased the affinity of TIP47 for MPRs *in vitro* (Carroll *et al.*, 2001). However, TIP47 was also suggested to have a role as lipid-droplet-binding protein (Wolins *et al.*, 2001), thus the involvement of TIP47 in the recycling pathway is controversial. Hence, it seems as there are parallel pathways mediating retrieval from endosomes to the Golgi. The cell is probably sorting different cargos along parallel and maybe partially redundant recycling pathways that use different sorting machineries.

I.5.2: AP-3 mediated traffic

Studies on AP-3-mediated pathways and function were greatly facilitated by the discovery of naturally occurring AP-3 mutants in *Drosophila*, mouse and humans, and by the creation of AP-3 mutants in yeast (Boehm and Bonifacino, 2002). In contrast to lethal AP-1 null-mutants, AP-3 null-mutants are viable but show altered trafficking to lysosomes and related organelles, in particular melanosomes and platelet dense granules. Physiological outcomes of AP-3 mutations in mammals are hypopigmentation, prolonged bleeding and pulmonary fibrosis (Boehm and Bonifacino, 2002; Starcevic *et al.*, 2002). AP-3 localizes mainly to an early endosomal tubular network and the TGN (Dell'Angelica et al., 1997; Peden et al., 2004; Simpson et al., 1997). Despite the fact that AP-3 mutations as well as AP-3 knock-down experiments lead to an increased plasma membrane appearance of AP-3 dependent lysosomal proteins like Lamp-1 (Dell'Angelica et al., 1999b; Le Borgne et al., 1998), the exact sorting event that requires AP-3 remains controversial. Especially the pathway taken by newly synthesized Lamp-1 to the lysosome and the site where Lamp-1 traffic involves AP-3 is a matter of debate. Two different pathways, referred to as ‘direct’ and ‘indirect’, have been proposed for Lamp-1 traffic. The direct pathway is a completely intracellular route that involves transport of newly synthesized lysosomal membrane proteins like Lamp-1 from the TGN to endosomes and then to the lysosome without appearing at the plasma membrane

(Cook et al., 2004; Harter and Mellman, 1992; Ihrke et al., 2004; Rous et al., 2002). In the indirect pathway, in contrast, lysosomal proteins like Lamp-1 are first transported from the TGN to the plasma membrane, after which they are internalized and sequentially delivered to early endosomes, late endosomes and lysosomes (Gough *et al.*, 1999; Janvier and Bonifacino, 2005). It has also been proposed that both pathways could contribute to the delivery of newly synthesized lysosomal proteins to lysosomes in parallel, however the majority is transported along the direct pathway (Carlsson and Fukuda, 1992). In addition, there are evidences that the indirect pathway via the plasma membrane is used upon higher expression levels of Lamp-1 (Harter and Mellman, 1992), thus suggesting that lysosomal targeting at endogenous levels involves a saturable direct intracellular pathway for lysosomal proteins like Lamp-1 on their way to lysosomes.

In addition, the exclusive role of AP-3 over AP-1A in Lamp-1-sorting is questioned, mainly based on *in vitro* assays (Crottet et al., 2002; Honing et al., 1996). However, the clear effects of Lamp-1 missorting to the plasma membrane in AP-3 deficient cells (Dell'Angelica et al., 1999b; Le Borgne et al., 1998) were not detected in fibroblasts lacking functional AP-1A (Meyer *et al.*, 2000), thus making a role of AP-1A in targeting of Lamp-1 *in vivo* unlikely.

Another open question in the context of AP-3 is whether the function of AP-3 is clathrin dependent in higher eukaryotes. Even though the β-subunit of human AP-3 contains a clathrin interaction motif and partially colocalizes with clathrin (Dell'Angelica *et al.*, 1998; Peden *et al.*, 2004), AP-3 is not enriched in CCVs (Simpson *et al.*, 1996). In addition, genetic studies in yeast (Cowles *et al.*, 1997) as well as rescue of naturally occurring AP-3 mutants in mice suggested that AP-3 function is independent of clathrin (Peden *et al.*, 2002).

Taken together, the results about trafficking of Lamp-1 as well as AP-3 localization suggest two roles of AP-3. AP-3 could be responsible for the recruitment of lysosomal proteins into a direct transport pathway from the TGN to endosomes. In addition, AP-3 could function at an early or recycling endosomal compartment, in which Lamp-1 must be segregated from other integral membrane proteins that recycle back to the plasma membrane, allowing an efficient Lamp-1 sorting to the late endosomes/lysosomes. For review see (Owen et al., 2004; Robinson and Bonifacino, 2001).

I.5.3: AP-2 mediated endocytosis

AP-2 is probably the best studied AP complex and the predominant clathrin adaptor responsible for clathrin-mediated endocytosis (CME) leading to AP-2-containing CCV formation (Conner and Schmid, 2003; Mousavi *et al.*, 2004; Perrais and Merrifield, 2005; Traub, 2005). CME is essential in all mammals. It carries out the continuous uptake of nutrients like cholesterol containing low-density lipoprotein (LDL) particles that bind to the LDL receptor, or iron-transferrin complexes that bind to transferrin receptors. CME also

modulates signal transduction by controlling levels of surface signaling receptors and plays a central role in synaptic vesicle recycling. CME occurs where cytosolic coat proteins including clathrin and AP-2 polymerize and form clathrin coated pits. Coated pits that cover up to 2% of the cell surface concentrate transmembrane receptors, which contain signals for internalization in their cytoplasmic domains. The mechanism defining the assembly site of clathrin-coated pits are not fully understood. However, recent findings suggest that cargo molecules (Ehrlich *et al.*, 2004) and phosphoinositides, in particular PI-4,5P_2 at the inner plasma membrane (Collins *et al.*, 2002; Honing *et al.*, 2005), are critical for this process because they directly interact and stabilize AP-2 on the plasma membrane. After formation, coated pits invaginate and pinch off to form endocytic CCVs. More than 20 accessory proteins are involved in the process of endocytic CCV formation, including AP180/CALM, epsin, Eps15, dynamin or synaptojanin (Kirchhausen, 2000b; Mousavi *et al.*, 2004; Perrais and Merrifield, 2005; Traub, 2005). Some of these accessory proteins are found in coated pits but not in purified CCVs, suggesting that they play assisting roles during formation including anchoring, scaffolding, lipid modification, scission, actin polymerization or uncoating. After scission, AP-2-containing plasma membrane derived endocytic CCVs are uncoated, probably due to the function of Hsc70 and auxilin (Lemmon, 2001), and fuse with endosomes.

I.5.4: AP-4 mediates basolateral traffic in polarized cells

The labs of Scotty Robinson and Juan Bonifacino discovered AP-4, the latest and presumably last member of the AP complexes, not a decade ago. In mammals, AP-4 localizes mainly to the TGN, as demonstrated by its colocalization with the TGN markers TGN38 and furin (Dell'Angelica *et al.*, 1999a; Hirst *et al.*, 1999). It has been recently proposed that AP-4 is involved, like AP-1B, in basolateral trafficking in polarized cells (Simmen *et al.*, 2002). AP-4 was shown to bind selected Tyr-based sorting motifs mediating basolateral sorting. In addition, depletion of the μ4 subunit induced missorting of several basolateral proteins to the apical membrane. Because AP-4 is not present in CCV and EM pictures localize AP-4 to non-clathrin coated vesicles, AP-4 is supposed to function in a clathrin-independent pathway (Hirst *et al.*, 1999). This hypothesis is further supported by the absence of a clathrin interaction motif in the hinge region of the β4 subunit. For review see (Owen et al., 2004; Robinson and Bonifacino, 2001).

I.6: Membrane targeting and AP-coat assembly

The accuracy of intracellular transport depends upon the correct recognition of the target membrane by the coat to form a coated carrier. Recent findings support the idea that several low affinity interactions are needed in a cooperative manner to support coat assembly in a temporal and spatial organized way. Both proteins and lipids participate in this regulation. Therefore, coats recognize their target membranes by cargo molecules carrying sorting signals in their cytoplasmic tails, membrane specific lipid species like phosphoinositides and activated GTPases of the Arf family. This combination between transient determinants and integral cargo molecules provide membranes with an identity that is unique and flexible to ensure controlled coat assembly. However, there are clear evidences that cargo molecules containing sorting signals are part of the determinants for efficient and selective coat assembly on membranes, whereas the others have minor but nevertheless important roles for directed membrane traffic and specific coat stabilization (Baust et al., 2006; Ehrlich et al., 2004; Honing et al., 2005).

I.6.1: Sorting Signals

Long before the identification of sorting signals and their recognition proteins, it had become clear that sorting occurs through coated areas of membranes. Based on these findings, it was hypothesized that sorting involve interactions between signals present within the cytosolic domains of transmembrane proteins and components of the protein coats (Pearse and Robinson, 1990). Thus, to ensure correct intracellular traffic of proteins, they carry sorting signals that are directly or indirectly bound by coat proteins. For transmembrane proteins, interaction of sorting signals with the coat is considered to be the key event leading to selective recruitment into the nascent transport carrier. Most of the sorting signals of transmembrane proteins are in their cytoplasmic domain. They consist of short, linear stretch of amino acids that fit one of several consensus motifs. Two major classes of sorting signals are referred to as ‘tyrosine-based’ and ‘dileucine-based’ owing to the identity of their most critical residues. Beside these two major sorting motifs, it seems that acidic clusters, which can be phosphorylated by casein kinase II, have also important functions in protein sorting (Bonifacino and Traub, 2003; Robinson, 2004). In addition to peptide motifs, ubiquitination of cytosolic lysine residues also serves as signal for sorting at various stages in the cell (Bonifacino and Traub, 2003; Katzmann et al., 2002; Raiborg et al., 2003). Sorting mediated by sorting signals is saturable, indicating that it relies on recognition of the signals by a limited number of receptors.

I.6.1.1: Tyrosine-based sorting motifs

The first identified endocytic sorting signal was the NPxY-motif in the cytoplasmic domain of the LDL receptor 20 years ago (Davis *et al.*, 1986). Several studies on this motif revealed that it mediates rapid internalization of a subset of transmembrane proteins at the plasma membrane but is not involved in other intracellular sorting events. Many other receptors like the MPRs or the transferrin receptor, however, lacked the NPxY motifs but were rapidly internalized, suggesting the existence of other types of endocytic signals. Lazarovits and Roth showed that tyrosine residues are nonetheless the key elements of these signals (Lazarovits and Roth, 1988). They found that the insertion of a single tyrosine in the cytoplasmic domain of the influenza hemaglutinin enabled the protein to undergo rapid internalization via clathrin-coated pits. Systematic mutational analysis of the cytoplasmic domain of the MPRs in the lab of Steward Kornfeld identified completely the second, more general, tyrosine-based endocytic sorting motif of the Yxxϕ-type, where ϕ is a bulky hydrophobic residue and x could be any amino acid (Canfield *et al.*, 1991; Jadot *et al.*, 1992). This by far best-characterized sorting motif is not only involved in endocytosis, but also in targeting transmembrane proteins from the TGN to the endocytic compartment as well as in basolateral sorting in polarized cells (Bonifacino and Traub, 2003). The Yxxϕ tetrapeptide is the minimal motif, however, the x residues and other residues flanking the motif also contribute to the strength and fine-tuning of the sorting motif. A prominent feature of Yxxϕ signals involved in lysosomal targeting is that most have a glycine before the critical tyrosine. The Yxxϕ is recognized by the μ subunit of AP complexes, as shown by a yeast-two-hybrid screen, in which the two AP subunits μ1 and μ2 were found as specific binding partners of the sequence SDYQRL (Ohno *et al.*, 1995). The μ2 subunit of AP-2 exhibited the highest affinity and broadest specificity for Yxxϕ motifs, but the μ subunits of AP-1, AP-3 and AP-4 were also shown to bind this sorting signal, however more weakly and with different preferences for residues at the x and ϕ position in the Yxxϕ motif (Aguilar et al., 2001; Ohno et al., 1998). The crystal structure of μ2 complexed with Yxxϕ signal peptides revealed that the critical Y and ϕ residues fit into two hydrophobic pockets on the surface of μ2 (Owen and Evans, 1998). Because of their homology, the μ subunits of the other AP-complexes are expected to have a similar structure of the Yxxϕ-binding pocket.

I.6.1.2: Dileucine-based sorting motifs

During the time when the field of intracellular protein sorting was focused on the study of tyrosine-based sorting signals, Lobel and Kornfeld provided first evidence for other types of sorting signals that are not utilizing tyrosine residues (Lobel *et al.*, 1989). They studied mutants of the large MPR were all tyrosines in the cytoplasmic domain were replaced by alanine. Even though, this mutant was defective in endocytosis, it was only partially inhibited in intracellular sorting, thus suggesting, that there must exist other types of sorting signals. Letourneur and Klausner identified this novel type of sorting motif in the cytosolic domain of the T-cell antigen receptor and showed that it relied on two leucines (Letourneur and Klausner, 1992). They could demonstrate that the dileucine-based sorting motif is sufficient for endocytosis and lysosomal delivery. Shortly later, Johnson and Kornfeld published that dileucince-based sorting motifs in the cytoplasmic domain of the MPRs are as well responsible for MPR sorting from the TGN to the endosomal system (Johnson and Kornfeld, 1992a; Johnson and Kornfeld, 1992b). Further analysis revealed that the dileucine-based sorting motifs of the T-cell receptor and MPRs represent two different consensus sequences that are both often preceded by one or more acidic residues. Both dileucine-based consensus motifs were also shown to be recognized by different binding proteins (Bonifacino and Traub, 2003).

I.6.1.2.1: [D/E]xxxL[L/I]-type motif

The dileucine-based signal of the T-cell receptor belongs to the consensus sequence [D/E]xxxL[L/I], which plays especially a role in the sorting to late endosomes, lysosomes and lysosomal related organelles like melanosomes. This dileucine-based sorting motif has been shown to bind AP-complexes *in vitro*. However, each [D/E]xxxL[L/I] motif shows distinct preferences for the different AP-complexes, like the DERAPLI signal of LimpII that binds AP-3 but not AP-1 or AP-2 (Honing *et al.*, 1998), a notion consistent with the observation that LimpII is missorted in AP-3-deficient cells. The structural basis for the [D/E]xxxL[L/I] interaction with AP-complexes has not been solved yet, but recent yeast three-hybrid systems provided strong evidence that [D/E]xxxL[L/I] motifs bind to γ-σ1 hemicomplexes of AP-1 and δ-σ3 hemicomplexes of AP-3 (Janvier *et al.*, 2003).

I.6.1.2.2: DxxLL-type motif

The dileucine-based signals of the MPRs belong to a second type of dileucine motif with the consensus sequence DxxLL. This motif is found in several transmembrane proteins that cycle between the TGN and endosomes, as it is the case for MPRs. The DDSDEDLL motif of the large MPR is a combination of a DxxLL motif and a casein kinase II site. Both have been shown to be a major sorting motif for proper MPR traffic (Chen *et al.*, 1997). DxxLL motifs

do not detectably bind to AP complexes but they bind GGAs. The GGA interaction was shown to be necessary for the incorporation of MPRs into CCVs that bud at the TGN for transport to the endosomal system. Noteworthy, it was shown that GGAs do not bind the other type of dileucine-based sorting motif [D/E]xxxL[L/I] or the tyrosine-based sorting motif Yxxϕ (Puertollano *et al.*, 2001; Zhu *et al.*, 2001). X-ray crystallography revealed that DxxLL motifs bind the N-terminal part of GGAs, the so called VHS domain (Vps27, Hrs, STAM domain), where the critical residues fit into an electropositive and two shallow hydrophobic pockets (Misra *et al.*, 2002; Shiba *et al.*, 2002).

I.6.1.3: Acidic clusters

Serine or threonine residues fitting the [S/T]xx[D/E] consensus motif (x residues are generally acidic) of caseine kinase II (CKII) (Meggio and Pinna, 2003), are often found one to three positions N-terminal to DxxLL motifs as it is the case in the cytoplasmic domains of the MPRs. The MPR acidic cluster-DxxLL motif has been shown to be phosphorylated both *in vivo* and *in vitro*. Crystallographic analysis have revealed that phosphorylation of this motif enhances interactions of the acidic cluster-DxxLL with its recognition module, the VHS domain of GGAs (Kato *et al.*, 2002). Acidic clusters may therefore be conserved to provide CKII recognition sites for the regulation of GGA interaction with DxxLL motifs.

Acidic clusters containing sites for phosphorylation by CKII have also been shown to serve as sorting determinants by themselves. This motif is often found in transmembrane proteins that are localized to the TGN at steady state including furin or the glycoprotein I of the *vesicular zoster virus*. These proteins cycle between the TGN and endosomes, and it is thought that the acidic cluster plays a role in retrieval from endosomes. A monomeric protein named PACS-1 (phosphofurin acidic cluster sorting protein 1) was identified to bind acidic clusters in a CKII-phosphorylation-dependent manner (Wan *et al.*, 1998). PACS-1 was shown to bind mainly AP-1, thus functioning as linker between the phosphorylated acidic cluster and the AP-1-mediated protein sorting. Since deletion of functional PACS-1 resulted in an accumulation of MPRs or furin in the endosomal compartment, PACS-1 is believed to function in retrograde transport from endosomes to the TGN (Crump *et al.*, 2001).

I.6.1.4: Ubiquitin as sorting signal for lysosomal degradation

Ubiquitin is a conserved 76-amino acid globular protein that can be conjugated by an isopeptide linkage to lysine side chains of target proteins. The conjugation of ubiquitin to a protein can regulate its stability, activity, or location. Ubiquitin conjugation occurs by the sequential action of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase (E3) (Pickart, 2001; Weissman, 2001). Proteins can be modified by a single ubiquitin moiety (monoubiquitination) or by lysine-

linked polymeric chains of ubiquitin (polyubiquitination). Polyubiquitination of cytoplasmic proteins with a chain of at least four moieties mainly serves as signal for protein degradation via the 26S proteasome, whereas monoubiquitination is an important signal for intracellular transport of proteins. It was shown that monoubiquitination can serve as signal for both, endocytosis and sorting within the endosomal pathway (Bonifacino and Traub, 2003; Hicke and Dunn, 2003).

The role of monoubiquitination in protein traffic has been intensively studied because it is a well-known signal for lysosomal targeting. Monoubiquitination regulates the sorting of some transmembrane proteins like the epidermal-growth-factor-receptor (EGFR) into luminal vesicles of multivesicular bodies (MVB) for subsequent transport to the lysosome and degradation (Katzmann *et al.*, 2002; Raiborg *et al.*, 2003). The detailed mechanisms are not clear at the moment but it seems as the ubiquitin-binding protein hepatocyte-growth-factor-regulated tyrosine-kinase substrate (Hrs) and the endosomal sorting complexes required for transport-I, -II and –III (ESCRT-I, -II and –III) are involved in this process (Babst, 2005; Gruenberg and Stenmark, 2004; Hurley and Emr, 2006). Hrs is localized to early and late endosomes and its localization depends on its FYVE domain, which binds to the endosome-specific lipid PI-3P. Hrs binds ubiquitinated transmembrane proteins and through its interaction with clathrin, the ubiquitinated proteins are sorted into flat lattices of 'bilayered' clathrin-coated domains on the limiting membrane of endosomes. In addition, Hrs recruits ESCRT-I via its interaction with the ESCRT-I protein TSG101/Vps23 (Katzmann *et al.*, 2003; Lu *et al.*, 2003). The ubiquitinated proteins are then transferred to ESCRT-I, and then sequentially to ESCRT-II and –III, which are implicated in the formation of luminal MVB vesicles and the sorting of ubiquitinated proteins into these vesicles. In addition to the ESCRT machinery, there is evidence that also the phospholipid lysobisphosphatic acid (LBPA) and Alix (ALG-2 interactor x) are involved in the process of intraluminal budding (Matsuo *et al.*, 2004). The final step of the degradation pathway is the fusion of the limiting membrane of the MVB with lysosomal membranes results in the delivery of the luminal MVB vesicles and their contents to the hydrolytic interior of the lysosome, where they are degraded. Noteworthy, not all intraluminal vesicles of MVB are doomed for degradation. Antigen presenting cells (APCs) were found to secrete MHC class II loaded exosomes, which are intraluminal vesicles of specialized MVBs. Exosomes are released by fusion of these multivesicular MHC class II compartments with the plasma membrane by a thus far unknown mechanism. For review see (Fevrier and Raposo, 2004).

Interestingly, recent studies in mammalian cells have shown that GGA3 binds ubiquitin and is involved in sorting of ubiquitinated transmembrane proteins into MVBs. Beside with clathrin and ubiquitin, GGA3 was shown to interact with TSG101, a component of the ESCRT-1 complex (Puertollano and Bonifacino, 2004). Therefore, GGA3 could be able to recruit the

ESCRT-I complex onto endosomes, thus having a similar function as Hrs. These observations suggest that GGAs may function in the MVB pathway in addition to their role as clathrin-adaptors for sorting of DxxLL motifs at the TGN. Interestingly, recent studies in yeast were suggesting that the GGA-mediated sorting step on the TGN might also involve ubiquitin and ubiquitin binding by GGA for subsequent delivery of cargo molecules to the vacuole (Scott *et al.*, 2004).

I.6.2: Phosphoinositides, regulators of membrane traffic

A role for phosphoinositides (PI) metabolism in the secretory process was first discovered during the 1950s. Stimulation of secretion resulted in an increased amount of ^{32}P incorporation into phospholipids, primarily phosphoinositolphosphates (PIPs). Further analysis led to the widely accepted role of PI metabolism in signal transduction, leading to the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) as second messengers (Berridge, 2005; Berridge and Irvine, 1989). The identification of SEC14 by a genetic screen in yeast for secretion mutants provided initial support for a role of PIs in fundamental aspects of vesicular transport (Novick *et al.*, 1980). However it took 15 years from the initial cloning to the functional identification of SEC14 as a PI transfer protein that helps to maintain the appropriate lipid composition in the Golgi membranes for normal post-Golgi secretory traffic (Bankaitis et al., 1990; Bankaitis et al., 1989; McGee et al., 1994; Skinner et al., 1995). Based on these findings the role of PIPs as key regulators in membrane traffic expanded rapidly (Behnia and Munro, 2005; Cremona and De Camilli, 2001; De Camilli et al., 1996; De Matteis et al., 2005; De Matteis and Godi, 2004; Rusten and Stenmark, 2006; Wenk and De Camilli, 2004).

PIPs are derivates of phosphatidylinositol with phosphate groups attached to the 3, 4 or 5 position of the inositol ring. The metabolism of PIPs is highly compartmentalized in the cell, as every organelle is equipped with a distinct set of PIP kinases and PIP phosphatases. The localized synthesis and rapid turnover prevent PIPs to spread over the cell and localize them to specific cellular organelles, where they are recognized by proteins having PIP-specific binding domains and exert their function as temporal and spatial regulators of membrane traffic (Figure 6).

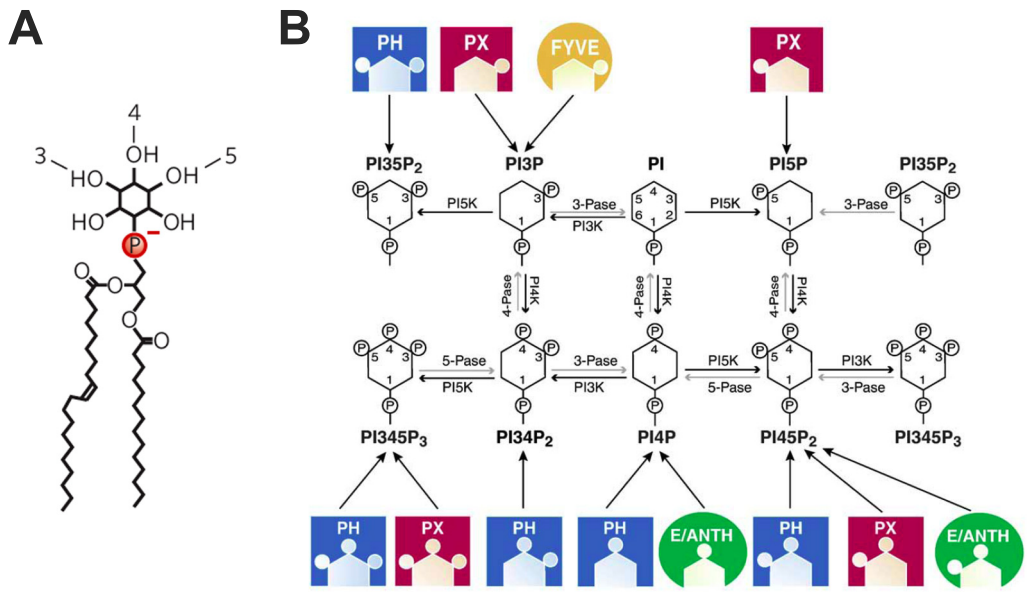

Figure 6: Phosphatidylinositolphosphate metabolism.

Panel A: Phosphatidylinositolphosphates are generated by phosphorylation of phosphatidylinositol on the 3, 4 or 5 positions of the inositol ring; taken from (Behnia and Munro, 2005). Panel B: Metabolism of the PIPs and PIP-specific binding domains; taken from (De Matteis et al., 2005).

Two of the seven PIPs identified in mammals function mainly as second messengers and are synthesized only in response to external signals at the plasma membrane (PI-3,4P_2 and PI-3,4,5P_3). The others are constitutively present in cells, however at low abundance. Most of them have emerged as vectorial regulators of distinct vesicular trafficking steps in the biosynthetic and endocytic compartments (Figure 7). The first direct evidence for an essential role of PIPs and PI-kinases in vesicle-mediated transport was provided when the yeast VPS34 gene was found to encode a phosphatidylinositol-3-phosphate kinase that is essential for sorting and delivery of vacuolar hydrolases from the late Golgi to the vacuole (Herman and Emr, 1990; Hiles *et al.*, 1992; Schu *et al.*, 1993; Stack and Emr, 1994). Starting with these findings, the role of PI-3P in membrane traffic was further studied. Nowadays, PI-3P and its function is probably the best-characterized example of PIPs in the cell.

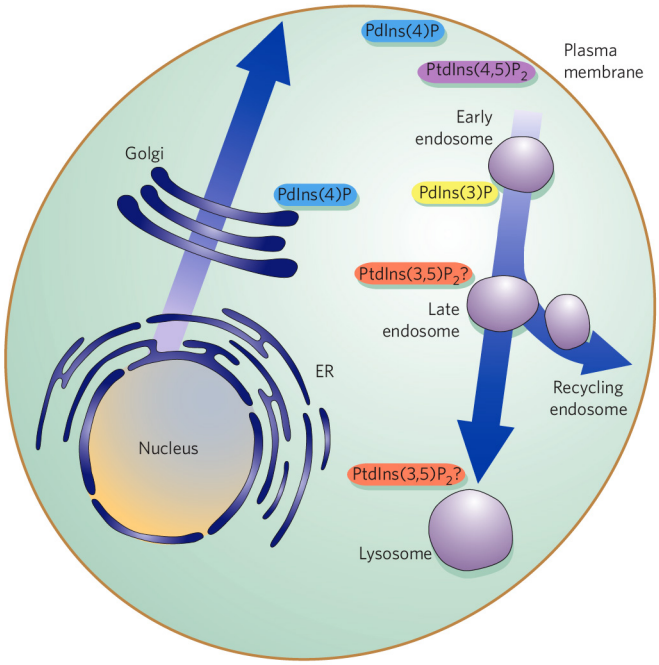

Figure 7: Restricted distribution of phosphatidylinositolphosphates.

Illustration about where the different PIPs localize within the biosynthetic and endocytic compartments of the cell to exert their function in the regulation of membrane traffic; taken from (Behnia and Munro, 2005).

PI-3P, which is present on early endosomes and internal vesicles of MVBs, is recognized by a wide range of peripheral membrane proteins that have key roles in endosomal function (Lindmo and Stenmark, 2006; Zerial and McBride, 2001). Many of these proteins bind PI-3P through one of two small PI-3P-binding domains: the Fab1/YOTP/Vac1/EEA1 (FYVE) domain and the PHOX homology (PX) domain. PI-3P is also used as a substrate for the synthesis of PI-3,5P_2, which is found on late endosomal compartments, however, not that many effectors have been identified. PI-3,5P_2 was shown to interact with Vps24, a subunit of the ESCRT-III complex (Whitley *et al.*, 2003). Hence, PI-3,5P_2 might function as an activator of ESCRT-mediated protein sorting into luminal vesicles of MVBs (Babst, 2005; Gruenberg and Stenmark, 2004; Katzmann et al., 2002). PIPs are also involved in the exocytic pathway like PI-4P at the Golgi complex (De Matteis *et al.*, 2005). Several TGN-associated proteins that bind PI-4P contain a pleckstrin homology (PH) domain or an epsin or AP180 N-terminal homology (ENTH or ANTH) domain. Among the PI-4P-binding proteins, there are vesicle coat proteins like AP-1 (Wang *et al.*, 2003) and EpsinR (Hirst *et al.*, 2003; Mills *et al.*, 2003) or proteins involved in lipid metabolism at the Golgi. PI-4P is also found at the plasma membrane, where it is a substrate for the synthesis of PI-4,5P_2. PI-4,5P_2 is mainly located to the cytoplasmic leaflet of the plasma membrane. However, using electron microscopy, PI-4,5P_2 was also detected on other intracellular membranes including the Golgi, endosomes and

the ER (Watt *et al.*, 2002). Beside being a source for second messengers at the plasma membrane (Berridge and Irvine, 1989) and having a role in the regulation of the actin cytoskeleton (Yin and Janmey, 2003), PI-4,5P_2 anchors proteins through PH-domains and other PI-4,5P_2-binding modules including ENTH and ANTH domains to the plasma membrane (De Matteis and Godi, 2004). Using such interactions, PI-4,5P_2 is recognized at the plasma membrane by several proteins involved in membrane traffic including AP-2, AP180 or Epsin. Most of these proteins are involved in the formation of clathrin-coated pits and endocytosis. In the case of AP-2, PI-4,5P_2 was shown not only to be a binding partner, but also to render AP-2 more competent for the recognition of sorting signals (Honing *et al.*, 2005).

I.6.3: The Arf family of small GTPases, regulators of coat formation

ADP-ribosylation factors (Arfs) are a family of ~20kDa guanine nucleotide-binding proteins, initially identified by their ability to enhance cholera toxin-catalyzed ADP-ribosylation of a stimulatory component of the adenylylcyclase system (Schleifer *et al.*, 1982). 10 years later Serafini and colleagues identified Arf to be a component COP-I coats (Serafini *et al.*, 1991). They could show that Arf is highly enriched in coated vesicles and that Arf is removed from transport vesicles through uncoating during transport. These results led to the suggestion that Arf proteins may modulate vesicle budding and uncoating through controlled GTP hydrolysis. Several small GTPases that belong to the Arf family have been identified and all of them are implicated in the regulation of membrane traffic. The Arf family belongs to the Ras superfamily and comprises Sar1, Arf1-6 and a number of Arf-like GTPases that are similar to Arfs but more distantly related (Burd et al., 2004; Lee et al., 2004; Nie et al., 2003b).

Arfs are divided into three classes based on their sequence similarity: class I, Arf1-Arf3; class II, Arf4 and Arf5; and class III, Arf6. They are all myristoylated at their N-terminus and have a N-terminal amphipathic α helix. Arf1 and Arf6 are probably the best characterized members (D'Souza-Schorey and Chavrier, 2006). Arf6 localizes to the plasma membrane and endosomes and influences the actin cytoskeleton at the plasma membrane as well as endosomal membrane traffic, probably via the metabolism of PI-4,5P_2 (Donaldson, 2003). Arf1 localizes to the Golgi complex and has a well-established role in the recruitment of proteins involved in vesicle formation, lipid metabolism and actin polymerization. Arf1-dependent coats include three of the AP-complexes (AP-1, AP-3 and AP-4), GGAs (GGA1-3) and COP-I. In each case, Arf has been found to bind the coat proteins directly (Donaldson et al., 2005; Nie et al., 2003b). Beside coat interaction, Arf1 regulates the recruitment of PI-4 Kinase IIIβ to the Golgi, thereby regulating the production of the established regulator of membrane traffic at the Golgi, PI-4P (Godi *et al.*, 1999). Arf1 was also found to interact with

the Rac interactor arfaptin, thereby providing a possible link between coat formation and actin polymerization at the Golgi (Tarricone *et al.*, 2001). Because a single Arf isoform functions at multiple sites in the cell, other proteins that interact with Arf must confer the specificity of Arf function.

I.6.3.1: Regulation of Arf GTPases

In general, small GTPases are molecular switches that can alternate between a GTP-loaded active state and a GDP-loaded inactive state. The exchange of GDP for GTP is mediated by interaction with guanine nucleotide exchange factors (GEFs), whereas GTPase-activating proteins (GAPs) stimulate the weak intrinsic GTP-hydrolysis activity of the GTPase, thereby inactivating them. Hence, GEFs are activators and GAPs are negative regulators. The inactive state of small GTPases is cytosolic, whereas the active state is associated with membranes. This characteristic enables small GTPases including the Arf family to recruit effectors like peripheral membrane proteins only when they are active and associated with membranes (Figure 8).

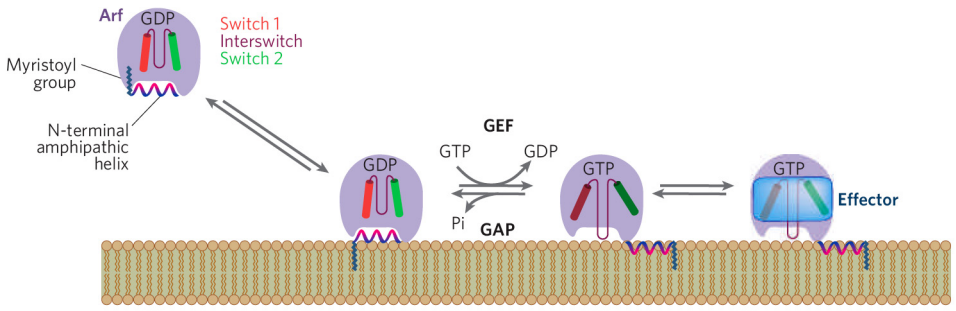

Figure 8: Membrane recruitment of Arf GTPases.

Inactive cytosolic Arf GTPases burry a N-terminal amphipathic helix in a hydrophobic pocket. The N-terminal myristoyl group interacts reversibly with membranes, where GEFs could activate the GTPase. The GTP-loading induces a conformational change of the N-terminal amphipathic helix, which flips out of its pocket to interact stably with membranes and renders the effector domains (switch1/2) competent for effector interactions. Taken from (Behnia and Munro, 2005).

I.6.3.1.1: Arf GEFs, activators for Arf GTPases

Despite the fact that a GEF activity on Arf was identified in Golgi preparations more than 15 years ago, it was in 1996, when the first Arf GEFs were identified in yeast and mammals (Chardin *et al.*, 1996; Peyroche *et al.*, 1996). All identified Arf GEFs contain a Sec7 domain, a region of around 200 amino acids with strong homology to the yeast protein Sec7p that is sufficient to catalyze the exchange of GDP for GTP. The mammalian Arf GEFs are a family of 14 members in five subfamilies with different Arf specificities and intracellular

localizations. The BIG1/BIG2/Sec7 and the GBF/Gea/GNOM family were shown to activate Arf1/Arf3 but not Arf6. The ARNO/cytohesin/GRP family is Arf1 and Arf6 specific, while the EFA6 and the Arf GEP 100 families are exclusively Arf6 specific. Only the Big1/Big2/Sec7 and GBF/Gea/GNOM families localize to the Golgi and are inhibited by the fungal metabolite brefeldin A (BFA), while the others are more peripheral or localize to the plasma membrane and are BFA insensitive (D'Souza-Schorey and Chavrier, 2006; Jackson and Casanova, 2000; Nie et al., 2003b).

The Golgi localized Arf1 GEFs of the BIG1/BIG2/Sec7 and the GBF/Gea/GNOM families are associated with different regions of the Golgi complex. In particular, GBF1 is associated with the cis-Golgi and probably with COP-I traffic, while BIG1 and BIG2 are localized to the TGN and recycling endosomes (Donaldson et al., 2005; Shin and Nakayama, 2004). BIG2 overexpression or expression of a BIG2 dominant negative mutant affects the TGN recruitment of AP-1 and GGA coat proteins but not COP-I, thus suggesting that BIG2 is involved in AP-1 and GGA mediated membrane traffic via the activation of Arf1 (Shinotsuka *et al.*, 2002a; Shinotsuka *et al.*, 2002b).

I.6.3.1.2: Arf GAPs, negative regulators of Arf GTPases

Since the first identification of ArfGAP1 as the first GAP for Arf (Cukierman *et al.*, 1995), 23 other mammalian Arf GAPs have been found. All of these GAPs contain a common GAP domain that includes a characteristic Zinc-finger motif as well as an arginine that is critical for the GAP activity. The Arf GAPs have been categorized into three different groups based on their structural homologies: the ArfGAP1 type (ArfGAP1/3) and the Git type (Git1/2) both have the GAP domain at the extreme N-terminus. The third group comprises the AZAP type, which has a PH domain N-terminal to the GAP domain and ankyrin repeats immediately C-terminal to the GAP domain. The AZAPs, which are also called centaurins (Jackson *et al.*, 2000), are further subdivided into ASAPs, AGAPs, ARAPs and ACAPs that differ in their non-catalytic regions (Nie et al., 2003b; Nie and Randazzo, 2006; Randazzo and Hirsch, 2004). Like the different Arf GEFs, Arf GAPs also show restricted localizations within the cell as well as selected Arf-isoform specificities. While the ArfGAP1/3 type localizes to the Golgi complex and especially ArfGAP1 was shown to influence Arf1 activity in the context of COP-I traffic (Donaldson *et al.*, 2005), the Arf1 specific AGAP1 and AGAP2 were localized to endosomes, where they were found to regulate AP-3 and AP-1 dependent traffic, respectively (Nie *et al.*, 2003a; Nie *et al.*, 2005). The other Arf GAPs localize mainly to the cell periphery or focal adhesions, were they are probably implicated in actin remodeling or in the regulation of membrane traffic (Nie *et al.*, 2003b; Nie and Randazzo, 2006). Of special interest are the ARAPs that are bifunctional and contain a Rho GAP domain in addition to the Arf GAP domain, thus probably connect Arf GTPases with Rho GTPases, which are involved

in the organization of the cytoskeleton (Van Aelst and D'Souza-Schorey, 1997). Beside their peripheral localization of ARAPs, ARAP1 was also shown to be Golgi associated (Miura *et al.*, 2002), thus probably connecting membrane traffic and cytoskeletal rearrangements at the Golgi.

I.7: The vesicle tethering and membrane fusion machinery

The current working models about vesicle formation and fusion imply that after the formation at the donor membrane and the movement of the transport intermediates along cytoskeletal elements, the coat components are released for subsequent targeting and fusion with the acceptor membrane. Several lines of evidence imply that the tethering and fusion processes depend on small GTPases of the Rab family, tethering factors as well as SNAREs (soluble N-ethylmaleimide-sensitiv factor attachment protein receptors) (Behnia and Munro, 2005; Bonifacino and Glick, 2004). Rab GTPases function as membrane organizers but also mediate membrane tethering via tethering factors upstream of SNARE-mediated membrane fusion (Figure 9).

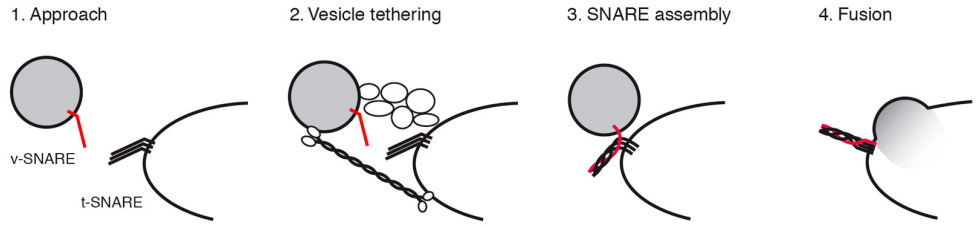

Figure 9: Vesicle tethering and fusion

After the uncoating and movement to the acceptor membrane, tethering factors form physical links between the transport intermediates and the acceptor membrane before the engagement of SNAREs and subsequent membrane fusion. Taken from (Whyte and Munro, 2002).

I.7.1: The Rab family of small GTPases

The role of Rab GTPases in membrane traffic was first demonstrated in yeast during the late 1980s by several studies on Sec4p and Ypt1p, which function in the exocytic pathway (Pryer *et al.*, 1992). In the meanwhile, at least 60 different mammalian Rab GTPases have been identified (Bock *et al.*, 2001). Like other small GTPases, Rab proteins can switch between their cytosolic inactive GDP-bound and membrane associated active GTP-bound state. Proteins that regulate the Rab GDP/GTP cycle are mediating the correct membrane recruitment of Rab GTPases (Pfeffer and Aivazian, 2004; Seabra and Wasmeier, 2004). Rab-GDP forms a complex in the cytosol with a GDP-dissociation inhibitor (GDI) that masks the

C-terminal prenyl groups of the Rab protein. A set of membrane proteins known as GDI displacement factors (GDFs) catalyze the dissociation of Rab from GDI at the target membrane, resulting in the anchoring of the prenyl groups in the lipid bilayer. On the membrane, GEFs are catalyzing the exchange of GDP by GTP on the Rab proteins, which induces a conformational change of the Rab GTPases rendering them competent to bind specific Rab effectors. A GAP stimulates the hydrolysis of GTP and the Rab is retrieved from the membrane by a free GDI to the cytosol (Figure 10). According to this scheme, GDFs and GEFs are the critical determinants in mediating organelle specific activation of Rab proteins. Even though there are some organelle specific GDFs and GEFs, their precise contribution to Rab location is at present poorly understood as it is the case for the few Rab GAPs identified (Behnia and Munro, 2005).

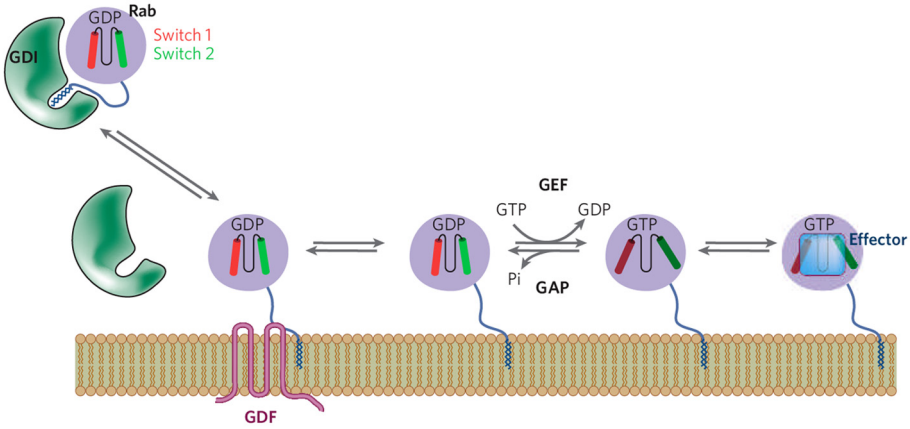

Figure 10: Membrane association of Rab GTPases.

Rab-GDP forms a complex with GDI in the cytosol. GDF displaces GDI from Rab-GDP, and the Rab is anchored at the membrane by its C-terminal prenyl groups. A GEF at the membrane activates Rab by the exchange of GDP by GTP, which induces a conformational change of the Rab. The Rab-GTP is now able to interact with its effectors. A GAP stimulates the hydrolysis of GTP and the Rab is retrieved by GDI to the cytosol. Taken from (Behnia and Munro, 2005).

I.7.2: Rab GTPases as membrane organizers

The different Rab proteins are associated with specific organelles, where they coordinate several functions including vesicle budding, tethering and fusion as well as movement of vesicles along cytoskeletal elements (Jordens et al., 2005; Zerial and McBride, 2001). Rab5, Rab4, and Rab11 for example occupy domains of endosomal compartments that are involved in endocytosis and recycling of cargo receptors (Sonnichsen *et al.*, 2000), while late endosomes show populations of separate Rab7 and Rab9 domains (Barbero *et al.*, 2002). It was shown that the highly compartmentalized Rab proteins have central roles in the

regulation of membrane traffic by determining when and where peripheral proteins are recruited to membranes. Rab5 is one of the best-studied Rab proteins, regulating the recruitment of several effectors onto PI-3P containing early endosomes, thereby establishing a molecular network that drives a Rab5 microdomain formation and regulates early endosomal membrane traffic (Zerial and McBride, 2001). Recently, it was reported that during vesicular transport Rab conversion of organelles is taking place (Rink *et al.*, 2005; Vonderheit and Helenius, 2005). In other words, transport intermediates accumulate sequentially different Rab GTPases on their way to their destination, which provides a vectorial sorting mechanism. This finding is further supported by the identification of divalent Rab effectors that regulate protein sorting in endosomal organelles by connecting different Rab domains (de Renzis *et al.*, 2002).

I.7.3: Tethering factors are anchored by Rab GTPases

Beside their role as membrane organizers, Rab proteins mediate membrane tethering. Tethering is the first event of contact between transport intermediates and the target membrane (Guo et al., 2000; Lupashin and Sztul, 2005; Whyte and Munro, 2002). The initial, loose tethering of vesicles with their targets over distances of more than 200nm is mediated by a group of highly specific tethering factors with distinct subcellular localizations. Distinct tethering factors are involved in different trafficking steps and are localized to the membrane via their direct or indirect interaction with Rab-GTP. They are divided into a group of rod-like coiled-coil proteins that form long homodimers and a group of multisubunit complexes. The coiled-coil tethers Uso1/p115, GM130, giantin and golgin84 are involved in ER to Golgi and intra-Golgi transport, golgin97 participates in traffic from endosomes to the Golgi and EEA1 is involved in endosomal traffic. The multisubunit TRAPPI/II and COG complexes mediate ER-Golgi and intra-Golgi traffic, while the HOPS and GARP complexes are involved in TGN-endosome traffic, and the exocyst complex facilitates fusion of secretory vesicles with the plasma membrane. After vesicle tethering, the tethering factors interact either directly or indirectly with the SNARE fusion machinery that mediates the fusion of the transport intermediate with the target membrane (Lupashin and Sztul, 2005; Whyte and Munro, 2002).
Beside the yeast exocyst complex and its function at the plasma membrane (Guo *et al.*, 2000), the role of Rab5 and EEA1 is probably the best-studied example in mammals. Hence, Rab5 recruits directly the coiled-coil tethering factor EEA1, that is needed for homotypic fusion of early endosomes as well as heterotypic fusion of CCVs with early endosomes (Christoforidis et al., 1999; Rubino et al., 2000; Simonsen et al., 1998). Subsequently EEA1 interacts directly with the t-SNARE syntaxin 13 that mediates endosomal fusion (McBride *et al.*, 1999). For review see (Guo *et al.*, 2000; Zerial and McBride, 2001).

I.7.4: SNAREs mediate membrane fusion

The final step in membrane traffic is the fusion of the transport intermediate with is target membrane. This step is mediated by a protein family called SNAREs that show specific enrichment in different organelles (Bonifacino and Glick, 2004; Chen and Scheller, 2001; Jahn et al., 2003). The human genome encodes 35 SNARE proteins, which are mainly C-terminal anchored transmembrane proteins with their functional N-terminal domain facing the cytosol. Each of these proteins contain a 60-70 amino acid SNARE motif that participates in coiled-coil formation (Bock *et al.*, 2001). With their discovery and purification in 1993, the SNARE hypothesis was postulated as a first working model explaining vesiclular docking and fusion (Rothman, 1994; Sollner et al., 1993). It proposed that each type of transport vesicle carries a specific v-SNARE that binds to a unique cognate t-SNARE on the target membrane and that this specific interaction targets the vesicle to the correct membrane for subsequent fusion. However, most of the recent studies indicate that the targeting of transport intermediates to their target membranes is mediated by upstream tethering factors and only partially by the SNARE machinery.

The synaptic SNAREs synaptobrevin/VAMP (on vesicles) and syntaxin 1 and SNAP-25 (on the plasma membrane) are mechanistically the best studied SNARE proteins and have served as models to understand SNARE function (Chen and Scheller, 2001; Rizo and Sudhof, 2002). When appropriate SNARE motifs of normally one v-SNARE and three t-SNAREs interact with each other, they form spontaneously a four-helix bundle in which the four SNARE motifs are in a parallel orientation. This simple mechanism of zippering from the extended N-terminus to the membrane anchored C-terminus of SNARE proteins pulls the two membranes close together, thereby overcoming the energy barrier for fusion. Therefore, v- and t-SNAREs in separate membranes pair to form a trans-SNARE complex that persists throughout the fusion reaction to become a cis-SNARE complex in the fused membrane. The cis-SNARE complex is then bound by α-SNAP (Soluble NSF-attachment Protein), which in turn recruits NSF (N-ethylmaleimide Sensitive Factor). ATP hydrolysis by NSF untwists the four-helix bundle of the cis-SNARE complex to recycle the SNAREs for further rounds of membrane fusion. To keep the SNAREs inactive during recycling, cytosolic factors bind them to keep them separated (Figure 11). The crystal structure of a trans-SNARE complex revealed that SNAREs provide partially a layer of selectivity for membrane fusion. One arginine and three glutamines, contributed by each of the four helices, form a central ionic layer in the otherwise hydrophobic core of the trans-SNARE complex (Sutton *et al.*, 1998) (Figure 12). Thus, SNAREs are partially selective for their partners to form a stable four-helix bundle (McNew *et al.*, 2000). The finding that SNARE-complex formation is dependent on the identity of the individual SNAREs has led to an alternative nomenclature, where the SNAREs are categorizes as R- or Q-SNAREs, reflecting the contribution of the critical amino acid to the

ionic core. Even though the nomenclatures follow different principles, there is a rough correspondence of R-SNAREs with v-SNAREs and Q-SNAREs with t-SNAREs.

Interestingly, not only all intracellular fusions seem to involve SNARE proteins, also the fusion of viruses seem to use a similar mechanism. Despite the fact that viruses use their own fusion protein, which operates only as a "single shot device", the mechanism of membrane fusion is structurally and functionally highly similar to SNARE-mediated fusion (Jahn *et al.*, 2003; Sollner, 2004).

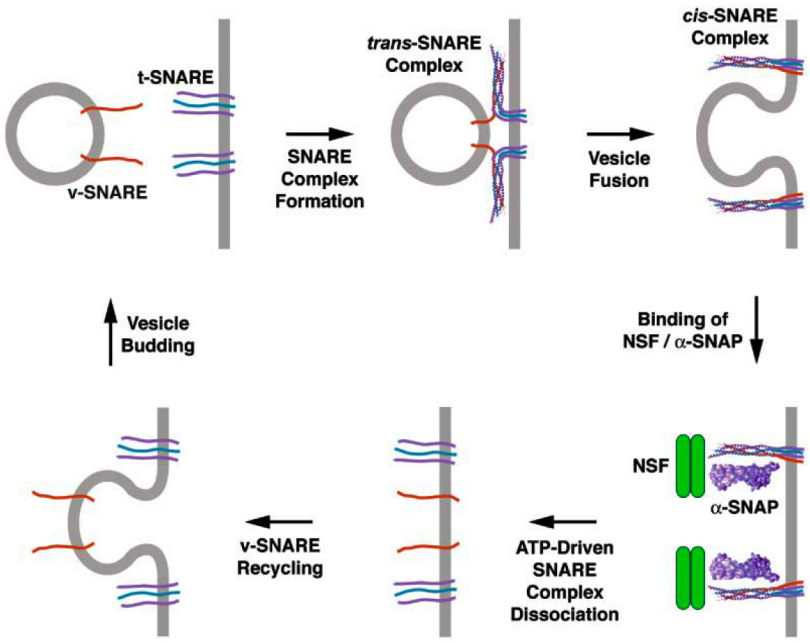

Figure 11: The SNARE cycle.

A trans-SNARE complex assembles when a monomeric v-SNARE on the vesicle binds to an oilgomeric t-SNARE on the target membrane, forming a stable four-helix bundle that promotes fusion. The result is a cis-SNARE complex in the fused membrane. α-SNAP binds to this complex and recruits NSF, which hydrolysis ATP to dissociate the complex. Unpaired v-SNAREs can then be packed into vesicles for recycling. Taken from (Bonifacino and Glick, 2004).

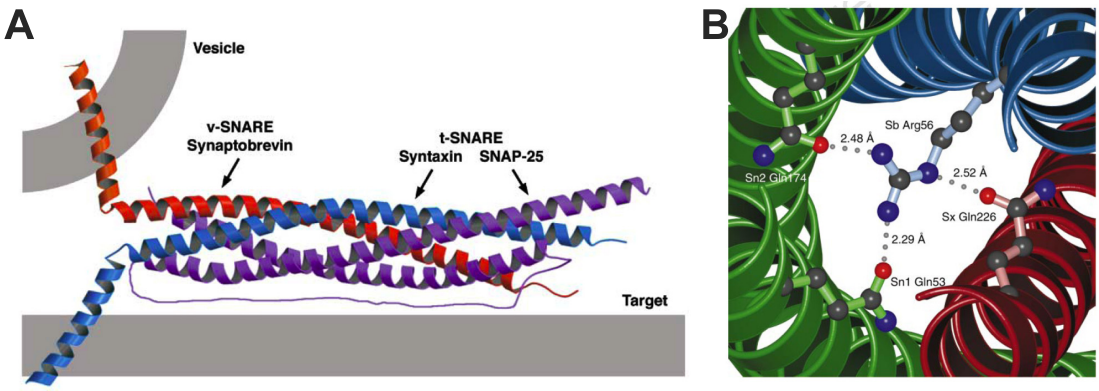

Figure 12: Crystal structure of a synaptic trans-SNARE complex.

Panel A: Hypothetical model of a trans-SNARE complex, build of Synaptobrevin-II (red), Syntaxin-1A (blue) and SNAP-25 (purple), the two membrane anchors and the peptide that links the two SNAP-25 α-helices are hypothetical; Panel B: Ionic layer of the four-helix bundle, the critical arginine and the three glutamines are depicted as balls and sticks; modified from (Sutton et al., 1998).

II. Rationale of the PhD thesis

The completion of genome sequencing of several eukaryotic organisms from yeast (Goffeau *et al.*, 1996) to humans (Lander *et al.*, 2001; Venter *et al.*, 2001) has provided an invaluable tool to discover new genes. However, it is the decoding of this genomic information into proteins and their interactions and functions that are key to understand cellular processes. Studies on proteins and protein networks have dramatically increased in complexity due to the development of novel sophisticated techniques. Just 20 years ago, studies on protein-protein interactions were limited to biochemical techniques such as cross-linking, coimmunoprecipitation and cofractionation by chromatographic methods. During the late 1980s, several techniques for the screening of large libraries of genes or fragments of genes whose products may interact with a protein of interest have been developed including phage display and yeast two-hybrid systems (Phizicky and Fields, 1995). With the instrumentation of mass spectrometry for proteomic studies within the last two decades (Aebersold and Mann, 2003; Domon and Aebersold, 2006), the analyses peaked in genome wide high-throughput screens for protein-protein interactions (Phizicky *et al.*, 2003). Using yeast two-hybrid systems, protein microarrays or mass spectrometry-based proteomic methods, several groups published the interactome of complete eukaryotic organisms including yeast, flies and worms (Gavin et al., 2002; Giot et al., 2003; Ho et al., 2002; Li et al., 2004; Uetz et al., 2000; Zhu et al., 2001). However, there is still need to integrate the broad but shallow proteomic data into deeper understanding of biological processes.

Nevertheless, advances in mass spectrometry-based methods have greatly increased the ability to identify the protein components of biological samples but the problem of complexity is still limiting its application for proteome-wide analyses of complete organisms. It became apparent that complete cells and tissues are not ideal because they are too complex. Therefore, isolated organelles, cellular subcompartments and large protein complexes have been shown to be attractive targets for proteomic analysis since their protein complexity is reduced and they can be highly purified. Such proteomic analyses included among others mitochondria, phagosomes, lysosomes, exosomes as well as the splicosome or the nuclear core complex (Taylor *et al.*, 2003; Yates *et al.*, 2005). Interestingly, also brain derived CCVs as well as vesicles enriched for the AP-3 cargo zinc transporter 3 or *in vitro* assembled AP-1A coated liposomes have been subjected to proteomic analysis (Baust et al., 2006; Blondeau et al., 2004; Ritter et al., 2004; Salazar et al., 2005). In each case, characterized proteins not previously known to associate with such vesicles and novel proteins have been identified.

Thus, combining proteomics with traditional cell biological techniques is providing a strategy for the functional characterization of biological processes.

Beside the isolation of cellular compounds to high purity and their characterization, another approach was utilized to study the protein machineries involved in vesicular traffic. This approach took advantage of the reconstitution of purified coat components on chemically defined liposomes. Randy Schekman established this *in vitro* approach to reconstitute the biogenesis of COP-I and COP-II coated transport vesicles (Matsuoka *et al.*, 1998b; Spang *et al.*, 1998). Steward Kornfeld was using this approach to study the assembly of AP-1 and AP-3 coated structures on synthetic liposomes (Drake et al., 2000; Zhu et al., 1999). In a next step, the method was further developed by using proteoliposomes that contain specific cytoplasmic domains of SNAREs or cargo receptors to study COP-I and COP-II coat formation from purified coat components (Bremser et al., 1999; Matsuoka et al., 1998a), highlighting the importance of cargo molecules for coat assembly.

All of these liposome-based studies were using purified coat components to define the minimal machinery needed for coat assembly. Since sophisticated mass spectrometry-based tools are available, I set up a highly controlled *in vitro* assay that recapitulates specific AP-1A and AP-3 coat formation on proteoliposomes from cytosol in order to identify the complete protein networks involved. In the first part of this study, the physiological behavior of the liposome-based affinity matrix is addressed and in the second part, the proteomic screen and the protein networks identified in the context of AP-1A and AP-3 coat formation are analyzed.

III. Results

III.1: Coupling of synthetic peptides to lipid anchors via hydrazone bonds

Different procedures have been used to introduce cytoplasmic domains of membrane proteins into liposomes including lipopeptides (Bremser *et al.*, 1999; Crottet *et al.*, 2002) or GST-fusion proteins which binding to a lipid anchor containing glutathione (Matsuoka *et al.*, 1998). In order to introduce synthetic cytoplasmic domains of type I and type III transmembrane cargo proteins into liposomes, another chemistry was used that is easy to handle, stable under reducing conditions and provides the spatial arrangement of the sorting signal relative to the membrane analog to the *in vivo* situation (Bourel-Bonnet *et al.*, 2005). A lipid anchor containing an aldehyde-derivatized head group reacts with a hydrazine group present at the N-terminus of synthetic peptides, thus forming a stable hydrazone bond (Figure 1).

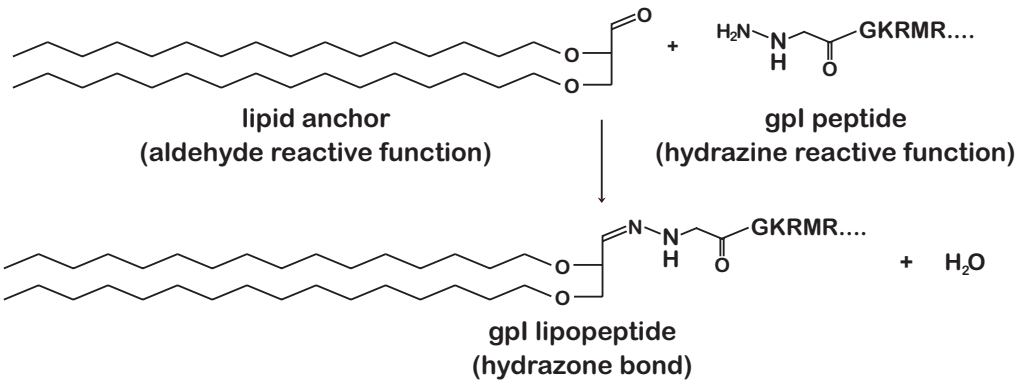

Figure 1: Coupling of hydrazino peptides with aldehyde lipid anchors.

Chemical reaction of coupling synthetic peptides to a lipid anchor incorporated into liposomes.

Therefore, liposomes having a well-defined phospholipid, cholesterol and lipid anchor composition were incubated with different hydrazino peptides corresponding to cytoplasmic domains of cargo proteins sorted either along an AP-1A or an AP-3 mediated pathway *in vivo*. In this manner, the synthetic cytoplasmic domains of the gpI envelope glycoprotein of the *Varicella Zoster* virus, an AP-1A cargo protein (Alconada *et al.*, 1996), Lamp-1 and LimpII, both are lysosomal transmembrane proteins of the limiting membrane and AP-3 cargos (Dell'Angelica et al., 1999; Le Borgne et al., 1998), as well as corresponding peptides containing mutations in the relevant sorting signals, were covalently linked to the outer surface of liposomes (Table I).

amino acid sequence	name
H2N-G-GKRMRVKAYRVDKSPYNQSMYYAGLPVDDFEDSESTDTEE-OH	gpI wt
H2N-G-GKRMRVKAYRVDKSPYNQSMYAAGLPVDDFEDSESTDTEE-OH	gpI Y23A
H2N-G-GKRMRVKAARVDKSPYNQSMYYAGLPVDDFEDSESTDTEE-OH	gpI Y10A
H2N-G-GKRMRVKAYRVDKSPYNQSMYYAGLPVDDF-OH	gpI ΔAC
H2N-G-GKRMRVKAARVDKSPYNQSMYAAGLPVDDF-OH	gpI Y10,23A ΔAC
H2N-G-GRKRSHAGYQTI-OH	Lamp-1 wt
H2N-G-GRKRSHAGAQTI-OH	Lamp-1 Y10A
H2N-G-RGQGSTDEGTADERAPLIRT-OH	LimpII wt
H2N-G-RGQGSTDEGTADERAPAART-OH	LimpII L18A,I19A

Table I: Amino acid sequences of cytoplasmic domains used in this study.

Note that the gpI tail referred here as wild type contains a truncation at the C-terminus devoid of any trafficking signals as described (Alconada et al., 1996).

III.1.1: GpI, a cargo molecule of the AP-1 pathway

Varicella Zoster virus (VZV) is a human herpes viruses. Its viral envelope has at least five major glycoproteins designated gpI to gpV. Out of those, gpI is the predominant virion envelope glycoprotein and also the major glycosylated VZV cell surface antigen (Grose, 1990). GpI localizes to the TGN and cycles between this compartment and the cell surface, when expressed in HeLa cells in the absence of additional virally encoded factors (Alconada *et al.*, 1996). Like the mannose 6-phosphate receptors (Ghosh *et al.*, 2003), gpI can leave the TGN in AP-1 clathrin-coated vesicles for subsequent transport to endosomes. Its return from the cell surface to the TGN occurs as well through endosomes. Mutational analysis of gpI has shown that the proper localization and cycling depends on the 62 amino acid cytoplasmic domain containing two sorting determinants, a classical tyrosine-based sorting motif and an acidic cluster with an casein kinase II phosphorylation site (Alconada et al., 1996; Yao et al., 1993). Based on the mutational analysis, and the ability of gpI to recruit selectively AP-1 onto Golgi membranes after overexpression (Le Borgne *et al.*, 1998), five different gpI peptides were designed to study the mechanism of selective and efficient AP-1 recruitment onto lipid-defined membranes. The synthetic 41 amino acid gpI peptide, referred here as wild type (gpI wt), contains a C-terminal truncation devoid of any sorting motifs when compared with the full length cytoplasmic domain. The truncation showed no change when compared to the full length cytoplasmic domain in steady state distribution of gpI and colocalization with TGN38 (Alconada *et al.*, 1996). The gpI wt peptide contains a tyrosine-based sorting motif starting with amino acid 23, an acidic cluster starting with amino acid 32 and a putative tyrosine-based sorting motif starting with amino acid 10, which was not further analyzed in previous studies. The sorting motifs were individually mutated either by replacing the critical tyrosines by alanines, gpI Y10A and gpI Y23A respectively, or by truncating the acidic cluster leading to a 31 amino acid long peptide (gpI ΔAC). In addition, all motifs were mutated altogether leading to a 31 amino acid peptide devoid of any sorting motifs (gpI Y10,23A ΔAC).

III.1.2: Lamp-1 and LimpII, two cargo molecules using the AP-3 pathway

Lamp-1 (lysosomal associated membrane protein 1), formerly known as lgp120, is a heavily glycosylated integral membrane protein enriched at steady state in the limiting membranes of late endosomes and lysosomes (Eskelinen et al., 2003; Howe et al., 1988; Lewis et al., 1985). Newly synthesized Lamp-1 is transported from the TGN to endosomes/lysosomes mainly via a direct intracellular route without appearing at the cell surface (Cook et al., 2004; Harter and Mellman, 1992). Lamp-1 traffic is dependent on the AP-3 adaptor complex as shown by selective AP-3 recruitment onto Golgi membranes after Lamp-1 overexpression or disturbing Lamp-1 sorting by partial AP-3 inactivation (Le Borgne *et al.*, 1998). In addition, immuno EM studies revealed a co-localization of Lamp-1 and AP-3 on early endosomal exit sites (Peden *et al.*, 2004). Mutational analysis of the short cytoplasmic domain of Lamp-1 has revealed that a single tyrosine-based sorting motif at the very C-terminus and its position relative to the membrane is necessary for proper sorting of Lamp-1 to lysosomes (Guarnieri et al., 1993; Honing and Hunziker, 1995; Hunziker et al., 1991; Rohrer et al., 1996; Williams and Fukuda, 1990). Based on these findings, the 13 amino acid long cytoplasmic domain of Lamp-1 (Lamp1 wt) was chosen as a model cargo to study selective AP-3 recruitment onto synthetic membranes dependent on a single tyrosine-based sorting motif. To interfere with AP-3 recruitment in control experiments, the critical tyrosine at position 10 was substituted by an alanine (Lamp1 Y10A).

LimpII (lysosomal integral membrane protein II) was discovered to be highly glycosylated and located by immuno electron microscopy to lysosomes, the Golgi and vesicles next to the TGN (Barriocanal *et al.*, 1986). Characterization of LimpII, which is also known as LGB85, showed that it possesses a transmembrane domain near the C-terminus, that together with the uncleaved N-terminal signal peptide anchors the protein to the membrane through two distant segments (Eskelinen et al., 2003; Vega et al., 1991b). Mutational analysis indicated that the tyrosine-lacking C-terminal tail of LimpII is sufficient to target the protein directly from the Golgi to lysosomes (Vega *et al.*, 1991a). Further analysis identified a di-leucine-based sorting motif of the Leu-Ile type at position 18 and 19 in the 21 amino acid long C-terminal cytoplasmic domain and its position relative to the membrane to be responsible for proper targeting of LimpII to lysosomes (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994). The implication of AP-3 in LimpII traffic was first shown by the fact that the di-leucine-based sorting motif specifically interacts with AP-3 but not with AP-1 or AP-2. In addition, the AP-3 interaction was shown to be modulated by Asp-Glu, two acidic amino acids in the cytoplasmic domain of LimpII at position 13 and 14 respectively (Honing *et al.*, 1998). In addition, overexpression of LimpII specifically recruited AP-3 to Golgi membranes and partial inhibition of AP-3 synthesis was shown to interfered with LimpII sorting to lysosomes *in vivo* (Le Borgne *et al.*, 1998). Based on these findings, the 21 amino acid long C-terminal

cytoplasmic domain of LimpII (LimpII wt) was chosen as a model cargo to study selective AP-3 recruitment dependent on a single di-leucine-based sorting motif. The critical Leu-18 and Ile-19 were substituted by Ala (LimpII L18A,I19A) for negative control experiments.

III.2: AP-1A and AP-3 recruitment requires selected transmembrane proteins

To study specific AP coat recruitment onto liposomes *in vitro*, gpI wt, Lamp-1 wt, LimpII wt and liposomes without any peptide on the surface (-cd) were incubated with pig brain cytosol under various conditions. The conditions tested included different incubation temperatures (37°C and 4°C) and incubations with or without the presence of GTP-γS, a non-hydrolysable analog of GTP, which stabilizes small GTPase including Arf1 in their active, membrane-bound conformation (Walker *et al.*, 1992). After the incubation, liposomes were recovered by centrifugation, washed and analyzed for their recruited protein content by SDS-PAGE and Western blotting using specific antibodies against AP-1A, AP-2, AP-3, COP-I, clathrin heavy chain, and Arf1. The amounts of liposome-bound coats were quantified by comparison of the liposome-bound signal with the signal obtained from cytosol representing 1% of input. Figure 2 shows that liposomes without peptide tails (-cd) are able to recruit Arf1, AP-1A, AP-3, clathrin heavy chain, and COP-I in the presence of GTP-γS as previously described (Drake et al., 2000; Spang et al., 1998; Zhu et al., 1999). This recruitment was decreased when the incubations were performed in the absence of GTP-γS or at 4°C. However, when the same experiment was performed with liposomes carrying cytoplasmatic domains of transmembrane proteins, AP-1A and AP-3 recruitment was more than ten times increased and became specific. Thus, gpI wt-containing liposomes recruited AP-1A while little AP-3 binding was observed and liposomes exhibiting Lamp-1 wt or LimpII wt peptides recruited AP-3 but only little AP-1A. The amount of Arf1 recruited onto liposomes exposing cargo molecule cytoplasmic domains increased drastically as well, when compared to liposomes without peptide tails. Interestingly, clathrin was efficiently recruited when AP-1A was bound onto gpI wt-containing liposomes, but poorly recruited when AP-3 was bound onto Lamp-1 wt and LimpII wt-containing liposomes or on liposomes devoid of cytoplasmic domains. Under all conditions, AP-2 and COP-I binding remained negligible. Similar results were obtained when theses experiments were done with Arf-depleted cytosol complemented with recombinant, myristoylated Arf1 (Figure 3). Furthermore, AP-1A, AP-3 and Arf1 binding was prevented by brefeldin A (BFA), which blocks the exchange of GDP for GTP or GTP-γS on Arf1, thereby preventing Arf1 stabilization on membranes (Donaldson *et al.*, 1992; Helms and Rothman, 1992). These experiments demonstrate that efficient and selective recruitment of AP-1A and AP-3 onto liposomes requires both selected cargo proteins sorted along AP-1A or AP-3-dependent pathways *in vivo* and the small GTPase Arf1.

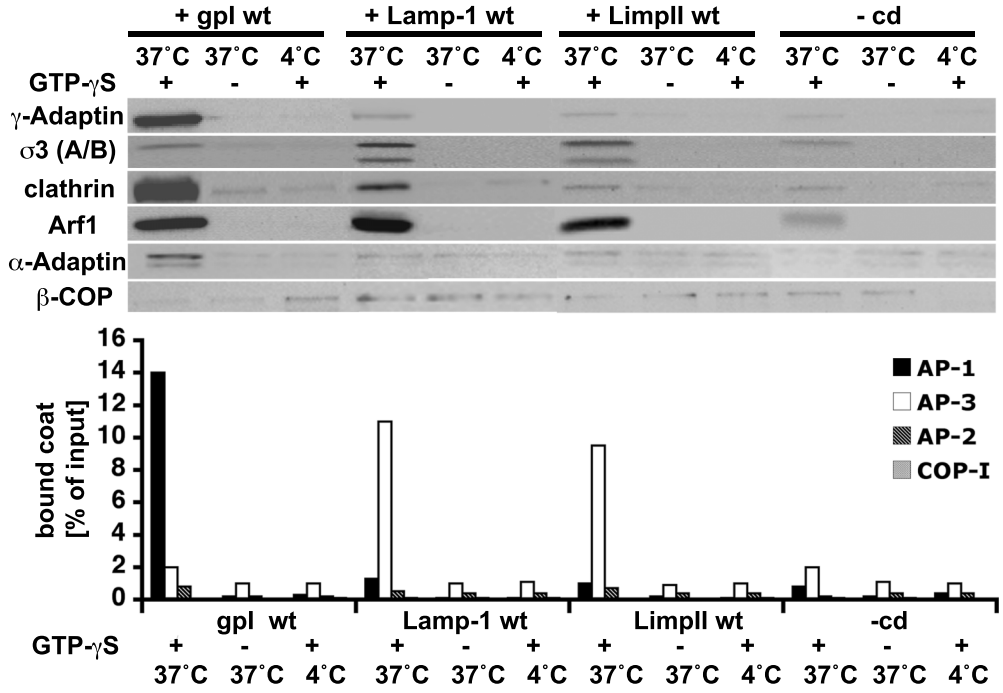

Figure 2: AP-1A and AP-3 recruitment is cargo specific.

Liposomes with gpI wt, Lamp-1 wt, LimpII wt or without cytoplasmic tails (-cd) were incubated with cytosol in the presence of GTP or GTP-gS at 37°C or at 4°C. After the incubation, AP-1A, AP-2, AP-3, COP-I, clathrin heavy chain, and ARF1 bound to liposomes were detected after SDS-PAGE and Western blotting using specific antibodies. AP-1A, AP-3, AP-2, and COP-I signals were quantified.

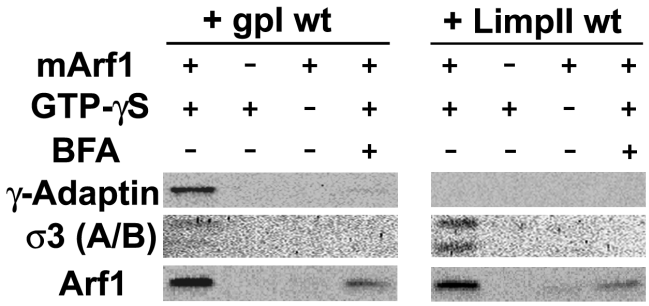

Figure 3: AP-1A and AP-3 recruitment is Arf1 dependent.

Liposomes with gpI wt or LimpII wt cytoplasmic domains were incubated with Arf depleted cytosol [3mg/ml] and supplemented or not with recombinant, myristoylated Arf1 [30µg/ml] with or without GTP-γS and with or without brefeldin A [100µg/ml]. AP-1A, AP-3 and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting.

III.3: AP-1A and AP-3 recruitment requires intact sorting signals

Accurate gpI trafficking *in vivo* requires tyrosine-based sorting signals and an acidic cluster in its cytoplasmic domain (Alconada *et al.*, 1996). The *in vivo* targeting of Lamp-1 (Hunziker et al., 1991; Williams and Fukuda, 1990) and LimpII (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994) relies on single tyrosine or dileucine-based sorting signals, respectively. To monitor whether these sorting signals were important for efficient and selective AP-1A and AP-3 coat recruitment, the key residues of the sorting motifs in the gpI, Lamp-1 and LimpII cytoplasmic domains were replaced by alanine and the acidic cluster of gpI was truncated, leading to peptides devoid of any sorting motifs. Figure 4 shows that AP-1A was no longer recruited on liposomes when all sorting motifs of the gpI tail were absent. Similarly, AP-3 was no longer efficiently recruited on liposomes with Lamp-1 and LimpII cytoplasmic domains containing mutated sorting signals. As shown before, AP-2 and COP-I were not significantly recruited on the different liposomes. Thus, the efficient and specific recruitment of AP-1A and AP-3 onto liposomes is dependent on intact sorting signals in the cytoplasmic domain of selected cargo proteins.

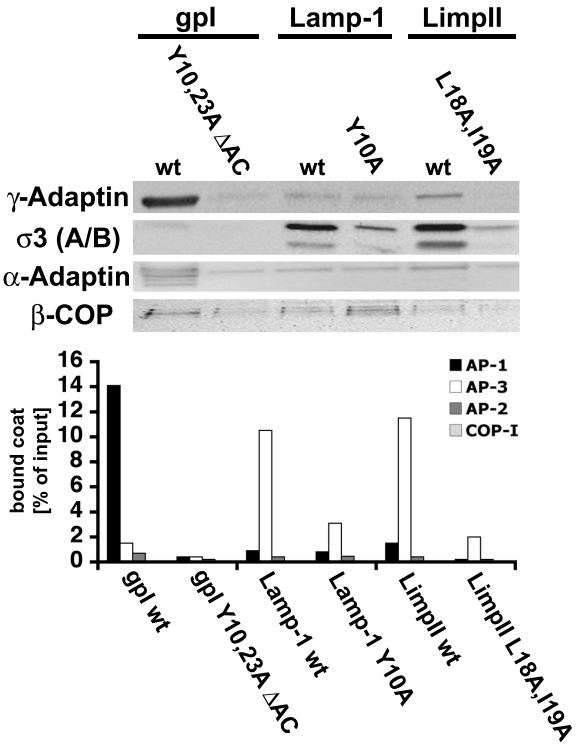

Figure 4: AP-1A and AP-3 recruitment requires intact sorting signals

Liposomes with wild type or mutated cytoplasmic domains of gpI, Lamp-1, and LimpII were incubated with cytosol under standard conditions in the presence of GTP-γS. AP-1A, AP-2, AP-3, and COP-I bound to liposomes were detected after SDS-PAGE and Western blotting using specific antibodies. AP-1A, AP-3, AP-2, and COP-I signals were quantified.

III.4: AP-1A recruitment onto liposomes mainly depends on acidic clusters

The implication of the tyrosine-based sorting motifs and the acidic cluster in the gpI cytoplasmic domain and especially their impact on AP-1A recruitment was further analyzed. Therefore, liposomes exhibiting gpI cytoplasmic domains with single deletions of the tyrosine-based sorting motifs or truncation of the acidic cluster as well as wild type and negative control gpI cytoplasmic domains were tested for their ability to recruit AP-1A. As shown in Figure 5, AP-1A recruitment onto liposomes was only partially inhibited by gpI tails mutated on a single tyrosine-based sorting motif at position Tyr-10 or Tyr-23, 10% and 40% reduction respectively. However, when the acidic cluster was absent, AP-1A recruitment was drastically reduced by 90%, giving similar values as those obtained with gpI tails devoid of any trafficking signals or liposomes without any peptide tail (-cd). Interestingly, gpI cytoplasmic domains do not only influence AP-1A recruitment but also that of Arf1. Arf1 was drastically increased in the presence of intact sorting signals in the gpI cytoplasmic domain, in particular acidic clusters. These results highlight the importance of acidic clusters in AP-1A recruitment and Arf1 stabilization on liposomes exposing gpI cargo tails.

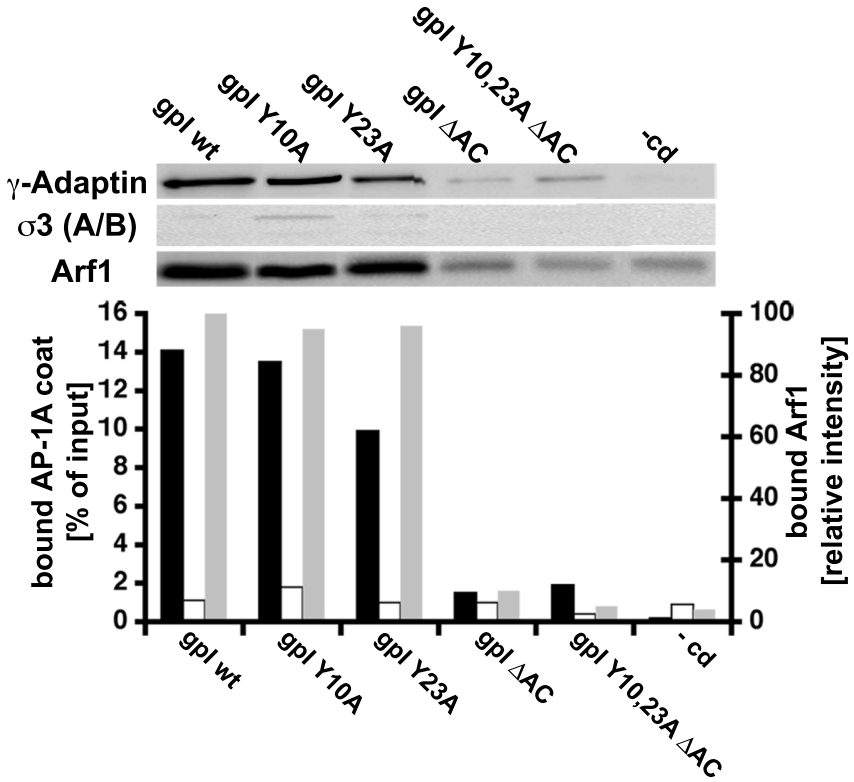

Figure 5: Role of sorting motifs in AP-1A coat recruitment and Arf1 stabilization.

Liposomes with wild type or mutated (Y10A, Y23A, ΔAC, Y10,23A ΔAC) gpI cytoplasmatic domains or without any peptide tails (-cd) were incubated under standard conditions in the presence of GTP-γS. AP-1A, AP-3, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting using specific antibodies. AP-1A (black bars), AP-3 (white bars), and Arf1 (grey bars) signals were quantified.

III.5: Phosphorylation of the gpI acidic cluster enhances AP-1A recruitment

The acidic cluster in the cytoplasmic domain of gpI contains a typical phosphorylation site for caseine kinase 2 (CK2) (Yao *et al.*, 1993). After phosphorylation of two serine residues in the acidic cluster of gpI, the sorting motif is recognized by PACS-1 (Wan *et al.*, 1998), an AP-1 accessory protein, thereby increasing the indirect interaction of AP-1 with its cargo molecule ensuring an accurate sorting (Crump *et al.*, 2001). To monitor the influence of a phosphorylated gpI acidic cluster on AP-1A recruitment, gpI cytoplasmic domain-containing liposomes were phosphorylated *in vitro* using recombinant CK2 before the incubation with cytosol and GTP-γS. Figure 6 shows that AP-1A binding increased by 40% when the gpI wt tail was phosphorylated. This phosphorylation dependent increase of AP-1A binding was still present on liposomes with a mutated tyrosine-based sorting motif. No significant difference in AP-1A binding was observed when liposomes exhibited gpI cytoplasmic domains lacking the acidic cluster. This result leads to the conclusion that the phosphorylation of serine residues in the acidic cluster of gpI is among the determinants required for high affinity binding of AP-1A.

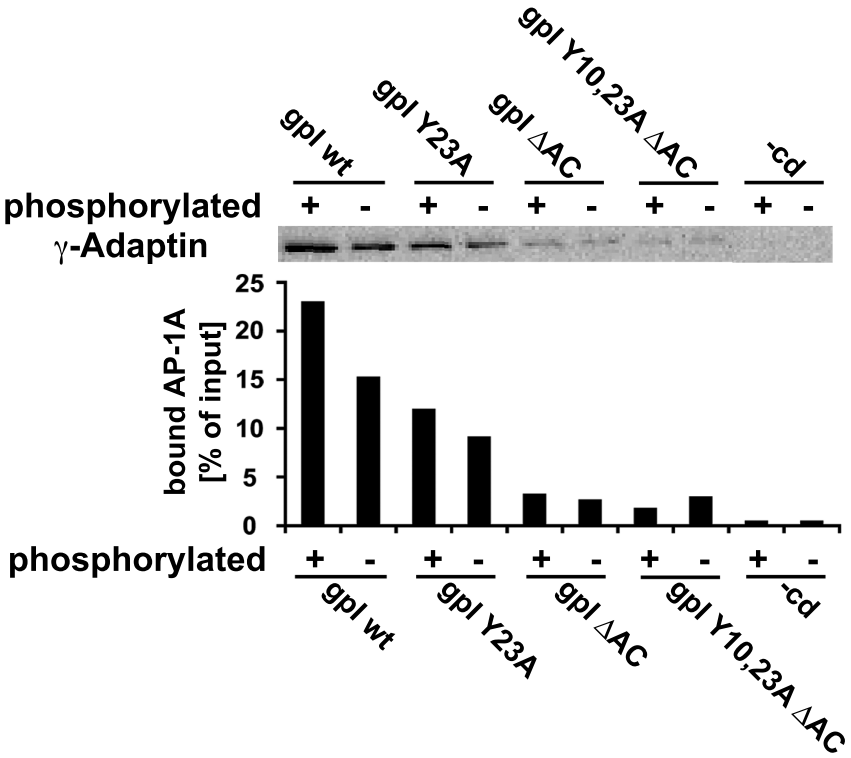

Figure 6: AP-1A recruitment is enhanced by acidic cluster phosphorylation.

Liposomes with wild type or mutated (Y10A, Y23A, ΔAC, Y10,23A ΔAC) gpI cytoplasmatic domains or without any peptide tails (-cd) were phosphorylated or not by caseine kinase 2 before being incubated with cytosol in the presence of GTP-γS. AP-1A bound to liposomes was detected after SDS-PAGE and Western blotting and then quantified.

III.6: AP-1A and AP-3 recruitment is saturable: implication for clathrin recruitment

To show that the numbers of binding sites for AP-1A and AP-3 at the surface of liposomes is saturable, as expected for specific biochemical binding and recruitment reactions, liposomes were incubated with cytosol of increasing concentrations. Maximal AP-1A binding onto liposomes with gpI wt domains was reached at cytosol concentrations of around 10mg/ml, while AP-3 or COP-I binding to these liposomes remained mainly unaffected at low signal intensities (Figure 7). As expected, the signals for clathrin and Arf1 reached as well a saturation plateau around 10mg/ml. Similarly maximal AP-3 and Arf-1 binding onto liposomes with Lamp-1 wt domains was reached at concentrations around 10mg/ml of cytosol while AP-1A or COP-I signals stayed at background values (Figure 8). Clathrin binding to Lamp-1 wt liposomes was by factor 5 less efficient, even at high cytosol concentrations when compared to clathrin recruitment onto gpI wt liposomes. This might reflect the role of clathrin in AP-3 mediated pathways and their partial colocalization *in vivo*, when compared to the strong clathrin signal in the context of AP-1A. As expected, all specific interactions were not observed when GTP-γS was absent.

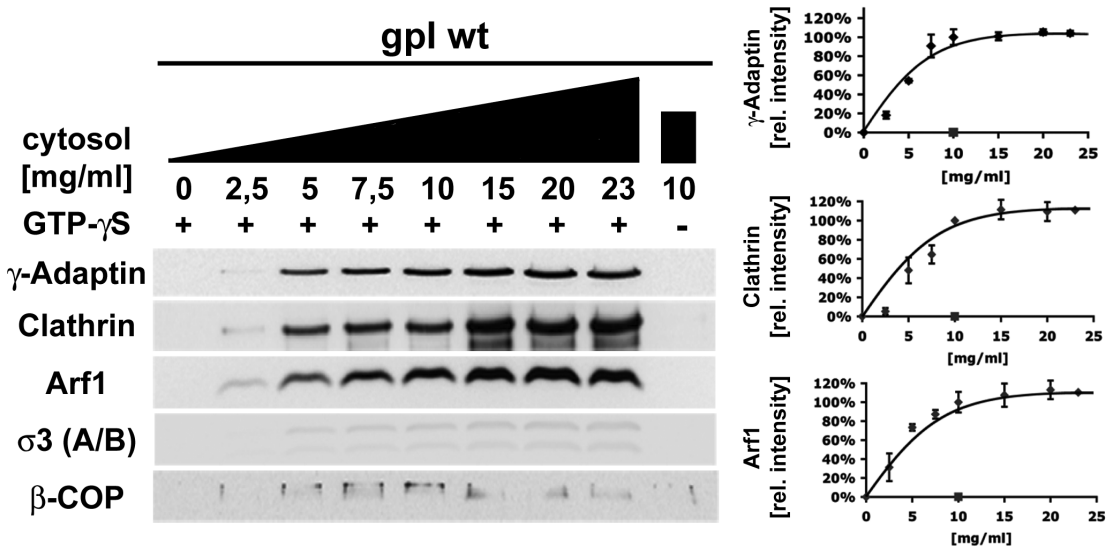

Figure 7: AP-1A binding sites on liposomes are saturable.

Liposomes with gpI wt cytoplasmic domains were incubated with (◆) or without (■) GTP-γS and increasing concentrations of cytosol. AP-1A, AP-3, β-COP, clathrin heavy chain, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-1A, clathrin heavy chain and Arf1 were quantified.

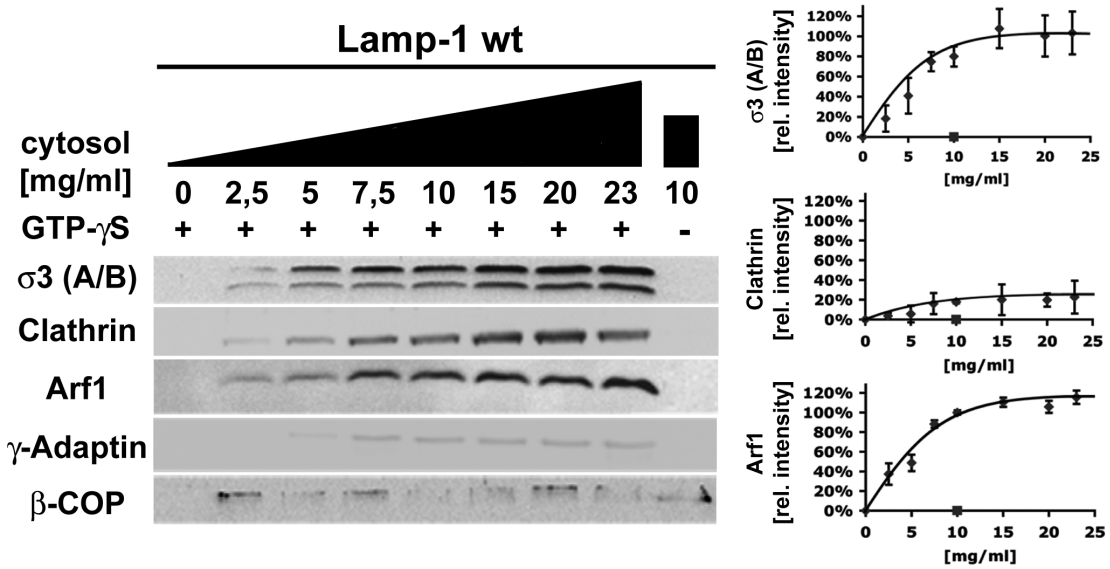

Figure 8: AP-3 binding sites on liposomes are saturable.

Liposomes with Lamp-1 wt cytoplasmic domains were incubated with (◆) or without (■) GTP-γS and increasing concentrations of cytosol. AP-1A, AP-3, β-COP, clathrin heavy chain, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-3, clathrin heavy chain and Arf1 were quantified. The quantification of clathrin is relative to the maximal signal obtained with gpI wt containing liposomes (Figure 7) assigned as 100%.

III.7: Arf1 recruitment is influenced by cargo molecules

While studying the *in vitro* assay, an interesting observation was made: the cytoplasmic domains of cargo molecules do not only influence AP recruitment but also seem to stabilize Arf1 on membranes. The observation that intact gpI sorting signals, especially the presence of acidic clusters, increase the amount of Arf1 on liposomes was already detected in Figure 2 and Figure 5. The titration of gpI wt cytoplasmic domains indicates that this effect is clearly dependent on the presence of the cargo tail containing intact sorting signals (Figure 9). Under the conditions used, the specific recruitment of Arf1 and AP-1A were saturable by increasing concentrations of cytoplasmic domains on the surface of liposomes. This effect was probably due to a complete coverage of the liposome surface area by AP coats, rather than a limited amount of AP coats in the cytosol. Arf1 and AP-1A were selectively recruited onto liposomes exposing increasing concentrations of gpI wt cytoplasmic domains on their surface. Maximal binding was reached at peptide concentrations around 500nM of peptide (soluble peptides added during peptide ligation). Under peptide saturation conditions, around 15% of the cytosolic AP-1A was recruited onto gpI wt containing liposomes. Clathrin was also recruited in similar, saturating manner. In agreement with the results described above, the recruitment of AP-2, AP-3, and COP-I were not significant, even at high gpI wt peptide concentrations and Arf1 and AP-1A were not recruited onto liposomes without cytoplasmic domains or liposomes with gpI mutant peptides devoid of any trafficking signals.

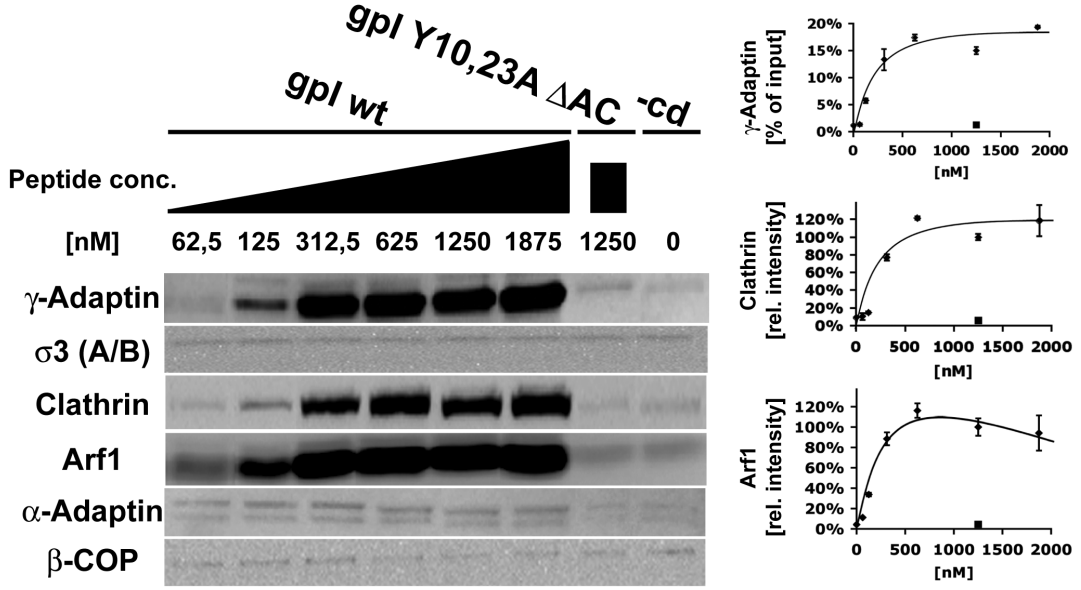

Figure 9: Saturation of AP-1A recruitment onto liposomes with increasing amounts of gpI peptide tails.

Liposomes with increasing amounts of gpI wt (◆), gpI mutant devoid of trafficking signals, gpI Y10,23A ΔAC (■), or no peptide tail (-cd) (◆) were incubated with cytosol in the presence of GTP-γS. AP-1A, AP-2, AP-3, COP-I, clathrin heavy chain, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-1A, clathrin heavy chain, and Arf1 binding was quantified.

Similarly, AP-3 was recruited onto liposomes containing LimpII wt cytoplasmic domains and reached maximal binding at peptide concentrations around 750nM (Figure 10). AP-3 recruitment stayed around 12% of cytosolic input under saturation conditions of LimpII wt peptides. The effect of increasing LimpII wt cytoplasmic domains on Arf1 stabilization was as well detectable as already shown in Figure 2. However, Arf1 recruitment was not as strong as in the context of AP-1A, maybe highlighting the role of different sorting motifs in Arf1 stabilization on membranes. Nevertheless, the presence of cargo tails containing intact sorting signals is increasing the amount of Arf1 recruited onto liposomes, even in the context of dileucine-based sorting signals. No specific AP-3 and Arf1 recruitment was observed onto liposomes containing LimpII domains lacking the dileucine-based sorting motif or liposomes without cytoplasmic domains. As expected, AP-1A, AP-2, and COP-I recruitment remained at background values. Clathrin recruitment was not remarkably enhanced, despite the peptide-dependent recruitment of AP-3 and was around 20% when compared to maximal clathrin recruitment together with AP-1A onto gpI wt containing liposomes.

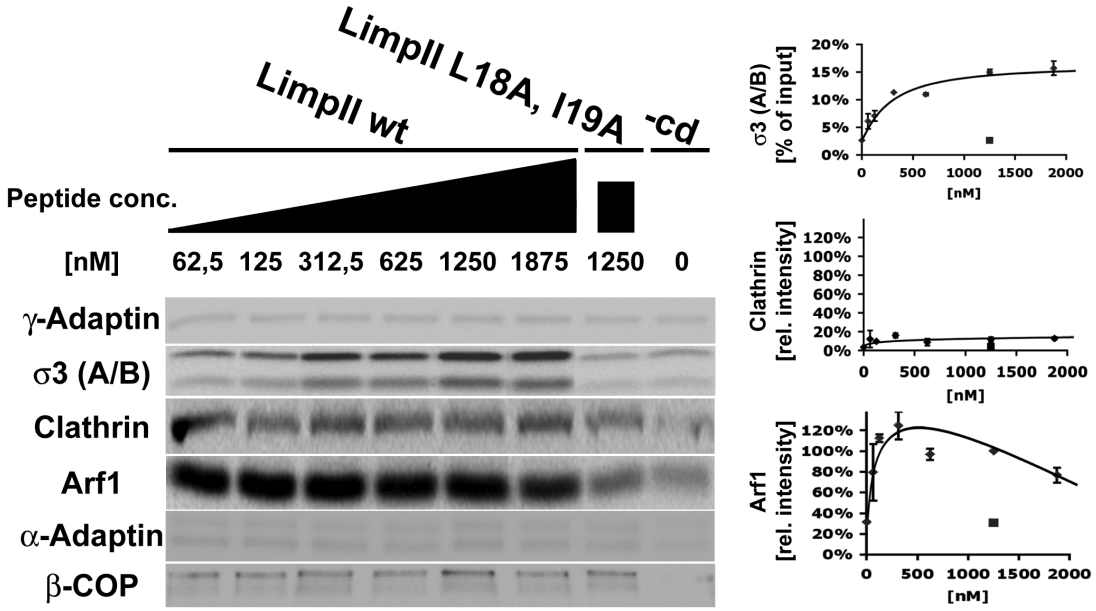

Figure 10: Saturation of AP-3 recruitment onto liposomes with increasing amounts of LimpII peptide tails.

Liposomes with increasing amounts of LimpII wt (◆), LimpII mutant devoid of trafficking signals, LimpII L18A,I19A (■), or no peptide tail (-cd) (◆) were incubated with cytosol in the presence of GTP-γS. AP-1A, AP-2, AP-3, COP-I, clathrin heavy chain, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-3, clathrin heavy chain, and Arf1 binding was quantified. The quantification of clathrin is relative to the maximal signal obtained with gpI wt containing liposomes (Figure 9) assigned as 100%.

III.8: Role of phosphatidylinositolphosphates in AP-coat recruitment

Phosphatidylinositolphosphates (PIPs) have been shown to play an important role in the regulation of membrane traffic and protein sorting (Cremona and De Camilli, 2001; De Camilli et al., 1996; De Matteis and Godi, 2004; Zerial and McBride, 2001). To investigate the role of PIPs in the context of AP-1A and AP-3 dependent trafficking pathways, gpI wt and LimpII wt containing liposomes were analyzed for their capability of recruiting AP-coats in the presence of different PIPs. For these experiments, liposomes were incubated in the presence of phosphatase inhibitors with sub-limiting concentrations of cytosol so that changes in the avidity of AP-1A or AP-3 for their binding sites could be evaluated by measuring an increase in AP-1A or AP-3 recruitment onto liposomes. As Figure 11 shows, two PIPs, namely PI-4P and PI-3P, had selective effects on AP-1A and AP-3 recruitment respectively. The other PIPs tested showed no significant influence.

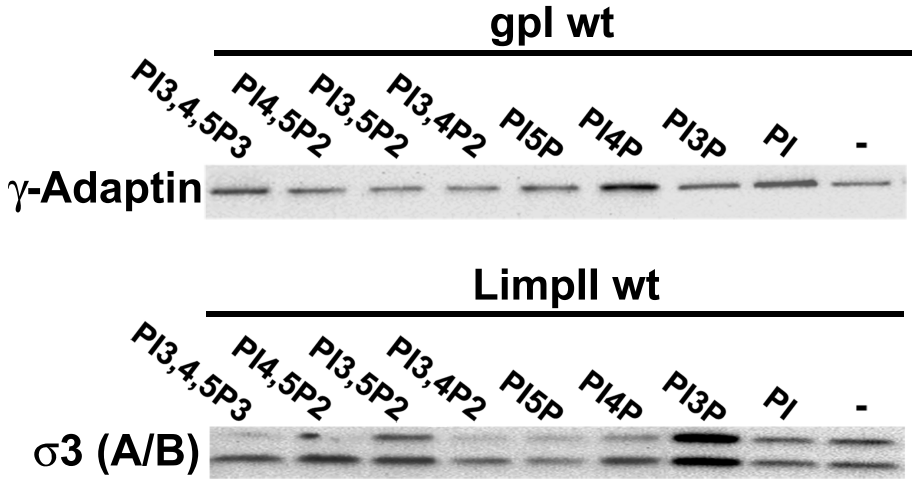

Figure 11: Effects of PIPs on AP-1A or AP-3 recruitment.

Liposomes with gpI wt or LimpII wt cytoplasmic domains containing different or no PIPs were incubated with cytosol [3mg/ml] and GTP-γS. AP-1A and AP-3 bound to liposomes were detected after SDS-PAGE and Western blotting.

Figure 12 show that AP-1A binding was two times increased when PI-4P was present in gpI wt liposomes, whereas AP-2, AP-3 and COP-I remained at background values. Such an increase was not observed when liposomes without cytoplasmic domains were used. In contrast, AP-3 recruitment was two fold increased, when PI-3P was present in LimpII wt liposomes, whereas that of AP-1A, AP-2 and COP-I remained unaffected (Figure 13). It should be mentioned that PI-3P has by itself a significant effect on AP-3 recruitment. These results show that the presence of specific PIPs, PI-4P and PI-3P respectively, induces a two-fold increase in AP-1A or AP-3 recruitment onto liposomes carrying specific cytoplasmic

domains of cargo proteins. Under optimal conditions, around 30% of the cytosolic pool of AP-1A or AP-3 were recruited onto liposomes. Thus, PIPs are providing additional specific binding sites for AP complexes beside cargo molecules, thereby providing an additional determinant for specific AP recruitment onto membranes.

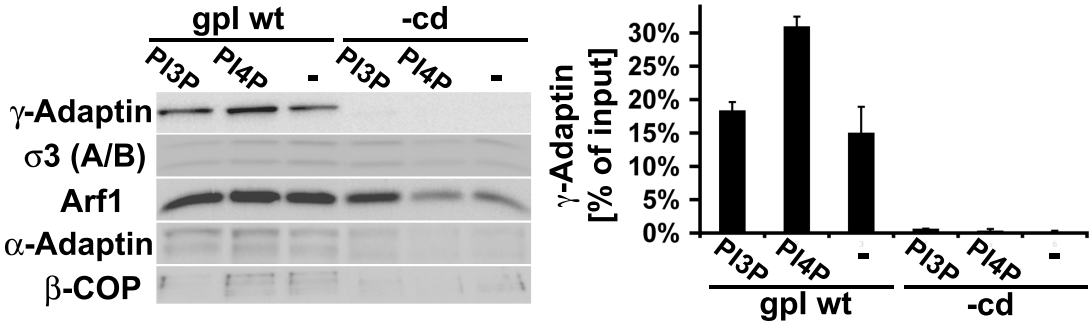

Figure 12: PI-4P increases AP-1A recruitment onto gpI wt containing liposomes.

GpI wt or no cytoplasmic domain exhibiting liposomes either containing PI-3P, PI-4P, or no PIP were incubated with cytosol [3mg/ml] in the presence of GTP-γS and phosphatase inhibitors. AP-1A, AP-2, AP-3, COP-I, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-1A binding was quantified.

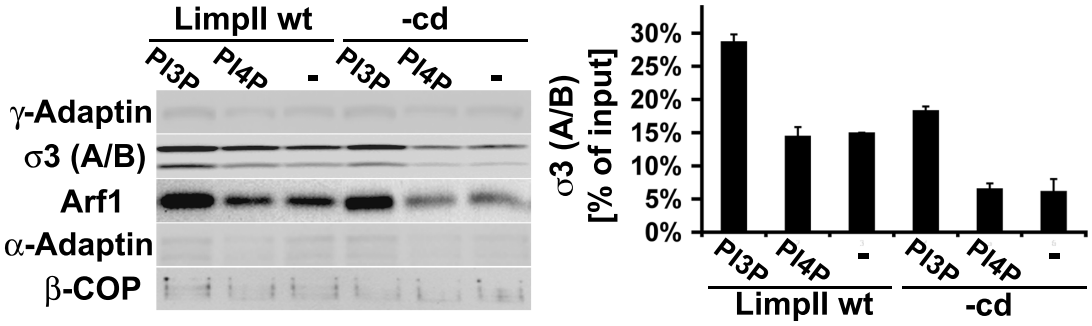

Figure 13: PI-3P increases AP-3 recruitment onto LimpII wt containing liposomes.

LimpII wt or no cytoplasmic domain exhibiting liposomes either containing PI-3P, PI-4P, or no PIP were incubated with desalted cytosol [3mg/ml] in the presence of GTP-γS and phosphatase inhibitors. AP-1A, AP-2, AP-3, COP-I, and Arf1 bound to liposomes were detected after SDS-PAGE and Western blotting. AP-3 binding was quantified.

III.9: PI-3P is required for proper sorting of AP-3 dependent cargo *in vivo*

Since PI-4P is regulating AP-1 recruitment (Wang *et al.*, 2003) and PI-(4,5)P_2 is involved in AP-2 recruitment (Honing *et al.*, 2005) *in vivo*, it was important to illustrate the role of PI-3P in AP-3-dependent traffic *in vivo*. Electron and fluorescence microscopy using a PI-3P specific probe have located PI-3P to the limiting and intralumenal membranes of endosomes (Gaullier *et al.*, 2000), thus PI-3P localization is similar to the endosomal localization of AP-3 (Peden *et al.*, 2004). To analyze the implication of PI-3P in AP-3-dependent protein sorting *in vivo*, a Lamp missorting assay was used to detect whether Lamp was misrouted to the plasma membrane while manipulating the PI-3P pool *in vivo* (Le Borgne *et al.*, 1998). Thus, a Lamp-1 antibody was added to the cell culture medium, which is internalized only when Lamp-1 is missorted to the plasma membrane. In a first experiment, the classical PI-3 kinase inhibitor wortmannin was used to prevent PI-3P-production in HeLa cells. Figure 14 shows that 100nM wortmannin induced an increased plasma membrane appearance of Lamp-1 resulting in an increased anti-Lamp-1 antibody uptake. Internalized anti-Lamp-1 antibody signals mainly localized to swollen vacuolar like structures, probably enlarged endosomes as expected for wortmannin treatment (Reaves *et al.*, 1996).

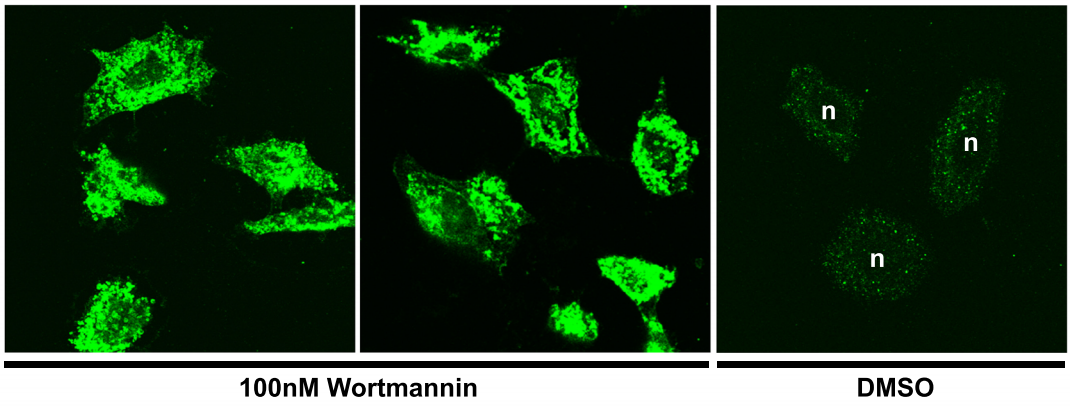

Figure 14: Anit-Lamp-1 antibody uptake after wortmannin treatment of HeLa cells.

HeLa cells were treated with 100nM wortmannin or only the buffer for 2 hours in media containing an anti-Lamp-1 antibody. The cells were then processed for confocal microscopy and the endocytosed anti-Lamp-1 antibody was detected. (n: nucleus)

In a second approach, the AP-3 dependent pathway was disturbed by overexpression of the PI-3P-specific probe 2xFYVE (Gillooly *et al.*, 2000), thus competing out other PI-3P binders. The overexpression resulted in an increased anti-Lamp-1 antibody uptake in HeLa cells when compared to non-transfected cells, thus in an increased missorting of the AP-3 cargo Lamp-1 to the plasma membrane (Figure 15). Both results indicate an important role for PI-3P in proper Lamp-1 traffic along an AP-3-dependent pathway to the lysosome *in vivo*.

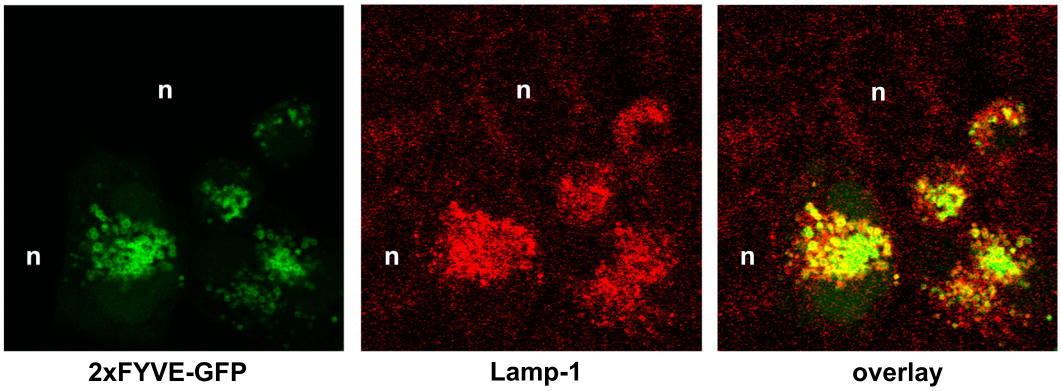

Figure 15: Anti-Lamp-1 antibody uptake after overexpression of 2xFYVE-GFP.

Hela cells were transfected with a 2xFYVE-GFP construct, after 24 hours an anti-Lamp-1 antibody was added to the cell culture medium. 3 hours later the cells were processed for confocal microscopy and the endocytosed anti-Lamp-1 antibody was detected. (n: nucleus of a non transfected cell)

III.10: Morphology of coated liposomes

AP-1A, AP-3 and clathrin assemble *in vivo* as organized structures (Kirchhausen, 2000; Owen *et al.*, 2004). Therefore, the liposomes were examined by electron microscopy. Most of the gpI wt exposing liposomes were coated and showed large clathrin lattices with typical pentagonal and hexagonal structures of clathrin coats, as well as typical spike like structures or profiles of still attached clathrin-coated buds with a diameter of 80 – 100nm (Figure 16).

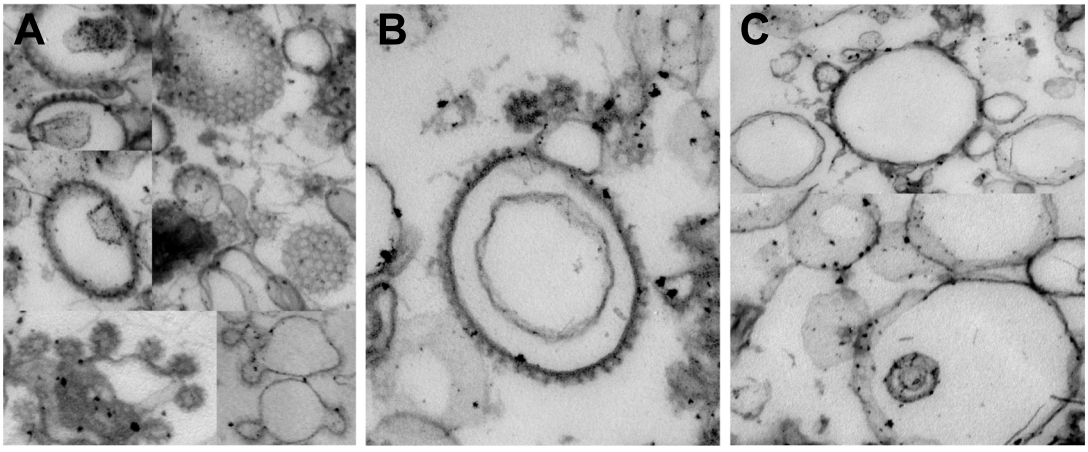

Figure 16: Morphology of gpI liposomes.

Panel A: Liposomes with gpI wt tails obtained under standard conditions were fixed and processed for electron microscopy. Magnification: 44k. Panel B: Same as Panel A. Magnification: 71k. Panel C: Liposomes with gpI tails devoid of any sorting motifs obtained under standard conditions were fixed and processed for electron microscopy. Magnification: 44k.

Isolated vesicles were occasionally observed, depending on the sections examined. However, due to the use of GTP-γS, a complete pinching of the vesicles from the synthetic liposomes should be prevented, since GTP-γS prevents GTP hydrolysis by the pinchase dynamin (Praefcke and McMahon, 2004). Blocking the release of clathrin-coated vesicles induced in some cases a total collapse of gpI wt liposomes, showing several clathrin-coated buds on the collapsed liposome. In contrast, such clathrin-coated budding profiles or clathrin lattices were not observed on liposomes exposing gpI cytoplasmic domains devoid of any sorting signals or on liposomes without any cytoplasmic domains or on liposomes, which were incubated in the absence of GTP-γS. Thus, clathrin is efficiently recruited onto AP-1A-coated liposomes, in agreement with the biochemical data described above. In sections of Lamp-1 wt liposomes, such typical clathrin-coated structures were rarely detected. Clathrin-coated lattices were not detected at all, but around 5% of the Lamp-1 wt liposomes showed spike like structures and profiles of typical clathrin-coated buds (Figure 17). No electron dense coat was detected on liposomes without any cytoplasmic domains or on liposomes containing Lamp-1 cytoplasmic domains devoid of sorting signals. These results further illustrate that clathrin is efficiently recruited onto liposomes that allow efficient AP-1A binding but is poorly recruited on those that allow an efficient AP-3 binding.

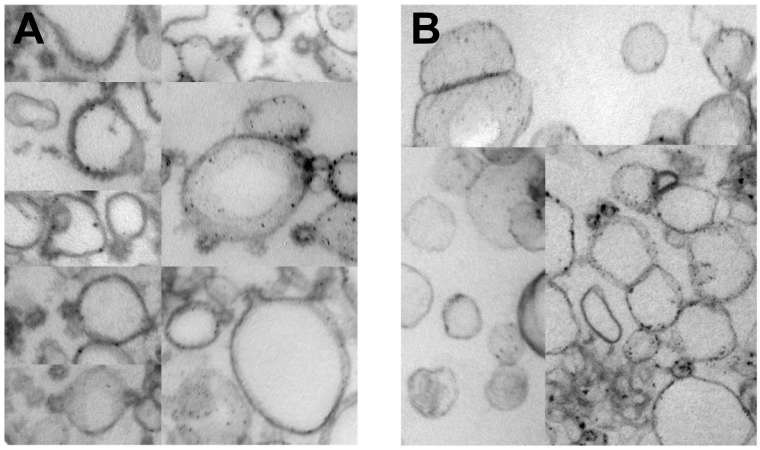

Figure 17: Morphology of Lamp-1 liposomes.

Panel A: Liposomes with Lamp-1 wt tails obtained under standard conditions were fixed and processed for electron microscopy. Magnification: 44k. Panel B: Liposomes without any peptide tails obtained under standard conditions were fixed and processed for electron microscopy. Magnification: 44k.

III.11: Proteomic analysis of AP-1A coated liposomes

The protein content of AP-1A-coated liposomes was analyzed by a 7% and a 15% SDS-PAGE in parallel to get optimal resolution in the high and low molecular weight range with a maximal separation length. The analysis revealed that several proteins were specifically recruited concomitant to the AP-1A complex onto liposomes containing PI-4P and exhibiting gpI wt cytoplasmic domains. The major bands, indicated with numbers on the scanned protein intensity profile, were identified by MALDI-TOF/TOF mass spectrometry (Figure 18).

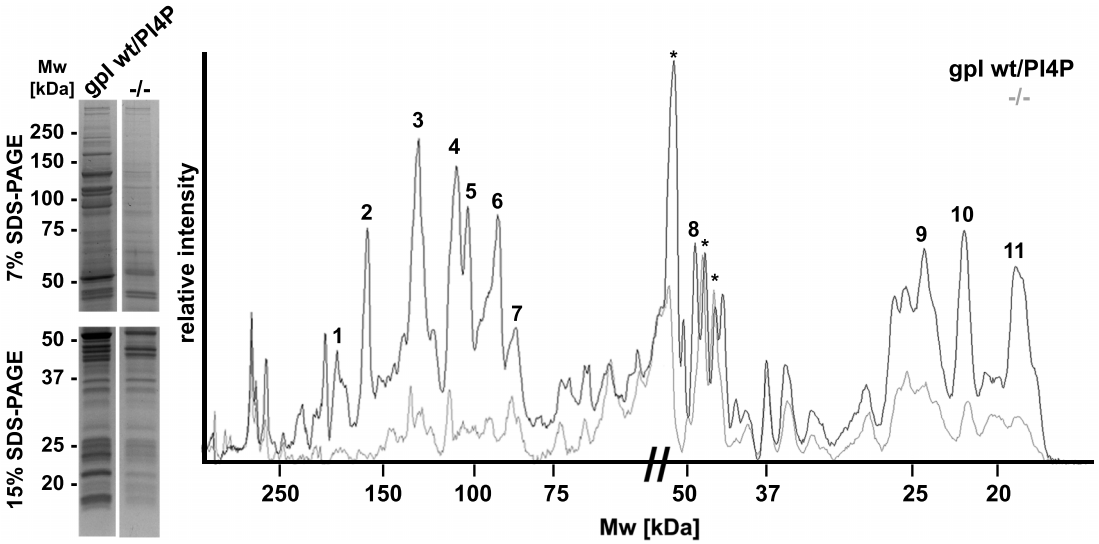

Figure 18: Protein profile of AP-1A-coated liposomes.

Liposomes containing gpI wt cytoplasmic domains and PI-4P or liposomes without cytoplasmic domains and without PI-4P (-/-) were incubated with cytosol and GTP-γS, then purified by floatation on a density gradient and analyzed on 7% and 15% SDS-PAGE. The peak numbers on the reconstituted protein profiles indicate the major proteins identified by MALDI-TOF/TOF mass spectrometry. The asterisks represent major contaminants like glutamate dehydrogenase, tubulin and actin.

In addition, the lanes were cut into 50 slices, which were then analyzed by more sensitive LC-MS/MS to detect minor recruited proteins. Both analyses identified around 40 different proteins recruited along with the AP-1A complex (Table II). The proteins were classified into three major groups. First, coat components i.e. clathrin heavy and light chains with the uncoating chaperone Hsc70, the four AP-1A complex subunits and γ-synergin, an AP-1A γ-subunit accessory protein (Page *et al.*, 1999), were identified. As well Arf1/Arf3 (Arf1 and Arf3 could not be distinguished by mass spectrometry) with its brefeldin A-inhibited exchange factor 2, also called Big2 (Togawa *et al.*, 1999), and its GTPase activating proteins Git1 and Git2 (Premont *et al.*, 1998; Premont *et al.*, 2000) were detected. Arfaptin 1 and 2, which equally bind to Arf1 and Rac1 were also present (Tarricone *et al.*, 2001). Second, the MS analysis identified a Rac1-dependent actin nucleation module i.e. the different subunits of

the Wave/Scar complex including CYFIP2 (PIR121), Nck-associated protein 1 (HEM-2), Abi-1 and 2 as well as WAVE1 and WAVE3, which activate the ARP2/3 complex (Stradal *et al.*, 2004). Six of the seven ARP2/3 complex subunits were also detected (Millard *et al.*, 2004). Rac1 as well as its exchange factor Rho-GEF7, also known as β-PIX (Manser *et al.*, 1998), and its GTPase activating protein SLIT-ROBO Rho-GAP3, also called Wave-associated Rac-GAP protein (Soderling *et al.*, 2002), was identified. Third, two major Rab GTPases, Rab11B and Rab14 were detected. Finally, two kinases, the lipid modifying PI-4 Kinase IIIβ and the p21-activated serine/threonine kinase PAK3, which is known to interact with the PIX-family of Rho-GEFs (Manser *et al.*, 1998), were recovered on gpI wt/PI-4P-containing liposomes.

Protein	NCBI GI number	Predicted Mr (Da)	LC-MS/MS analysis	MALDI-TOF/TOF analysis			
			No. of sequenced peptides	Mascot score	Matched/total peptides	Sequence coverage	Peak number
Coat components							
Clathrin heavy chain	66773801	191,557	33	317	47/75	38%	2
Clathrin light chain B	62510439	25,172	3				
Clathrin light chain A	2493731	25,557	3				
Hsc70	123648	70,871	9				
AP-1 β1	21541948	104,540	26	215	28/52	36%	5
AP-1 γ1	113349	91,202	21	203	27/47	43%	6
AP-1 μ1	543817	48,512	18	161	22/60	53%	8
AP-1 σ1B	21541960	18,917	4				
AP-1 σ1A	48428720	18,721	5				
AP-1 γ-subunit binding protein 1 (γ-synergin)	34996507	122,005		100	16/37	18%	
ARF-1/ARF-3	51316986	20,553	9	75	9/39	56%	11
	47117658	20,457					
ARF-GEF 2 (brefeldin A-inhibited) (BIG2)	63492672	181,465	8	78	25/81	22%	1
G protein-coupled receptor kinase-interactor 1 (ARF-GAP Git1)	58864889	84,195	11	94	14/48	32%	7
G protein-coupled receptor kinase-interactor 2 (ARF-GAP Git2)	18203126	78,747	9				
Arfaptin 1	63501125	41,518	3				
Arfaptin 2	67460562	37,773	5				
Actin nucleation							
Nck-associated protein 1 (HEM-2;NAP-1)	26986194	128,784	25	69	12/29	14%	4
CYFIP2 (PIR121)	19526988	145,589		388*	46/86	41%	3
Abi-1	50400517	52,255	5				
Abi-2	50400259	49,356	7				
SH3 adapter protein SPIN90 (Nck-interacting protein with SH3 domain)	57015413	78,523	6				
WASP-family protein member 1 (WAVE1)	16877274	61,471	6				
WASP-family protein member 3 (WAVE3)	20071942	55,170	4				
Actin-like protein 2 (ARP2)	47117649	44,761	4				
Actin-like protein 3 (ARP3)	47116573	47,327	4				
ARP 2/3 complex subunit 1A	59797974	41,600	4				
ARP 2/3 complex subunit 2	23621467	34,357	4				
ARP 2/3 complex subunit 4	38372626	19,523	5				
ARP 2/3 complex subunit 3	62899893	20,525	2				
p21-Rac1	51702788	21,436	4	108	5/51	31%	10
Cdc42 homolog	46397379	21,297	3				
SLIT-ROBO Rho-GAP3 (WAVE-associated Rac-GAP; WRP)	48428625	124,341	32	85	18/60	21%	
Rho-GEF 7 (PAK-interacting exchange factor β; β-PIX)	18202873	79,782	13	178	13/20	16%	
PAK 3	47117898	62,358	11				
Lipid modification							
PI-4-Kinase IIIβ	51262078	89,887		78†	7/30	14%	
Membrane fusion							
Rab11B	1172815	24,474	11	86	9/47	54%	9
Rab14	46577103	23,882	10	165	15/47	74%	9
Rab4	15986733	23,539	4	131‡	6/50	38%	

* Combined mascot score: 2 sequenced peptides ([M+H]$^+$ 1420.83 ^{1053}YAPLHLVPLIER^{1064} and 1824.00 ^{477}NTIYAALQDFAQVTLR^{492}).
†Combined mascot score: 1 sequenced peptide ([M+H]$^+$ 1187.63 ^{482}EPVFIAAGDIR^{492}).
‡Combined mascot score: 1 sequenced peptide ([M+H]$^+$ 1406.75 ^{80}GAAGALLVYDITSR^{93}).

Table II: Mass spectrometric analysis of AP-1A coated liposomes.

Data sets of MALDI-TOF/TOF analysis and LC-MS/MS analysis of AP-1A coated liposomes. Peak numbers refer to Figure 18.

The proteomic data suggesting a connection between AP-1A coats and the Wave/Scar complex involved in actin nucleation is further supported by the colocalization of CI-MPR-GFP, a major AP-1A cargo (Le Borgne *et al.*, 1996) and CYFIP2-myc in the perinuclear region of transfected HeLa cells (Figure 19). The myc signal was detected in tubular structures of the perinuclear region, probably resembling the Golgi as well as tubules in the

cell periphery. The MPR signal shows as expected a tubular perinuclear staining of the Golgi as well as punctuate structures in the cell periphery.

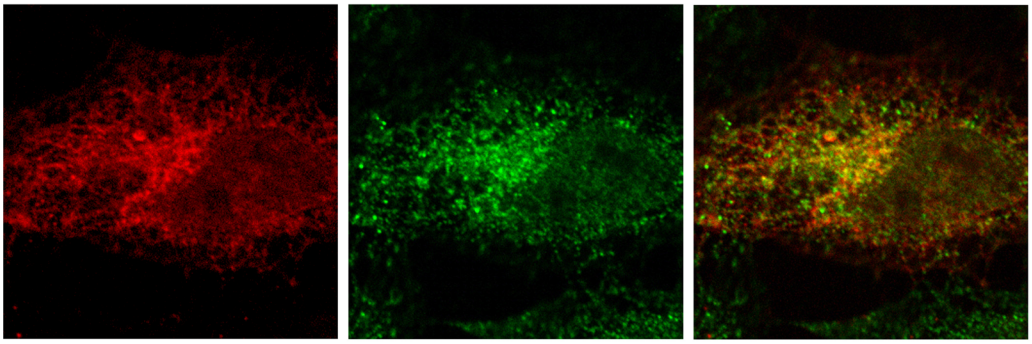

Figure 19: Localization of CYFIP2 and CI-MPR in the perinuclear region of HeLa cells.

HeLa cells stably expressing CI-MPR-GFP were transfected with a CYFIP2-myc construct. 48 hours after the transfection, the cells were fixed and processed for confocal microscopy.

III.12: Stoichiometry of the proteins recruited onto AP-1A coated liposomes

An estimation of the stoichiometry based on the intensity of the Coomassie blue staining of the protein bands indicates that 1 clathrin heavy chain, 1 AP-1A complex, and 1 Wave/Scar complex were found together with roughly 3 Arf1, 3 Rac1, and 3 Rab11B/Rab14 GTPases (Figure 20). The different GTPase effectors were found in sub-stoichiometric amounts, however among them, the Arf1 effectors were the most prominent. Thus, machineries controlling AP-1A coat formation, actin nucleation and membrane fusion are selectively recruited onto gpI wt/PI-4P-containing liposomes. The efficient and specific recruitment of the different protein machineries was drastically reduced on liposomes devoid of gpI wt tails and PI-4P. Contamination by AP-2 was estimated to be less than 5% in Coomassie staining when compared to AP-1A, based on comparing the AP-2 α-subunit with the AP-1A γ-subunit. The contamination by other coats was below this value.

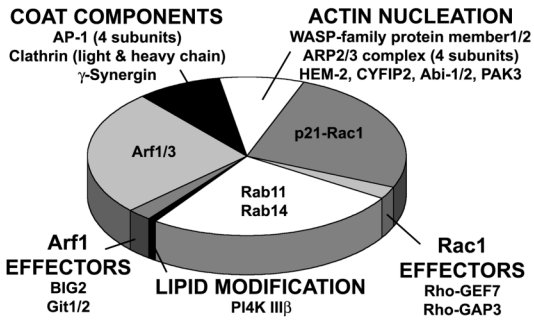

Figure 20: Stoichiometric representation of the proteins recruited together with AP-1A.

The surface areas on the diagram reflect an estimated stoichiometry between the different protein complexes and GTPases recruited onto gpI wt/PI-4P-containing liposomes obtained after Coomassie blue staining. The AP-1 γ-subunit and CYFIP2 were used as markers of the AP-1A complex and the Wave/Scar complex respectively.

III.13: Proteins with unknown function on AP-1A coated liposomes

In addition to the proteins involved in AP-1A coat formation, actin nucleation and membrane fusion, several proteins with thus far unknown functions in these processes were identified. Table III shows some of the proteins, which were grouped into 3 categories: proteins containing GAP activities (GTPase activating RapGAP domain-like 1; KIAA1219), proteins known to be involved in membrane traffic but not related to AP-1A pathways (Protein kinase C, epsilon and gamma type; 14-3-3 protein gamma; cytoplasmic domain of VAM-B) and other proteins (KIAA1576). It needs of course to be evaluated whether these proteins are selectively recruited and if they have a function in AP-1A coat formation.

Other proteins identified on AP-1A coated liposomes	NCBI GI number	Predicted Mr (Da)	LC-MS/MS analysis No. of sequenced peptides
GAP-domain containing			
GTPase activating RapGAP domain-like1	51315849	229,389	16
KIAA1219	45477128	165,200	5
Membrane traffic			
Protein kinase C, epsilon type	125555	83,561	7
Protein kinase C, gamma type	54037693	78,358	5
14-3-3 protein gamma	48428722	28,171	4
Cytoplasmic domain of VAMP-B	24638341	26,815	3*
Other proteins			
Probable oxidoreductase KIAA1576	52783066	45,817	10

* Only peptides of the cytoplasmic part.

Table III: Proteins with unknown function.

List of proteins recruited on AP-1A coated liposomes with thus far unknown function.

III.14: AP-1A coat assembly stabilizes machineries for actin nucleation and membrane fusion

It was shown that gpI cytoplasmic domains containing intact sorting signals, in particular acidic clusters, do not only influence AP-1A recruitment but also induce Arf1 stabilization on liposomes. Therefore, the question remained open as to whether extend the recruitment of the different protein machineries identified by the proteomic screen, are influenced by gpI wt cytoplasmic domains and PI-4P. Figure 21 shows that gpI wt cytoplasmic domains and PI-4P stabilize in addition to AP-1A and Arf1 the membrane association of Rac1, CYFIP2 (taken as a marker of the Wave/Scar complex), Rab11, and PI-4 kinase IIIβ. Although PI-4P alone is able to stabilize to some degree the different components on membranes, the most efficient recruitment was observed in the presence of gpI wt domains in combination with PI-4P. In contrast, AP-3, Rab5, and PI-4 kinase IIα were not recruited. Thus, gpI wt cytoplasmic domains stabilizes not only AP-1A and Arf1, a process enhanced in the presence of PI-4P, but also contributes to the stabilization of machineries required for actin nucleation and membrane fusion.

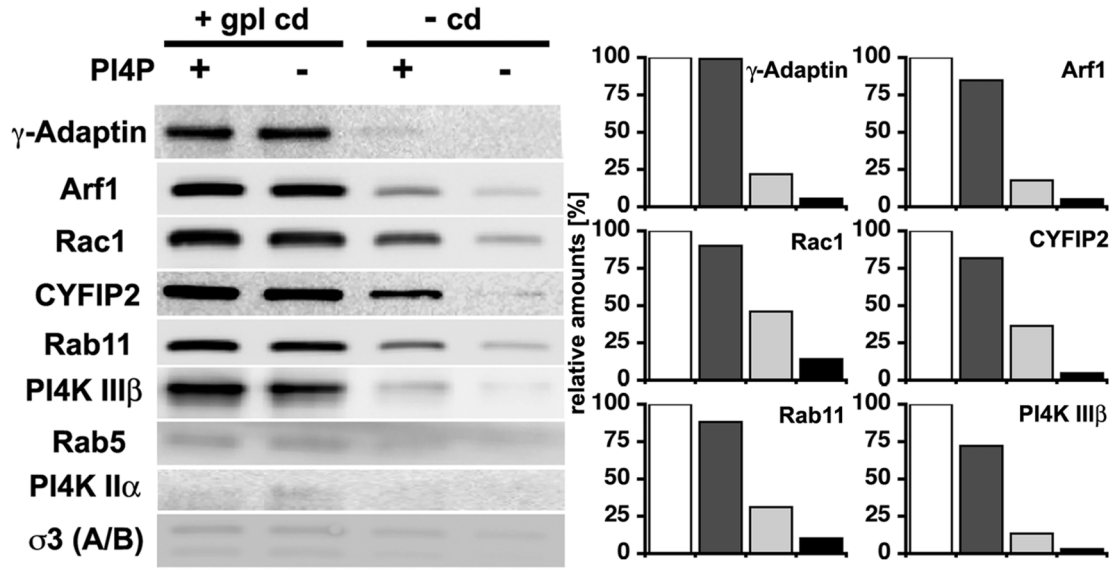

Figure 21: AP-1A coat assembly stabilizes machineries required for actin nucleation and membrane fusion.

Liposomes with or without gpI wt cytoplasmic domains and with or without PI-4P were incubated under standard conditions in the presence of GTP-γS. After incubation and purification by flotation gradients, AP-1A, AP-3, Arf1, Rac1, CYFIP2, Rab11, Rab5, PI-4 Kinase IIIβ, and PI-4 Kinase IIα bound to liposomes were detected after SDS-PAGE and Western blotting. AP-1A, Arf1, Rac1, CYFIP2, Rab11, and PI-4 Kinase IIIβ binding was quantified (white bars: gpI wt and PI-4P; dark grey bars: gpI wt no PI-4P; light grey bars: PI-4P no gpI wt; black bars: no gpI wt and no PI-4P).

III.15: Bioinformatic analysis of protein networks involved in AP-1A coat formation

One possibility to analyze the links between the protein networks involved in AP-1A coat formation is the use of bioinformatic tools. Bioinformatics allows now predictions on protein-protein interactions based on several informations: published literature, predicted interactions based on protein homology or sequence similarity. Therefore, the proteins identified by the proteomic screen on AP-1A-coated liposomes were analyzed in order to map their known and potential interactions, probably identifying the missing link between the Rab machinery and the actin nucleation machinery and/or the AP-1A coat itself. In collaboration with the bioinformatics group of Prof. Schroeder at the TU Dresden, the protein dataset was analyzed using SCOPPI (Winter et al., 2006). The *in silico* analysis revealed by several lines of evidence that the proteins found on AP-1A coated liposomes form a interconnected network regulating AP-1A coat formation, actin nucleation and membrane fusion, thus supporting the biochemical data and the proposed network based on the proteomic screen (Figure 22). Interestingly, the bioinformatic analysis suggested an interaction of PAK3, a kinase involved in the regulation of the Wave/Scar complex via its interaction with β-PIX (Manser *et al.*, 1998), with the Arf-GEF Big2 as well as with the two identified Rab proteins. Hence, PAK3

could be the link between coat formation, actin nucleation and the membrane fusion machinery, an attractive direction to follow after the experimental confirmation of the proposed PAK3 interactions. The used program SCOPPI (Structural Classification of Protein-Protein Interfaces) is a comprehensive database that classifies and annotates all domain-domain interactions and their interfaces derived from PDB structure files and SCOP domain definitions. It allows to identify interaction partners based on published direct or indirect interactions using the NetPro databank, predicted interactions of proteins by structural similarity using PSI-BLAST and predicted interactions of protein domains using the SCOPPI databank. Interactions based on the literature database NetPro are depicted in red solid lines for direct interactions, and in broken red lines for indirect interactions. Interactions based on structural protein similarity using PSI-BLAST to proteins shown to interact in the NetPro database are shown in yellow and protein interactions based on structural similarities of protein interaction domains found in the SCOPPI databank are depicted in blue.

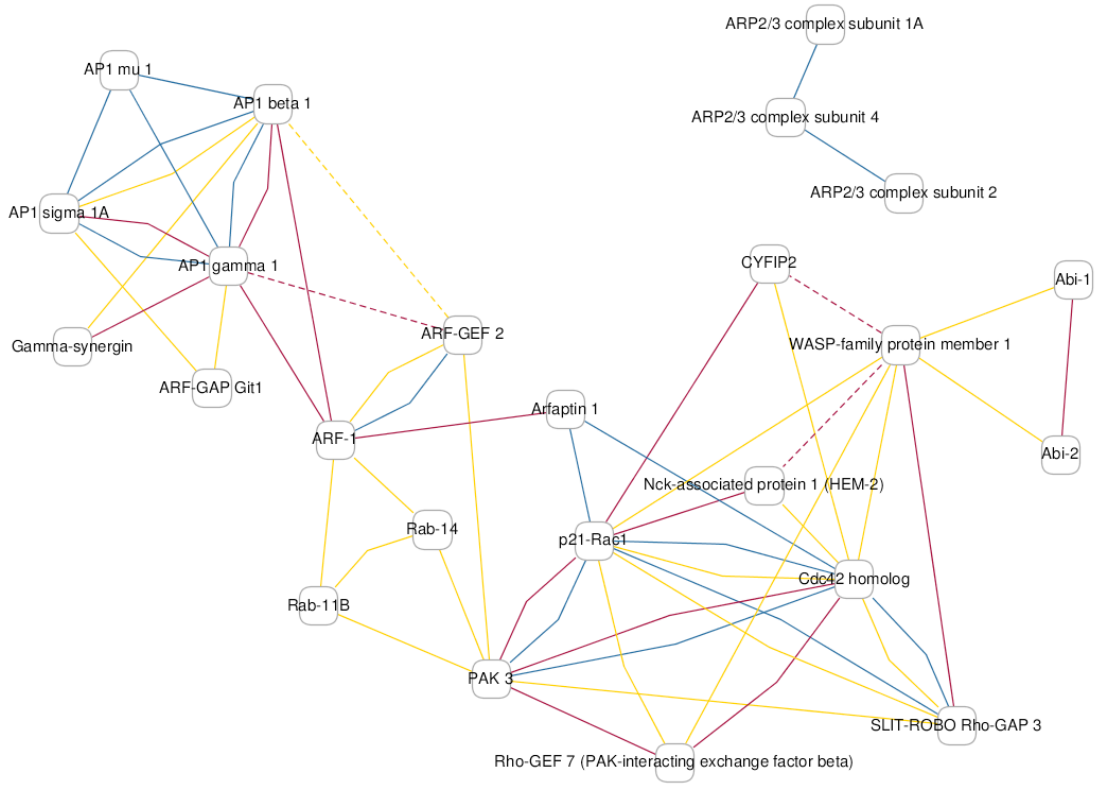

Figure 22: Bioinformatic interaction analysis of proteins found on AP-1A-coated liposomes.

The proteins identified on AP-1A-coated liposomes were analyzed for interactions using SCOPPI. Direct interactions (solid red lines) and indirect interactions (broken red lines) are based on the literature databank NetPro. Potential interactions based on structural protein similarity (PSI-BLAST) and potential interactions based on structural domain similarity (SCOPPI databank) to proteins known to interact in NetPro are depicted in yellow and blue respectively.

III.16: Proteomic analysis of AP-3 coated liposomes

Similarly, LimpII wt/PI-3P containing AP-3 coated liposomes were analyzed for their protein content by 7% and 15% SDS-PAGE. To favor AP-3 recruitment over other coats, the cytosol concentration was decreased to 5mg/ml during the recruitment assay. The analysis revealed however an increased background staining of control liposomes. In protein mass, the unspecific background recruitment of AP-1A was actually comparable to the amount of selectively recruited AP-3 on LimpII wt/PI-3P containing liposomes. The protein amounts of COP-I and AP-2 were still negligible. Despite the higher background recruitment, the scanned profile clearly indicates the selective recruitment of several proteins specifically onto LimpII wt/PI-3P containing liposomes (Figure 23).

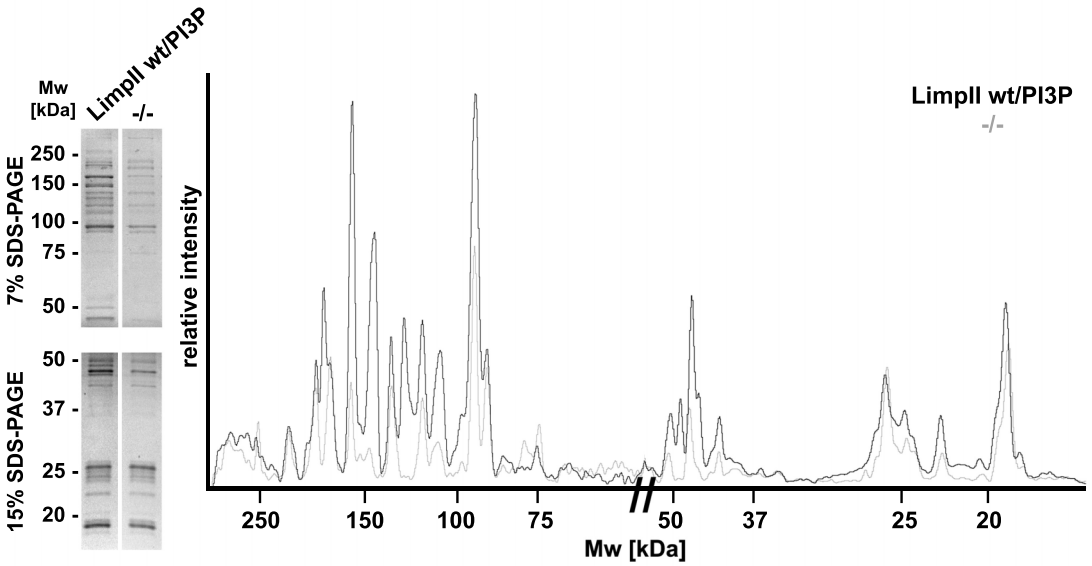

Figure 23: Protein profile of AP-3-coated liposomes.

Liposomes containing LimpII wt domains and PI-3P or liposomes without cytoplasmic domains and without PI-3P (-/-) were incubated with cytosol and GTP-gS, then purified by floatation on a density gradient and analyzed on 7% and 15% SDS-PAGE.

MALDI-TOF/TOF analysis of the major protein bands identified components of the AP-3 complex and proteins probably associated with AP-3 coat formation but also proteins identified on AP-1A coated liposomes. Therefore, after LC-MS/MS analysis of the LimpII wt/PI-3P lane only those proteins were taken into consideration, which were not identified in the context of AP-1A. Hence, 45 proteins that were exclusively recruited in the context of AP-3-coated liposomes are listed in Table IV. 31 of these proteins were identified by two repeated LC-MS/MS analysis or were in addition identified by an independent mass spectrometric analysis of AP-3-derived vesicles (Salazar *et al.*, 2005).

AP-3 associated proteins	NCBI GI number	Predicted Mr (Da)	No. of sequenced peptides	Information
Coat components				
AP-3 δ1	81882150	135,081	17	Adaptor complex 3 subunit; moucha mouse
AP-3 β1	18203657	122,798	14	Adaptor complex 3 subunit; HPS type 2; pearl mouse
AP-3 β2	61219108	119,118	39	Adaptor complex 3 subunit; brain specific
AP-3 μ2	1703029	46,844	8	Adaptor complex 3 subunit; brain specific
AP-3 σ1	33112223	21,718	5	Adaptor complex 3 subunit
AP-3 σ2	33112221	22,003	5	Adaptor complex 3 subunit
Brefeldin A-inhibited GEP 1 (BIG1)	13123997	208,576	11	Arf1-GEF, BFA-sensitiv, Sec7-domain containing, PKA substrate
ADP-ribosylation factor 1 GTPase-activating protein 1 (AGAP1)	51338821	45,260	7	AP-3 specific Arf1-GAP; Centaurin γ2
ARAP1	73620948	128,406	4	Arf1-GAP domain, Rho-GAP-domain; Centaurin δ2
Endosomes				
Early endosomal antigen 1 (EEA1)	76363511	160,915	30	FYVE-domain, Rab5 interactor
Rabaptin-5	47605961	99,552	12	Rab5 & Rab4 effector, complex with Rabex-5; γ-adaptin interactor, GGA-interactor
Rabex-5	56405101	56,896	5	Rab5-GEF, VPS9-domain, complex with Rabaptin-5; Ub-Ligase activity
Vacuolar protein sorting-associated protein 45	23396903	65,012	10	Sec1/Munc18 protein family; SNARE ineractor (Syntaxin-binding protein)
Vacuolar ATP synthase catalytic subunit A	1718086	68,268	6	Peripheral subunit of the vacuolar ATPase; acidicfiaction of endosomes
Vacuolar ATP synthase catalytic subunit E	1718091	26,571	2	Peripheral subunit of the vacuolar ATPase; acidicfiaction of endosomes
GRIP1-associated protein 1 (GRASP-1)	76363168	92,658	7	Ras-GEF, colocalization with EEA1
Rab machinery				
Rab5C	38258917	23,398	10	GTPase regulating early endosomal organization; major Rab-GTPase detected
Rab7	46397834	23,465	12	GTPase regulating late endosomal organization; major Rab-GTPase AP-3 specific
Rab3C	51338605	25,856	10	GTPase involved in synaptic vesicle release; major Rab-GTPase AP-3 specific
TBC1 domain family member 10	20454885	56,167	9	RabGAP involved in vesicular transport; probably interacts with p50RhoGAP
Rho-GTPase-activating protein 1 (p50RhoGAP)	3024550	50,404	4	Cdc42GAP, sec14-domain, link between Rab & RhoGTPases
CDC42 effectors and actin organization proteins				
Cdc42 effector protein 4 (BORG4)	21362404	37,846	7	Actin reorganization; interacts with WD-repeat proteins; probably septin interactor
Neuronal-specific septin 3	13124538	39,320	4	GTPase; involved in cytokinesis; colocalizes with dynamin I
Septin-4	114978	54,901	7	GTPase; involved in cytokinesis; interacts with septin-5/8
Septin-5	83305642	40,215	7	GTPase; involved in cytokinesis; interacts with septin-2/4/7/8; syntaxin-binding protein
Septin-6	20178348	49,588	5	GTPase; involved in cytokinesis; interacts with MAP4
Septin-7	9789726	50,518	7	GTPase; involved in cytokinesis; interacts with septin-2/5/9/11; Sec6 & BORG1 interactor;
Septin-8	45477305	49,781	8	GTPase; involved in cytokinesis; interacts with septin-4/5
Septin-11	50401563	49,663	9	GTPase; involved in cytokinesis; interacts with septin-7/9
Kinases & effectors				
CDC42 binding protein kinase alpha	81174934	196,940	8	Ser/Thr-kinase; PKC-domain; PH-domain; cdc42-binding domain
Protein kinase C, beta type	55977813	76,713	5	Ser/Thr-kinase
Protein kinase C-alpha-binding protein (PICK1)	22095978	46,487	5	Arfaptin-domain; PDZ-domain; involved in AMPA-receptor traffic; GRIP-interactor
14-3-3 zeta/delta (Protein kinase C inhibitor protein 1)	52000885	27,754	6	Phosphoserine/Phosphothreonine-binding
A kinase anchor protein 10	71153491	73,586	13	Contains 2 RGS-domains, 1 PKA-binding motif and 1 PDZ-binding motif
Others				
Syntaxin binding protein 1	48429206	67,526	4	Sec1/Munc18 protein family; SNARE-interactor
Vesicular-fusion protein NSF	1171774	82,513	8	Involved in SNARE recycling; PICK1-interactor; contains 2 cdc48 domains and ATPase activity
WD-repeat protein 13	20140638	53,630	11	Scaffold protein for multi-protein complexes
Coronin-1C (Coronin-3)	54041071	53,087	3	WD-repeat protein; actin binding; phosphorylation-substrate
Myosin 18A	33301327	230,765	37	Motor protein
Islet cell autoantigen 1 (ICA69)	51316204	54,348	7	Arfaptin-domain; insulin secretion
Synaptic vesicle membrane protein VAT-1 homolog	52788286	43,069	9	Synaptic vesicle transport; ATPase activity; only cytoplasmic domain
Fused toes protein	54035927	32,921	4	Ubiquitin-conjugating enzyme E2
Unknown function				
Protein FAM49B	52782789	36,753	3	
Protein FAM49A	52782782	37,319	5	
Protein C14orf24 homolog	46395843	23,563	3	

Table IV: Proteins recruited selectively onto AP-3 coated liposomes.

Data set of LC-MS/MS analysis of AP-3-coated liposomes. Proteins already identified on AP-1A coated liposomes were not listed again.

Interestingly, it turned out that beside AP-3 coat components including potential AP-3-specifc Arf effectors, also protein machineries were recruited that are known to be specifically involved in endosomal dynamics including the endosomal Rab5 and Rab5 effectors. In addition, the analysis revealed the specific recruitment of Cdc42 effectors of the septin family as well as a set of kinase effectors and proteins involved in vesicular traffic beside a set of proteins with unknown functions. Thus, the proteomic analysis revealed the potential connections between AP-3 and protein machineries that are involved in endosomal dynamics, cytoskeletal rearrangements and other aspects of vesicular traffic. However, the direct links between the different machineries have to be identified. The AP-3-specific proteins were assigned into 7 groups, according to their functions. In detail, the proteins were grouped into: First, AP-3 coat components, containing all AP-3 subunits and AP-3-specific Arf1 effectors like AGAP1 (Nie *et al.*, 2003). It will be of special interest to study whether Big1 represents an Arf1-specifc GEF in the context of AP-3, similar to the role of Big2 in the context of AP-1 (Shinotsuka *et al.*, 2002). Second, endosomal associated proteins, especially Rab5 effectors involved in early endosomal dynamics like EEA1, Rabaptin-5, Rabex-5 or Vps45 (Zerial and McBride, 2001), thus suggesting an involvement of AP-3 in early endosomal membrane

traffic. Third, the Rab machinery including the endosomal associated Rab5 and Rab7 beside a Rab-GAP protein, which could reflect a Rab conversion mechanism for vectorial AP-3 traffic. Fourth, Cdc42 effectors and actin remodeling proteins like BORG-4 as well as several members of the septin family that could probably function as AP-3 specific devices for cytoskeletal rearrangements. Fifths, kinases and kinase-effectors, mainly PKA and PKC associated. Sixth, other proteins with thus far not AP-3-related functions like WD-repeat proteins, a motor protein or an ubiquitin-conjugating enzyme that could be involved together with the ubiquitin-ligase Rabex-5 in specific lysosomal sorting in the context of AP-3. And seventh, the screen identified proteins with unknown functions and unknown homologies to other proteins. Thus, in analogy to the results of the analysis of AP-1A coated structures, the proteomic analysis of AP-3 coated structures revealed the involvement of new and partially unexpected machineries in the context of AP-3 mediated traffic, which are targets to further analyze AP-3 mediated traffic *in vivo*.

IV. Discussion

The data provided demonstrate that the high affinity interaction of AP-1A, AP-3 and their associated machineries requires the combinatorial use of several low affinity components, namely Arf1 in its active membrane associated conformation, intact sorting motifs present in the cytoplasmic domain of selected cargo molecules and selected PIPs. Proteomic analysis of the AP-1A-coated liposomes revealed interesting insight into the connection between AP-1A-coat formation, actin nucleation and membrane fusion machineries, while the analysis of AP-3-coated liposomes supported the role of AP-3 in endosomal traffic and revealed a potential connection to the septin protein family.

IV.1: Cargo molecules and Arf1 induce coat stabilization on membranes

Previous studies have shown that AP-1 (Zhu *et al.*, 1999a) and AP-3 (Drake *et al.*, 2000) can be recruited onto protein-free liposomes in an Arf1-dependent manner. According to these results, it was proposed that cargo transmembrane proteins are not essential for the interaction of AP-1 or AP-3 with membranes. The results of this study indicate that cytosolic Arf1 can modestly recruit AP-1A and AP-3 onto protein-free liposomes in the presence of GTP-γS, a non-hydrolysable analog of GTP, which stabilizes Arf1 on membranes in its active conformation. On the other hand, the data clearly show that AP-1A and AP-3 recruitment were drastically increased (at least by factor ten) and became selective upon the presence of selected cargo cytoplasmic domains of proteins, which are transported *in vivo* along an AP-1A or an AP-3 dependent pathway. These findings support previous findings showing that overexpression of selected cargo molecules, either transported along an AP-1 or an AP-3 dependent pathway, enhances the recruitment of AP-1 or AP-3 onto cellular membranes (Le Borgne et al., 1998; Le Borgne et al., 1996) or the observation that mouse fibroblasts lacking both MPRs are not as efficient in AP-1 recruitment onto membranes as wild type cells (Le Borgne and Hoflack, 1997), clearly indicating an involvement of cargo molecules beside Arf1 in the stabilization of AP-1A and AP-3 coats on membranes. Even though this proposal has been questioned (Zhu *et al.*, 1999b), the data show that selective and efficient AP-1A and AP-3 recruitment onto liposomes is strictly dependent on the presence of Arf1 and selected cargo molecules, gpI and Lamp-1 or LimpII respectively. Even more, the recruitment of AP-1A and AP-3 is dependent on intact sorting signals present in the cytoplasmic domains of the cargo molecules, as shown by mutational analysis of the sorting motifs. In the case of gpI wt domains, it was even possible to identify the acidic cluster as the prime motif for AP-1A interaction and to modulate the specific AP-1A interaction by phosphorylation of the acidic cluster. The simplest explanation for any apparent discrepancy is that probably AP-1A and

AP-3 coats can be recruited randomly onto membranes in an Arf1-dependent but cargo-independent process. Such membrane interactions of AP coats would be rather weak and would collapse unless stabilized by cytoplasmic domains of selected cargo as proposed for the formation of stable clathrin-coated pits in the context of AP-2 during endocytosis (Ehrlich *et al.*, 2004). Similar observations have been reported as well for COP-I coats, where stable association of coatomer with synthetic lipid bilayers and subsequent COP-I-coated vesicle budding was dependent on the tripartite interaction of coatomer with membrane bound Arf1, coupled cytoplasmic tails of putative cargo molecules and the lipid bilayer (Bremser *et al.*, 1999).

IV.2: Clathrin association of AP-1A and AP-3

It is well established that AP-1 and AP-2 complexes function together with clathrin and that they interact directly with clathrin through a clathrin box sequence in the flexible hinge region of their β1 and β2 chains (Owen *et al.*, 2004). The mammalian AP-3 complex contains as well a clathrin box sequence in its β3 chain, which can interact with clathrin *in vitro* (Dell'Angelica *et al.*, 1998). However, AP-3 is not enriched in clathrin-coated vesicles and appears to be able to function independently of clathrin. It is actually a matter of debate whether and to what extend AP-3 can bind clathrin *in vivo* and whether this interaction is necessary for proper AP-3 function (Robinson and Bonifacino, 2001). The biochemical and morphological analysis of the results obtained with this *in vitro* assay indicate that clathrin is efficiently recruited onto liposomes in an AP-1A dependent manner, whereas the recruitment in an AP-3 dependent manner was weak, even under saturation with cytosol and peptide tails. This observation is in agreement with EM studies showing that AP-3 colocalizes *in vivo* with clathrin, but to a much lower extent than AP-1A (Peden *et al.*, 2004), thus suggesting a minor role of clathrin in AP-3 dependent pathways and AP-3 function *in vivo*.

IV.3: PIPs provide additional binding sites and specificity for of AP coats

During the past decade, it has become clear that PIPs play an important role in membrane traffic and due to their rapid and spatially restricted turnover, they are key molecules in providing organelle identities (Behnia and Munro, 2005; De Camilli et al., 1996; De Matteis and Godi, 2004). PIPs can provide weak binding sites or induce conformational changes of adaptor complexes, thereby rendering them more competent for interactions with sorting signals of transmembrane proteins as proposed from structural studies on AP-2 (Collins *et al.*, 2002). Recently, it was shown that the recognition of sorting motifs by recombinant AP-2 is dependent on the co-recognition with PI-4,5P_2, thereby explaining in part the selective recruitment of AP-2 to the plasma membrane (Honing *et al.*, 2005). A similar mechanism could also hold for AP-1A and AP-3, since the provided data demonstrate that high affinity

interactions of AP-1A or AP-3 with membranes require the combinatorial use of several low affinity binding sites, including PI-4P or PI-3P, respectively.

There are several lines of evidence that PI-4P is involved in the regulation of Golgi derived membrane traffic, in particular in AP-1A and EpsinR binding on the TGN (Hirst *et al.*, 2003; Mills *et al.*, 2003; Wang *et al.*, 2003). The distribution of AP-1A further supports the notion that AP-1A is functioning in the context of probably PI-4P-rich membranes, since AP-1A is located mainly to the TGN or to post-TGN sites, most likely recycling endosomes (Folsch et al., 2003; Folsch et al., 2001). It was shown that the production of PI-4P is controlled by Arf1 which recruits the PI-4 kinase IIIβ to the Golgi complex (Godi *et al.*, 1999). Interfering with PI-4 kinase IIIβ by overexpression of a dominant-negative mutant disrupted the structural integrity of the Golgi (Godi *et al.*, 1999), providing additional evidence for PI-4P in the regulation of Golgi function. Interestingly, PI-4 kinase IIIβ was identified in the proteomic analysis as a component of AP-1A-coated liposomes together with Rab11B, a major GTPase found on AP-1A coated liposomes, whose Golgi recruitment was shown to be dependent on the direct interaction with PI-4 kinase IIIβ (de Graaf *et al.*, 2004).

Since membrane association of Arf1 is increased by gpI wt cytoplasmic domains, especially by the presence of acidic clusters, it is possible that specific transmembrane proteins could indirectly favor the production of additional PI-4P, an idea consistent with the recovery of PI-4 kinase IIIβ on AP-1A coated liposomes. Such an amplification loop could expand PI-4P and Arf1 containing microdomains around cargo molecules on membranes that then serve as additional sites for efficient assembly of AP-1A coats. There are evidences that cargo transmembrane proteins might not simply be passive passengers, but play crucial roles in regulating coat formation via Arf1 (Spang, 2002). Cargo transmembrane proteins as well as vesicle-SNAREs were shown to interact with the Arf1 GTPase-activating protein (Arf-GAP), thereby probably regulating the formation of COP-I coats (Aoe *et al.*, 1997; Rein *et al.*, 2002). In addition, the cytoplasmic domain of a member of the p24 family was shown not only to bind ArfGAP1 but also to inhibit the ArfGAP1-mediated GTP hydrolysis on Arf1 bound to liposomes and Golgi membranes, thereby favoring COP-I coat formation (Lanoix *et al.*, 2001). During a later stage of vesicle formation, this inhibition of GTP hydrolysis might be terminated by the tripartite interaction of coatomer, ArfGAP and Arf1, which accelerates GTP hydrolysis on Arf1, thus triggering the disassembly of COP-I coats (Goldberg, 1999). Such an interplay between several coat components could be a general mechanism to regulate vesicular transport.

There are several lines of evidence that PI-3P, whose production is regulated by the GTPase Rab5 and the PI-3 kinase hVps34 (Christoforidis *et al.*, 1999), is an important component of early endosomes (Zerial and McBride, 2001). In the light of the presented data, which show that PI-3P is a binding partner for AP-3, one might expect to find AP-3 predominantly on PI-

3P-rich domains of membranes, thus early endosomes. In mammalian cells, immunofluorescence localization experiments of the AP-3 complex have revealed a concentrated perinuclear staining, partially overlapping with markers of the TGN, but also a significant staining in the cell periphery, partially co-localizing with endocytic markers such as TfR (Dell'Angelica *et al.*, 1997; Simpson *et al.*, 1997). EM localization analysis also indicated dual localization of the AP-3 complex on the TGN, as well as on endosomes (Dell'Angelica et al., 1998; Simpson et al., 1996). However, recent EM studies could not confirm a significant TGN localization and proposed that AP-3 complexes mainly define an endosomal exit site from endosomes to lysosomes (Peden *et al.*, 2004). The information about AP-3 localization *in vivo* would support the finding that PI-3P is increasing AP-3 membrane association, therefore it is likely that AP-3 functions *in vivo* mainly in the context of PI-3P-rich membranes, probably early endosomes.

IV.4: Trafficking pathways controlled by AP-1A and AP-3

Although a tremendous amount of information has been collected on molecular interactions between sorting signals and AP subunits (Bonifacino and Traub, 2003; Owen *et al.*, 2004), it is still difficult to interpret trafficking pathways followed by transmembrane proteins in terms of single interactions with sorting machineries. Such interaction studies, even very detailed down to the atomic level, provide just one state of highly dynamic and complex processes which are necessary to drive vesicle genesis at the correct time and place in the cell. The results provided with this *in vitro* assay illustrate the complexity of sorting processes since membrane recruitment of AP-1A and AP-3 complexes is regulated not by single protein-protein or protein-lipid interactions, but probably by the combinatorial use of several low affinity binding sites. To add an additional layer of complexity, these low affinity binding sites can be modulated in several ways: first, by the combination of different sorting motifs and different phosphorylation statuses of acidic clusters, as show for the gpI cytoplasmic domain. Second, due to the regulation of the Arf1 GTP/GDP cycle by Arf1-GEFs and Arf1-GAPs. Third, by the rapid turnover and spatial restriction of selected PIPs, and fourth, by conformational changes of APs upon phosphorylation or binding to selected lipids. Nevertheless, this complex combinatorial behavior can probably be used to elucidate the trafficking pathways controlled by AP-1A and AP-3, simply by identifying cellular membranes showing the highest concentration of suitable AP binding sites at a given time point, thus sites where AP coat formation is most likely to occur.

Even though AP-1A has been located to the TGN and endosomal structures, probably recycling endosomes (Folsch et al., 2003; Folsch et al., 2001), the precise AP-1A dependent trafficking pathways are not clear at the moment. AP-1A together with GGAs were shown to cooperate in TGN-to-endosome transport of MPRs (Doray *et al.*, 2002). On the other hand,

based on knock-out experiments in yeast and mammals, AP-1A has been suggested to function as well in endosome-to-TGN transport (Meyer *et al.*, 2000; Valdivia *et al.*, 2002). Disruption of functional AP-1A resulted in AP-1-cargo accumulation in endosomal structures as shown by colocalization with the early endosomal marker EEA1, concluding that AP-1 is required in endosome-to-TGN traffic. However, such an accumulation does not exclude the possibility that AP-1 cargo was sorted indirectly to endosomes using an AP-1-independent rout via the plasma membrane and subsequent endocytosis, thereby hiding the need for a direct and AP-1-dependent pathway from the TGN to endosomes. In addition, a role of AP-1 in endosome-to-TGN traffic has been suggested by studies on PACS-1, a linker between AP-1 and acidic clusters of proteins like MPRs or furin. Depletion of PACS-1 or PACS-1 variants that cannot bind AP-1 redistribute furin and MPR to the endosomal compartment (Crump et al., 2001; Wan et al., 1998). Taken together there are evidences that AP-1A is involved in both directions, TGN to endosomes and endosomes to the TGN.

Without excluding a role of AP-1A in endosome-to-TGN traffic, the TGN provides, in addition to membrane associated Arf1 and specific cargo cytoplasmic domains, PI-4P, which was shown to increase AP-1A recruitment. In respect of the provided data about efficient AP-1A membrane association using several low affinity binding sites and the spatial distribution of them, it is likely that AP-1A functions predominantly in TGN-to-endosome traffic. On membranes without PI-4P, the interactions between AP-1A and its cargo would be weaker, allowing AP-1A dissociation, thereby leaving a partial access of AP-1A cargo transmembrane proteins to the cell surface, as expected for MPRs and gpI, probably from endosomal structures.

A similar explanation could actually shed some light on the pathway controlled by AP-3. Although AP-3 is clearly involved in trafficking to lysosomes, the precise pathway that it mediates is still not clear. The major missorting defect that has been observed in AP-3 deficient cells is that certain lysosomal membrane proteins, including Lamp-1 and LimpII, show increased trafficking via the plasma membrane, although their steady state distribution is still mainly lysosomal (Dell'Angelica et al., 1999; Le Borgne et al., 1998). Studies in yeast have proposed that AP-3 mediates the sorting of selected transmembrane proteins in the TGN for direct transport to the vacuole (Cowles *et al.*, 1997). However, without excluding a role in TGN-to-endosome traffic, the localization of AP-3 and the finding that PI-3P is increasing AP-3 membrane association makes it likely that AP-3 functions predominantly in the context of PI-3P-rich membranes, probably early endosomes. The finding that PI-3P and a PI-3 kinase are critical for proper traffic of AP-3 cargo to lysosomes *in vivo*, as shown by wortmannin treatment or overexpression of PI-3P binding probes, also supports this notion (Reaves *et al.*, 1996). The cargo binding property of AP-3 would be enhanced in PI-3P-rich membranes of early endosomes, thereby allowing an efficient AP-3-cargo sorting from early

endosomes to lysosomes, supporting EM studies where AP-3 was detected on endosomal exit sites together with its cargo Lamp-1 (Peden *et al.*, 2004). The stabilization of AP-3 binding on PI-3P-rich membranes could therefore prevent the access of lysosomal proteins to the cell surface and permit their selective, intracellular transport from the TGN to endosomes and finally to lysosomes.

IV.5: Proteomic analysis of AP-1A coated liposomes reveals coordination between AP-1A coat formation, actin nucleation and membrane fusion

IV.5.1: The AP-1A coat machinery

The proteomic screen of gpI wt and PI-4P containing liposomes identified components involved in early stages of AP-1A coat assembly stabilized by GTP-γS. The identified proteins were grouped into 3 different groups: AP-1A coat, actin nucleation and membrane fusion. The first group includes not only the expected clathrin light and heavy chains, the four subunits of the AP-1A complex and Arf1/Arf3, but also γ-synergin, an AP-1 γ-ear accessory protein with unknown function (Page *et al.*, 1999), the Arf-GEF Big2, two Arf-GAPs Git1 and Git2, as well as Arfaptin1 and Arfaptin2, two Arf1-interacting proteins that also interact with Rac1 (Tarricone *et al.*, 2001). In addition, Hsc70 was detected, a protein involved in clathrin-coated vesicle uncoating (Chappell *et al.*, 1986), which was also shown to be necessary for uncoating of AP-1 coated structures (Hannan *et al.*, 1998). The presence of Hsc70 could reflect the incorporation of parts of the uncoating machinery already during early steps of AP-1A coat formation. Beside γ-synergin no other AP-1A accessory protein was detected, probably illustrating an exclusive recruitment mechanism of the different AP-1A γ-subunit accessory proteins. The lack of GGAs can be explained by the absence of the appropriate di-leucine-based sorting motifs in the gpI wt tail (Bonifacino, 2004) and the absence of PACS-1 can be explained due to the non-phosphorylated acidic cluster of gpI wt, which is necessary for PACS-1 recruitment (Wan *et al.*, 1998). The reason why significant amounts of EpsinR (one peptide was sequenced but failed the criteria to be listed in Table II) are missing on AP-1A coated liposomes, could probably be explained be the fact that appropriate SNAREs are not present on the liposomes used. It was shown that the ENTH domain of EpsinR interacts specifically with the N-terminus of a SNARE protein involved in endosome-to-TGN traffic thereby probably linking SNARE recruitment and AP-1A coat formation (Chidambaram *et al.*, 2004; Hirst *et al.*, 2004). The identified Arf-GEF Big2 was shown to localize to the TGN and Rab11-positive recycling endosomes and to regulate the structural integrity of recycling endosomes by the specific activation of Arf1 and Arf3 *in vivo* (Shin *et al.*, 2004). In addition, Big2 co-localizes in HeLa cells with the AP-1A subunit γ-

adaptin, making it an interesting candidate regulating Arf1 membrane association in the context of AP-1A on Rab11-positive membranes. This notion is further supported by the finding that Big2 is an AP-1 specific Arf-GEF (Shinotsuka *et al.*, 2002). Git1, which is probably involved in Rab11-positive membrane organization as shown for its avian homolog p95-APP1 (Di Cesare *et al.*, 2000; Matafora *et al.*, 2001), and Git2 are mainly described in Arf6 dependent effects in the cell (Turner *et al.*, 2001). However, their role in Arf1 mediated cellular events might be under estimated since Git1 and Git2 have an identical GAP activity for Arf1 and Arf6 (Vitale *et al.*, 2000), and both were originally identified as Arf1 GTPase activating proteins (Premont *et al.*, 1998; Premont *et al.*, 2000). Arf6 was not substantially detected on AP-1A-coated liposomes by LC-MS/MS and not at all detected by MALDI-TOF/TOF, whereas Arf1/Arf3 were significantly detected by both methods. Thus, it is very likely that the recovery of Git1 and Git2 on gpI wt and PI-4P-containing liposomes reflects their implication in Arf1 mediated AP-1A-coat formation. The fact that dynamin was not detected, which is involved beside scission of clathrin-coated vesicles at the plasma membrane in the formation of transport vesicles at the TGN (Jones *et al.*, 1998), could reflect the expected role of dynamin during late stages of AP-1A-coated transport intermediate formation at the TGN and therefore its absence during initial steps of AP-1A coat formation.

IV.5.2: The actin nucleation machinery

Although the actin cytoskeleton is widely believed to play an important role in intracellular protein transport, this role is poorly understood, especially in the context of clathrin coated vesicle traffic emerging at the TGN. Several lines of evidence show that the actin cytoskeleton is linked to protein traffic, mainly by the use of actin toxins and the identification of actin binding and actin regulatory proteins on Golgi membranes (Stamnes, 2002). There are clear indications that different actin pools are assembled on Golgi membranes in an Arf1-dependent manner mainly by the recruitment of different actin-binding proteins upon Arf1 activation (Fucini *et al.*, 2000). It has also been proposed that members of the Rho family of small GTPases are controlling Golgi function in coordination with Arf1, possibly through the regulation of the Arp2/3 complex and actin polymerization on Golgi membranes (Dubois et al., 2005; Miura et al., 2002; Wang et al., 2005). Interestingly, the proteomic screen and the biochemical data of AP-1A coated liposomes revealed the selective recruitment of a Rac1-dependent actin nucleation machinery. This machinery consists of the Rho-GTPase Rac1 with its effectors β-PIX and the Wave-associated Rac-GAP, the complete Wave/Scar complex consisting of WAVE1/3, Abi-1/2, Hem-2, and CYFIP2 as well as the Arp2/3 complex. In addition the kinase PAK-3 was recruited, a member of the PAK-family (Zhao and Manser, 2005), which is known to regulate the interaction between Rac1 and β-PIX (Ten Klooster *et al.*, 2006). Comparison of the Coomassie stained protein bands

indicated that the AP-1A complex and the Wave/Scar complex were selectively recruited in similar stoichiometric amounts, indicating an intense recruitment of the Rac1-dependent actin nucleation machinery. Cdc42 and Rac1 initiate both actin polymerization through the Arp2/3 complex, both activate Arp2/3 indirectly, Cdc42 by the WASP complex and Rac1 by the Wave/Scar complex (Stradal and Scita, 2006). Even though the Wave/Scar complex was mainly described to function at the plasma membrane, its recruitment and activation is crucially dependent on Rac1 binding, thus targeting and activation of Rac1 could in turn define the site of activation of the Arp2/3 complex via the Wave/Scar complex on several membranes. Targeting and activation of Rac1 was shown to be mediated by the exchange factor β-PIX (Ten Klooster *et al.*, 2006), and β-PIX was identified to be in a complex with the ArfGAP Git1 (Premont *et al.*, 2004), localizing to the cell periphery but also to the perinuclear region as shown in fibroblasts (Botrugno *et al.*, 2006). In addition, avian Git1 was shown to be involved in Rab11-positive membrane organization (Matafora *et al.*, 2001). This idea is further supported by the colocalization of CI-MPR, an AP-1A cargo, and CYFIP2, taken as a marker of the Wave/Scar complex, in the perinuclear region, most likely the TGN of HeLa cells. This implies that interactions between the Rac1-GEF β-PIX and the Arf1-GAP Git1 could couple AP-1A coat assembly and actin nucleation by the Wave/Scar complex on the Golgi. This hypothesis is further supported by the presence of Arfaptins on AP-1A coated liposomes, since Arfaptins are mediating the cross-talk between Arf and Rac GTPases (Tarricone *et al.*, 2001). Actin polymerization could provide forces required for clustering of membrane-bound AP-1A complexes or for budding of AP-1A-coated transport intermediates. Taken together, it is possible that Rac1-mediated actin nucleation could be associated with the Golgi and probably with AP-1A function *in vivo*. It will be important to address this issue in more detail because actin depolymerization prevents the formation of MPR-containing, AP-1A-coated, TGN-derived transport intermediates in HeLa cells (Waguri *et al.*, 2003), thus further highlighting the involvement of an AP-1A associated actin nucleation machinery *in vivo*.

IV.5.3: The Rab machinery

Finally, the proteomic screen revealed that two major Rab GTPases, Rab11b and Rab14, were found on AP-1A-coated liposomes. It is well established that different Rabs localize to distinct membrane-bound cellular compartments in mammalian cells, where they are involved in the regulation of membrane dynamics (Zerial and McBride, 2001). The two identified Rabs are both implicated in the membrane traffic between the TGN and endosomes. It has been proposed that Rab14 controls protein traffic between the TGN and endosomes, since endogenous Rab14 has been shown to localize to the TGN as well as endosomal compartments and expression of mutant versions of Rab14 interfered with vesicular traffic

between these compartments (Junutula *et al.*, 2004). There are clear evidences that Rab11 regulates the recycling of cargo from early endosomes to the cell surface via perinuclear recycling endosomes (Ren et al., 1998; Ullrich et al., 1996). Interestingly, studies in yeast have shown that Ypt31p, the Rab11 homolog in yeast, is essential for Golgi function and is required for the exit from the yeast trans-Golgi compartment (Benli *et al.*, 1996; Jedd *et al.*, 1997). In addition, synthetic genetic arrays of Pik1, the yeast PI-4 Kinase IIIβ, have identified a mechanistic involvement for Pik1 with Ypt31p to regulate protein traffic in the secretory pathway (Sciorra *et al.*, 2005), similar to the finding in mammals, where the PI-4 Kinase IIIβ was shown to be functionally connected to Rab11 (de Graaf *et al.*, 2004). The reason why Rab11 and Rab14 are equally recovered together with AP-1A is actually unknown. However, their presence could highlight the transport pathways controlled by AP-1A and PI-4P and emphasizes the importance of recycling endosomes as a connection between the biosynthetic and endocytic pathway. AP-1A and AP-1B have both been found on recycling endosomes in polarized cells (Folsch et al., 2003; Folsch et al., 2001) and it has been proposed that recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells (Ang *et al.*, 2004), thus suggesting a significant role of Rab11-positive recycling endosomes in protein sorting to several destinations.

The molecular links bridging the selected Rab machinery with the AP-1A coat or the actin nucleation machinery are undefined at the moment and even less is known about how their activity is controlled. It is possible that the few proteins with unknown functions identified in the proteomic screen provide the molecular links coupling Rab11 and Rab14 with AP-1A coat assembly and/or the actin nucleation machinery. Especially the two proteins containing GAP domains with so far undefined specificities, GTPase activating Rap/RanGAP domain-like 1 and KIAA1219, are potential candidates maybe involved in the regulation of the Rab GTPases found on AP-1A coated liposomes, but this needs to be specified in further experiments. The first analysis using bioinformatics of the two GAP-domain containing proteins recruited onto AP-1A coated liposomes revealed that they do not contain a Sec14 domain. This domain was shown to target GAP-domain proteins to endosomes, providing a link between Rab11 and Rho-GTPases (Sirokmany *et al.*, 2006). However, the bioinformatic analysis revealed that the GTPase-activating proteins contain beside their GTPase regulatory domain also various conserved protein-domains mediating protein-protein or protein-lipid interactions, which represent potential sites for regulation and targeting of these proteins to their site of function (Bernards and Settleman, 2004).

IV.6: Bioinformatic analysis of the AP-1A protein networks

Understanding protein interactions provides the key to understand cellular processes. Therefore, the set of proteins identified by the proteomic screen on AP-1A-coated liposomes was analyzed in addition to biochemical and immune histochemical methods with bioinformatic tools in collaboration with the bioinformatics group of Prof. Schroeder at the TU Dresden. The analysis using SCOPPI (Winter *et al.*, 2006) revealed that the proteins identified on AP-1A-coated liposomes are interconnected members of three different machineries, AP-1A coats, actin nucleation and membrane fusion, thus agreeing with the provided biochemical and immune histochemical data. Interestingly, the *in silico* analysis identified PAK3 to be the link between the three machineries, a notion that will be followed by biochemical studies. Unfortunately, the bioinformatic analysis failed in the prediction of some already published connections. SCOPPI missed the link between the Arp2/3 complex and the Wave/Scar complex despite the known regulative interaction (Stradal and Scita, 2006). The connection between Git1 and β-PIX (Rho-GEF 7) was also not detected, even though several publications are providing experimental data for this interaction (Botrugno et al., 2006; Premont et al., 2004). Detailed analysis of this missing link revealed that the database NetPro, which is used by SCOPPI, contains only PubMed publications until May 2005 (until PubMed ID15878932), thus missing all recent publications. In addition, NetPro is also missing the second publication about the Git1/β-PIX complex for unknown reasons. Hence, bioinformatics is at least at the moment not an absolute method to identify protein-protein interactions or molecular networks because it relies always on the quality of the databases used. Nevertheless, bioinformatics provides quite useful tools to proof and to refine datasets obtained by biochemical methods.

IV.7: Proteomic analysis of AP-3 coated liposomes

The proteomic analysis of liposomes containing LimpII wt and PI-3P identified several components selectively recruited along AP-3 adaptor complexes. Proteins already identified in the context of AP-1A-coated liposomes were deducted from the proteins identified by two independent LC-MS/MS analyses of AP-3-coated liposomes. Interestingly, out of the 45 AP-3 specific proteins, 14 were already described to be part of AP-3-derived vesicles (Salazar *et al.*, 2005). These include the four subunits of the AP-3 complex, the early endosomal marker EEA1, the three GTPases Rab5, Rab7 and Rab3, two peripheral subunits of the vacuolar ATPase, the β subunit of PKC and the PKC binder PICK1 as well as WD-repeat protein 13 and the cytoplasmic domain of a VAT-1 homolog. However, two independent LC-MS/MS analyses identified 31 more proteins associated with AP-3-coats. The most interesting among them are the AP-3 specific Arf1 effectors. The Arf1GEF Big1 (Togawa *et al.*, 1999) was repeatedly identified to be part of AP-3 coats and its recruitment was even detectable on the

Coomassie-stained protein level. In addition, two Arf1GAPs of the centaurin family (Jackson *et al.*, 2000) were identified on AP-3 coated liposomes. AGAP1 (Centaurin γ2), which was already published as an AP-3-specific GTPase activating protein (Nie *et al.*, 2003) and ARAP1 (Centaurin δ2), an Arf1-GAP protein which contains as well a Rho-GAP domain, thereby linking Arf and Rho signaling (Miura *et al.*, 2002). Interestingly, BIG1 was shown to bind the RhoGAP domain of myosin IXb (Saeki *et al.*, 2005). Sequence alignments of myosin IXb with ARAP1 showed a significant homology of both RhoGAP-domains, thus a potential binding site between the ARF1-GEF BIG1 and the ARF1-GAP ARAP1, thereby linking AP-3 coats and Rho-GTPase signaling. In addition, it will be interesting to address the question, whether Big1 is an AP-3-specific Arf-GEF. This idea is coming from the finding that Big2 is an AP-1-specific Arf-GEF (Shinotsuka *et al.*, 2002). Thus far it remained largely unknown how and when the regulators of Arf GTPases are recruited onto the nascent vesicle. Thus, it would be a highly interesting finding since it would demonstrate the direct coordination between Big1 and ARAP1 to regulate specifically AP-3 coat formation. Biochemical and *in vivo* analysis like pull-down assays and siRNA experiments in combination with the already explained Lamp-1 missorting assay have already started to shed some more light on this proposal in order to prove the mechanistically novel hypothesis. Beside the already mentioned endosomal components, several proteins of the early endosomes were identified like Rabaptin-5, Rabex-5, Vps45, and GRASP-1, a RasGEF which was shown to colocalize with EEA1 (Stinton *et al.*, 2005) and functions in AMPA receptor traffic control (Ye *et al.*, 2000). Beside the endosomal RabGTPases, the RabGAP-domain containing protein TBC1-domain family member 10 (EPI64) (Reczek and Bretscher, 2001) was identified as well as the Cdc42GAP protein p50RhoGAP, a sec14-containing protein, which was proposed to provide the link between Rab and RhoGTPases (Sirokmany *et al.*, 2006). In analogy to the results of AP-1A coated structures, this could suggest that the formation of AP-3 coats already stimulates the recruitment of proteins involved in early endosomal dynamics including Rab5 that would facilitate either the fusion with early endosomes, or the formation of early endosomal like vesicles that will fuse with the late endosomal compartments after Rab conversion. In the light of the Rab conversion theory and the identification of two TGN-endosome specific Rab proteins in the context of AP-1A, the identification of Rab5 and Rab7 would suggest a similar mechanism in the context of AP-3 mediating traffic to the late endosomal compartment. Thus, similar to the findings of AP-1A, the proteomic results of AP-3 coated structures imply that the cytoplasmic domain of LimpII and PI-3P are sufficient to reconstitute selectively the protein machineries involved in AP-3 coat formation and early endosomal dynamics including the Rab5 machinery. However, the direct links between AP-3 and the Rab5 machinery are still elusive. If the Rab5 machinery is stabilized due to the presence of PI-3P or due to direct interaction with the AP-3 machinery has to be analyzed.

Interestingly, several members of the septin-family (septin3/4/5/6/7/8/11), GTPases involved in cytokinesis but also having other functions (Kartmann and Roth, 2001), together with the Cdc42 effector protein 4, a member of the Borg-family (Joberty *et al.*, 1999), were identified in the context of AP-3. Borg proteins were found to control septin organization, specifically the Borg Homology Domain 3 (BD3 domain) was shown to mediate Borg-septin interactions (Joberty *et al.*, 2001). The identification of the septin machinery could support the idea that AP-3 is using another way to regulate the cytoskeletal elements, when compared with AP-1A. Thus, it will be important to analyze whether septins and Borg4 are involved in AP-3-mediated traffic of Lamp-1 and LimpII *in vivo* by regulating the actin and/or microtubule cytoskeleton. Additionally, beside PKC another serine/threonine kinase, Cdc42 binding kinase α was detected together with an A kinase anchor protein and a member of the 14-3-3 protein family, which are phosphoserine/phosphothreonine-binding proteins with several functions (Dougherty and Morrison, 2004). In addition, several other proteins were identified whose roles in AP-3-dependent traffic have to be studied in more detail. Likewise, Syntaxin binding protein 1, Vesicular fusion protein NSF, Coronin-1C, Myosin 18A, Islet cell autoantigen1, or the Fused toes protein were identified. Interestingly, bioinformatic sequence analysis of the Fused toes protein identified it as a potential Ubiquitin-conjugating enzyme E2. Together with the recently identified Ubiquitin-ligase Rabex-5 (Mattera *et al.*, 2006), which was detected as well on AP-3-coated liposomes, both proteins could actually function as part of a Ubiquitin-dependent protein sorting machinery in the context of AP-3-dependent cargo traffic. The role of such a ubiquitination machinery could either be the ubiquitination of AP-3 cargo molecules or of parts of the AP-3 machinery itself during transport, using ubiquitin as a sorting signal for delivery to the endosomal compartment, or the ubiquitination of non AP-3 cargo molecules as a way to exclude them from AP-3 coated structures in order to sort them classically into intraluminal vesicles of MVBs via the ESCRT complexes. Both hypotheses are of course pure hypothetical but can be tested experimentally, like the role of the three proteins with unknown function. Taken together, the proteomic approach to study AP-3 coats, which were selectively assembled on LimpII wt/PI-3P-containing liposomes, identified several proteins involved in cargo sorting to the endosomal compartment. Thus, this method is a powerful tool to identify new components of AP-3 coated structures and to identify functional protein networks involved in protein sorting along AP-3 mediated pathways as well as the formation of AP-3 microdomains *in vivo*.

V. Conclusion and Perspectives

Overall, the results of this thesis research have provided important insight into the formation of AP-1A and AP-3 coated structures. The biochemical analyses indicate that several components, namely cargo molecules containing intact sorting signals, Arf GTPases and phosphoinositides are needed for the selective formation of AP coated structures. The proteomic analyses of AP-1A and AP-3 coated structures revealed a probably general mechanism of interconnection between AP coats, actin remodeling devices and proteins of a fusion machinery that are involved in later stages of vesicular traffic.

Beside biochemical characterization of the stabilization of the machineries involved in AP-3 coat formation, the functional analysis of the proteins identified by both proteomic screens, especially of those with thus far unknown function are immediate experimental objectives from this research. The most promising experiments to do are the analysis of the AP-1A accessory protein γ-synergin as a potential acidic cluster sensor and the analysis of Big1 as an AP-3 specific Arf GEF as well as the interaction of Big1 with ARAP1 to identify a potential functional interconnection between an AP-3 specific Arf GEF and Arf GAP. In addition, it is important to test whether ARAP1 is linking AP-3 coats and the actin nucleation machinery. In addition, the implication of the identified protein machineries can be tested *in vivo* by loss of function studies in combination with missorting assays. RNAi experiments targeting proteins involved in AP-1A mediated traffic should lead to a missorting of cathepsin D, a soluble lysosomal hydrolase that is normally transported via MPRs directly to endosomes, and targeting of protein involved in AP-3 mediated traffic should lead to a missorting of Lamp-1, as already used to demonstrate the implication of PI-3P in AP-3 mediated traffic *in vivo*. Conceptually, however, the development of a liposome-based *in vitro* assay that recapitulates the fidelity of protein sorting *in vivo*, has paved the way for new approaches. It opens the doors in various fields including comparative studies of coat components in higher and lower eukaryotes, the possibility to analyze the influence of SNARE molecules on coat composition and stabilization or the utilization of this approach to study cell signaling complexes. This approach could also be used for the *in vitro* analyses of coat and microdomain dynamics, maybe leading to an *in vitro* budding assay. Initial experiments with GFP-labeled proteins in collaboration with the biophysics group of Prof. Petra Schwille (TU Dresden) indicate that the method can be applied to investigate this aspect. It will be interesting to study how the dynamic interplay between Arf, Rho and Rab GTPases is coordinated during coat formation, vesicular movement and membrane fusion. One possibility is the direct interaction between their effectors. In the light of this plethora of possibilities, this is just the starting point for further studies, either to analyze the identified proteins involved in AP-1A and AP-3 coat formation or to use this *in vitro* assay in different areas to gain further understanding of cell biology.

VI. Publication record of this PhD thesis

Parts of the results of this PhD thesis have been published in the following scientific articles:

1) **Baust, T.**, C. Czupalla, E. Krause, L. Bourel-Bonnet, and B. Hoflack. 2006. Proteomic analysis of adaptor protein 1A coats selectively assembled on liposomes. *Proc Natl Acad Sci U S A.* 103:3159-64.

2) Bourel-Bonnet, L., E.I. Pecheur, C. Grandjean, A. Blanpain, **T. Baust**, O. Melnyk, B. Hoflack, and H. Gras-Masse. 2005. Anchorage of synthetic peptides onto liposomes via hydrazone and alpha-oxo hydrazone bonds. preliminary functional investigations. *Bioconjug Chem.* 16:450-7.

VII. Material and Methods

Note: catalog numbers are not given for materials were product names are sufficient for assignment.

VII.1: Lipids, phosphoinositolphosphates & chemicals

L-α-Phosphatidylcholine (PC, cat. # 840053), Phosphatidylethanolamine (PE, cat. # 840022), Phosphatidylserine (PS, cat. # 840032), Cholesterol (cat. # 700000) were from *Avanti Polar Lipids, Inc.* D-*myo*-Phosphatidylinositol (PI, cat. # P-0016), D-*myo*-Phosphatidylinositol 3-phosphate (PI-3P, cat. # P-3016), D-*myo*-Phosphatidylinositol 4-phosphate (PI-4P, cat. # P-4016), D-*myo*-Phosphatidylinositol 5-phosphate (PI-5P, cat. # P-5016), D-*myo*-Phosphatidyl inositol 3,4-bisphosphate (PI-3,4P_2, cat. # P-3416), D-*myo*-Phosphatidyl inositol 4,5-bisphosphate (PI-4,5P_2, cat. # P-4516), D-*myo*-Phosphatidyl inositol 3,5-bisphosphate (PI-3,5P_2, cat. # P-3516), D-*myo*-Phosphatidyl inositol 3,4,5-trisphosphate (PI-3,4,5P_3, cat. # P-3916) were from *Echelon Biosciences, Inc.* Complete Protease Inhibitor Cocktail Tablets and GTP-γS were from *Roche Biochemicals*. Brefeldin A (BFA) was from *Calbiochem*. Other reagents and chemicals were from *Sigma* unless otherwise stated.

VII.2: Antibodies

Antibodies used were: polyclonal antibodies against the AP-3 σ-subunit (Le Borgne *et al.*, 1998), Rab5 (gift of M. Zerial), PI4 Kinase IIα and PI4 Kinase IIIβ (gifts of A. De Matteis) and Rab11 (*USBiological*); monoclonal antibodies against AP-1 γ-subunit (100/3, *Sigma*), AP-2 α-subunit (100/2, *Sigma*), clathrin heavy chain (*Transduction Laboratories*), COP-I β-subunit (maD, *Sigma*), ARF-1 (1D9, *Dianova*), Rac1 (*Cytoskeleton*), CYFIP2 (Mayne *et al.*, 2004) and Lamp1 (CD107a, *BD Biosciences*). The secondary antibodies used were goat anti-mouse and goat anti-rabbit conjugated with horseradish peroxidase (*Dianova*), Alexa fluor 488 or Texas Red (*Invitrogen*).

VII.3: Hydrazino peptides, aldehyde lipid anchor & liposomes

The α-hydrazino acetyl peptides and the Di-O-hexadecyl-rac-glyceraldehyde lipid anchor were synthesized as described (Bourel-Bonnet *et al.*, 2005). After preparative RP-HPLC, peptide purity was routinely >95% and peptide identities were controlled by mass spectrometry. Standard liposomes were prepared as follows: A mixture of PC:PE:PS:Cholesterol:lipid anchor (40:30:10:10:10 molar ratio; phosphoinositides were added as 1% molar ratio when indicated) in chloroform/methanol 1/1 (vol/vol) solution was evaporated to dryness under a steam of nitrogen and resuspended in LPL-Buffer (Liposome-

Peptide-Ligation Buffer: 15.4mM citric acid, 69.2mM dibasic sodium phosphate, pH 6.4) by vigorous vortexing. Homogeneous unilamella liposomes were formed from the lipid emulsion by ten cycles of freeze/thawing and finally eleven cycles of extrusion through a polycarbonate membrane with pore diameters of 400nm using a LiposoFast (*Avestin*). For further coupling to peptides, 250µl of suspension containing 1.25µmol of total lipids and, among them, 0.125µmol of anchor were added to 250µl of 0.5mM (1 equivalent considering total anchor, i.e., 2 equivalents toward outer exposed aldehydes) of hydrazino peptides dissolved in LPL-Buffer. 10mM Glycine instead of peptide was used in control experiments. The liposome/peptide mixture was left for 16h at 25°C in the dark for coupling to occur. To prevent competitive binding reactions, unbound peptides were removed by gel filtration over a Sephadex G-25 column (NAP 5 columns, *Amersham Biosciences*) equilibrated with R-Buffer (Recruitment Buffer: 25mM HEPES-KOH pH 7.2, 125mM potassium acetate, 2.5mM magnesium acetate, 1mM DTT; 5mM glycerol-2 phosphate was added when phosphoinositides or phosphorylated peptides were present). The coupling yield was checked by amino acid analysis with ninhydrine detection after total acid hydrolysis of a liposomes/peptide sample in a sealed tube with HCl 6N/phenol 10/1 (v/w) at 110°C. All coupling reactions had similar efficiencies. Typically 80% of the anchor exposed on the outside of the liposome was coupled to peptides. In a typical assay 10µl of liposomes were used corresponding to approximately 0.5nmol of coupled peptide or 12.5nmol of total lipid (~8.8µg) per reaction.

VII.4: Recombinant myristoylated Arf1 production

Co-expression of bovine ARF-1 and yeast N-myristoyl-transferase (yNMT) was carried out in *E. coli* strain BL21 (DE3) (*Stratagene*). 50µl electrocompetent BL21 (DE3) were co-electroporated (0.2cm electrode gap cuvette, 2 pulses of 2500V, *BIORAD Gene Pulser*) with 0.5µg pET11d/Gly2-bARF-1 (ampicillin-resistance) and 0.5µg pBB131-yNMT (kanamycine-resistance) and plated after 1hour at 37°C in LB-medium on L-Agar plates containing ampicillin [50µg/ml] and kanamycine [50µg/ml]. A single colony was picked for the inoculation of 5ml LB-medium containing the appropriate antibiotics. After incubation at 37°C overnight under constant agitation (220rpm), 1ml of the overnight culture was used for the inoculation 600ml fresh LB-medium containing appropriate antibiotics. 5x 600ml were incubated at 37°C under constant agitation until optical density reading at 600nm reaches 0.4, the cultures were then shifted to 27°C under constant agitation. When the optical density reached 0.6, a 100x stock solution of myristate (sodium-myristate [10mM] and BSA [0.6mM] in water) was added. After 10min protein co-expression was induced with IPTG to 0.3mM final. Cultures were incubated at 27°C under constant agitation. After 3.5hours, bacteria were pelleted by centrifugation at 4°C at 9000g for 10min (JLA-8.1000), the pellet was washed

once with ice-cold PBS, pelleted again and resuspended in ice-cold bacterial lysis buffer (50mM Tris pH 8.0, 5mM $MgCl_2$, 1mM DTT, 200µM GDP, complete protease inhibitors). Pressure homogenization of the suspension was done by two passages using a pre-cooled EmulsiFlex (*Avestin*). The solution was clarified at 4°C at 180 000g for 60min (45Ti) and the supernatant was adjusted to 35% (w/v) ammonium sulfate at 4°C under continues stirring for 30min. The precipitate was pelleted at 4°C at 10 000g for 30min (SW32 Ti) and resuspended in 5ml Dialysis Buffer (10mM Tris pH 8.0, 5mM $MgCl_2$, 1mM DTT, 5µM GDP). The solution was dialyzed against Dialysis Buffer at 4°C overnight (4 buffer exchanges) using a dialysis membrane with a molecular cut off of 12-14000Da (*Spectrum*) and clarified at 4°C at 160 000g for 60min (SW40 Ti). Myristoylated ARF-1 was purified by anion exchange chromatography using a DEAE column (HiTrap DEAE FF 5ml, *Amersham Biosciences*) equilibrated with dialysis buffer connected to a ÄKTA system (Explorer 10S, *Amersham Biosciences*). A linear KCl gradient (20CV) at 0.3ml/min was used for elution. 200µl fractions were collected and peak fractions were pooled after SDS-PAGE analysis, aliquoted, snap-frozen and stored at -80°C. Purified myristoylated ARF-1 [1mg/ml] was more that 95% pure and mainly contaminated by non-myristoylated ARF-1.

VII.5: *In vitro* phosphorylation using casein kinase II

The standard phosphorylation mixture contained 20µl of liposomes corresponding to approximately 1nmol of peptide and 300 units of caseine kinase II (*Calbiochem*). The 300 units of caseine kinase II were pre-incubated on ice for 15min in R-Buffer containing 10µM poly-L-lysine, 10mM Manganese(II) chloride and 5mM ATP, before they were incubated with the liposomes at 30°C for 30min. After the *in vitro* phosphorylation of the peptides, the liposomes were used in standard coat protein recruitment assays containing 5mM β-glycero-phosphate.

VII.6: Coat protein recruitment assay

Pig brain cytosol was prepared from fresh brains obtained at the local slaughterhouse. Brain slices were transferred to homogenization buffer (25mM HEPES-KOH pH 7.2, 125mM potassium acetate, 2.5mM magnesium acetate, 1mM DTT supplemented with complete protease inhibitors) on ice. Homogenization was done with a blender in a 2-to-1 tissue-to-buffer (w/w) ratio. The crude homogenate was centrifuged at 4°C at 10 000g for 45min (JLA-8.1000) and finally at 140 000g for 90min (SW 32 Ti). The final supernatant was snap-frozen in liquid nitrogen before storage at -80°C. Before use, cytosol was rapidly thawed, if needed desalted over Sephadex G-25 (PD-10, *Amersham Biosciences*) columns previously equilibrated with R-Buffer, and aggregated material was removed by centrifugation at 4°C at

280 000g for 45min (SW 40 Ti). ARF-depleted cytosol was obtained by gel filtration over a self-made Sephadex G-50 column. ARF-depletion was checked by Western blotting.
Recruitment reactions were performed in siliconized Eppendorf tubes in a total volume of 200µl. Typical recruitment reaction mixtures contained R-Buffer, cytosol [10mg/ml], 10µl of liposomes, GTP [1mM] or GTP-γS [0.15mM], and when indicated recombinant myristoylated bovine ARF-1 [15µg/ml]. Tubes were kept on ice, and binding reactions were initiated by transfer to 37°C. After 20min, reactions were stopped on ice, and membranes were recovered by centrifugation at 4°C at 20 000g for 15min. The pellets were washed once with R-Buffer and recovered again by centrifugation. The pellet was analyzed by SDS-PAGE followed by Western-blotting using the relevant antibodies.

VII.7: Floatation of coated liposomes for mass spectrometric analysis

To remove unspecific bound or co-pelleted protein aggregates and to enrich for specifically recruited coat proteins, liposomes were floated on sucrose density gradients. Mouse brain cytosol was used as protein source prepared as follows: brains of 40 CD1 mice were homogenized in 20ml of R-Buffer (supplemented with complete protease inhibitors) using a tight dounce tissue grinder. The homogenate was clarified twice at 4°C at 280 000g for 60min (SW40 Ti). The final supernatant was snap-frozen and stored at -80°C. Standard recruitment assays for MS analysis were performed in siliconized Eppendorf tubes in a total volume of 2ml R-Buffer containing mouse brain cytosol [7.5mg/ml], 180µl of liposomes, GTP [1mM] or GTP-γS [0.15mM]. After 20min at 37°C, 2 volumes of ice-cold R-Buffer containing 60% (w/v) sucrose was added to the reaction mixture and transferred to centrifuge tubes (ultra-clear, 14x95mm, *Beckman*). The resulting 40% (w/v) sucrose mixture was overlaid with 3ml R-Buffer containing 30% (w/v) sucrose and finally with 3ml R-Buffer. The liposomes were floated with slow acceleration and no brake at 4°C at 280 000g for 12h (SW 40 Ti). The liposomes band at the interface between 30% sucrose and R-Buffer was removed completely together with 2ml of R-Buffer and diluted with 2ml of R-Buffer. The liposomes were pelleted at 4°C at 25 000g for 45min, washed once with R-Buffer and recovered again by centrifugation before SDS-PAGE and mass spectrometric analysis.

VII.8: SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Mini gels (5.5cm x 8.8cm x 1mm) were cast and run using the Protean 3 system (*Amersham Biosciences*) and running buffer (25mM Tris pH 8.3, 192mM Glycine, 0.1% (w/v) SDS) according to the manufactures protocol. Separating gels of the appropriate acrylamide concentrations (12% gels for standard SDS-PAGE analysis followed by Western blotting; 7%, 10% and 15% gels for mass spectrometric analysis) were prepared using 4x separating gel buffer (1.5M Tris-HCl pH 8.8), 30% (w/v) acrylamide stock solution (acrylamide/bisacrylamide 37.5/1 (w/w)), 10% (w/v) SDS stock solution, TEMED and 10% (w/v) ammonium persulfate stock solution. Stacking gels, having a final acrylamide concentration of 4%, were prepared using 4x stacking buffer (0.5M Tris-HCl pH 6.8) and the same stock solutions as used for the separating gel.

Liposome pellets after coat protein recruitment assays were mixed with 1x sample buffer (50mM Tris pH 6.8, 100mM DTT, 2% (w/v) SDS, 0.01% (w/v) bromphenole blue, 10% (v/v) glycerol) and heated to 65°C for 15min under constant agitation. Other samples were mixed with 0.2 volumes of 5x sample buffer (250mM Tris pH 6.8, 500mM DTT, 10% (w/v) SDS, 0.05% (w/v) bromphenole blue, 50% (v/v) glycerol) and heated to 95°C for 5min under constant agitation. Samples were centrifuged briefly and loaded. Mini gels were run at a constant voltage of 120V. Protein bands were stained for mass spectrometric analysis with staining solution (50% (v/v) MeOH, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie blue G-250) for 1h and de-stained using de-staining solution (50% (v/v) MeOH, 10% (v/v) acetic acid) with several exchanges and finally water. Band quantification was done using the LAS 3000 CCD camera system and the AIDA software (*Raytest*).

VII.9: Immunoblotting

Minigels were removed from the SDS-PAGE chamber, soaked in transfer buffer (25mM Tris pH 8.3, 192mM Glycine, 15% (v/v) MeOH, 0.01% (w/v) SDS) for 5min and blotted onto transfer buffer soaked nitrocellulose membrane (pore size 0.45µm, *Schleicher&Schuell*) using a semi-dry transfer cell (*BIORAD*) at constant current of 100mA per membrane for 1h. Transfer efficiency was checked by Ponceau S Red staining (0.5% (w/v) Ponceau S Red, 1% (v/v) acetic acid). The membrane was blocked in 5% (w/v) dry milk powder for 1h at 25°C. Incubations with primary and HRP-coupled secondary antibodies in blocking solution were done for 1h each at 25°C with 3x 10min PBS washes after the primary and secondary antibody incubation. Bands were detected with the ECL Western blotting detection reagents (*Amersham Biosciences*) and the LAS 3000 CCD camera system and the AIDA software (*Raytest*).

VII.9: Sample preparation for mass spectrometry

Coomassie stained protein bands were excised from the gel, cut into 1mm-cubes and washed twice with ultra-pure water. The gel pieces were then washed twice with 50% (v/v) acetonitrile (ACN) in 50mM ammonium bicarbonate (ABC) for 15min and shrunk by dehydration in ACN. The ACN was removed and the gel pieces were re-hydrated in 50mM ABC. After 5min the same volume of ACN was added for 15min and finally removed completely. The gel pieces were shrunk again in ACN for 5min, ACN was removed and gel pieces were dried in a vacuum centrifuge. Disulfide bonds were reduced by incubation with 10mM DTT in 50mM ABC for 45min at 56°C. Alkylation was performed by replacing the DTT solution with 55mM iodoacetamide in 50mM ABC. After 30min at 25°C in the dark, the gel pieces were washed with twice 50% (v/v) ACN in 50mM ABC, shrunk by dehydration in ACN, and dried in a vacuum centrifuge. The gel pieces were incubated with 100ng trypsin (sequencing grade, *Promega*) at 37°C overnight in 20µl of 25mM ABC. To extract the peptides, 20µl of 0.5% (v/v) trifluoroacetic acid (TFA) in ACN were added, the samples were sonicated and vortexed for 5min each. The supernatant was transferred into new tubes and the gel pieces were washed, sonicated and vortexed again with 20µl ACN. The supernatants were combined and dried in a vacuum centrifuge. For mass spectrometric analysis of the peptide mixture, samples were re-dissolved in 5µl 0.1% (v/v) TFA in water, referred as analyte solution.

VII.10: Protein identification by mass spectrometry

MALDI-MS measurements were performed with an Ultraflex MALDI-TOF/TOF mass spectrometer (*Bruker Daltonics*) in reflection mode using α-cyano-4-hydroxycinnamic acid as matrix. 1µl of the analyte solution was mixed with 1µl of α-cyano-4-hydroxycinnamic acid matrix solution consisting of 5mg of matrix dissolved in 1ml of 0.3% TFA in acetonitrile/water (1:1, v/v). 2µl of the resulting mixture was applied to the sample plate. Samples were air-dried at 25°C. All mass spectra were internally calibrated with trypsin autolysis peaks. Spectra obtained represented the accumulation of approximately 800 laser shots and were processed using FlexAnalysis software (*Bruker Daltonics*). Protein identification, both by peptide mass fingerprint and fragment ion analysis, was performed using MASCOT (*Matrix Sciences*). Search criteria were: taxonomy, mouse; mass accuracy, 50ppm for peptide mass fingerprinting and 0.5Da for fragment analysis; modifications, carbamidomethylation and mehtionine oxidation; maximum one missed cleavage site. The NCBI non-redundant protein database was used for protein identification.

VII.11: Electron microscopy

After incubation of a standard recruitment assay, liposomes were recovered by centrifugation. Membrane pellets were fixed overnight at 4°C with 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH7.4 (*Electron Microscopy Sciences*) and then post-fixed with 1% (v/v) osmium tetroxide. Membranes were then dehydrated with increasing concentrations of ethanol and then embedded in Epon. After polymerization of the resin at 60°C for 48hours, the sample was thin sectioned (50nm) using an ultramicrotom (*Leica Microsystems*). Thin sections were mounted on grids and further contrasted with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate. Analyses were done with a Morgagni transmission electron microscope (*FEI company*).

VII.12: Cell culture

HeLa cells were grown in D-MEM (1000mg/l glucose, 4mM L-glutamine, 1mM sodium pyruvate) supplemented with 10% (v/v) fetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator (all tissue culture reagents were from *Invitrogen* unless otherwise stated). Confluent cells were passaged by two washes with PBS followed by incubation with trypsin-EDTA at 37°C for 10min. Trypsin was inhibited by addition of normal culture medium and cells were transferred into new cell culture dishes.

VII.13: FuGENE 6 transfection

For transient lipofection, 35 000 HeLa cells were directly plated onto 11mm-diameter cover slips in 500µl medium in 24 well plates. After 24h, FuGENE 6 (*Roche Biochemicals*) was used for transfection with a FuGENE/DNA ratio of 6µl/1µg. The amount of DNA per cover slip varied between 0.2µg and 0.8µg, depending on the plasmid used. Briefly, FuGENE was diluted into 20µl D-MEM without any supplements, after 5min DNA was added followed by 10min incubation at 25°C. The FuGENE solution was added drop wise to the well and cells were normally incubated for 24h until fixation.

VII.14: Calcium-phosphate transfection

For more homogenous and milder expression levels, 30 000 HeLa cells were plated onto 11mm-diameter cover slips in 500µl medium in 24 well plates. After 24h, between 0.1µg and 0.8µg plasmid DNA was diluted in 10mM Tris pH 6.5, 500mM calcium phosphate in a final volume of 50µl. An equal volume of 2x HBS (50mM HEPES pH 7.07, 280mM NaCl, 1.43mM NaH_2PO_4) was added and the resulting 100µl solution containing calcium phosphate precipitates was added to the well. After 24 h, the cells were washed twice with PBS, new cell culture medium was added and the cells were again incubated for 24h until fixation.

VII.15: Immunostaining

Cells grown on cover slip were washed 3 times with PBS (PBS washing steps were done in between all following steps), fixed in 3% (w/v) paraformaldehyde in PBS for 20min at 25°C. Free paraformaldehyde was chemically quenched with 50mM NH_4Cl in PBS for 10min followed by permeabilization of the cells with 0.1% (v/v) Triton X-100 in PBS. Cells were blocked with blocking solution (5% (w/v) gelatine, 3% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS) for 1h at 25°C. Cover slips were inverted on a drop of 30µl of appropriate primary antibody diluted in blocking solution and incubated for 1h at 25°C in a moist chamber. Following three PBS washing steps, cover slips were incubated with the appropriate secondary antibody as described above. Cover slips were washed three times with PBS, once with water and mounted on glass slides by inverting them onto a droplet of mowiol containing 10µg/ml DAPI (*Invitrogen*).

VII.16: Lamp1-antibody uptake assay

The assays were performed in 24 well plates with 80% confluent HeLa cells. HeLa cells were transfected with 0.3µg pEGFP-2xFYVE (gift of M. Zerial) by calcium phosphate transfection. After 24h, cells were washed and fresh culture medium was added. 48h post transfection, cells were washed and culture medium was replaced by culture medium containing 1% (v/v) FCS, 20mM HEPES pH 7.25 and 2µg/ml anti Lamp1 antibody. In a modified Lamp1-antibody uptake assay, Hela cells were treated with wortmannin (*Sigma*). 50 000 HeLa cells were plated on cover slips, 24h later cells were washed and the culture medium was replaced by culture medium containing 1% (v/v) FCS, 20mM HEPES pH 7.25, 2µg/ml anti Lamp1 antibody and 100nM wortmannin. In both assays, cells were allowed to take up the anti Lamp1 antibody at 37°C for 2.5h before they were washed with PBS, fixed and processed for immunostaining to detect the relative amount of bound and internalized antibody.

VII.17: Confocal microscopy

Cover slips with stained and/or transfected cells were mounted in mowiol. An inverted confocal laser scanning microscope LSM510 META (*Carl Zeiss*) with a 63x or 100x oil Apochromat objective was used for image acquisition. Images were generally taken as 12 Bit 1024/1024 image frames at pin hole settings yielding 0.8 µM-optical sections in all channels. Image processing after acquisition was done with Adobe Photoshop.

VII.18: Equipment

Centrifuge 5417R (*Eppendorf*); Concentrator 5301 (*Eppendorf*); SW32 Ti, SW40 Ti, SW60 Ti, 45Ti for Optima LE-80K Ultracentrifuge (*Beckman/Coulter*); JLA8.1000 for Avanti J-20 XP (*Beckman/Coulter*); Protean 3 system (*BIORAD*), Semi-dry transfer cell (*BIORAD*); LAS-3000 CCD camera system (*Raytest*); LSM510 META confocal microscope (*Carl Zeiss*); Morgagni transmission electron microscope (FEI company); HERA cell 150 incubator (*Heraeus*); Electroporator 2510 (*BIORAD*); EmulsiFlex-C5 (*Avestin*); ÄKTA explorer 10S (*Amersham Biosciences*); Ultraflex MALDI-TOF/TOF (*Bruker Daltonics*)

VII.19: Frequently used buffers, media & solutions

LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl); PBS (155mM NaCl, 1.54mM KH_2PO_4, 2.71mM Na_2HPO_4-$7H_2O$ pH 7.2); 5x SDS-PAGE sample buffer (250mM Tris pH 6.8, 500mM DTT, 10% (w/v) SDS, 0.05% (w/v) bromphenole blue, 50% (v/v) glycerol); 10x SDS-PAGE running buffer (250mM Tris pH 8.3, 1.9M glycine, 1% (w/v) SDS); semi-dry blotting transfer buffer (25mM Tris pH 8.3, 192mM Glycine, 15% (v/v) MeOH, 0.01% (w/v) SDS); recruitment buffer (25mM HEPES-KOH pH 7.2, 125mM potassium acetate, 2.5mM magnesium acetate, 1mM DTT; 5mM glycerol-2 phosphate was added when phosphoinositides or phosphorylated peptides were present); immunostaining blocking solution (5% (w/v) gelatine, 3% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS)

VIII. Abbreviations

APs	adaptor complexes
APCs	antigen presenting cells
Arf1	ADP ribosylation factor protein 1
BFA	brefeldin A
CCVs	clathrin coated vesicles
CME	clathrin mediated endocytosis
COP	coatomer coat protein
DAG	diacylglycerol
ER	endoplasmatic reticulum
ESCRT	endosomal sorting complexes required for transport
FCCS	fluorescence cross correlation spectroscopy
FCS	fetal calf serum
FYVE domain	Fab1/YOTP/Vac1/EEA1 domain
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GGAs	Golgi localized γ-ear containing Arf binding proteins
gpI	glycoprotein I
LPCs	large pleiomorphic carriers
MALDI	matrix assisted laser desorption ionization
MPRs	mannose 6-phosphate receptors
MS	mass spectrometry
MVBs	multivesicular bodies
PACS-1	phosphofurin acidic cluster sorting protein 1
PH domain	pleckstrin homology domain
PI	phosphoinositide
PIPs	phosphatidylinositolphosphates
PX domain	PHOX homology domain
SNAREs	soluble N-ethylmaleimide sensitive factor attachment protein receptors
SNX	sorting nexin
TGN	trans-Golgi network
VAMP	vesicle associated membrane protein
wt	wild type

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