A Role for Amphiphysin in AP-1/Clathrin Coat Formation

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Summary

Transport of cargo within the endocytic and secretory pathway is generally mediated by coated vesicles. Clathrin, in combination with different adaptor proteins, is the major coat protein for vesicle formation at the plasma membrane, endosomes, and the trans-Golgi network (TGN). Best characterized is the formation of clathrin coats for endocytosis at the plasma membrane involving the adaptor protein complex AP-2. Clathrin and AP-2 were shown to be at the centre of a complex interactome of proteins accessory to vesicle formation. Considerably less is known about the formation of clathrin coated carriers at the TGN and endosomes, where the adaptor protein complex AP-1 plays a major role.

In vitro studies showed the minimal requirements for association of AP-1 to liposomal membranes to be activated ARF1, phosphoinositides, and either sorting signals or unknown cytosolic factors. We have used a liposome floatation assay to identify cytosolic proteins collaborating with AP-1 at the membrane. Separation of proteins from bovine brain cytosol with several chromatographic methods yielded an active fraction containing amphiphysin 1, amphiphysin 2, and endophilin A1. All three proteins are expressed in brain and known to be involved in AP-2/clathrin coat formation. They consist of an N-terminal N-BAR (Bin, amphiphysin, Rvs) domain for dimerization and membrane binding and a C-terminal SH3 (Src homology 3) domain for interaction with dynamin and synaptojanin. Amphiphysin 1 and 2 in addition contain a middle domain with binding sites for adaptors and clathrin. It was proposed that amphiphysins and endophilin are targeted to membranes with high curvature, such as the neck of a forming vesicle, where they recruit dynamin and synaptojanin in preparation for vesicle fission and uncoating.

In this thesis, I bacterially expressed and purified all three proteins and tested them in the floatation assay for AP-1 membrane binding activity. Only amphiphysin 2 showed activity, both as a homodimer and as a heterodimer with amphiphysin 1. Activity depended on a motif that was shown to bind to AP-1, AP-2, and clathrin in GST pull-down experiments.

Endogenous amphiphysins in primary neurons, as well as transiently expressed in neuronal or fibroblast cell lines, co-localized with AP-1 at the TGN. In addition, when expressed at high levels in neuronal cells, amphiphysins aggregated and interfered dominantly with the TGN localization of AP-1. Both phenomena depended on the presence of the clathrin and adaptor interaction sequence in the amphiphysins. Furthermore, both amphiphysins could be cross-linked to AP-1 *in vivo*.

Our results indicate that amphiphysin 1 and 2 function not only in clathrin coated vesicle formation for endocytosis at the plasma membrane, but are also part of the machinery forming AP-1/clathrin coats at the TGN and endosomes. This suggests that the machineries for CCV formation with AP-1 and AP-2 at different locations in the cell share more components than previously anticipated.

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Abbreviations

AAK1	**A**daptor **a**ssociated **k**inase 1
AP-1, -2, -3, - 4	**A**daptor **p**rotein complex 1, 2, 3, 4
ARF	**A**DP-**r**ibosylation **f**actor
ARP2/3	**A**ctin-**r**elated **p**rotein **2/3**
BAR	**B**in, **a**mphiphysin, **R**vs
BDNF	**B**rain-**d**erived **n**eurotrophic **f**actor
BFA	**B**re**f**eldin A
BIN1	**B**ox-**d**ependent myc-**in**teracting protein-**1**
Bip	**B**inding **i**mmunoglobulin **p**rotein
CALM	**C**lathrin **a**ssembly **l**ymphoid **m**yeloid leukemia
CCP	**C**lathrin **c**oated **p**it
CCV	**C**lathrin **c**oated **v**esicle
CHC	**C**lathrin **h**eavy **c**hain
CLASP	**Cl**athrin-**a**ssociated **s**orting **p**rotein
CLC	**C**lathrin **l**ight **c**hain
CME	**C**lathrin **m**ediated **e**ndocytosis
COPI/II	**Co**at **p**rotein I/II
DAPI	4’,6-**Dia**midino-2-**p**henyl**i**ndole
EEA1	**E**arly **e**ndosome **a**ntigen 1
EGF	**E**pidermal **g**rowth **f**actor
EH	**E**psin **h**omology
EM	**E**lectron **m**icroscopy
ENTH	**E**psin **N**-**t**erminal **h**omology

EPS15 **E**pidermal growth factor **p**rotein **s**ubstrate 15

Epsin1 **EPS**15 **in**teracting protein

ER **E**ndoplasmic **r**eticulum

ERAD **ER**-**a**ssociated **d**egradation

ERC **E**ndocytic **r**ecycling **c**ompartment

ERES **ER** **e**xit **s**ite

ERGIC **ER**-**G**olgi **i**ntermediate **c**ompartment

ESCRT **E**ndosomal **s**orting **c**omplex **r**equired for **t**ransport

FCHo **F**er/**C**ip4 **h**omology domain-**o**nly

GAE γ-**a**daptin **e**ar

GAG **G**lycos**a**mino**g**lycan

GAK cyclin-**G**-**a**ssociated **k**inase

GAP **G**TPase-**a**ctivating **p**rotein

GAT **G**GA **a**nd **T**OM

GBF1 **G**olgi-specific **b**refeldin A resistance **f**actor 1

GEF **G**uanine nucleotide **e**xchange **f**actor

GGA **G**olgi-localized, γ-ear containing, **A**RF-binding protein

GMP-PNP Guanylyl imidodiphosphate

GPCR **G**-**p**rotein **c**oupled **r**eceptors

GPI **G**lyco**p**hosphatidyl**i**nositol

HSC70 **H**eat **s**hock **c**ognate 70

HSP **H**eat **s**hock **p**rotein

IF **I**mmuno**f**luorescence

IPTG **I**so**p**ropyl β-D-1-**t**hio**g**alactopyranoside

LAMP1 **L**ysosome-**a**ssociated **m**embrane **p**rotein **1**

LDL **L**ow-**d**ensity **l**ipoprotein

LDLR **L**ow-**d**ensity-**l**ipoprotein receptor

MMCC-DOPE	(N-((4-**m**aleimidyl**m**ethyl)**c**yclohexane-1-**c**arbonyl)-1,2-**d**ioleolyl-sn-glycero-3-**p**hospho-**e**thanolamine
MPR	**M**annose 6-**p**hosphate **r**eceptor
MTOC	**M**icro**t**ubule **o**rganizing **c**enter
MVB	**M**ulti**v**esicular **b**ody
NCS	**N**ewborn **c**alf **s**erum
NGF	**N**erve **g**rowth **f**actor
NTID	**N**-**t**erminal **i**nsert **d**omain
N-WASP	**N**eural **W**iskott-**A**ldrich **s**yndrome **p**rotein
PA	**P**hosphatic **a**cid
PC	**P**hosphatidyl**c**holine
PCR	**P**olymerase **c**hain **r**eaction
PDI	**P**rotein **d**isulfide **i**somerase
PE	**P**hosphatidyl**e**thanolamine
PFA	**P**ara**f**orm**a**ldehyde
PH	**P**leckstrin **h**omology
PMSF	**P**henyl**m**ethyl**s**ulfonyl **f**luoride
PRD	**P**roline **r**ich **d**omain
PS	**P**hosphatidyl**s**erine
PtdIns(3)P	**P**hospha**tid**yl**in**o**s**itol-**3**-**p**hosphate
PtdIns(3,5)P_2	**P**hospha**tid**yl**in**o**s**itol (**3,5**)-bis**p**hosphate
PtdIns(4)P	**P**hospha**tid**yl**in**o**s**itol-**4**-**p**hosphate
PtdIns(4,5)P_2	**P**hospha**tid**yl**in**o**s**itol (**4,5**)-bis**p**hosphate
PtdIns(3,4,5)P_3	**P**hospha**tid**yl**in**o**s**itol (**3,4,5**)-tris**p**hosphate
PtdIns(5)P	**P**hospha**tid**yl**in**o**s**itol-**5**-**p**hosphate
PTEN	**P**hosphatase and **ten**sin homologue
PX	**P**ho**x** homology

RME-1/EHD1	**R**eceptor **m**ediated **e**ndocytosis/**E**ps15 **h**omology-**d**omain containing **1**
RNAi	**RNA i**nterference
SH3	**S**rc **h**omology 3
SNARE	**S**oluble **N**-ethylmaleimide-sensitive factor **a**ttachment protein **re**ceptor
SNX	**S**orting **nex**in
SRP	**S**ignal **r**ecognition **p**article
TfR	**T**rans**f**errin **r**eceptor
TGN	***T**rans*-**G**olgi **n**etwork
TIRF	**T**otal **i**nternal **r**eflection **f**luorescence
TOM	**T**arget **o**f **m**yb
Tris	**Tris**(hydroxymethyl)aminomethane
UIM	**U**biquitin **i**nteraction **m**otif
UPR	**U**nfolded **p**rotein **r**esponse
VHS	**V**ps, **H**rs, **S**tam
VPS	**V**acuolar **p**rotein **s**orting
VSVG	**V**esicular **s**tomatitis **v**irus **G**
WB	**W**estern **b**lot

Introduction

1. Intracellular transport

Membrane traffic in higher eukaryotes is a fundamental function of every cell to maintain its metabolism and proceeds in a highly regulated and specific manner. Cargo proteins and lipids are transported through the secretory and the endocytic pathway via different membrane-enclosed organelles (Figure 1). Transport between these organelles occurs via vesicles and tubules or via fusion and maturation of transport intermediates and involves an array of different proteins to ensure specificity.

Even though the molecular details of distinct pathway steps are not fully understood, great progress has been made in the last decades to understand the basic mechanisms of protein transport through the cell.

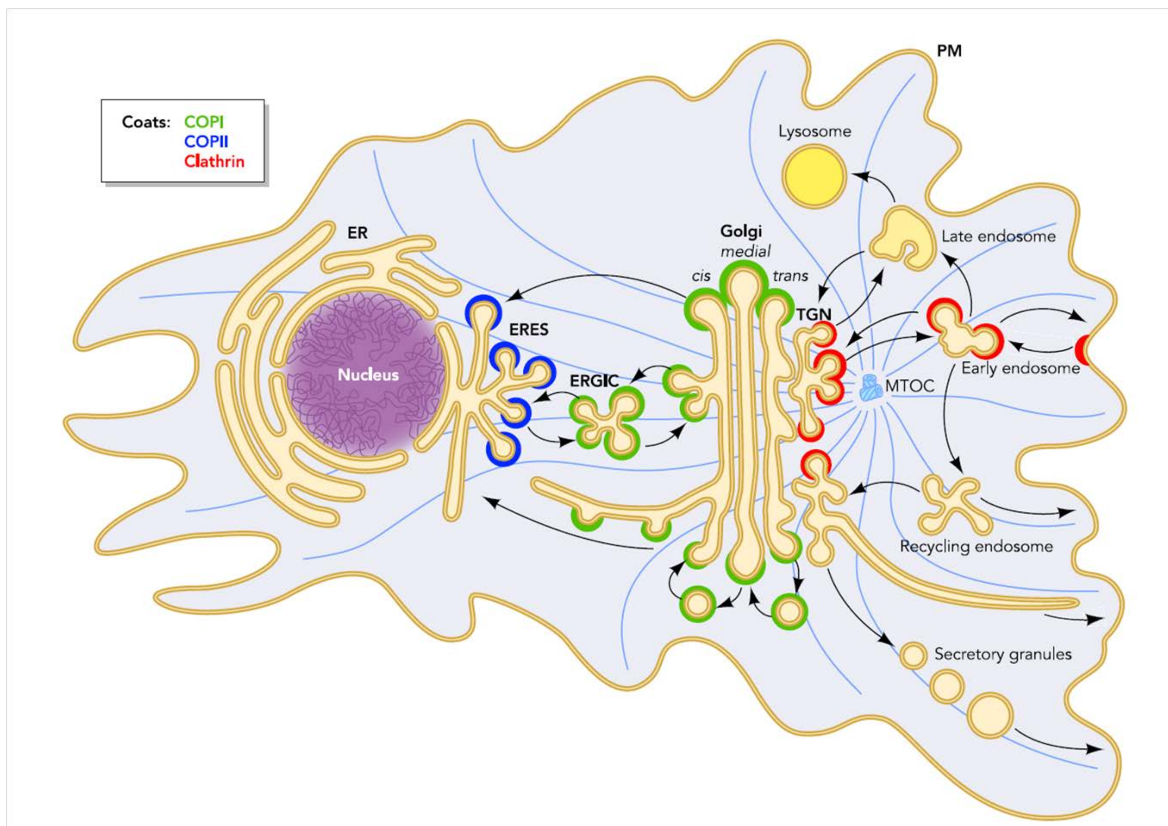

Figure 1: Intracellular transport pathways.

The scheme depicts the compartments of the secretory and endocytic pathway. Transport steps are indicated by arrows and colors indicate the vesicle coats: COPII (blue), COPI (green), and clathrin (red) ((Szul and Sztul, 2011).

1.1. The secretory pathway

In mammalian cells, cytosolic proteins, proteins localized in the nucleus, the peroxisomes, and the mitochondria are synthesized in the cytosol by free ribosomes as well as in the mitochondria itself. In contrast, proteins of the endoplasmic reticulum (ER), the Golgi-apparatus, the endosomal-lysosomal system, the plasma membrane, as well as secretory proteins are transported into the ER by membrane-bound ribosomes to enter the secretory pathway. The distinct steps of the secretory pathway include the translocation of cargo into the ER where it is folded and transported via ER-Golgi intermediate compartment (ERGIC) to the Golgi apparatus for further processing. After arriving in the TGN, cargo is sorted into *post*-Golgi carriers to be transported to its place of operation.

1.1.1. ER transport

The ER is the largest cell organelle and forms a tubular network throughout the cell consisting of smooth and rough (ribosome associated) regions. The functions of the ER involve biosynthesis of secretory and membrane proteins, protein quality control, protein glycosylation (N-glycosylation), lipid synthesis, as well as calcium storage.

Secretory proteins and most membrane proteins are co-translationally translocated into the ER via an aqueous translocation channel. These proteins contain a hydrophobic signal sequence of 7-25 amino acids, which is recognized by the signal-recognition particle (SRP) as the nascent poly-peptide chain emerges from the ribosome. The ribosome-nascent chain-SRP complex is targeted to the ER membrane by interaction of the SRP with its membrane receptor, which allows docking of the ribosome to the Sec61 channel (Gilmore et al., 1982; Walter et al., 1982) and subsequent translocation of the growing polypeptide directly into the ER lumen.

Once in the ER, proteins fold with the assistance of chaperones and different modifications such as signal-peptide cleavage, N-linked glycosylation, disulfide bond formation, and glycosylphosphatidyl-inositol (GPI)-anchor attachment occur. A quality control system consisting of different chaperones and heat shock proteins (Hsp) ensures that only correctly folded proteins can leave the ER. As these chaperones bind to incorrectly folded proteins they prevent them from leaving the ER and, at the same time, facilitate folding reactions to produce mature proteins ready to be released from the ER (Helenius et al., 1992). Chaperones used in the quality control include Binding immunoglobulin protein (Bip), calnexin/calreticulin and protein disulfide isomerases (PDI). Proteins not able to fold correctly are retro-translocated back to the cytosol by the ER-associated degradation (ERAD) machinery where they

undergo proteasomal degradation. An accumulation of unfolded proteins leads to ER stress and induces the unfolded protein response (UPR), which includes transcriptional up-regulation of specific UPR genes, down-regulation of global protein synthesis, as well as ERAD (Ron and Walter, 2007; Yoshida, 2007). In contrast, correctly folded and modified proteins and protein complexes are sorted to specific ER exit sites (ERES), where they are integrated into COPII vesicles and transported to the ERGIC (Barlowe et al., 1994).

Sorting of cargo from the ER is carried out by bulk-flow mechanism, where secretory proteins are packaged into transport vesicles by default (Wieland et al., 1987) or by selective export using signal sequences. Transmembrane cargo proteins contain sorting motifs (eg. Aromatic or dihydrophobic) on their cytoplasmic domains, which are recognized by the Sec24 subunit of the COPII coat. Transport of soluble cargo proteins is mediated by signal sequences (eg. dihydrophobic or dilysine residue), which bind to sorting receptors such as ERGIC-53, the p24 proteins, and a set of ER vesicle (ERV) proteins. These receptors interact with COPII components and cycle between the ER and Golgi (Dancourt and Barlowe, 2010; Szul and Sztul, 2011).

1.1.2. ER to Golgi transport

After leaving the ER, COPII vesicles transport their cargo to the ERGIC, a structure which is characterized by tubulovesicular membrane clusters and the presence of the marker protein ERGIC-53 (Hauri et al., 2000). In the now favored model, newly synthesized cargo proteins as well as ERGIC-53 are transported from ERES to stationary ERGIC clusters nearby, which serve as a sorting station that discriminates between anterograde transport and retrograde transport back to the ER (Ben-Tekaya et al., 2005). ER-resident membrane proteins contain a cytosolic ER retention signal (eg. KKXX), leading to their binding to COPI coat components and subsequent packaging into COPI vesicles for retrograde transport to the ER (Pelham, 1994). Soluble ER proteins terminating in the sequence KDEL or a related sequence are recognized by the transmembrane receptors KDEL or Rer1 and transported back to the ER (Pelham, 1996; Sato et al., 2003).

Anterograde cargo is transported from the ERGIC to the *cis*-Golgi via rather large anterograde carriers, which move rapidly towards the Golgi (Ben-Tekaya et al., 2005). However, the mechanism for the formation of these carriers remains unknown.

1.1.3. Intra-Golgi transport

In mammalian cells, the Golgi complex consists of a network of stacks, which are composed of flattened cisternae and linked by tubular connections. Unlike in yeast cells, where unstacked Golgi cisternae are distributed in the cytosol, the 40-100 Golgi stacks present in a mammalian cell are normally localized in a single perinuclear region near the microtubule organizing center (MTOC) (Duran et al., 2008). The Golgi can be subdivided into *cis*-, *medial*- and *tran*s-Golgi cisternae. Cargo enters the Golgi from the *cis*-side and during its transport to the *trans*-Golgi, it undergoes cisternae-specific modifications in every compartment. Modifications are executed by Golgi-resident enzymes and include O-linked glycosylation, addition of galactose and sialic acid, as well as synthesis and attachment of glycosaminoglycans (GAG) to form proteoglycans. An important function of the Golgi is also the labeling of lysosomal proteins with mannose-6-phosphate, which is recognized later by the mannose-6-phosphate receptor.

Anterograde intra-Golgi transport of cargo proteins is accomplished by cisternal maturation. Cargo stays within a single Golgi cisterna, which assembles at the *cis*-Golgi, matures along the Golgi apparatus and finally disassembles at the *trans*-Golgi (Losev et al., 2006; Matsuura-Tokita et al., 2006). Retrograde transport of Golgi-resident enzymes to their cisterna of origin is mediated by COPI vesicles (Love et al., 1998; Orci et al., 1997; Sonnichsen et al., 1996). COPI vesicles are also responsible for retrograde transport of ER-specific proteins, which are recognized by the same signal sequence as in ERGIC-to-ER-transport. As secretory proteins finally reach the *trans*-Golgi side, they are sorted to their final destination in the TGN.

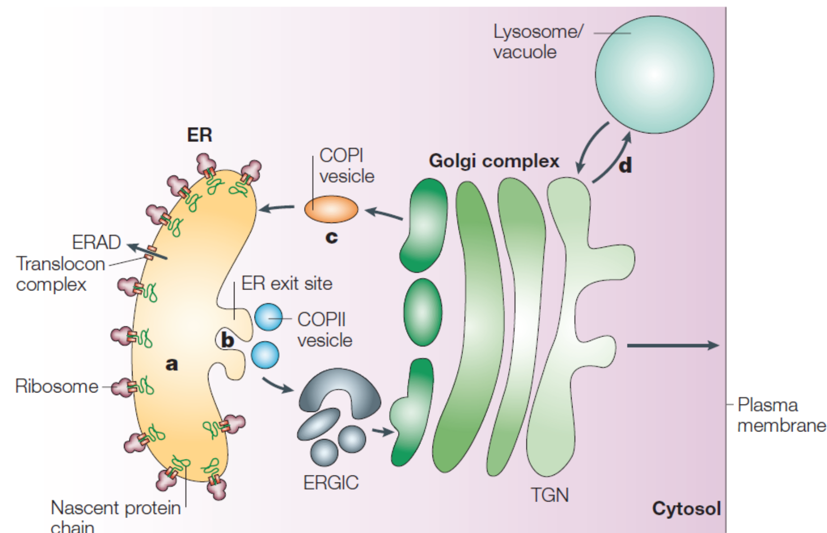

Figure 2: Organelles of the early secretory pathway.

The ER is the site of synthesis and maturation of proteins (**a**). Once they are correctly folded, they enter ERES (**b**), where they are packaged into COPII vesicles and are transported via ERGIC to the Golgi. The retrieval of ER resident or misfolded proteins from the Golgi to the ER occurs via COPI vesicles (**c**). Correctly folded and modified proteins are sorted in the TGN for the plasma membrane or the endosomal/lysosomal system (**d**) (Ellgaard and Helenius, 2003).

1.1.4. Post-Golgi transport at the TGN

The TGN is the cell compartment which combines secretory and endocytic routes, the destinations of cargo molecules released from the TGN are the plasma membrane, different parts of the endosomal-lysosomal system, and secretory granules (in endocrine cells), while the TGN receives cargo from endosomes and the plasma membrane (Figure 3).

Morphologically, the TGN is described as a tubular compartment adjacent to the *trans*-side of the Golgi stack that is continuous with the *trans*-most Golgi cisterna (Klumperman, 2011). Therefore, the TGN has a cisternal and tubular part, whose conversion is dependent on protein- and lipid-based mechanisms. The tubular part of the TGN has a distinctive and pleiotropic morphology, being a collection of branched tubules with budding regions and associated vesicles. This morphology shows a strong dependence on the cell type and also undergoes dynamic changes depending on the level of protein expression.

There are different exit routes for cargo proteins at the TGN. The region coated with clathrin gives rise to clathrin coated vesicles (CCVs) that transport e.g. mannose 6-phosphate-tagged lysosomal enzymes bound to mannose 6-phosphate receptors (MPRs) to endosomes. After delivery of their cargo, the MPRs recycle back to the TGN for new rounds of transport (Klumperman, 2011; Kornfeld and Mellman, 1989).

In general, sorting to the endosomal system and, in polarized cells, also to the basolateral membrane is carried out by clathrin coated carriers, specificity being accomplished by different sorting signals recognized by different types of clathrin adaptor proteins.

Proteins lacking a specific sorting signal are packed into vesicles of the constitutive pathway to the plasma membrane. Secretory cells contain an additional regulated secretory pathway where cargo is sorted into secretory granules which accumulate in the cytoplasm until an external stimulus triggers them to fuse with the plasma membrane (Huttner and Tooze, 1989; Kelly, 1985).

All these sorting processes occur during the formation of tubular-vesicular carriers whose exact structure and molecular components are not yet fully understood (Anitei and Hoflack, 2011). In contrast to the regularly shaped endocytic vesicles, these pleiomorphic carriers (1-8 μm long) extend from selected TGN regions, sometimes retracting before detaching and breaking into smaller elements. New findings now implicate that cargo is segregated and clustered into distinct membrane microdomains for bending, elongation, and fission of corresponding membranes and thus, specific carrier formation.

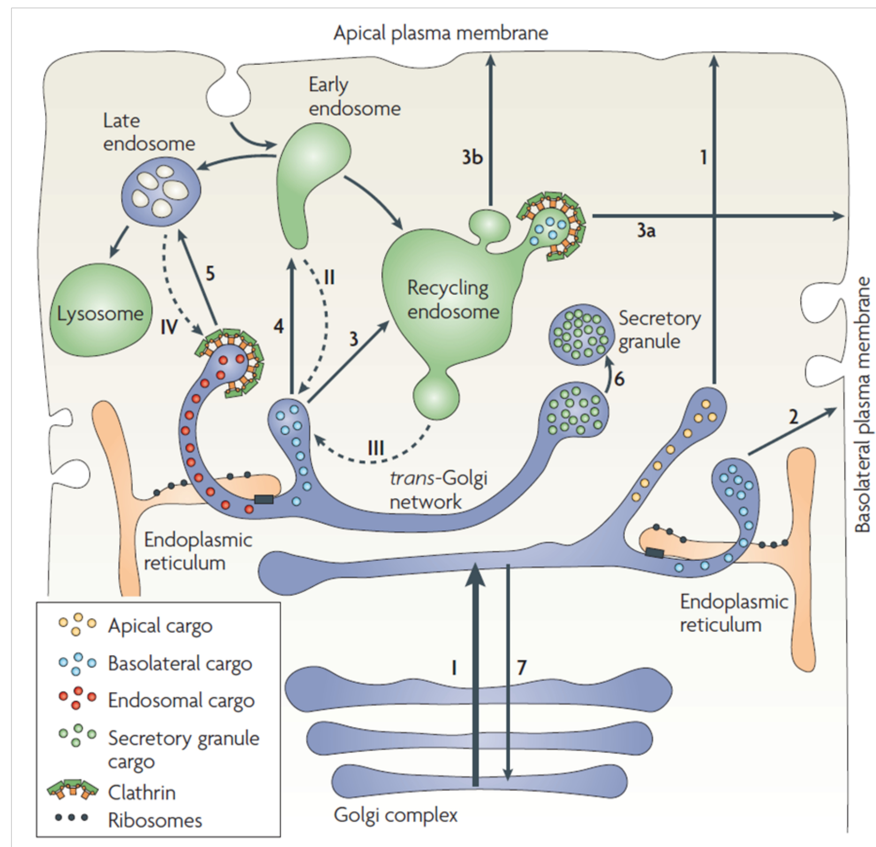

Figure 3: TGN sorting at the crossroads of the endocytic and secretory pathways.

The TGN sorts newly synthesized proteins that arrive from Golgi compartments (**I**) to different destinations as plasma membrane (**1**), basolateral membrane in polarized cells (**2**), recycling endosomes (**3**), early endosomes (**4**), late endosomes (**5**), and secretory granules in secretory cells (**6**). It also receives cargo from the endocytic pathway (**II-IV**) and sends back components to the Golgi cisternae (**7**) (De Matteis and Luini, 2008).

1.2. The endocytic pathway

Endocytosis is crucial for many cellular functions and plays a role in nutrient acquisition, antigen presentation, clearance of apoptotic cells, synaptic transmission, receptor regulation, as well as controlling the lipid and protein composition of the plasma membrane. There are several mechanisms how cargo can be internalized such as phagocytosis, macropinocytosis, and clathrin- and caveolin-dependent endocytosis (Figure 4). Some of these pathways are constitutive whereas others are triggered by external signals. The best studied endocytic process involves the internalization of receptors and their ligands by CCVs, a mechanism which is used by all eukaryotic cells and is fundamental to signal transduction.

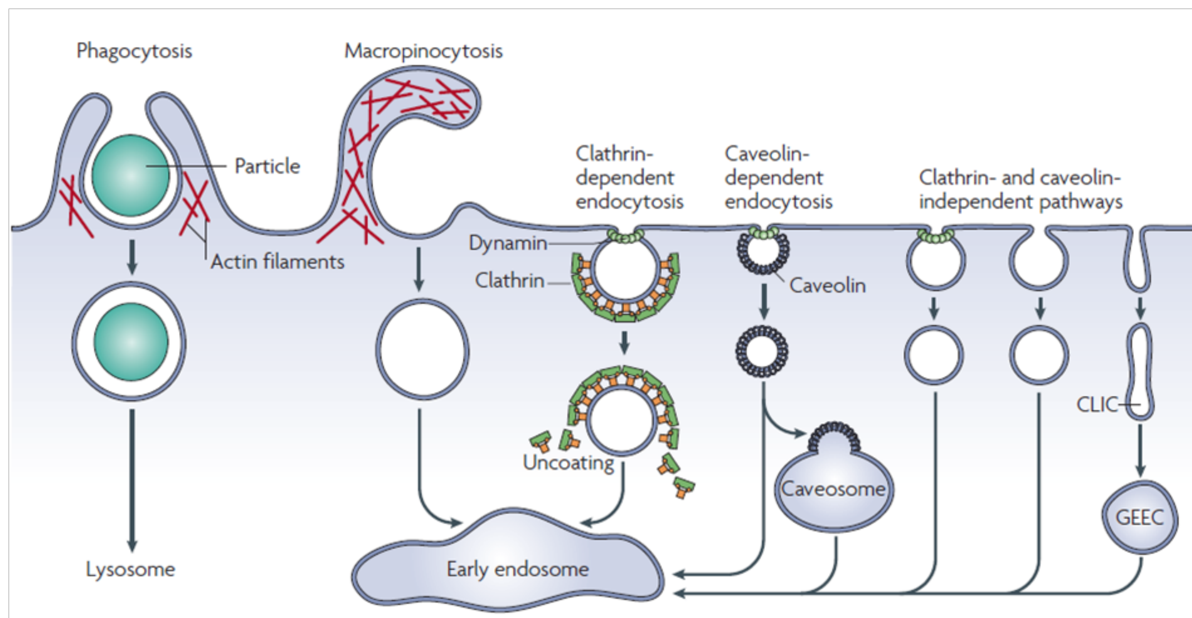

Figure 4: Pathways of entry into the cell.

Large particle and fluid uptake occurs via phagocytosis and macropinocytosis, respectively, while clathrin- and caveolin-dependent endocytosis generates much smaller, coated vesicles. In addition, numerous amount of cargo can be endocytosed by mechanisms that are independent of clathrin and caveolin (Mayor and Pagano, 2007).

Most cargo is delivered to early sorting endosomes after internalization. These endosomes consist of luminal and tubular parts, have a pH of ~6.0, are peripherally localized, and carry surface markers such as early endosome antigen 1 (EEA1) and Rab5. After stripping off their clathrin coats, endocytic vesicles fuse with one another and with pre-existing sorting endosomes. As a consequence of the low pH, most cargo receptors release their ligands and are either recycled back to the plasma membrane directly or indirectly via tubular recycling endosomes. The ligands and other soluble proteins stay in the luminal sorting endosome which starts to acquire acid hydrolases to become more acidic and takes on further properties of late endosomes as specific lipid composition (Phosphatidylinositol 3,5-bisphosphate) and surface proteins (Rab7, Rab9). This transition from sorting to late endosome is referred to as maturation (Maxfield and McGraw, 2004). Along the pathway to the lysosome, late endosomes gain a characteristic multivesicular appearance and are called multivesicular bodies (MVBs). Proteins sent for degradation and also receptors which are not recycled (eg. signaling receptors) are sorted into these vesicles by the endosomal sorting complex required for transport (ESCRT). This machinery is highly conserved between eukaryotes and mediates membrane invagination and vesicle fission for ubiquitin-dependent degradation of substrates (Saksena et al., 2007). Fusion of the MVB with the lysosomes finally delivers

the intraluminal vesicles and their content into the lumen of lysosomes where they are degraded by proteases (Figure 5).

1.3. The recycling system

As mentioned above, there are two main routes for internalized cargo from sorting endosomes back to the cell surface: some recycling molecules are delivered directly back to the plasma membrane from early sorting endosomes, while others are indirectly recycled over the recycling endosome or endocytic recycling compartment (ERC) (Figure5). The ERC is a tubular network of membranes which is microtubule associated and contains specific surface proteins as Rab11. Depending on the cell type, the ERC can be perinuclear or dispersed throughout the cytoplasm. Two well studied receptors using the slow recycling pathway are the low-density-lipoprotein receptor (LDLR) with its ligand low-density lipoprotein (LDL) and transferrin. LDL is released from the LDLR in the sorting endosomes and transported to the lysosome for degradation, while the LDLR recycles back to the plasma membrane via ERC. Transferrin, unlike most other ligands, is not released from the transferrin receptor (TfR) in the acidic environment of sorting endosomes, but it releases its two bound iron ions. Iron-free transferrin remains bound to its receptors until it is recycled to the cell surface (Maxfield and McGraw, 2004). It is presumed that the recycling endosomes consists of narrow diameter tubules that extend from sorting endosomes (tubular endosomal network) and are pinched off from the main body of the sorting endosomes which matures into the late endosomes.

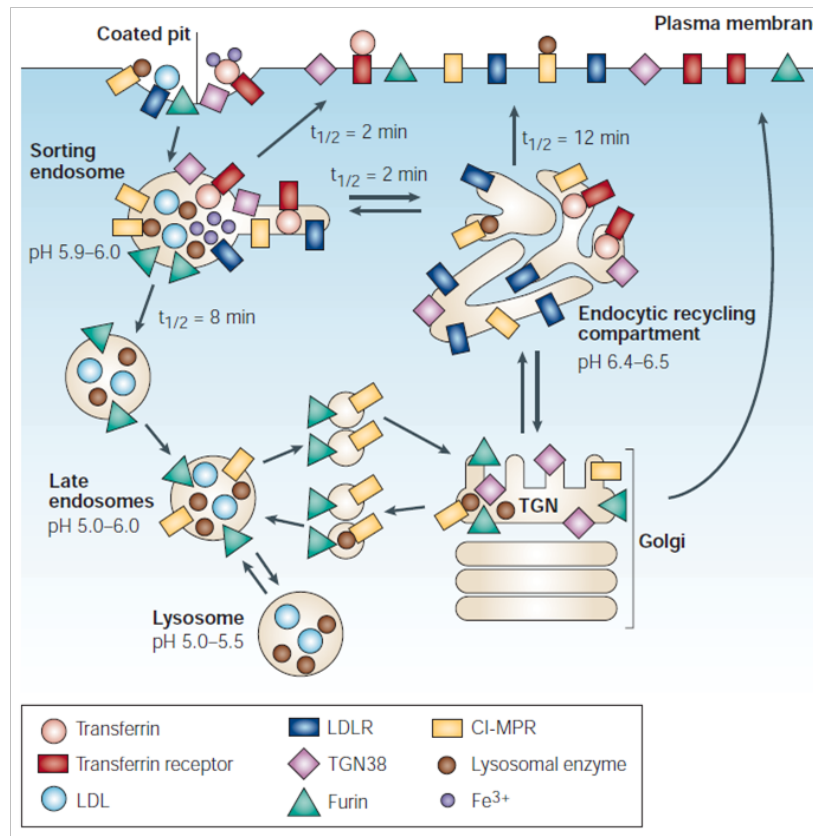

Figure 5: Endocytic recycling pathways.

Transferrin and the LDLR are internalized and transported into sorting endosomes where they release the bound iron and the LDL, respectively. The LDLR and transferrin bound to its receptor are recycled to the plasma membrane via endocytic recycling compartment, while LDL is sent to the lysosome for degradation (Maxfield and McGraw, 2004).

1.4. Retrograde transport from endosomes to the TGN

The retrograde transport system from the endosomes to the TGN is used by different intracellular transmembrane proteins as well as extracellular toxins such as shiga and cholera toxin and plays a role in transportation of membranes and organelle specific proteins back to their compartment of origin (Bonifacino and Rojas, 2006). For some cargo as acid-hydrolase receptors, SNARES and different transmembrane enzymes, retrograde transport from the endosomal system to the TGN is mediated by the retromer complex which was first identified in yeast (Seaman et al., 1998) (Figure 6B). In mammalian cells, the retromer complex comprises a vacuolar protein sorting-26 (VPS26)-VPS29-VPS35 trimer (including two isoforms of VPS26) and Sorting Nexins (SNX), SNX1, SNX2, SNX5 and SNX6 (McGough and Cullen, 2011). It is thought that the SNXs are recruited to endosomal membranes by binding of their

Phox-homology (PX)-domains to phosphatidylinositol-3-phosphate (PtdIns3P), a phosphoinositide which is enriched in endosomes. On the membrane, they form specific homo-and heterodimers via their BAR-domain which also induces high membrane curvature. The VPS26-VPS29-VPS35 complex is then recruited through interactions with the N-termini of the SNXs. After formation of the complex, the VPS35 subunit captures retrograde cargo proteins into retromer coated membrane domains (Bonifacino and Rojas, 2006; McGough and Cullen, 2011).

There are also other mechanisms which are involved in retrograde transport such as clathrin and its associated proteins, which cover different regions of the tubular endosomal network as the retromer complex (Figure 6A). They give rise to clathrin coated carriers involved in transport of Shiga toxin, TGN markers TGN38 and TGN46, as well as MPRs from endosomes to the TGN (Saint-Pol et al., 2004).

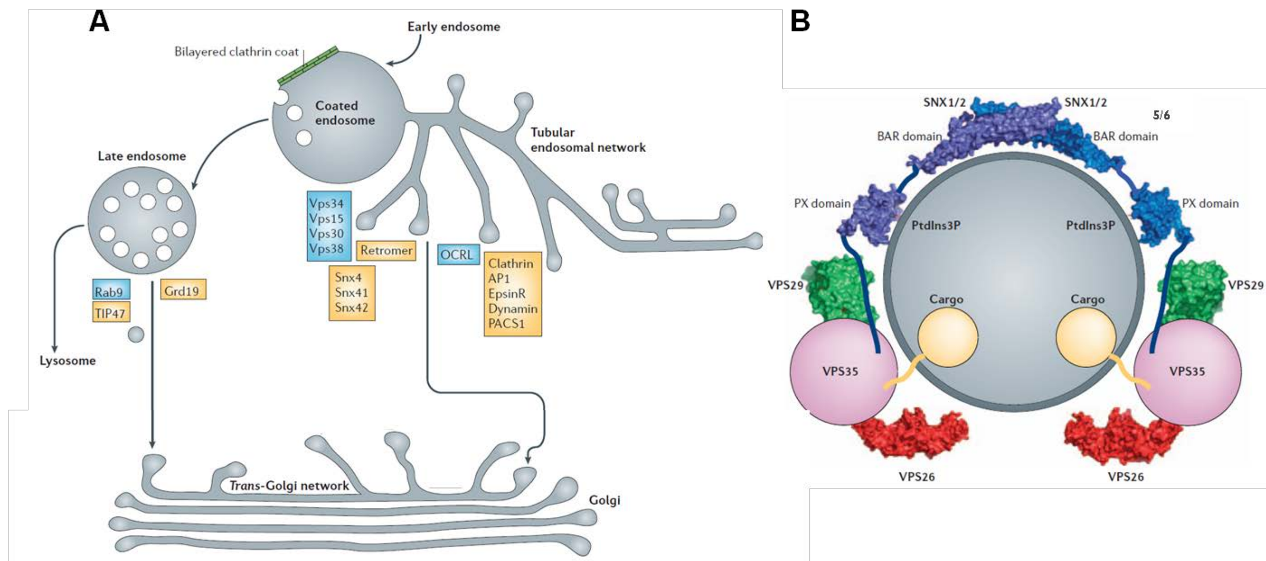

Figure 6: Retrograde transport from endosomes to the TGN.

(**A**) Schematic overview of mammalian and yeast components involved in retrograde transport. Proteins involved in recruitment are depicted in blue, proteins for budding and sorting are depicted in orange. The retromer complex and clathrin coated carriers emerge from the tubular endosomal network to transport retrograde cargo to the TGN while other cargo remains in the vacuolar part of the early endosomes as this matures to the late endosome and is then transported to the TGN by vesicles. (**B**) Model of the retromer complex with the SNX1/2 and the VPS26-VPS29 and VPS35 subcomplexes (Bonifacino and Rojas, 2006).

2. Coated transport vesicles

As mentioned above, transport of molecules within the endocytic and secretory pathway is typically mediated by coated vesicles which travel from donor to acceptor compartments. Transport vesicles are classified according to the components of their coats, the best understood being COPI, COPII, and clathrin coated vesicles. The function of the coat is cargo selection by recognition of specific cytosolic sorting signals, the deformation of the flat membrane to form a pit, as well as the scission of the final vesicle.

2.1. Steps of vesicular transport

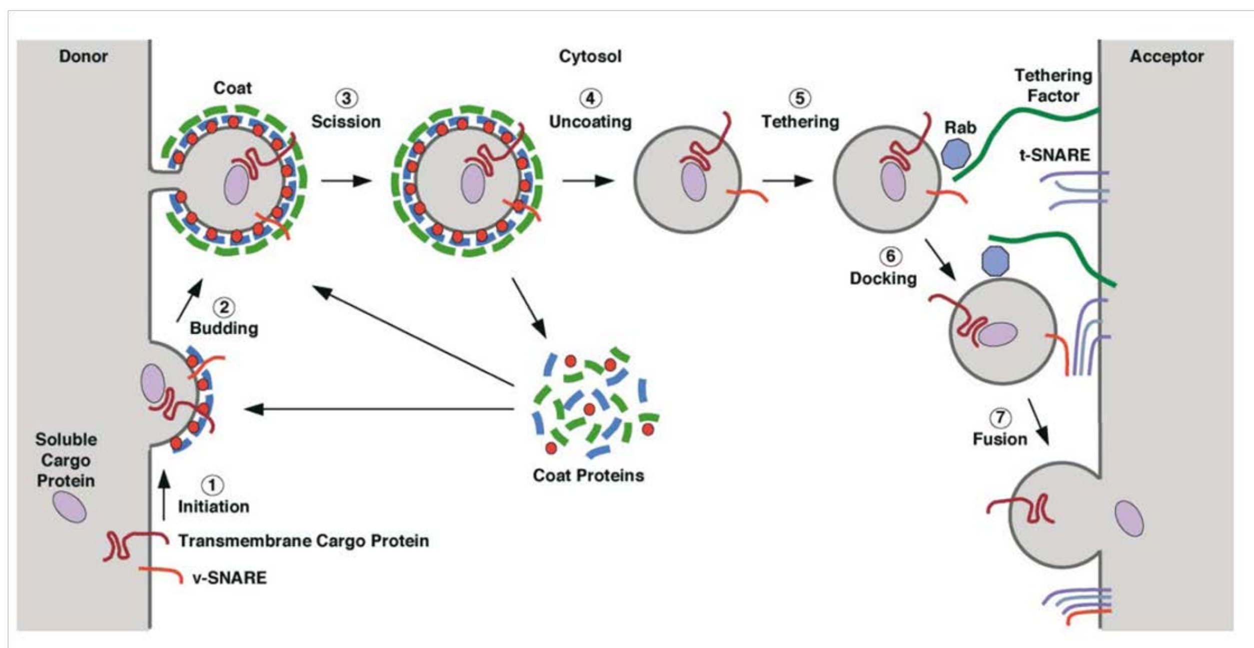

Figure 7: Steps of vesicle budding and fusion.

The different steps of vesicle formation include: coat initiation (**1**), budding (**2**) and scission (**3**) of the vesicle, followed by uncoating (**4**), tethering (**5**) and docking (**6**) at the target membrane and finally vesicle fusion (**7**) (Bonifacino and Glick, 2004).

Initiation of coat assembly

As a first step of vesicle formation, the coat components which are proximate to the membrane are recruited by binding to a membrane associated small GTPase, specific phosphoinositides, cytoplasmic tails of cargo proteins, as well as accessory factors.

Small GTPases such as Sar1 and ADP-ribosylation factor (ARF) 1-3 are involved in coat formation of the intracellular transport pathway. They exist in a GTP-bound and in a GDP-bound form and undergo cycles

of GTP binding and hydrolysis mediated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Figure 9A). The GTP-bound form is the membrane-bound active one and carries out G protein function through interactions with specific effectors, coat components and adaptor proteins (Donaldson and Jackson, 2011).

Through interactions with the small GTPases and signal sequences of cargo proteins gathering at the side of vesicle formation, components and building blocks of the inner coat are recruited and membrane curvature is induced (Bonifacino and Glick, 2004; Kirchhausen, 2000).

Budding

After initiation of coat formation, cargo proteins concentrate at the side of vesicle budding by binding of their signal sequences directly to coat components or adaptor proteins. Besides cargo, also specific SNARE proteins, which are crucial for later fusion of the vesicle with the acceptor membrane, are recruited to the side of vesicle formation. The membrane curvature increases by the action of BAR domain containing proteins or by a continuous process that is coupled to the growth of the coat. Finally, the outer coat components are recruited and polymerize to form a grid-like structure (Bonifacino and Glick, 2004; Kirchhausen, 2000).

Scission

To release the vesicle from the membrane, the constricted neck which is the connection between the membrane and the vesicle must be severed. There might exist more than one mechanism for the scission process. One mechanism is the recruitment of a large GTPase to the neck of the vesicle which undergoes a GTP-hydrolysis dependent conformational change that triggers membrane scission. However, there is also evidence that Sar1 contributes to membrane fission. The N-terminal helix of Sar1 invades the neck by aligning along its main axis which leads to further constriction of the membrane. Upon GTP hydrolysis, Sar1 is released, leaving the neck in an unstable state due to strong lipid packing defects which is resolved by scission (Antonny, 2006).

Uncoating

After scission, vesicles are transported to their final destination by motor mediated-transport along microtubules or actin. The molecular motors kinesin, dynein, and myosin have all been implicated in this process (Hammer and Wu, 2002; Matanis et al., 2002; Short et al., 2002). Before fusion with the target

membrane, the coat components are released from the vesicle. This process is believed to be mediated by cytosolic accessory factors, Rab proteins and their effectors, as well as GAPs for ARF and Sar1, promoting their GTP hydrolysis activity. Recently, also tethering factors (Zink et al., 2009) and even SNARES are presumed to be involved.

The exact time point of uncoating is still a matter of debate. Originally it was believed that uncoating takes place soon after budding, but newer data show that various interactions of coat proteins with tethering factors are required for vesicle targeting (Trahey and Hay, 2010). This suggests that the coat stays on (at least partially) until the tethering step.

Tethering

Tethering is a term used to describe the initial contact between a vesicle and its target membrane. Proteins or protein complexes called tethering factors together with Rabs, small GTPases of the Ras superfamily, were found to play a role in nearly all membrane-trafficking events and are located on different compartments throughout the intracellular trafficking pathway. They are not only required to bring the vesicle in close proximity with the acceptor membrane but also play a critical role in specificity of membrane targeting through interactions with coat components and SNAREs (Cai et al., 2007a).

Tethering factors and Rabs also have additional functions in trafficking such as stacking of Golgi cisternae (GRASPS, Golgins) (Ramirez and Lowe, 2009), endosome fusion (EEA1, Rab5), and sorting endosome to late endosome maturation (Rab5, Rab7) (Grosshans et al., 2006).

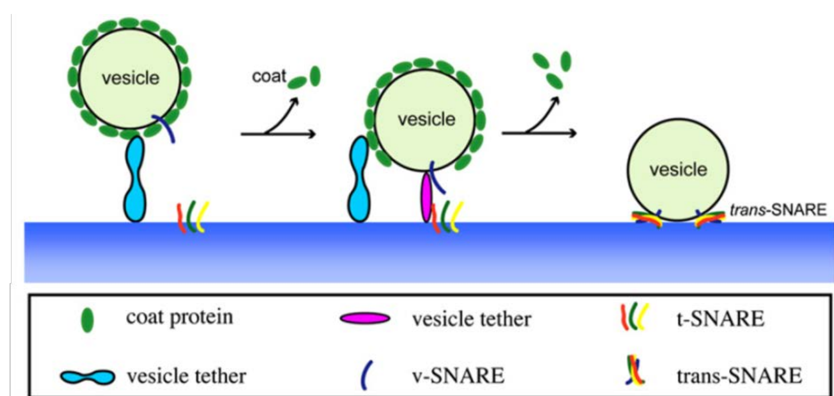

Figure 8: Vesicle tethering.

The interaction of coat proteins with specific tethering factors brings the vesicle in close proximity to the acceptor compartment leading to subsequent fusion of the vesicle with the target membrane (Cai et al., 2007a).

Docking and fusion

A set of SNARE proteins is involved in the final docking of a vesicle with its target membrane and catalyzes membrane fusion. SNAREs are classified into Qa-, Qb-, Qc- and R-SNARES, and they all contain transmembrane domains and evolutionary conserved SNARE motifs. If the SNAREs are monomeric, these motifs are unstructured, however, when appropriate sets of SNARES are combined, the SNARE motifs associate and form a complex of extraordinary stability. For vesicle fusion, Q-SNARES, which are organized in clusters in the target membrane, form an acceptor complex consisting of a Qa-, a Qb- and a Qc-SNARE. This complex assembles with the vesicular R-SNARE into a four helical trans-complex. Trans-complexes proceed from a loose state to a very tight state which results in the opening of the fusion pore and finally vesicle fusion with the membrane (Jahn and Scheller, 2006).

It was also proposed that the vacuolar H^+-ATPase is involved in fusion events following SNARE complex assembly. After formation of close contact of two membranes by the SNARE-complex, a dimer of the proteolipid V_0-subunit of the vacuolar H^+-ATPase can work as a fusion channel which allows invasion of lipids (Peters et al., 2001) .

2.2. COPI

COPI is required at multiple stages in intra-Golgi and Golgi to ER transport, primarily for retrograde transport. COPI coated vesicles are formed at the Golgi cisternae, the ERGIC, as well as from anterograde carriers as they move towards the Golgi. These vesicles transport cargo back to the ER or back to the next proximal compartment, being from *trans*-to *medial*-Golgi, from *medial*-to *cis*-Golgi, from *cis*-Golgi to ERGIC and from ERGIC to ER.

The COPI coatomer is a complex of seven proteins (α, β, β',ε, γ, δ and ζ), the present understanding is that a β, γ, δ , ζ tetrameric subcomplex forms the inner core while the α, β',ε, trimeric subcomplex forms the outer layer of the COPI coat (Kirchhausen, 2000; Szul and Sztul, 2011).

The first step of COPI vesicle formation is the activation of the small GTPase ARF1 by a specific GEF. The association of ARF1 with the appropriate GEF ensures its targeting to the correct membrane. Several GEFs of ARF1 have been identified with GBF1 being probably the main GEF for the COPI pathway (Manolea et al., 2010; Manolea et al., 2008; Szul et al., 2007). ARF1-GDP reversibly associates with the membrane surface via a myristoyl moiety of its N-terminal amphipathic helix. The exchange of GDP to GTP leads to a conformational change of the amphipathic α-helix of ARF1 which allows stable association with the membrane (Figure 9) (Antonny et al., 1997; Franco et al., 1996).

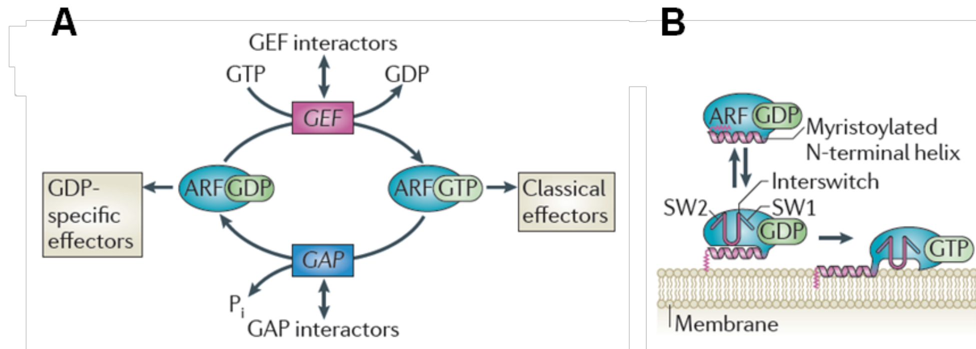

Figure 9: Regulation of small GTPases as ARF.

(**A**) ARF family proteins switch between their active GTP-bound form and their inactive GDP-bound form, which is mediated by GEFs and GAPs, respectively. (**B**) The myristoylated group and associated N-terminal amphipathic helix of ARF are inserted into the membrane upon a GTP-dependent conformational change that brings them into very close contact with the membrane (Donaldson and Jackson, 2011).

Activated ARF1, together with members of the p24 family recruits the pre-assembled coatomer (Hara-Kuge et al., 1994) by interacting directly with the β- and γ- subunits (Eugster et al., 2000; Zhao et al., 1997). This complex concentrates cargo by interaction of the coat subunits with specific cytoplasmic cargo tails such as KKXX and KXKXX (Cosson and Letourneur, 1994). In addition to transmembrane proteins that recycle back to the ER, soluble proteins that contain the C-terminal KDEL-motif are also retrieved via COPI vesicles. The KDEL sequence is recognized by the KDEL receptor which continuously cycles between ER and Golgi and directly interacts with COPI components (Townsley et al., 1993). Membrane deformation occurs at the same time as coat maturation, when the coat is complete, the vesicle buds from the membrane. The exact mechanism of COPI vesicle scission is not known but it is assumed that ARF1 plays a role in this process. In consistence with this, a recent study suggested that dimerization of ARF1 is required for separation of the vesicle from the donor membrane (Beck et al., 2011).

The formation of COPI vesicles also seems to involve the activity of ARF GAPs, which are recruited to budding COPI vesicles by interaction with active ARF1, cytoplasmic cargo tails and coat components. ARF-GAP activity, which is stimulated by the presence of cargo and coat components, leads to increased GTP hydrolysis of ARF1 and the release of ARF1 from the membrane. This coordination ensures that only vesicles containing cargo leave the membrane (Luo et al., 2009).

It is not clear whether ARF-GAP remains a component of the COPI coat but it is accepted that GTP hydrolysis of ARF1 is insufficient to cause uncoating of the vesicle. The exact mechanism and time point of coat dissociation remains to be characterized (Szul and Sztul, 2011). After reaching their acceptor compartment, COPI vesicles are tethered to the membrane by specific tethering factors, as the Dsl1 complex which is involved in tethering of COPI vesicles to the ER (Meiringer et al., 2011) or tethers acting within the Golgi as p115 and Golgin-84 (Szul and Sztul, 2011), before fusion and cargo release.

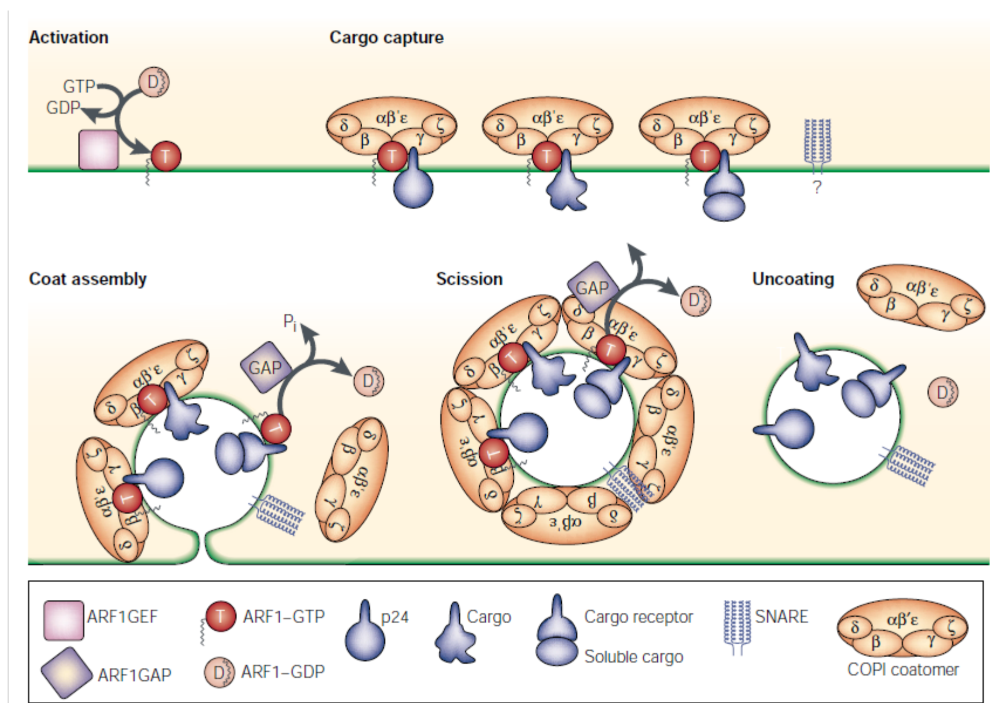

Figure 10: COPI coated vesicle formation.

The different steps of COPI coat formation are activation of ARF1 followed by recruitment of coat components and cargo proteins. After the coat has assembled, the vesicle is released from the membrane and uncoating takes place (Kirchhausen, 2000).

2.3. COPII

The sorting of newly synthesized proteins from the ER occurs exclusively at ERES and is mediated by COPII coated vesicles which transport cargo to the Golgi. The COPII coat consists of five cytosolic proteins in total: the Sec23/Sec24 complex, the Sec13/31 complex and the small GTPase Sar1 (Barlowe et al., 1994).

COPII vesicle formation starts with the activation of Sar1, mediated by the ER localized transmembrane GEF Sec12 (Nakano et al., 1988; Nakano and Muramatsu, 1989) (Figure 11). The activated Sar1-GTP undergoes a conformational change which exposes the N-terminal amphipathic α-helix. In contrast to ARF1, the amphipathic helix of Sar1 binds directly to the membrane, leading to its stable association with the ER (Huang et al., 2001). Active Sar1-GTP binds to Sec23, recruiting the heterodimeric Sec23/Sec24 subcomplex to the membrane (Bi et al., 2002). As a next step, ER membranes with Sar1-GTP and Sec23/Sec24 recruit the outer layer of the COPII coat, the heterotetramer Sec13/Sec31 (Barlowe et al., 1994; Lederkremer et al., 2001), which acts as a scaffold and cross-links adjacent pre-budding complexes. Sar1 also activates Sec23 to bind SNARE proteins, which are involved in the later, specific targeting and fusion of the vesicle with acceptor membranes (Springer and Schekman, 1998). Sequestering of transmembrane cargo proteins into the side of vesicle formation is mediated by Sec24, which recognizes distinct sorting signals on the cytoplasmic tails. It has been shown that different isoforms of Sec24 bind different sorting motifs, expanding the range of exported cargo. The Sec24a and Sec24b isoforms bind the DXE and the LXXL/ME motif, while the isoforms Sec24s and Sec24d recognize the IXM motif (Mancias and Goldberg, 2008). Soluble cargo proteins within the ER lumen bind to specific transmembrane receptors, whose cytoplasmic tail interact with the COPII coat.

Membrane curvature is locally induced by the insertion of the amphipathic α-helix of Sar1 into the membrane and the recruitment of Sec13/Sec31 is thought to propagate further curvature, finally leading to the formation of a vesicle (Lee et al., 2005). The exact mechanism which triggers fission is still under discussion, but Sar1 seems to be an important factor in this event. It has been proposed, that Sar1 facilitates vesicle scission in a similar manner as dynamin, which mediates scission of clathrin coated vesicles (see below). Another model also proposes that the amphipathic α-helix of Sar1 inserts into the membrane at the neck of the vesicle, leading to membrane destabilization and fission (Bielli et al., 2005; Lee et al., 2005).

The formation of ERES and packaging of cargo into COPII vesicles is regulated by additional factors such as Sec16, which localizes to ERES independently of COPII. Sec16 has been shown to bind directly to all four subunits of the COPII coats and is believed to stabilize COPII on the membrane during sorting and vesicle formation (Gimeno et al., 1996; Supek et al., 2002).

After vesicle budding, uncoating takes place before fusion with the target membrane. It is presumed that GTP hydrolysis of Sar1, which is stimulated by its GAP Sec23, participates in the disassembly of the COPII coat, although the details of uncoating remain under investigation (Szul and Sztul, 2011). The uncoated

vesicle is then tethered to its target compartment by the action of different tethering complexes such as the TRAPPI complex and p115 which are localized at the Golgi and the Golgi and ERGIC, respectively (Brandon et al., 2006; Cai et al., 2007b; Sacher et al., 2001).

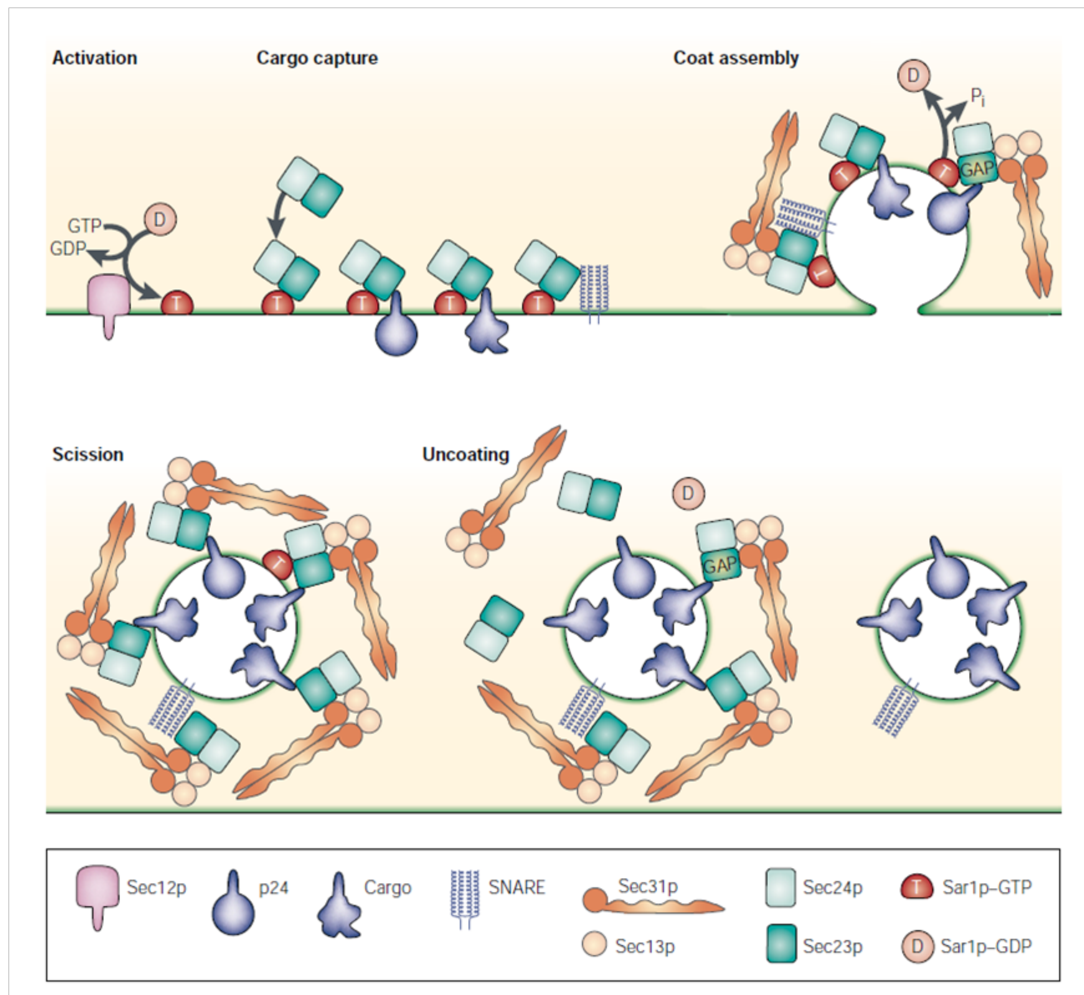

Figure 11: Formation of COPII coated vesicles.

Coat assembly is activated by the recruitment of Sar1-GTP to the membrane, which allows binding of the Sec23-Sec24 complex and cargo sequestering. Binding of Sec13-Sec31 leads to membrane deformation and finally, vesicle scission. The GTPase activity of Sar1 is stimulated by Sec23 and results in inactivation of Sar1 and uncoating (Kirchhausen, 2000).

2.4. Clathrin coated vesicles

CCVs are the most prominent and best characterized transport carriers and were the first to be discovered (Pearse, 1976). They mediate cargo transport at the plasma membrane, the TGN, and endosomes and, in contrast to COPI and COPII vesicles, have a large variety of associated proteins. So far,

more than 150 proteins have been identified to play a role in CCV formation. The most abundant protein in CCVs is clathrin itself (Blondeau et al., 2004), which forms a mechanical scaffold and is linked to the membrane by an inner layer of clathrin adaptors. The formation of these vesicles is a highly regulated and complex process and requires a perfect interplay of clathrin, clathrin adaptors, cargo proteins, and accessory factors.

2.4.1. Clathrin

Clathrin monomers assemble into triskelia, which form a lattice surrounding the central membrane vesicle. Each triskelion is made of three clathrin heavy chains (CHCs) and three clathrin light chains (CLCs) (Ungewickell and Branton, 1981) (Figure 12A). The heavy chain can be subdivided into a C-terminal proximal domain at the trimerization zone, a middle domain forming a typical knee, and a globular N-terminal domain (Figure 12B and C). The two light chain isoforms only existing in higher eukaryotes, LCa and LCb, were shown to bind to the proximal domain of the heavy chain and localize outside of the lattice (Fotin et al., 2004b). The heavy and the light chain have two contact sites (CHC-K1326-CLC-W108 and CHC-K1514-CLC-W130) (Chen et al., 2002). However, the role of the CLCs are still unclear; it seems probable that they have a regulatory function.

Purified clathrin triskelia can spontaneously assemble into cages at low pH (Keen et al., 1979), however, since clathrin does not interact with the lipid bilayer, adaptor proteins are absolutely required to form a clathrin coat *in vitro* (Lindner and Ungewickell, 1991). Clathrin binding to adaptor proteins is mediated by the clathrin N-terminal domain, which forms a seven bladed β-propeller. The first identified clathrin binding motif was the clathrin binding box (Dell'Angelica et al., 1998), a short consensus sequence of LΦxΦ[DE], where Φ represents a bulky hydrophobic residue. This motif binds to a site between blades 1 and 2 of the clathrin N-terminal β-propeller (ter Haar et al., 2000). Sequence analysis of different clathrin binding proteins showed that also an additional sequence, [SD]LL, also termed DLL motif, may serve as a clathrin binding motif (Morgan et al., 2000).

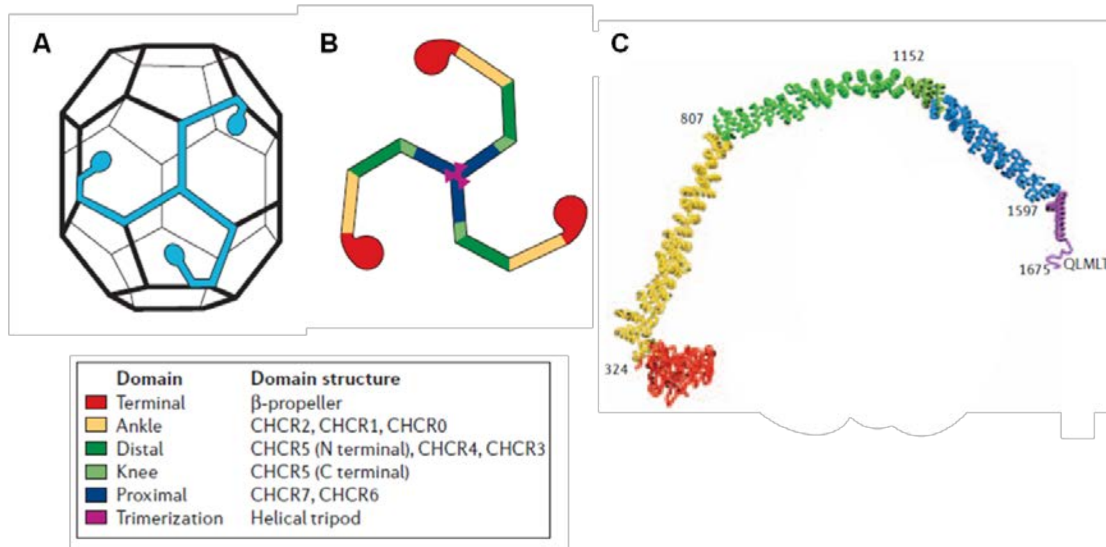

Figure 12: The architecture of clathrin.

(**A**) A clathrin barrel with a single triskelion highlighted in blue (**B**) A clathrin triskelion which highlights the various domains in different colors (**C**) A single clathrin heavy chain with its different domains (Edeling et al., 2006).

2.4.2. CCV formation

The formation of clathrin coated vesicles (Figure 13a) starts with a membrane invagination called a pit. Traditionally, it was thought that this step was induced by the recruitment of an adaptor protein to the membrane. However, previous studies in yeast and mammalian cells showed that the initiation stage may involve other factors, such as FCHo1/2 and EPS15, to define the site of the membrane where the vesicle will bud (see below)(Henne et al., 2010; Stimpson et al., 2009). These early stage proteins are involved in recruiting clathrin adaptor proteins to the membrane. Adaptors, together with cargo-specific accessory proteins such as AP180 and β-arrestins, mediate cargo selection by binding directly to specific motifs in the cytoplasmic tail of transmembrane cargo receptors. As cargo is sequestered to the pit by adaptors or accessory proteins, the clathrin coat can be assembled. Clathrin triskelia are directly recruited from the cytosol to sites of high adaptor concentrations at the membrane through the interaction with adaptors or clathrin-binding accessory factors. In the absence of clathrin, adaptor proteins are also found at the membrane, although the pit cannot mature. Clathrin polymerization seems to stabilize the coat proteins as well as membrane curvature. During formation of the clathrin coat, accessory factors such as Epsin 1 and amphiphysins are recruited to the edge of the vesicle where they mediate further membrane invagination by actions of specialized curvature domains. Vesicle scission depends on the large GTPase dynamin which is recruited to coated pits by BAR domain

containing proteins as SNX9, endophilin, and amphiphysin. Dynamin accumulates rapidly at the neck of the vesicle and polymerizes around it. A GTP-hydrolysis dependent conformational change of the dynamin polymer leads to vesicle fission. After release from the membrane, the clathrin coat disassembles from its lattice arrangement by the action of different uncoating factors as auxilin and the heat shock cognate 70 (HSC70), allowing the vesicle to fuse with the target endosome (Bonifacino and Glick, 2004; Kirchhausen, 2000; McMahon and Boucrot, 2011).

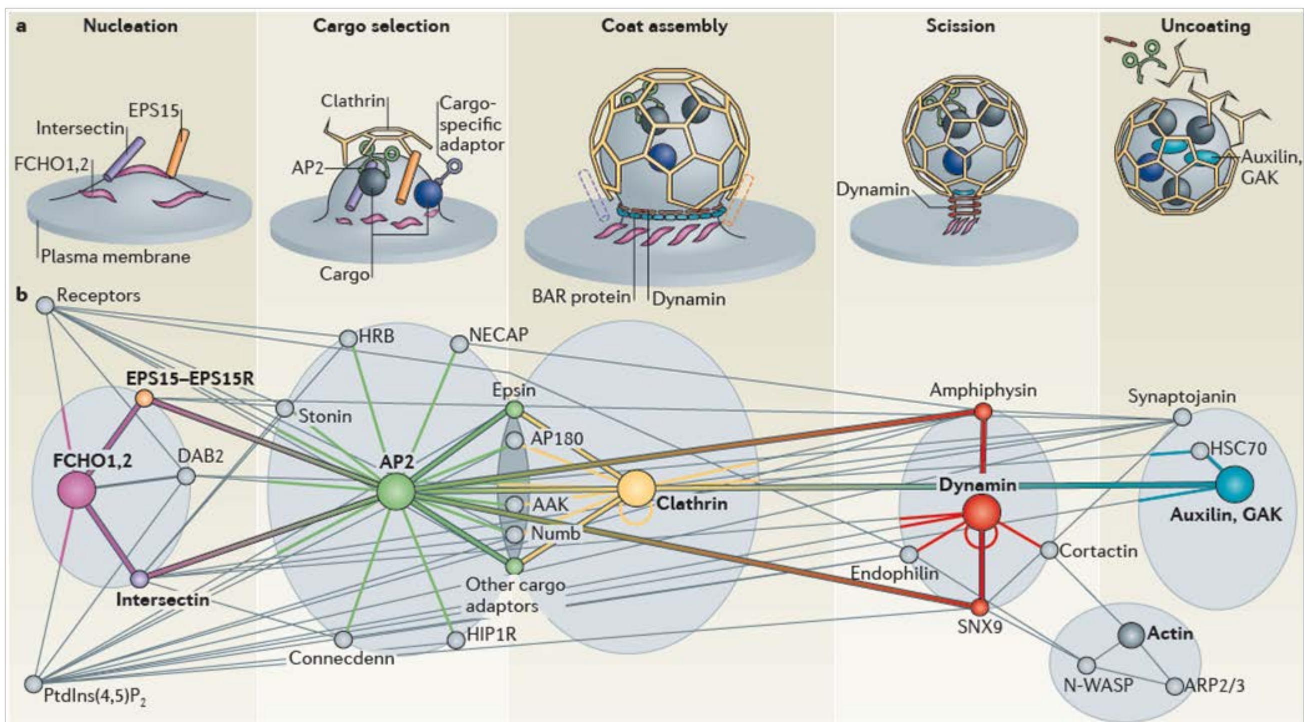

Figure 13: Clathrin coated vesicle formation.

(**a**) The five steps of clathrin coated vesicle formation: Proteins such as FCHo1/2 and EPS15 lead to initiation of the process and recruitment of adaptor proteins, cargo and clathrin. As the coat assembles, BAR domain containing proteins and the GTPase dynamin are recruited to the neck of the vesicle, followed by fission. Uncoating is mediated by auxilin and HSC70. (**b**) The interactome: a protein interaction network underlining the different stages of clathrin coated vesicle progression is depicted. The essential hubs and interactions are emphasized in color (McMahon and Boucrot, 2011).

2.4.3. The clathrin-adaptor interactome

In many models, CCV formation is seen as pathway of cargo recruitment to distinct membrane patches and subsequent vesicle formation and budding. On a closer look, this linear illustration is strongly oversimplified, because on the molecular level, many of the involved processes and mechanisms occur at

the same time and in a highly regulated fashion. To understand the many parallel interactions of the involved proteins, these interactions can be organized as a protein network termed the clathrin mediated endocytosis (CME) interactome (Figure 13b). There are several major hubs in the CME interactome such as AP-2, clathrin, and dynamin. These proteins are the most common interaction points in the network and surrounded by many accessory proteins. During the process of vesicle formation, the interactome undergoes dynamic changes and it becomes obvious that different accessory factors and adaptors become the major hubs. At an early stage, the AP-2 hub is important to concentrate cargo at the site of vesicle formation and to mediate recruitment of the clathrin hub. Upon polymerization of the clathrin lattice on the membrane, the AP-2 hub loses its importance, as clathrin now drives vesicle formation. During vesicle scission, dynamin is the central point of the interactome.
Knowing what status a protein has in the network, one can predict if it might be essential or not. It has been suggested that depletion of proteins with many interaction partners (as AP-2 and clathrin) is more probable to give strong phenotypes (Jeong et al., 2001). Furthermore, the CME interactome is likely to be slightly different in each cell type and, dependent on the cargo proteins and the speed of endocytosis, the accessory factors may vary (Schmid and McMahon, 2007; Wieffer et al., 2009).

2.4.4. Clathrin adaptors

Clathrin adaptors are proteins or protein complexes which link clathrin to the membrane through binding to phospholipids and/or cargo proteins. Over 20 different adaptor proteins have been identified so far which all share two common characteristics: interaction with the N-terminal domain of clathrin and a common structural organization. Adaptor proteins are divided into two main groups: multimeric adaptor protein complexes (APs) (Figure 14), of which there are five, and monomeric adaptor proteins such as the clathrin associated sorting proteins (CLASPs) (Reider and Wendland, 2011).
The most prominent of clathrin adaptors are the APs, with AP-2 being the longest-studied and best-understood one. All APs have two large subunits of ~ 100 kDa, one medium size subunit of ~ 50 kDa and one small subunit of ~ 20 kDa. They are organized in the typical structure of the APs with a core consisting of the small subunit, the medium subunit, and the N-terminal trunk domains of the large subunits. The two appendage domains of the large subunits are connected with the core via a flexible hinge domain (Owen et al., 2004). All APs recognize the same cytosolic sorting signals containing the YXXΦ (Φ represents a large hydrophobic amino acid) motif or the dileucine motif [DE]XXXL[LIM],

although each complex has individual preferences for the residues at the X and Φ positions (Kelly et al., 2008; Ohno et al., 1998; Traub, 2009).

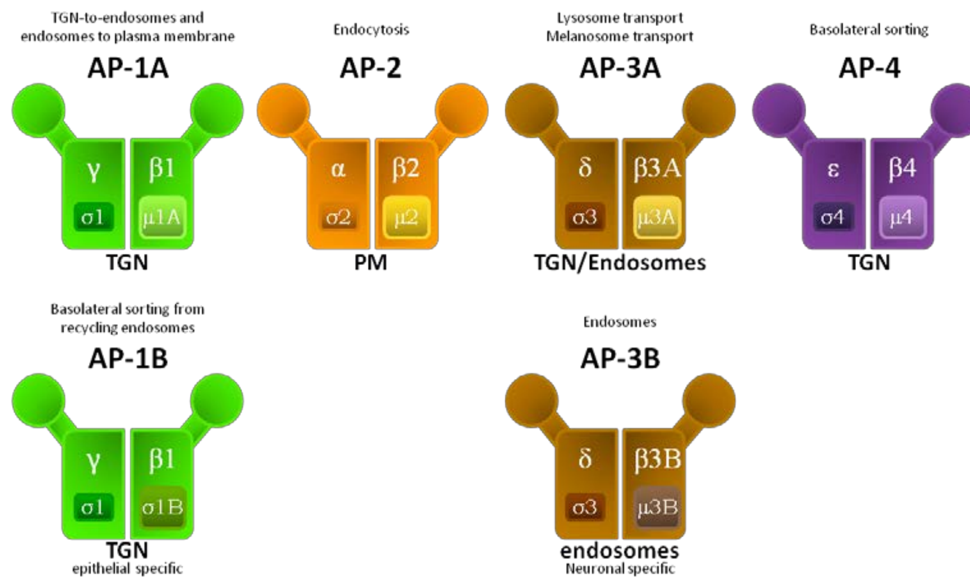

Figure 14: The clathrin adaptor protein complexes.

Schematic representation of the four major adaptor protein complexes and their isoforms, which are expressed in specialized cells (Nakatsu and Ohno, 2003).

AP-2

As mentioned above, the most detailed analyses have been carried out with AP-2 which promotes the formation of endocytic CCVs destined for early endosomes. AP-2 consists of the 100 kDa α-subunit, the 100 kDa β_2-subunit, the 50 kDa μ_2-subunit, and the 17 kDa σ-subunit (Figure 16B). Targeted disruption of the genes encoding these subunits is lethal in several species (Mitsunari et al., 2005; Shim and Lee, 2000).

AP-2 is targeted to the membrane by interactions of the α-subunit with phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3)(Gaidarov et al., 1996; Gaidarov and Keen, 1999). A mutation in the α-subunit which abolishes PtdIns(4,5)P_2 binding was shown to prevent AP-2 membrane binding even in the presence of sorting signals (Honing et al., 2005). However, it was shown that also the μ_2-subunit has binding sites for phosphoinositides (Rohde et al., 2002). In contrast to other adaptors, the role of a small GTPase for recruitment of AP-2 is controversial. AP-2 binding to membranes is insensitive to brefeldin A (BFA), which inhibits the activation

and therefore membrane association of ARF1-5. However, ARF6 remains a possible candidate, since its membrane localization is not affected by BFA (Paleotti et al., 2005).

At the membrane, AP-2 binds to cargo proteins via sorting signals. The μ_2-subunit was the first to be identified as cargo-binding subunit (Ohno et al., 1995). The C-terminal β-sandwich subdomain of μ_2 binds to YXXΦ-type sorting signals. Binding depends on the phosphorylation status of residue Thr156: Adaptor associated kinase 1 (AAK1)-mediated phosphorylation of this residue shifts the equilibrium of the μ_2-subunit to the open, YXXΦ-binding state which is further stabilized by binding of μ_2 to PtdIns(4,5)P_2 allowing simultaneous interaction with sorting signals and the bilayer (Olusanya et al., 2001; Ricotta et al., 2002). In addition, also the α/σ2-hemicomplex has been shown to mediate cargo binding via the [DE]XXXL[LIM]-motif (Doray et al., 2007).

Once stabilized at the membrane by interactions with lipids and cargo proteins, AP-2 can bind to additional key players of endocytosis (Figure 15A). The β2-subunit is particularly important for binding of clathrin through the clathrin-box motif LΦxΦ[DE] located in the hinge domain (Owen et al., 2000; Shih et al., 1995). The role of the α-appendage is to bind the DP[FW] and the FxDxFx motifs which are present in many other clathrin adaptors or accessory factors that perform regulatory functions in CCV formation. The α-appendage binding site for these sequences is a hydrophobic pocket formed by Trp840 (Brett et al., 2002).

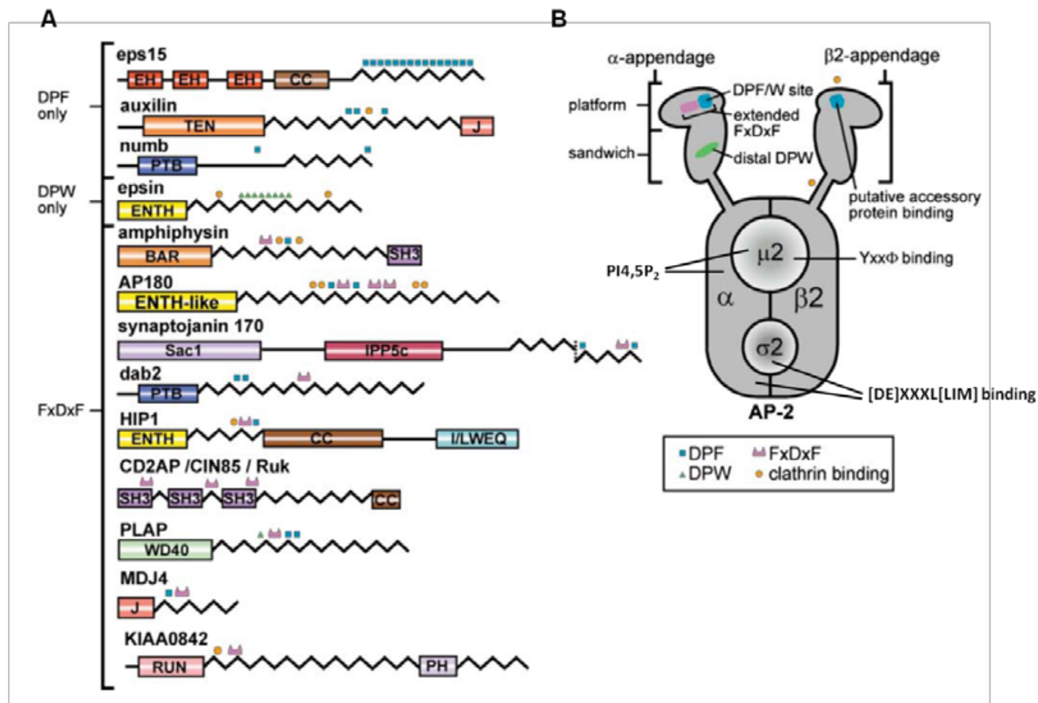

Figure 15: The AP-2 adaptor complex.

(**A**) Overview of adaptor proteins and accessory factors which interact with AP-2. (**B**) AP-2 subdomains and the location of the different binding motifs (Brett et al., 2002).

AP-1

AP-1 has two different isoforms which share the β1, γ, and σ1 subunits and differ in the μ1 subunit (μ1A and μ1B). AP-1A is ubiquitously expressed and involved in the assembly of CCVs at the TGN and endosomes (Traub et al., 1993), while AP-1B was exclusively found in polarized epithelial cells (Ohno et al., 1999) where it mediated basolateral sorting of cargo (Folsch et al., 1999). Within cells, AP-1A was found to be associated with the Golgi and post-Golgi vesicles in immunofluorescent studies (Ahle et al., 1988). In contrast, AP-1B colocalized well with internalized transferrin present in endosomes and only poorly with TGN marker TGN38 in epithelial cells transfected with the μ1B subunit (Cancino et al., 2007; Gan et al., 2002).

Since the TGN region of a cell is not as easy accessible as the plasma membrane, studies on how AP-1 is recruited to the membrane were performed using *in vitro* liposome recruitment assays. It was shown that the minimal machinery for AP-1 recruitment to membranes consists of myristoylated ARF1 activated by a GEF, tyrosine sorting signals, and specific lipids (Crottet et al., 2002). Mixed adaptors isolated from

calf brain cytosol were incubated with ARF1, GTP or its non-hydrolyzable analog GMP-PNP, and liposomes coupled to the tyrosine-containing signal peptide of LAMP-1 (LY). The mixture was supplemented with sucrose to a concentration of 40% below a 30% sucrose cushion. Peptidoliposomes and bound proteins were then separated from unbound material by high-speed centrifugation and liposome floatation. Using this method, it was shown that AP-1 is most efficiently recruited to phosphatidylcholine (PC) liposomes containing phosphoinositides, in contrast to liposomes consisting of PC only or containing phosphatic acid (PA), phosphatidylethanolamine (PE) or phosphatidylserine (PS). Furthermore, among the phosphorylated phosphoinositides, PtdIns(4,5)P_2 and PtdIns(5)P were the most efficient liposomes for AP-1 recruitment (Crottet et al., 2002). When liposomes of different sources containing no LY peptide were used in recruitment assays, AP-1 could only be recruited from full cytosol and not from CCV coat fractions (Crottet et al., 2002; Meyer et al., 2005; Zhu et al., 1999a). This observation suggests that docking factors which are present in the cytosol mediate AP-1 binding to liposomes also in absence of sorting signals. Using liposomal *in vitro* assays, it was furthermore demonstrated that AP-1 recruited to liposomes forms high-molecular-weight complexes even in the absence of clathrin and that this AP-1 oligomers disassemble upon GTP hydrolysis stimulated by ARF-GAP activity (Lee et al., 2008a; Meyer et al., 2005).

Based on these and other data, a model for AP-1/clathrin coat formation was proposed (Figure 16) (Crottet et al., 2002; Meyer et al., 2005; Seaman et al., 1998; Zhu et al., 1999a; Zhu et al., 1998): myristoylated and GEF-activated ARF1-GTP, which localizes to sites of coat initiation, interacts with cytosolic docking factors to generate a binding platform for AP-1. Under these conditions, AP-1 remains monomeric and recruitment to the membrane is short-lived. Nevertheless, if sorting signals are present, AP-1 stably associates with the membrane leading to its subsequent oligomerization. In turn, clathrin triskelia bind to immobilized AP-1 and laterally assemble into the characteristic lattice. GTP hydrolysis induced by a GAP leads to fast membrane dissociation of the AP-1/ARF1/cytosolic factor complex in absence of cargo, suggesting that it is highly susceptible to cytosolic GAPs. However, GAP stimulation of AP-1/cargo oligomers is weaker providing enough time to assemble the coat.

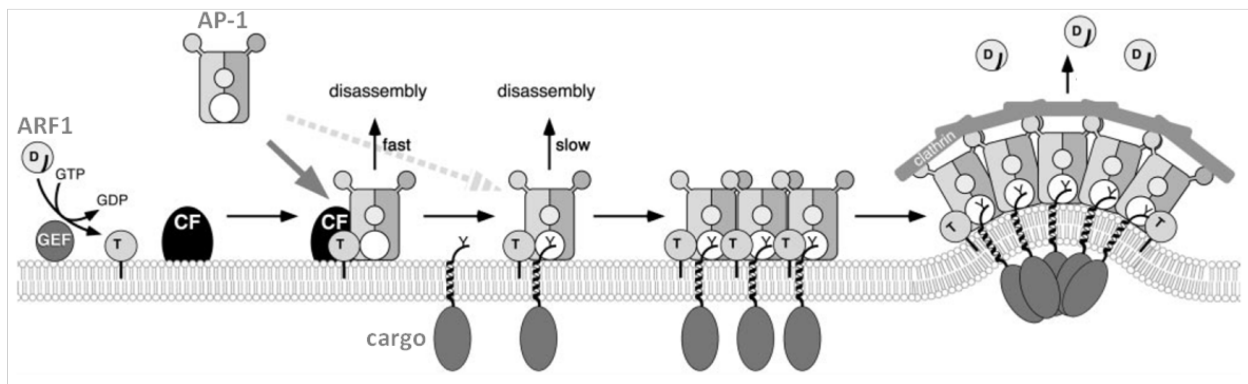

Figure 16: Minimal machinery for the recruitment of AP-1 to liposomal membranes.

Activated ARF1, together with unknown cytosolic factor(s) forms a binding platform for AP-1 recruitment to liposomes. Upon binding to cargo, AP-1 association with the membrane is more stable and oligomerization takes place. As a next step, clathrin is recruited to the membranes and the vesicle can form. Grey arrows indicate the recruitment of AP-1 via unknown cytosolic factors (CF) or directly to cargo proteins with tyrosine motifs (Y) (Meyer et al., 2005).

At the membrane, the different domains of AP-1 are engaged in binding to various components of the clathrin coat. AP-1 binds signal peptides with two different types of sorting signals. The μ1 domain recognizes signal peptide sequences containing the YXXΦ motif (Bremnes et al., 1998; Ohno et al., 1995) which is present on MPRs, LAMP-1, and furin, for example. The AP-1 γ/σ1 hemicomplex recognizes the [DE]XXXL[LIM] motif (Doray et al., 2007) present for instance in the lysosomal transmembrane protein LIMPII (Fujita et al., 1999). The interaction with phosphoinositides is mediated by the γ subunit (Heldwein et al., 2004), while ARF1 binding requires the trunk regions of both γ adaptin and β1 adaptin (Austin et al., 2000). Binding motifs for clathrin were found in the hinge regions of β1 and γ (Doray and Kornfeld, 2001; ter Haar et al., 2000) and the γ-appendage mediates binding to accessory factors as amphiphysins (Bai et al., 2004), γ-BAR (Neubrand et al., 2005), and the γ-synergin/aftiphilin complex (Hirst et al., 2005; Mattera et al., 2004; Page et al., 1999).

It is still a matter of debate where AP-1 exactly functions. Originally, AP-1 was proposed to be involved in MPR sorting at the TGN (Klumperman et al., 1998; Zhu et al., 1999b), but there is also evidence that AP-1 functions in endosome-TGN-transport (Meyer et al., 2000), basolateral sorting in polarized cells (Folsch et al., 1999), and receptor recycling to the plasma membrane (Deneka et al., 2003; Pagano et al., 2004).

By immunoelectron microscopy, AP-1 was found to co-localize with MPRs on TGN vesicles and tubules and associated to clathrin coated buds that emerge from the TGN (Klumperman et al., 1998). Other studies showed a co-localization of AP-1 and GGA in clathrin coated buds at the TGN (Dell'Angelica et al.,

2000; Doray et al., 2002). Biochemical data provide further evidence that GGAs function as adaptor proteins that select cargo molecules as MPRs for incorporation into AP-1 CCVs at the TGN (Doray et al., 2002).

However, it has also been suggested that only GGAs and not AP-1 functions in anterograde transport and AP-1 is more involved in retrograde transport from endosomes back to the TGN. In µ1A knockout cells, it would be expected that MPRs are stuck in the TGN. On the contrary, MPRs exited the Golgi, were transported to the plasma membrane, from where they were re-endocytosed and finally accumulated in early sorting endosomes positive for EEA-1 (Meyer et al., 2000). This indicates that AP-1 might mediate retrograde transport between endosomes and the TGN which is supported by the observation that shiga toxin co-localized with AP-1 on sorting and recycling endosomes during a temperature-dependent block of retrograde transport (Mallard et al., 1998).

The epithelial specific isoform AP-1B was shown to mediate basolateral sorting in polarized cells (Folsch et al., 1999; Futter et al., 1998). Live cell imaging experiments of cells treated with an antibody against µ1B showed that the basolateral proteins vesicular stomatitis virus G (VSVG) and TfR exited the TGN normally but became blocked at the recycling endosomes after 3-5 min (Cancino et al., 2007). By contrast, the µ1B antibody did not block trafficking of LDLR from the TGN directly to the plasma membrane but stopped its recycling after internalization at the stage of the recycling endosomes. This demonstrates that AP-1B functions exclusively at the recycling endosomes while the adaptor AP-4 may be rather involved in direct transport from the TGN to the basolateral plasma membrane (see below). However, a recent study showed that also AP-1A may be involved in basolateral sorting at the TGN (Gravotta et al., 2012).

There is also evidence that AP-1A is involved in generation of vesicles at recycling endosomes. *In vitro* formation of recycling vesicles from endosomes was reduced when AP-1-depleted cytosol was used in comparison to control cytosol (Pagano et al., 2004). Furthermore, AP-1 was found to co-localize with the Rab4 effector rabaptin-5 on recycling endosomes and it has been suggested that the interactions between Rab4, rabaptin-5, and AP-1 γ regulate membrane recycling (Deneka et al., 2003).

Additionally, AP-1 was also found in clathrin coated carriers from immature secretory granules of endocrine and exocrine cells (Tooze, 1998).

AP-3

Even if AP-3 is more distantly related to AP-1 and AP-2, it was first identified by searching sequence databases and cDNA libraries for homologues of AP-1 and AP-2 (Pevsner et al., 1994; Simpson et al., 1996). AP-3 consists of the four subunits δ, β3, μ3 and σ3. β3, μ3 and σ3 each exists as two isoforms, among whom the σ3 isoforms are ubiquitously expressed while β3B and μ3B are restricted to neuronal and neuroendocrine tissue (Boehm and Bonifacino, 2001, 2002; Darnell et al., 1991; Gurkan et al., 2005). β3B and μ3B are thought to assemble into neuronal isoform complexes containing δ and σ3A or σ3B subunits while δ, β3A, μ3A, and σ3A or σ3B subunits are part of the ubiquitous AP-3 adaptor present in all cells including neurons.

As for other adaptor complexes, the mechanisms that control recruitment of AP-3 to membranes include accessory proteins and small GTPases (Crump et al., 2001; Dell'Angelica et al., 1998; Dell'Angelica et al., 1997). ARF1 seems to be the GTPase involved with AP-3 since ARF1 mutants locked in their GDP-bound form prevent binding of AP-3 to organelles (Ooi et al., 1998).

AP-3A was found to be localized to the TGN and endosomes by immunofluorescence and immunoelectron microscopy and able to interact with clathrin through its β3A subunit (Dell'Angelica et al., 1998; Dell'Angelica et al., 1997). Studies making use of naturally occurring AP-3 mutants in humans and mice have shown that AP-3A functions in transporting cargo to lysosomes and melanosomes (Dell'Angelica et al., 1999b; Kantheti et al., 1998).

There are numerous studies which suggest that AP-3B performs extra-synaptic functions in neurons (Newell-Litwa et al., 2007). One study showed that overexpression of AP-3B in mouse chromaffin cells led to a large number of small-volume vesicles which released only small amounts of neurotransmitters, while cells deleted of AP-3B produced large-diameter vesicles that released high amounts of neurotransmitters. AP-3B appeared to localize to the TGN or immature secretory vesicles in these cells (Grabner et al., 2006). These and other results indicate that AP-3 might have a function in regulation of synaptic vesicle protein sorting in neurons.

AP-4

As the other adaptor complexes, also AP-4 consists of four subunits: ε, β4, μ4 and σ4, which were shown by northern blotting to be expressed ubiquitously (Dell'Angelica et al., 1999a). Immunofluorescence and immunoelectron microscopy showed that AP-4 was localized to the TGN and colocalized with TGN38 and furin (Dell'Angelica et al., 1999a; Hirst et al., 1999). Sequence analysis demonstrated that the homology

of the β4 subunit to other β subunits is restricted to the trunk domain, which is thought to mediate interaction with other subunits of the AP complexes. Even if hinge-like and ear-like domains are found in β4, they seemed not to contain clathrin binding motifs. Consistent with this observation is the finding that AP-4 localized to non-clathrin-coated vesicles in the area of the TGN and that it could not be detected in preparations of clathrin coated vesicles (Hirst et al., 1999).

Not much is known about the recruitment of AP-4 to the TGN, but BFA treatment disrupted the punctuate signal at the TGN, indicating that the membrane association of AP-4 is regulated by a GTPase, possibly ARF1 (Boehm et al., 2001).

It was proposed that AP-4 is involved in basolateral sorting in epithelial cells. AP-4 binds basolateral sorting signals of furin, LDLR, MPR46, and TfR and it was shown in MDCK cells, that disruption of AP-4 led to a mis-sorting of these proteins to the apical surface (Simmen et al., 2002). In other experiments, placement of a μ4-specific tyrosine-based sorting signal onto the cytoplasmic tail of a plasma membrane reporter protein led to its transport to the endosomal-lysosomal system, suggesting a role for AP-4 also in this pathway (Aguilar et al., 2001).

The fifth adaptor complex

For many years it has been assumed that there are only four adaptor complexes. However, recently, the protein encoded by the C14orf108 gene was found to be homologous to μ-adaptins (Hirst et al., 2011). By yeast two-hybrid screen a specific interaction with the uncharacterized gene product DKFZp761E198 was identified. Sequence analysis with this gene showed the top homology hits to be all β-adaptins. In fractionation experiments it was shown that C14orf108 was present in cytosolic and membrane fractions indicating that it cycles on and off the membrane. In cells expressing GFP-tagged C14orf108, a punctate staining was observed that was concentrated in the perinuclear region and co-localized with LAMP1. In C14orf108 siRNA-treated cells an increase of MVBs, that appeared to be swollen and had tubules emanating from them were observed in immunogold electron microscopy. These data point to a role of the C14orf108/DKFZp761E198 (μ5/β5) complex in endosomal trafficking. Another study demonstrated that μ5 and β5 can be co-immunoprecipitated with two novel proteins KIAA0415/SPG48 and C20orf29 (Slabicki et al., 2010). These two proteins have a number of properties that suggest them to be the other large subunit (ζ) and the small subunit (σ5) of the AP-5 complex. In addition to these putative subunits, two further proteins were co-immunoprecipitated: SPG11 and SPG15. They both have features that are consistent with a function in the AP-5 pathway e.g. SPG15 which has a FYVE domain that bound to the

endosomal phosphoinositide PtdIns(3)P and localized to endosomes (Hirst et al., 2011). Thus, it was proposed that AP-5, as the other APs, is a heterotetramer consisting of two large subunits, a medium subunit and a small subunit and also binds to accessory proteins as SPG11 and SPG15 (Hirst et al., 2011).

CLASPs

In the last years, a picture is emerging that various types of adaptor proteins recruit distinct classes of cargo into forming vesicles. It has been observed that APs do not recognize all types of sorting signals, e.g. ligand induced phosphorylation and ubiquitylation do not use APs as principal adaptors. Furthermore, experiments have shown that uptake of epidermal growth factor (EGF) or LDL is not significantly reduced in cells after siRNA-mediated silencing of AP-2 (Hinrichsen et al., 2003; Motley et al., 2003). These findings indicate that APs act together with additional adaptors to sort various cargos into forming vesicles. The appendage domains of AP-1 and AP-2 were shown to bind to different groups of CLASPs which can, through their interaction with the APs, target cargo with distinct sorting signals to forming CCVs. Thus, CLASPs can account for different cargo types found within a single coated vesicle.

GGAs: Several CLASPs are known to be involved in sorting cargo into AP-1 containing vesicles. The most prominent of these are the Golgi-localized, γ-ear containing, ARF-binding proteins (GGAs) which were discovered in 2000 by several groups (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). There are three GGAs in mammals, GGA1, GGA2 and GGA3, which are all monomeric and ubiquitously expressed. They all consist of a tandem rearrangement of three folded domains, designated the VHS (Vps, Hrs, Stam), GAT (GGA and TOM (target of myb)), and GAE (γ-adaptin ear) domains (Figure 17). The VHS domain is found in proteins involved in trafficking, it is followed by a proline-rich linker sequence connecting two domains. The GAT domain, which is conserved in all GGAs, is followed by a long hinge sequence predicted to be largely unstructured. Finally, the GAE domain is homologous to the ear domain of AP-1 γ-adaptin (Bonifacino, 2004).

It is proposed that mammalian GGAs are involved in packaging MPRs and their ligands into CCVs or other clathrin coated carriers that emerge from the TGN and deliver cargo either to early or late endosomes. Indeed, coated vesicles containing GGAs together with clathrin, AP-1, and MPRs have been observed in the region of the TGN (Doray et al., 2002; Puertollano et al., 2003), even if GGAs were not enriched in purified CCVs (Hirst et al., 2000). This could be due to preparational reasons or could indicate that GGAs might only play a transient role in packaging cargo into vesicles.

Another function for GGAs was proposed to be the sorting of ubiquitinated proteins which need to be transported to the lysosomes for degradation. GGAs can bind with their GAT domain to ubiquitin linked to a protein and sequester it to the site of CCV formation (Pelham, 2004).

As is the case for other adaptor proteins, the recruitment of GGAs to membranes is probably initiated by ARF1, which serves as a docking protein through its interaction with the GAT domain (Shiba et al., 2003). The binding of the GAT domain places the VHS domain in close proximity to the membrane where it can interact with DXXLL-type signals on the cytosolic tails of MPRs and other cargo proteins. The GAE domain recognizes accessory proteins containing the DFGXØ sequence, for example p56 (Lui et al., 2003). Subsequent recruitment of clathrin to the site of vesicle formation is mediated by the interaction between clathrin-box-like sequences in the hinge segment of the GGAs and the clathrin heavy chain (Mullins and Bonifacino, 2001; Puertollano et al., 2001; Zhu et al., 2001). At present it is not fully clear if the GGAs alone can mediate clathrin coated vesicle formation at the TGN or if they act in cooperation with other adaptors as AP-1. It was shown that GGAs can bind the AP-1 γ-appendage (Bai et al., 2004) which could work as platform for the assembly of specific accessory proteins.

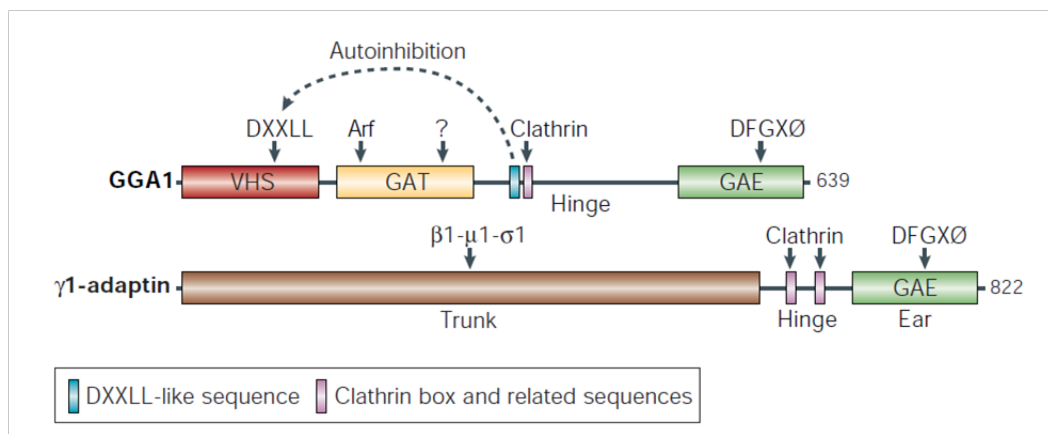

Figure 17: Domain organization of GGA1.

The structure of GGA1 is representative of that of other GGAs. The sequences or proteins that bind to each domain are indicated with arrows. The GAE domain of GGA1 is homologous to the ear domain of γ-adaptin, which is depicted for comparison (Bonifacino, 2004).

β-Arrestins: β-Arrestin 1 and 2 are cargo specific adaptors for G-protein coupled receptors (GPCR), seven-membrane-spanning receptors, which transmit various signals from the external environment to the interior of the cell. β-Arrestins consist of two domains made of β-sheets, the N-terminal part is

mainly responsible for GPCR binding while the C-terminal domain binds to PtdIns(4,5)P_2 (Gaidarov et al., 1999; Han et al., 2001; Milano et al., 2002; Oakley et al., 2001; Pulvermuller et al., 2000). Also located at the C-terminus is a short unstructured region, termed the C-terminal tail, which contains a clathrin box motif and binding sites for the AP-2 β2 subunit (Goodman et al., 1997; Krupnick et al., 1997; Laporte et al., 1999).

Through their interaction with phosphorylated, ligand-activated GPCRs, β-arrestins play a central role in controlling the duration and extent of GPCR signaling. Bound β-arrestin prevents the GPCRs from interacting with G proteins which stops further signaling (Lohse et al., 1990). In addition, GPCR binding induces a conformational change in β-arrestins that leads to the exposure of the C-terminal tail with its binding sites for clathrin and AP-2. This allows β-arrestins to target bound GPCRs to CCVs for endocytosis (Goodman et al., 1996; Laporte et al., 2000).

EpsinR: EpsinR (for epsin-related protein), a CLASP which is distantly related to the epsin family (see below), was identified based on its ability to interact with the γ-domain of AP-1 (Hirst et al., 2003). EpsinR contains an evolutionary conserved domain at the N-terminus known as the Epsin N-terminal Homology (ENTH) domain (Chen et al., 1998) which binds to PtdIns(4)P, a phosphoinositides mainly generated on the TGN, and is sufficient for stable membrane association (Ford et al., 2002; Hirst et al., 2003; Itoh et al., 2001; Mills et al., 2003). The ENTH domain is followed by a long unfolded polypeptide chain with binding motifs for clathrin, AP-1, and GGAs (Horvath et al., 2007).

EpsinR is ubiquitously expressed (Mills et al., 2003) and was found to localize in the Golgi region, often associated with coated budding profiles. Furthermore, epsinR colocalized with AP-1, however, it was shown that they do not recruit each other to membranes (Hirst et al., 2003). A possible function of epsinR might be the selective sequestering of SNARE proteins to AP-1 containing CCVs, since the SNARE protein vtib1 was reduced in CCV preparations isolated from epsinR-depleted cells. Furthermore, vtib1 was mislocalized in the absence of epsinR (Hirst et al., 2004).

A previous study demonstrated that epsinR might be involved in retrograde transport from endosomes to the TGN (Saint-Pol et al., 2004). EpsinR localized to endosomal membranes independently of AP-1 and was involved in the retrograde transport of exogenous Shiga toxin, endogenous TGN38/46, and MPR300. EpsinR might act as a structural adaptor between clathrin and lipids which suggests the existence of epsinR/clathrin coats on endosomes.

2.4.5. Accessory factors

In the last years, many new clathrin interacting proteins have been identified. In addition to coat components and adaptors, a large number of proteins have been described which only transiently interact with coated vesicles, since they are not enriched in CCV preparations. As part of the interactome, these accessory factors undergo multiple interactions with other components of the clathrin coat formation machinery and they regulate various steps in vesicle generation as membrane deformation, vesicle fission or uncoating of the budded vesicle (McMahon and Boucrot, 2011; Schmid and McMahon, 2007; Slepnev and De Camilli, 2000). In the following, some of the best characterized will be briefly reviewed.

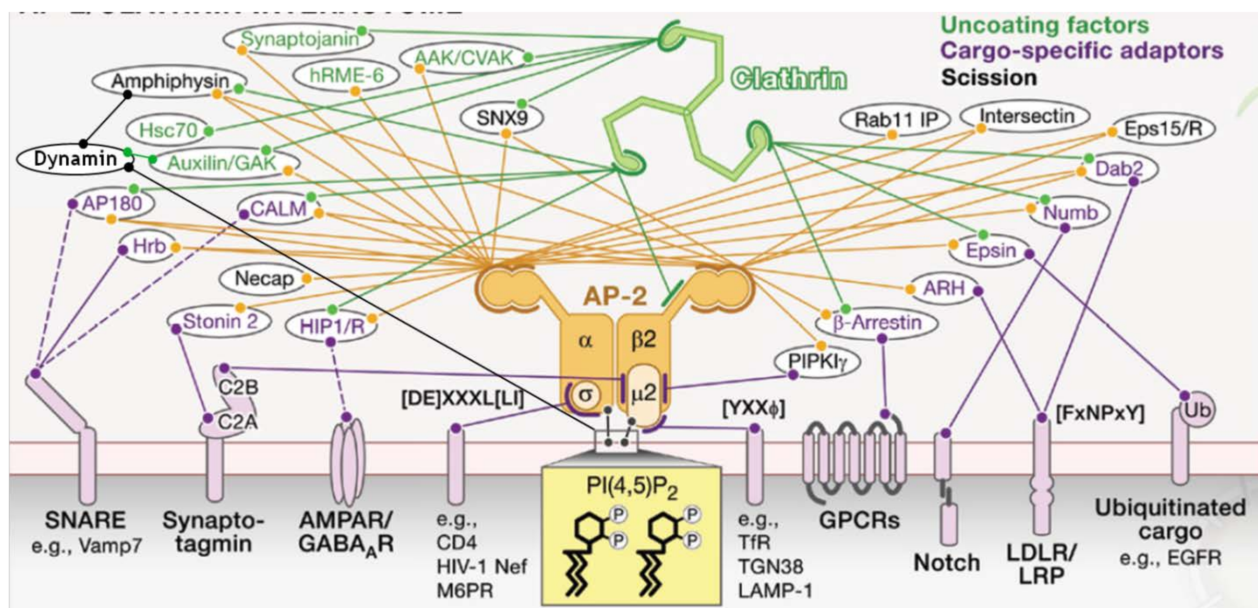

Figure 18: Network of accessory proteins involved in CCV formation at the plasma membrane.

Overview over accessory and adaptor proteins involved in clathrin coated vesicles at the plasma membrane. The ear domain of AP-2 and the clathrin terminal domain serve as interaction hub for the recruitment of accessory proteins, many of which have been shown to serve as cargo specific adaptors (purple) for the internalization of selected cargo proteins. Other accessory factors function in vesicle fission (black) and uncoating (green) (adapted from Wieffer et al., 2009)

EPS15

Epidermal growth factor protein substrate 15 (EPS15) was originally identified as substrate for the epidermal growth factor receptor tyrosine kinase (Salcini et al., 1999). Its N-terminal contains three Epsin homology (EH) domains which mediate binding to several endocytic proteins as epsin and synaptojanin

(Chen et al., 1998; Haffner et al., 1997). The central region of EPS15 is responsible for homodimerization and heterodimerization with intersectin (Sengar et al., 1999) while the C-terminal region is characterized by the presence of several DPF repeats which interact with the α-adaptin ear domain (Benmerah et al., 1996). EPS15 was found to be enriched in synapses (Chen et al., 1998) where it localized to the membrane with clathrin, dynamin, and AP-2. Interestingly, electron microscopy localization studies showed EPS15 to have a different distribution than AP-2. While AP-2 was evenly distributed on both clathrin coated pits and vesicles, EPS15 localized primarily to the rim of budding coated pits and is absent from vesicles (Tebar et al., 1996), indicating a possible function at the periphery of the coat. The localization of EPS15 to the plasma membrane depends on its N-terminal EH domain. Upon overexpression of a mutant which lacks the EH domain, EPS15 no longer localized to the plasma membrane and furthermore, endocytosis was disrupted (Benmerah et al., 1999). In addition to N-terminal EH domain deletion mutants, also overexpression of the C-terminal domain of EPS15 interfered with endocytosis, as uptake of transferrin and the EGF receptor were inhibited in HeLa cells (Benmerah et al., 1998). A more recent study suggests that EPS15 might be required for activating the clathrin assembly activity of AP180/CALM (Morgan et al., 2003). These results taken together suggest a scaffolding role for EPS15 to bring together the different components of the endocytic machinery and coordinate their tasks (Miliaras and Wendland, 2004).

Epsins

The epsin family consists of four members, epsin 1-3 and the already described epsinR. Epsin 1 was initially discovered as main binding partner for EPS15 and was named EPS15 interacting protein (Chen et al., 1998). Epsin 1 is abundantly expressed in most cell types, but is found to be enriched in brain, a pattern which is also described for epsin 2 (Rosenthal et al., 1999). In contrast, epsin 3 is exclusively associated with keratinocytes of wounded epithelial tissue (Spradling et al., 2001). As espinR, epsins 1-3 contain the ENTH domain at their N-terminus which is responsible for membrane binding. However, the region adjacent to the ENTH domain has different features than in epsinR. Downstream of the ENTH domain, several ubiquitin-interaction motifs (UIMs) are situated, responsible for ubiquitin binding. The central part is characterized by multiple DPW motifs, which are binding sites for AP-2, flanked by clathrin boxes (Legendre-Guillemin et al., 2004). The C-terminal comprises NPF repeats required for binding to EPS15 and other proteins (De Camilli et al., 2002).

Epsins were shown to play a role in CME at the plasma membrane. Epsin 1 is found to accumulate in puncta on the plasma membrane where it colocalizes with AP-2, clathrin, EPS15, and dynamin (Ford et al., 2002). Unlike the PtdIns(4)P-binding ENTH domain of epsinR, which has a different lipid specificity, the epsin ENTH domain binds to PtdIns(4,5)P_2 and drives plasma membrane deformation (Ford et al., 2002). While the major function of the ENTH domain might be to force membrane curvature, the C-terminal region of epsin 1 is crucial for recruiting clathrin coat components (Wendland, 2002). Since epsins and EPS15 recognize ubiquitinated cargo, especially transmembrane proteins, via UIM repeats, they are also good candidates for specialized CLASP adaptors, sorting ubiquitinated signaling receptors into CCVs for endocytosis (Horvath et al., 2007; Owen et al., 2004).

AP-180/CALM

AP-180 is a brain specific protein that is concentrated in nerve terminals. The ubiquitously expressed clathrin assembly lymphoid myeloid leukemia (CALM) protein shares considerable similarity with AP-180 and is thought to represent the functional homologue of AP-180 in non-neuronal cells (Slepnev and De Camilli, 2000). The N-terminal domain of AP-180 is characterized by an ANTH domain which is similar in structure as the epsin ENTH domain. The C-terminal part has no predicted secondary structure and contains α-appendage Dx[FW] binding motifs, epsin binding NPF motifs, and several clathrin boxes (Kalthoff et al., 2002).

Via interactions of the ANTH domain with PtdIns(4,5)P_2, AP-180 is recruited to the plasma membrane (Ford et al., 2001) where it interacts with clathrin, AP-2, and other accessory proteins. It was shown that a complex of AP-2 and AP-180 had a much stronger ability to assemble clathrin than each protein alone (Hao et al., 1999). This indicates a function for AP-180 in CCV formation, a possible role might be to assemble and maintain clathrin in large lattices on the side of the membrane from where cargo containing CCVs can bud off (Morgan et al., 1999). Additionally, it was demonstrated in *in vitro* experiments with purified proteins, that clathrin baskets polymerized in the presence of AP-180 are smaller and more homogenous than baskets formed when no AP-180 was present (Ye and Lafer, 1995a, b). This observation was also confirmed *in vivo* in *Drosophila melanogaster* where disruption of the gene encoding for the AP-180 homologue led to impaired efficiency of synaptic vesicle endocytosis and increased size of synaptic vesicles (Zhang et al., 1998). Thus, AP-180 might be involved in controlling vesicle size.

Auxilin

After endocytosis, newly formed CCVs rapidly shed their coat in a reaction involving heat shock protein cognate 70 (HSC70) and its cofactor auxilin, which is highly enriched in nerve terminals (Ahle and Ungewickell, 1990; Prasad et al., 1993; Ungewickell et al., 1995). Auxilin has an ubiquitously expressed homolog which is referred to as auxilin-2 or cyclin-G-associated kinase (GAK) (Kanaoka et al., 1997; Umeda et al., 2000). Mammalian auxilin has three domains. It's N-terminal domain comprises a PTEN (phosphatase and tensin homologue)-like domain that binds to PtdIns(4,5)P_2. PTEN was shown to have phosphoinositide phosphatase activity with specificity for the 3' position of the inositol ring (Lee et al., 1999). The central domain of auxilin binds to clathrin, AP-2 (Scheele et al., 2001), and dynamin (Newmyer et al., 2003). The C-terminal domain is characterized by the J-domain which interacts with HSC70 during clathrin uncoating (Jiang et al., 2003).

Through interactions with phosphoinositides as well as clathrin and AP-2, auxilin targets HSC70 to the assembled clathrin coat where it stimulates the HSC70 ATPase activity with the J-domain (Barouch et al., 1997; Ungewickell et al., 1995) finally leading to the uncoating of the vesicle. The exact mechanism of this process is not exactly known, however, it was proposed that auxilin binding to HSC70 promotes a local change of clathrin heavy chain contacts, creating a general deformation of the clathrin coat. This local destabilization of the lattice may lead to the falling off of the coat (Fotin et al., 2004a).

FCHo1/2

Previous studies in yeast and mammalian cells indicate that the initiation of CCV formation may involve the assembly of a putative nucleation module that defines the sites of the plasma membrane where clathrin will be recruited (McMahon and Boucrot, 2011). The Fer/Cip4 homology domain-only (FCHo) proteins 1 and 2 are good candidates. These proteins are ubiquitously expressed and are localized to clathrin coated pits (CCPs) only on the plasma membrane (Henne et al., 2010). They contain F-BAR domains in their N-terminal region and a μ-like domain at the C-terminus. BAR domains bind to membranes and it is assumed that they possess membrane-bending activity (see below). F-BAR domains can recognize very low curvature membranes which could be an evidence that they act early in vesicle formation. Indeed, in total internal reflection fluorescence microscopy (TIRF) and cryogenic immune-electron microscopy studies it was shown that the FCHo1/2 signal decreased before the clathrin signal intensity reached its maximum (Henne et al., 2010; Taylor et al., 2011). It was shown that the reduction of FCHo levels by RNAi led to a complete loss of CCPs while overexpression resulted in a dramatic

increase in CCP density (Henne et al., 2010). This correlation indicates that FCHo proteins appear as CCP nucleators.

As a very first step of CCV formation, FCHo1/2 bind to PtdIns(4,5)P_2-rich zones of the plasma membrane, where they induce membrane curvature and target EPS15 and intersectins to the nucleation module leading to the subsequent recruitment of AP-2 and clathrin (McMahon and Boucrot, 2011).

However, recent studies in zebrafish challenge the role of FCHo1/2 as nucleators (Umasankar et al., 2012). Furthermore, Cocucci et al., showed by live-cell TIRF imaging that coated pit initiation started with the arrival of clathrin and AP-2, while FCHo1/2 was not involved in this step (Cocucci et al., 2012).

Sorting nexins

Sorting Nexins (SNXs) are a family of proteins that are classified by the presence of a special type of PX domain, the SNX-PX domain (Teasdale et al., 2001). So far, 33 mammalian sorting nexins have been identified (Cullen, 2008). They function in diverse processes as endocytosis, endosomal transport, and signaling. Some examples are SNX1, SNX2, SNX5 and SNX6 which are, as mentioned above, involved in retromer transport from endosomes to TGN, and SNX4 that was proposed to play a role in endosomal recycling, possibly together with SNX30 and SNX7 (Traer et al., 2007).

A number of 12 mammalian sorting nexins contain BAR domains at the C-terminal region and were classified as the SNX-BAR subfamily (Cullen, 2008) (Figure 19A). They have the ability to target themselves to high-curvature membranes, mediate membrane deformation, and drive and stabilize formation of membrane tubules (Carlton et al., 2004; Carlton et al., 2005). SNX9, which has an N-terminal Src homology 3 (SH3) domain in addition to the BAR domain and the PX domain, has been shown to be essential for the late stages of clathrin mediated endocytosis (Yarar et al., 2007). It is targeted to the plasma membrane by binding of the PX domain to PtdIns(4,5)P_2 where it interacts with clathrin, AP-2, dynamin 2, and Neural Wiskott-Aldrich syndrome protein (N-WASP) (Badour et al., 2007; Lundmark and Carlsson, 2004; Soulet et al., 2005). The current model of SNX9 function suggests that it defines a high curvature region of the plasma membrane and, upon BAR domain-mediated oligomerization, drives membrane tubulation and clustering of N-WASP. N-WASP, which is an activator of the actin-related protein 2/3 (ARP2/3)-complex, induces ARP2/3-mediated filamentous-actin nucleation. The formation of actin filaments generates a force for further membrane remodeling and/or aids dynamin in the fission process (Yarar et al., 2007) (Figure 19B).

Another example is SNX18, a close relative of SNX9, which comprises the same domains and was shown to be associated with endosomal structures on which it colocalized with dynamin 2 and AP-1 (Haberg et al., 2008).

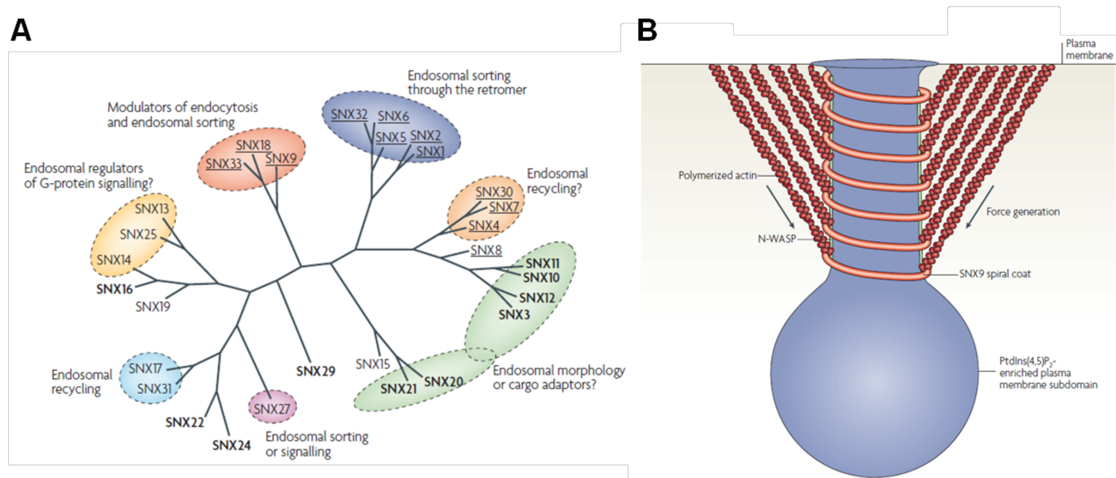

Figure 19: The Sorting Nexin Family.

(**A**) Phylogenetic analysis of the mammalian SNXs based on their amino acid sequence. To each group different functions in endocytosis, recycling and endosomal sorting are assigned. SNXs which belong to the SNX-BAR subfamily are in underlined text. (**B**) Model of SNX9 function: SNX9 deforms the membrane and stabilizes the formation of tubules. As it also binds N-WASP, tubule formation is coupled with clustering of this protein. This clustering leads to nucleation of filamentous actin and a force generation that induces further membrane remodeling (Cullen, 2008).

Synaptojanin

Synaptojanin is a dual-function phosphatase which is evolutionary conserved from yeast to human. Two mammalian synaptojanin genes have been identified so far. Synaptojanin 1 is characterized by an N-terminal Sac1-like polyphosphate phosphatase which converts PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, and PtdIns(3,5)P_2 to PtdIns and a central inositol polyphosphate 5-phosphatase that hydrolyzes the inositol ring of phosphoinositides (McPherson et al., 1996). The C-terminal region of synaptojanin 1 is subject to alternative splicing in adult versus developing neurons and exhibits a proline rich domain (PRD) which can mediate interaction with SH3 domain-containing proteins as amphiphysins, endophilin, and Grb2 (Cestra et al., 1999; McPherson et al., 1994; McPherson et al., 1996). Synaptojanin 1 is the predominant form in nerve terminals and localizes to coated endocytic intermediates (Haffner et al., 1997).

Synaptojanin 2 shows a broader tissue distribution than synaptojanin 1. Its catalytic domain is closely related to the one of synaptojanin 1 but the two C-terminal regions are unrelated. The synaptojanin 2

PRD domain binds only to one SH3-containing protein, Grb2, which is an adaptor for synaptic vesicle cycle and neurotransmitter release (Nemoto et al., 1997). This suggests that the PRDs of synaptojanin 1 and 2 are implicated in different protein-protein interactions and direct the two isoforms to distinct subcellular compartments.

Synaptojanin 1 and 2 exhibit different biological functions. The absence of synaptojanin 1 in mice led to a 100% mortality within 2 weeks after birth (Kim et al., 2002), a result of the impaired turnover of the PtdIns(4,5)P_2 pool at the plasma membrane which interferes with the disassembly of clathrin coats and a defect in synaptic transmission. Furthermore, it was shown using a cell-free assay with liposomes of different diameters, that synaptojanin 1 acts together with endophilin to preferentially remove PtdIns(4,5)P_2 from curved membranes rather than from flat ones. This suggests that elimination of spatially restricting PtdIns(4,5)P_2 by synaptojanin 1 at sites of high membrane curvature may cooperate with dynamin function to access the neck of the CCP and achieve vesicle fission (Chang-Ileto et al., 2011). Several studies also reported a role for synaptojanin in vesicle uncoating. In synaptojanin 1 knockout mice, CCVs accumulated in nerve terminals as an effect of the increased levels of PtdIns(4,5)P_2 (Cremona et al., 1999) and *Caenorhabditis elegans* synaptic termini, deletion of the gene encoding for the synaptojanin ortholog led not only to a defect in fission of the vesicle, but also in the uncoating process (Harris et al., 2000).

Synaptojanin 2 is assumed to be involved in an early step of the clathrin mediated endocytic pathway in non-neuronal cells. In lung carcinoma cells, RNAi knockdown of synaptojanin 2 caused a strong defect of clathrin mediated internalization of EGF and transferrin receptors. Moreover, electron microscopy showed a strong reduction of CCPs in these cells, confirming a role in an early stage of CCP formation (Rusk et al., 2003). These and other studies show that synaptojanins act at multiple steps of clathrin mediated endocytosis.

Dynamin

The mammalian genome contains three dynamin genes. The proteins encoded by these genes share the same domains and 80% homology but they display different tissue expression patterns. Dynamin 1 is expressed exclusively and at high levels in neuronal cells (Nakata et al., 1991), dynamin 2 shows a ubiquitous expression (Cook et al., 1994), and dynamin 3 is found primarily in brain and testis and at lower levels also in some other tissues as for example the lung (Cao et al., 1998).

Dynamins are cytosolic GTPases which are characterized by 5 domains: The N-terminal G domain, which mediates GTPase activity; a stalk domain; a pleckstrin homology (PH) domain; a GTPase effector domain, which can interact with the G domain; and a PRD at the C-terminus (Ferguson and De Camilli, 2012)(Figure 20A). The stalk domain dimerizes in a cross-like fashion which leads to a dynamin dimer in which the two G domains are oriented in opposite directions (Chappie et al., 2010; Faelber et al., 2011; Ford et al., 2011). The PH domain is responsible for binding of acidic phospholipids of the plasma membrane, in particular PtdIns(4,5)P_2 (Zheng et al., 1996). The PRD domain contains a number of PXXP motifs which interact with many SH3 domain-containing proteins to localize dynamin to endocytic sites and coordinate its function (Grabs et al., 1997; Lundmark and Carlsson, 2004; Shpetner et al., 1996).

It was demonstrated that purified dynamin spontaneously forms rings and helices in presence of liposomes or membrane tubules (Roux et al., 2010). The stalk-tip interactions of the dimers result in dynamin polymerization whose angle defines the diameter of the rings (Faelber et al., 2011; Ford et al., 2011).

It is generally agreed that the main function of dynamin is membrane fission during endocytosis, however, the exact mechanism of this process has been a matter of intense debate. Recent crystallographic and cryo-EM studies have shed more light on this open question. Assembly of dynamin dimers on membrane tubules and subsequent interaction of adjacent dynamin rings led to G domain dimerization which is critical for GTPase activity, indicating that dynamin function requires a polymer that wraps around a membrane template (Chappie et al., 2010; Gasper et al., 2009; Low and Lowe, 2010) (Figure 20B). It was demonstrated that GTP hydrolysis by a G domain led to a lever-like movement of the adjacent neck domain. Such a movement along its subunits could constrict the dynamin helix and result in fission (Chappie et al., 2011) (Figure 20C).

The question if dynamin is also involved in CCV formation at the TGN is not fully resolved. Several groups reported a prominent punctuate dynamin staining not only at the plasma membrane but also in the Golgi region (Cao et al., 2000; Jones et al., 1998). Furthermore, in a cell free assay of vesicle formation from the TGN after addition of cytosol, dynamin-depleted cytosol completely inhibited budding of vesicles (Jones et al., 1998). However, these data are contradicting to other studies where no effect of dynamin on sorting from the TGN could be shown (Altschuler et al., 1998).

It seems to be presumable that the ubiquitously expressed dynamin 2 isoform has housekeeping functions, as knockout mice die earl in embryonic development (Ferguson et al., 2009), while the neuronally enriched dynamin 1 and 3 have partially overlapping functions in synaptic transmission, as

double-knockout mice have a more severe phenotype than dynamin 1 single-knockout mice (Raimondi et al., 2011). It is proposed that this allows clathrin mediated endocytosis to function over a very broad range of neuronal activities and that the cooperation of dynamin 1 and 3 and their splice variants can fine tune this processes effectively (Ferguson and De Camilli, 2012).

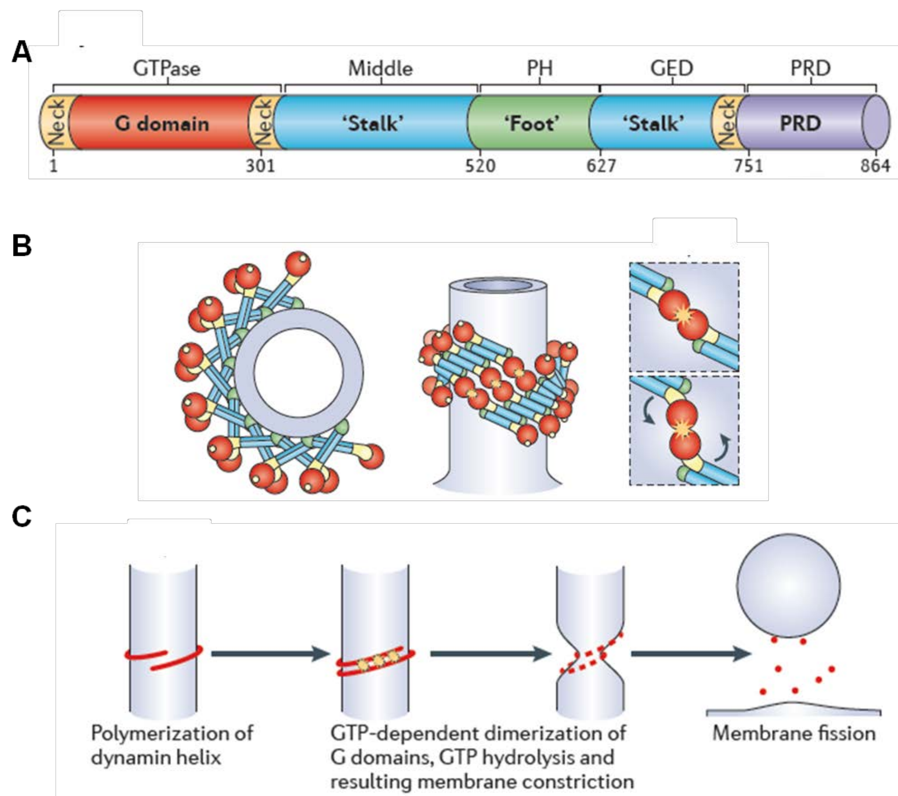

Figure 20: Dynamin structure and mode of action.

(**A**) Linear representation of the domain organization of dynamin. (**B**) Arrangement of dynamin dimers on a membrane tubule. Polymerization results of interactions between the stalk domains of monomers and between stalk dimers. Upon GTP-hydrolysis, the neck domain undergoes a lever-like movement. (**C**) Schematic view of key steps leading to dynamin-mediated membrane scission (Ferguson and De Camilli, 2012).

Endophilin

The endophilin family of proteins has five members: A1, A2, A3, B1, and B2. Endophilin A1 is brain specific while endophilin A2 is ubiquitously expressed. Endophilin A3 is highly enriched in brain and testis (Giachino et al., 1997). Endophilin B1 and B2 have no specific tissue distribution and are expressed in most organs including brain (Pierrat et al., 2001). At a subcellular level, endophilins A were concentrated at pre-synaptic terminals where they localized to synaptic vesicles and in the cytosol (Ringstad et al.,

1997). Endophilin A3 was also found to be associated with filamentous structures that co-localized with microtubules (Hughes et al., 2004). Endophilin B1 localization has been described as both diffuse and punctate and localized in the cytoplasm with a perinuclear enrichment (Pierrat et al., 2001; Wan et al., 2008), while the localization of endophilin B2 is not well known.

All endophilins share the same structural domain organization. They consist of an N-terminal BAR domain, a variable middle region and a C-terminal SH3 domain. BAR domains form crescent shaped homo – or heterodimers with highly conserved, positively charged residues at the concave side. The BAR domain preferentially binds to regions of specific membrane curvature in negatively charged membranes and has been proposed to sense, induce, and/or stabilize membrane curvature (Gallop and McMahon, 2005; Peter et al., 2004). Within the endophilin BAR domain dimer, each monomer consists of three kinked, anti-parallel alpha helices. The endoBAR belongs to the N-BAR class, characterized by the presence of an amphipathic helix at the N-terminus which is important for curvature generation (Gallop et al., 2006; Weissenhorn, 2005). The central domain structure is unknown, however, it was shown that this region is important for determining whether endophilin promotes or inhibits receptor-mediated endocytosis (Sugiura et al., 2004). The SH3 domain displays the typical β-barrel core with a hydrophobic groove that harbors proline rich sequences present in binding partners (Loll et al., 2008). The SH3 domain of endophilin was shown to bind to dynamin, synaptojanin (Ringstad et al., 1997), and amphiphysins (Micheva et al., 1997b).

An array of different studies has shown that endophilin A acts at multiple steps of endocytosis. Antibody-mediated disruption of endophilin function in a stimulated lamprey giant synapse led to a block in the invagination of clathrin coated pits and their subsequent accumulation, indicating a role for endophilin at an early stage of endocytosis (Ringstad et al., 1999). This was confirmed by TIRF live cell-imaging studies on turnover rates of abortive and productive CCPs (Mettlen et al., 2009).

In contrast to these findings, microinjection of a peptide blocking the binding of endophilin to dynamin into lamprey axons resulted in the accumulation of late-stage coated pits whose closer examination failed to reveal any dynamin localized around the neck (Gad et al., 2000). Furthermore, in mouse fibroblasts depleted of all dynamin isoforms, clathrin coated pits exhibit long narrow necks covered with endophilin. These and other findings promoted a model in which endophilin and other BAR domain proteins promote narrowing of the neck region prior to the recruitment of dynamin (Ferguson et al., 2009).

It is also suggested that endophilin works closely together with synaptojanin during the uncoating process. In *D. melanogaster*, endophilin and synaptojanin single mutants showed the same phenotypes as the double mutants and synaptojanin was mis-localized and destabilized at synapses devoid of endophilin (Verstreken et al., 2003) indicating that endophilin recruits synaptojanin to endocytic sites. Furthermore, upon deletion of all three endophilin genes in mice, a striking accumulation of CCVs at synapses without a change in the number of CCPs was observed (Milosevic et al., 2011). Though these triple knockout mice died within a few hours after birth, synaptic transmission was greatly reduced but not completely impaired showing that endophilins are not essential for this process. These and other data lead to the suggestion that endophilin may be involved in the coupling of endocytic vesicle fission (via dynamin) and uncoating (via synaptojanin).

A role for endophilin B1 in endocytosis has not been demonstrated so far. In contrast to endophilin A, endophilin B1 was not only found at the plasma membrane but also localized to intracellular structures as well as co-localized with Golgi specific markers in the perinuclear region (Farsad et al., 2001). It was also observed that a small subpopulation of endoB-YFP over-expressed in HeLa cells co-localized with mitochondria, which was confirmed by cell fractionation (Karbowski et al., 2004). When endophilin B1 was silenced with RNAi, alterations in mitochondrial morphology could be detected in a significant amount of cells which showed misshaped, often unusually interconnected mitochondria randomly distributed in the cytoplasm.

Furthermore, endophilin B1 was shown to participate in the trafficking of TrkA, a TRK receptor tyrosine kinase which serves as signaling receptor for Nerve growth factor (NGF). Endophilin B1 partially co-localized with TrkA itself, EEA1, and the lysosomal marker LAMP1 in PC12 cells. Knockdown of Endophilin B1 reduced targeting of NGF and TrkA to EEA1-positive structures and led to their enlargement after NGF treatment (Wan et al., 2008).

Amphiphysin 1

Amphiphysin 1 was first identified as brain-specific protein associated with synaptic vesicles (Lichte et al., 1992) and was connected with the rare, central nervous system disease Stiff-Man syndrome (De Camilli et al., 1993). Amphiphysin 1 expression is highest in neuronal tissue but also detectable to a much lesser extent in adrenal gland (Lichte et al., 1992).

Amphiphysin 1 comprises three regions: an N-terminal BAR domain, an unstructured middle domain and a C-terminal SH3 domain (Figure 21). The amphiphysin BAR domain forms elongated, banana-shaped

homo/heterodimers in which each monomer is a coiled-coil of three long α-helices which form a six-helix bundle. The concave surface of the dimer shows positively charged patches which mediate interaction with phospholipid membranes (Casal et al., 2006; Peter et al., 2004). The N-terminal residues form an additional amphipathic helix, which groups amphiphysin into the N-BAR family. It was shown that the amphiphysin BAR domain can sense membrane curvature upon insertion of the amphipathic α-helix into the lipid bilayer (Bhatia et al., 2009) and that it binds to highly curved membranes where it induces further membrane bending (Arkhipov et al., 2009; Blood and Voth, 2006). Furthermore, the BAR domain is able to tubulate liposomes *in vitro* (Peter et al., 2004; Takei et al., 1999) and molecular dynamics simulations demonstrated that the degree of membrane curvature of tubules was dependent on the type and density of the lattice formed by the amphiphysin 1 BAR domains (Yin et al., 2009).

The central insert domain comprises binding sites for clathrin, adaptors, and endophilin. The clathrin binding domain is located between amino acids 347 and 386 and comprises two distinct binding sites. The first stretch includes the sequence LLDLD, which fits the clathrin box motif present in many accessory proteins, and the second stretch includes the sequence PWDLW and was termed the W box (Drake and Traub, 2001; Miele et al., 2004; Slepnev et al., 2000). The crystal structure of a complex of the N-terminal β-propeller domain of clathrin and a peptide comprising the W box motif shows its binding to a different location in clathrin than the binding site for clathrin box motifs (Miele et al., 2004). The region of amphiphysin 1 necessary for AP-2 binding is partially overlapping with the clathrin binding domain and contains the core sequence FFED and the downstream located DPF motif, which is not essential, but enhances binding to the AP-2 α ear domain (Olesen et al., 2008; Slepnev et al., 2000; Wang et al., 1995). Surprisingly, in GST-pull down assays with a peptide comprising the clathrin binding sequence PWDLW, AP-2 and the β1, μ1, and γ subunits of AP-1 could be recovered in addition to clathrin (Drake and Traub, 2001). The region of amphiphysin displaying these binding motifs was termed the CLAP (clathrin and adaptor binding) domain. Upstream of the CLAP domain, amphiphysin 1 contains the conserved, proline-rich sequence RKGPPVPPLP which forms a consensus binding site for SH3 domains (Sparks et al., 1996) and was shown to bind to the endophilin SH3 domain in pull-down assays with different GST-amphiphysin 1 fusion proteins (Micheva et al., 1997b).

The C-terminal SH3 domain mediates binding to dynamin and synaptojanin (Ramjaun et al., 1997). *In vitro* binding assays mapped amphiphysin 1 binding to the PSRPNR sequence in the proline-rich region of dynamin which is distinct from the binding site of other SH3 domains (Grabs et al., 1997). In the case of

synaptojanin, amphiphysin 1 binds the two sequences LPIRPSR and PTIPPRA in the PRD with equal affinity (Cestra et al., 1999).

Various studies imply a role for amphiphysin 1 in synaptic vesicle recycling of neuronal cells. In amphiphysin 1 knockout mice, synaptic vesicle recycling is decreased and the pool of recycling vesicles is smaller compared to wildtype (Di Paolo et al., 2002). Furthermore, these mice have a reduced viability caused by an increased susceptibility to seizures. This shows that amphiphysin 1 is involved in synaptic transmission, but not essential.

The data on amphiphysin 1 suggest a model where amphiphysin recruits synaptojanin and dynamin to the curved neck of the endocytic CCP and thereby supports vesicle fission and uncoating. It was shown by several groups that the interaction of dynamin and amphiphysin 1 influences clathrin-mediated endocytosis. Injection of amphiphysin 1 into the lamprey giant synapse led to the accumulation of coated pits and decreased the number of synaptic vesicles (Shupliakov et al., 1997). Furthermore, when Cos-1 cells were transfected with the amphiphysin SH3 domain, uptake of transferrin and EGFR were blocked. This effect could be rescued by the co-transfection of dynamin with the amphiphysin SH3 domain (Wigge et al., 1997b). Such results indicate that the amphiphysin SH3 domain sequesters endogenous dynamin from endocytic pits and full-length amphiphysin is necessary to target dynamin to the pit neck. These data are supported by the finding that the presence of amphiphysin 1 enhanced dynamin-dependent vesicle formation from large liposomes *in vitro* by stimulation of the dynamin GTPase activity (Yoshida et al., 2004). This effect was influenced by the liposome size. The late function which was proposed for amphiphysin 1 in endocytosis was previously confirmed by TIRF studies demonstrating amphiphysin membrane recruitment during vesicle formation shortly before dynamin action caused vesicle release from the membrane (Taylor et al., 2011).

However, there is evidence that amphiphysin may not only act in endocytosis but also at other stages of the trafficking pathway. Disruption of the only amphiphysin gene of *C. elegans* led to a defect in recycling endosome morphology and function (Pant et al., 2009). Furthermore, endogenous amphiphysin was enriched on recycling endosomes and co-localized with RME-1 from the RME-1/EHD1 (receptor mediated endocytosis/Eps15 homology-domain containing 1) family. The interaction of these two proteins promoted recycling of transmembrane cargo.

Several studies indicate that amphiphysin 1 functions not as a homodimer but as a heterodimer with the neuronal isoform of amphiphysin 2. In amphiphysin 1 knockout mice, expression of amphiphysin 2 was nearly abolished in brain, while expression of amphiphysin 2 in other tissues as muscle was normal (Di

Paolo et al., 2002). In addition, Cos-1 cells could only uptake transferrin when expressing both amphiphysin 1 and 2 and not when they expressed only one of the two (Wigge et al., 1997a). This suggests that the functional entity of amphiphysin in brain is a heterodimer of amphiphysin 1 and amphiphysin 2.

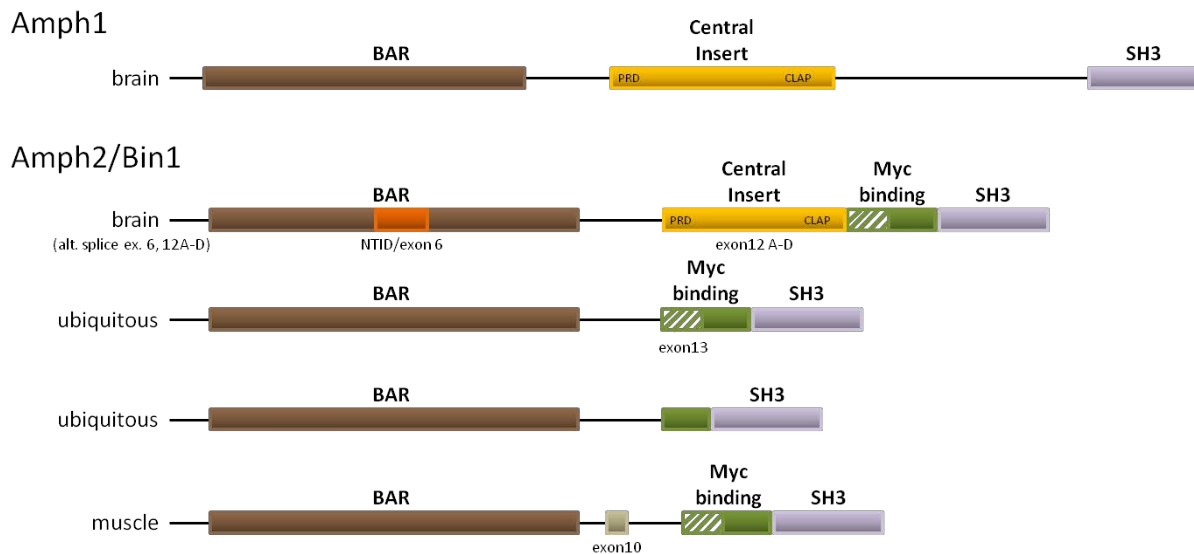

Figure 21: Domain organization of Amphiphysin 1 and Amphiphysin 2.

Amphiphysin 1 is brain-specific and contains an N-terminal BAR domain, a CLAP and a PRD domain in the middle part and a C-terminal SH3 domain. Amphiphysin 2 consists of the same domains as amphiphysin 1, however, different isoforms are expressed in different tissues, with the brain-specific isoform being the only one which contains the central insert domain (exon12A-D) where the CLAP domain is localized. Amphiphysin 2 exons 6, 10, 12A-D, and 13 undergo alternative splicing.

Amphiphysin 2

Amphiphysin 2, also known as BIN1, was described by several groups who cloned various splice variants on the basis of their similarity to amphiphysin 1 (Butler et al., 1997; Gold et al., 2000; Leprince et al., 1997; Ramjaun et al., 1997; Sakamuro et al., 1996; Tsutsui et al., 1997; Wechsler-Reya et al., 1997). So far, two ubiquitous isoforms, one muscle isoform, and several neuronal isoforms have been identified. The muscle isoform and one ubiquitous isoform of amphiphysin 2 were identified due to the presence of a myc binding domain and termed BIN1 (box-dependent myc-interacting protein-1)(Sakamuro et al., 1996). They localize to the nucleus and display features of a tumor suppressor (DuHadaway et al., 2001; Elliott et al., 2000; Elliott et al., 1999). The muscle isoform furthermore contains the exon 10, which is responsible for T-tubule association (Butler et al., 1997; Lee et al., 2002). The other ubiquitous isoform

was shown to associate with early phagosomes in macrophages and a mutant deficient in dynamin binding inhibited phagocytosis at the stage of membrane extension around bound particles (Gold et al., 2000). The brain specific isoforms of amphiphysin 2 are the only isoforms which contain exons 12A-D (central insert domain), where the CLAP domain is located and which undergo alternative splicing (Butler et al., 1997; Leprince et al., 1997; Ramjaun and McPherson, 1998; Ramjaun et al., 1997; Wechsler-Reya et al., 1997).

Brain amphiphysin 2 shares an average amino acid identity of around 50% with amphiphysin 1 (Tsutsui et al., 1997; Wigge et al., 1997a) and comprises the same domain organization, being an N-terminal BAR domain, different binding sites in the unstructured middle part, and a C-terminal SH domain. In a previous study with different brain specific splice variants of amphiphysin 2, it was shown that a 31-amino-acid sequence in the N-terminal BAR domain, termed the N-terminal insert domain (NTID), which is present only in some splice variants, mediated plasma membrane targeting and dimerization (Ramjaun et al., 1999).

The central insert domain contains the binding sites for endophilin, clathrin, and the adaptors. The CLAP region contains that same conserved clathrin binding motifs as amphiphysin 1, LLDLD and PWDLW, however, the flanking sequences show no similarity (McMahon et al., 1997; Miele et al., 2004; Ramjaun and McPherson, 1998). It was demonstrated in *in vitro* binding assays using GST-fusion peptides that appropriately spaced clathrin binding sequences enhance the binding affinity and facilitate interaction with clathrin (Drake and Traub, 2001). The AP-2 α binding sequences FED and DPL are overlapping with the clathrin binding sequences within the CLAP domain (Olesen et al., 2008; Slepnev et al., 2000). GST-fusion peptides of the PWDLW motif interacted with clathrin, AP-2, and AP-1, especially the γ-subunit, in pull-down assays (Bai et al., 2004; Drake and Traub, 2001). As in amphiphysin 1, the endophilin SH3 binding site is located upstream of the CLAP domain. Endophilin bound to full-length GST-amphiphysin 2, but not to GST-amphiphysin 2 lacking a 43 amino acid segment that includes the conserved proline-rich stretch RKGPPVPPPP, revealing this to be the binding site for the endophilin SH3 domain (Micheva et al., 1997b).

The C-terminal SH3 domain of amphiphysin 2 comprises a compact, five-stranded anti-parallel β-barrel. This core region provides a scaffold displaying a number of conserved hydrophobic residues, which mediate association to proline residues of binding partners (Owen et al., 1998). The amphiphysin 2 SH3 domain displays 51% amino acid identity to the one of amphiphysin 1 and as well mediates binding to dynamin and synaptojanin (Ramjaun et al., 1997). Amphiphysin 2 binds to the same PSRPNR sequencing

in the dynamin PRD as amphiphysin 1 (Owen et al., 1998). Furthermore, it was shown that dynamin displaced clathrin from amphiphysin 2 since binding of amphiphysin 2 to clathrin was reduced in the presence of dynamin (McMahon et al., 1997). *In vitro* binding studies with different synaptojanin constructs revealed that amphiphysin 2 binds synaptojanin via different binding sites than amphiphysin 1 and endophilin (Micheva et al., 1997a).

Disruption of the murine BIN1/amphiphysin 2 gene resulted in perinatal lethality indicating a role in embryonic development, most likely muscle differentiation. However, no effect on endocytosis in *BIN1* null mouse embryo fibroblasts was observed in transferrin uptake assays (Muller et al., 2003).

Together with amphiphysin 1, brain specific amphiphysin 2 was proposed to play a role in endocytosis. As shown for amphiphysin 1, also the SH3 domain of amphiphysin 2 inhibited endocytosis of transferrin when transfected into Cos-7 fibroblasts (Owen et al., 1998). Furthermore, co-immunoprecipitation experiments demonstrated that amphiphysin 2 interacts with SNX4 and the two proteins co-localized on transferrin positive structures (Leprince et al., 2003). In addition, amphiphysin 2 partially co-localized with EEA1, CD63, and LAMP-1 indicating that amphiphysin 2 can be associated with different early and late endosomal and lysosomal structures.

Aim of the Thesis

The minimal machinery for the recruitment of AP-1 to membranes was defined by *in vitro* assays using liposomal membranes (Crottet et al., 2002; Lee et al., 2008a; Lee et al., 2008b; Meyer et al., 2000; Zhu et al., 1999a). Purified proteins and nucleotides were incubated with liposomes of defined lipid composition with or without covalently coupled peptides mimicking cargo proteins. The liposomes were separated by sedimentation or floatation and analyzed for associated AP-1. It was observed that phosphoinositides, activated small GTPase ARF1, and membrane-bound cargo signals are necessary and sufficient. However, in the presence of cytosol, ARF1-dependent membrane association of AP-1 could be observed also without sorting signals. This suggested unknown cytosolic factors to contribute to formation of AP-1/clathrin coats.

We have used the liposome floatation assay to identify cytosolic proteins collaborating with AP-1 at the membrane. Separation of proteins from bovine brain cytosol yielded a final active fraction containing amphiphysin 1, amphiphysin 2, and endophilin A1.

The goal of this work was to confirm that these proteins are responsible for the stabilization of AP-1 on membranes *in vitro* and, in addition, to investigate if they might be involved in the formation of AP-1/clathrin coats at the TGN and/or endosomes. To address these questions, amphiphysin 1 and 2 (wildtype and mutants) and endophilin A1 were bacterially expressed and purified. Purified proteins were tested in the liposome floatation assay under different conditions to investigate which proteins are necessary and sufficient for AP-1 binding to liposomal membranes. Furthermore, mutant constructs of amphiphysins were analyzed to localize the sequence responsible for AP-1 association to liposomes.

To show an involvement of amphiphysin in AP-1/clathrin coat formation *in vivo,* localization of endogenous amphiphysin in primary neurons as well as exogenously expressed amphiphysin in neuronal cell lines was studied and the interaction of amphiphysin and AP-1 was analyzed using chemical cross-linker.

Material and Methods

Material

Reagents

Iproof High Fidelity DNA Polymerase was purchased from Bio-RAD. T4 ligase and restriction enzymes were from Roche. Bactotryptone, bactoagar, and yeast extract were from Applichem.

HisTrapp FF nickel columns and Glutathione Sepharose 4B were purchased from GE Healthcare. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Boehringer. Imidazole and L-glutathione reduced were from Sigma. Phenylmethylsulfonyl fluoride (PMSF) was from Applichem. Coomassie brilliant blue R-250 was from Sigma. The Bradford Standard Assay and Precision Plus (All blue Standards) molecular maker were from Bio-RAD. ECL reagent was from Millipore.

Soybean phospholipids containing 20% PC (azolectin, P-5638) were purchased from Sigma. (N-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dioleolyl-sn-glycero-3-phospho-ethanolamine (MMCC-DOPE) was from Avanti Polar Lipids. Peptides from Lamp-1Y (CRKRSHAGYQTI-COOH) were purchased at > 70% purity from NeoMPS. Guanylyl imidodiphosphate (GMP-PNP) was from Fluka. Superdex 75 (High Load 26/60 prep grade, 2.6 x 60 cm) was from GE Healthcare.

Dulbecco's modified Eagle's medium, trypsin from bovine pancreas, 4',6-Diamidino-2-phenylindole (DAPI), and retinoic acid were purchased from Sigma. Fugene HD Transfection reagent was from Promega. Digitonin was from Serva. Brefeldin A solution (1000x) was from BioLegend. Dithiobis[succinimidylproprionate] (DSP) was from ProteoChem. Pitstop 2 was from Abcam Biochemicals. DRAQ5 DNA Dye was from Biostatus. Mowiol 4-88 was from Hoechst.

Antibodies

Primary:

Origin	Antigen	Used for	Dilution	Source
Rabbit polyclonal	anti-myc (ab9106)	IF	1:1000	Abcam
Mouse monoclonal	anti-FLAG (M2)	IF	1:2000	Sigma
Mouse monoclonal	anti-amph1 (13)	WB	1:4000	Santa Cruz Biotech.
Mouse monoclonal	anti-amph1 (8)	Depl.	8 μg	Santa Cruz Biotech.
Mouse monoclonal	anti-amph1 (clone4)	IF	1:100	P. de Camilli, Yale
Goat polyclonal	anti-amph2 (N-19)	WB	1:4000	Santa Cruz Biotech.
Mouse monoclonal	anti- endo I-III (G-8)	WB	1:4000	Santa Cruz Biotech.
Goat polyclonal	anti-AP-1 γ	IF	1:100	Acris antibodies
Mouse monoclonal	anti-AP-1 γ (88)	IF/IP	1:1000	BD
Mouse monoclonal	anti-AP-1 γ (100/3)	WB	1:4000	Sigma
Mouse monoclonal	anti-AP-2 α (AP.6)	IF	1:500	ATCC
Mouse monoclonal	anti- AP-2 α (100/2)	WB	1:4000	Sigma
Mouse monoclonal	anti- clathrin (X-22)	IF	1:100	ATCC
Mouse monoclonal	anti-clathrin (clone 23)	WB	1:1000	BD transduction labs
Mouse monoclonal	anti-dynamin (Hudy 1)	IF	1:100	Upstate Cell Signaling
Rabbit monoclonal	anti-EEA1	IF	1:500	Cell Signaling
Sheep monoclonal	anti-TGN46	IF	1:100	SeroTec
Mouse monoclonal	anti- Giantin	IF	1:1000	H.P. Hauri, Biozentrum
Mouse monoclonal	anti-tubulin	WB	1:20’000	H. Farhan, Biozentrum

Secondary:

Origin	Antigen	labeled	used	Dil.	Source
Donkey polyclonal	α-mouse	A568	IF	1:200	Life Technologies
Donkey polyclonal	α-goat	A488	IF	1:200	Life Technologies
Donkey polyclonal	α-rabbit	A488	IF	1:200	Life Technologies
Donkey polyclonal	α-sheep	Cy5	IF	1:200	P. Scheiffele, Bioz.
Goat polyclonal	α-mouse	HRP	WB	1:10’000	Sigma
Rabbit polyclonal	α-goat	HRP	WB	1:4000	Sigma

Methods

Plasmid construction

All Polymerase Chain Reactions (PCR) were performed using the iproof high fidelity polymerase. PCR products were purified from 1% agarose gel using the gel extraction kit (Machery-Nagel) according to manufacturer's protocol. The purified constructs were cut with the corresponding restriction enzymes and ligated using T4 DNA ligase. The resulting constructs were transformed into *E. coli* UT580.

Wild-type human Amph1 and Amph2 were cloned into the bacterial expression vector pET24d (Novagen) by PCR thus adding a C-terminal His_6-tag. Wild-type human endophilin A1 was cloned into pGEX-4T-2 (GE Healthcare) fused to the C-terminus of glutathione-S-transferase (GST). To generate Amph1ΔSH3 and Amph2ΔSH3, codons 1–621 of Amph1 and 1–519 of Amph2, respectively, were cloned into pET24d. For Amph1WWAA and Amph2WWAA, the codons for W382 and W385 of Amph1 and W417 and W420 of Amph2, respectively, were substituted by alanine codons with PCR mutagenesis.

To generate the chimeric construct Amph2M1, the middle domain of Amph2 (M2, residues 277–512) was replaced with the homologous portion of the middle domain of Amph1 (M1, residues 241–495). For Amph1ΔM1', the second part of the Amph1 middle domain without a direct counterpart in the Amph2 sequence (M1', residues 496–612) was deleted. For Amph1M2 and Amph1M2ΔM1', M1 or the entire middle domain of Amph1 (M1+M1', residues 241-612), respectively, were replaced by M2.

To produce Amph1/2 heterodimers, Amph2 and C-terminally His_6-tagged Amph1 were cloned into pETDuet-1 (Novagen) containing two multiple cloning sites for co-expression of two target genes.

For mammalian cell expression, Amph1-myc and Amph2-FLAG were constructed by addition of the respective epitope-encoding sequences to the 3' end of the coding sequences and cloned into pcDNA3 (Invitrogen). For Amph1ΔCLAP-myc and Amph2ΔCLAP-FLAG, residues 321–387 and 359–423, respectively, were deleted by PCR mutagenesis. Amph1ΔSH3, Amph1WWAA, Amph2ΔSH3, and Amph2WWAA were fused to a myc-tag (Amph1) or a FLAG-tag (Amph2) and cloned into pcDNA3. Endophilin in pCMV6-ENTRY was purchased from OriGene. In addition to the pcDNA3 constructs, also wildtype Amph1 and Amph2 were cloned into pCMV6-ENTRY.

Expression and purification of Amph1, Amph2, endophilin A1, and Arf1

Amph1-His_6 and Amph2-His_6 wildtype and mutant constructs were expressed in a 2 l culture of *E. coli* BL21 (Novagen) upon induction with 0.5 mM IPTG at 30°C for 4 h (Amph1) or at 37°C for 6 h (Amph2). Cells were harvested and lysed in 20 ml His-buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol) supplemented with 10 mM imidazole, using a digital sonicator (Bransch). The lysate was clarified by centrifugation for 1 h at 150’000 x g and passed 3x through a 1-ml His-Trapp FF column using a pump (Bio-RAD). The column was washed with 20 volumes of His-buffer supplemented with 50 mM imidazole and eluted stepwise with 2 ml His-buffer containing 75 mM imidazole, 100 mM, 150 mM, 200 mM, and 250 mM imidazole. Protein concentration was determined using Bradford assay to be around 0.2 µg/µl for Amph1 and 0.5 µg/µl for Amph2.

To purify Amph1/2 heterodimers, Amph2 and C-terminally His_6-tagged Amph1 from pETDuet-1 were co-expressed in *E. coli* Rosetta 2 (DE3) pLysS (Novagen) upon induction with 0.5 mM IPTG for 4 h at 30°C and purified as above.

GST-Endophilin A1 was expressed in *E. coli* BL21 upon induction with 1 mM IPTG at 37˚C for 4 h. Cells were lysed in 20 ml GST-buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA) by sonication. Lysate was clarified by centrifugation and incubated with glutathione-Sepharose 4B for 1 h at 4˚C. The resin was washed with 20 volumes of GST-buffer and eluted with 40 mM glutathione (5 ml). Protein concentration was generally around 2 µg/µl as determined using Bradford assay. All purified proteins were stored at –80˚C.

Purified proteins were supplemented with SDS-sample buffer (4% SDS, 0.16 M Tris-HCl, pH6.8, 8.7% glycerol, 0.05% Bromo-phenolblue, 0.4% β-mercaptoethanol) and separated by 12.5% SDS-polyacrylamide gel electrophoresis. Separated Proteins were either stained with Coomassie blue or transferred to polyvinylidene fluoride membrane (Millipore) for immunoblot analysis with Anti-Amph1 (13), anti-Amph2 (N-19), and anti-endophilin (G-8).

Myristoylated Arf1-His_6 was purified as described by Liang and Kornfeld (Liang and Kornfeld, 1997) (Pascal Crottet).

Preparation of cytosol and clathrin coated vesicles (CCVs)

The preparation of cytosol and the isolation of CCVs have been described previously (Crottet et al., 2002; Suri et al., 2008). Briefly, six calf brains were purchased from the local slaughterhouse, cleaned from fat and meninges, supplemented with half their volume of buffer A (0.1 M MES-NaOH, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 0.5 mM PMSF) and mixed in a Waring blender. The homogenate was centrifuged at 8000 x g for 30 min and the supernatant was subjected to a high-speed centrifugation of 180’000 x g for 80 min to pellet the membranes. The resulting high-speed supernatant, which contains the cytosol, was collected, aliquoted, and shock-frozen in liquid nitrogen. Protein concentration was typically around 20-30 μg/μL as determined using the Bradford assay.

To prepare CCVs, the pellets resulting from the high-speed centrifugation were dissolved in 8 mL buffer A and homogenized in a medium Dounce homogenizer (Bellco Biotechnology). The homogenate was mixed with one volume of buffer B (0.1 M MES-NaOH, pH 7.0, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 12.5% (w/v) Ficoll400, 12.5% (w/v) sucrose), re-homogenized, and centrifuged at 60’000 x g for 40 min. The resulting supernatant was diluted with 3 volumes of buffer A and centrifuged at 180’000 x g for 80 min to pellet the CCVs. Pellets were resuspended in an equal volume of buffer A, homogenized in a small Dounce homogenizer (Bellco Biotechnology), and cleared by centrifugation at 15’000 x g for 12 min to remove aggregated material. The supernatant containing the CCVs was collected, shock-frozen in liquid nitrogen, and stored at -80°C.

Preparation of mixed adaptors

Mixed adaptors were essentially produced as described before (Crottet et al., 2002; Suri et al., 2008). Coat proteins were released by homogenizing CCVs in a small Dounce homogenizer with an equal volume of stripping buffer (1.5 M Tris-HCL, pH 7.0, 6 mM EDTA, 0.6 mM DTT). After addition of 0.5 mM PMSF and 1 x Protease inhibitor cocktail (PIC) (from a 500 x stock of 5 mg/mL benzamidine, 1 mg/mL pepstatin A, leupeptin, antipain, and chymostatin), the mixture was incubated on ice over night before membranes were pelleted for 35 min at 100’000 x g. The supernatant was loaded on a 1.6 x 60 cm Superose 6 column (GE Healthcare) that was pre-equilibrated in Superose buffer (0.5 M Tris-HCl, pH 7.0, 2 mM EDTA, 0.2 mM DTT) and 1 mL fractions were collected. AP-1 containing fractions were pooled,

supplemented with 1 x PIC and 2 mM PMSF, and stored at 4°C. Protein concentration was determined using Bradford assay.

Preparation of AP-1 from cytosol (Gregor Suri)

To purify cytosolic AP-1, 1.5 mg anti-γ-adaptin (100/3) was diluted in 50 mM Tris-HCl, pH 9.0, mixed with 1.2 ml packed protein A-Sepharose, and incubated at room temperature for 1 h with end-over-end rotation. The beads were washed twice with 50 mM Na-borate, pH 9.0, resuspended in 20 ml of 0.2 M Na-borate, pH 9.0, supplemented with 20 mM dimethylpimelidate, and incubated at room temperature for 30 min to covalently link the antibodies to the beads. After washing, the beads were incubated with 20 ml 0.2 M ethanolamine, pH 8.0, for 2 h to quench the reaction, washed again, and resuspended 1:1 in PBS. Ten ml of cytosol was supplemented with 1 x PIC and 0.5 mM PMSF and centrifuged for 1 h at 10’000 x g to remove insoluble aggregates. After centrifugation, the cytosol was incubated with the anti-γ-adaptin-coupled protein A-Sepharose overnight at 4°C and washed with 0.1 M MES-NaOH, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA. Bound AP-1 was eluted by incubating the beads three times with 1 ml buffer containing protease inhibitors and a 100-fold molar excess over antibody of the competing epitope peptide DLLGDINLTGAPAAAPA for 30 min at 37°C. The released AP-1 was supplemented with 0.5 mM PMSF and 1x PIC and stored at 4°C.

Preparation of peptidoliposomes

Liposomes were produced from 97.5% soybean phospholipids (azolactin, containing about 20% L-α-phosphatidylserine) as described previously (Crottet et al., 2002; Suri et al., 2008). Soybean lipids (3.8 mg) were dissolved in chloroform/methanol (2:1) to a concentration of 5 μmol and mixed with NBD-PE (1 mol%). If a signal peptide was added, the mixture was supplemented with 125 nmoles MMCC-DOPE (2.5 mole%). The organic solvent was evaporated under a stream of nitrogen before dichloromethane was added and evaporated twice. Dried lipids were resuspended in 1 mL liposome buffer (10 mM HEPES-NaOH, pH 6.5, 100 mM NaCl, 0.5 mM EDTA) and freeze-thawed five times by cycles of vortexing, shock-freezing in liquid nitrogen, and thawing. Finally, liposomes were prepared by extrusion through a 400 nm nucleopore polycarbonate membrane (Whatman) using a homemade hand-driven extruder.

If a signal peptide was added, 0.4 mg/ml peptide (i.e., about a fourfold excess over the coupling lipid, assuming half of it is exposed) was added to the liposomes immediately after extrusion. After incubation for 1 h at room temperature, peptidoliposomes were supplemented with 0.02% NaN_3 and then stored at 4°C for up to 3 days. When there was no peptide to be added, the liposomes were stored at 4°C immediately after extrusion.

Liposome floatation assay

In a total volume of 480 µl, 100 µl liposomes (0.5 µmol lipid) were mixed with 2 µg Arf1, 0.2 mM GMP-PNP, mixed adaptors (10 µg) or isolated AP-1 preparation (4 µg), 1.5 mg/ml bovine serum albumin to block unspecific binding, and 330 µl of either assay buffer (0.1 M MES-NaOH, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT) as the negative control, cytosol (1 mg protein diluted in assay buffer) as the positive control, or different concentrations of purified proteins. After incubation at 37°C for 30 min, the reaction was mixed with 480 µl of 60% (w/v) sucrose in assay buffer, overlaid with 3 ml 30% sucrose in assay buffer and 0.18 ml assay buffer, and placed into a 4-ml centrifuge tube. Liposomes and bound protein were separated from unbound material by centrifugation at 300,000 × g for 1.5 h at 4°C. The top 1-ml fraction containing the floated liposomes was collected and recruited proteins were precipitated with 17% (w/v) trichloroacetic acid after addition of 5 µg bovine serum albumin as carrier protein. Pellets were washed with acetone, subjected with SDS-sample buffer and analysed by immunoblot using anti-γ-adaptin (100/3) or anti-α-adaptin (AP.6) (Crottet et al., 2002; Suri et al., 2008).

Cytosol fractionation (Gregor Suri)

Cytosol was supplemented with protease inhibitors, mixed with increasing amounts of saturated ammonium sulfate solution for 1 h at 4°C, and centrifuged for 30 min at 10,000 × g. The final supernatant and re-dissolved pellet proteins were desalted into assay buffer.

Chromatography was performed at 4°C using an FPLC system and columns from GE Healthcare. Protein precipitated from 20 ml cytosol with 30% (sat.) ammonium sulfate was re-dissolved in assay buffer, desalted on a PD10 column, supplemented with 2 M NaCl, and loaded on a HiPrep Phenyl FF

hydrophobic interaction column (high sub, 1.6×10 cm). Bound proteins were eluted with a linear 200-ml gradient of 2–0 M NaCl. Fractions of highest activity were collected, concentrated to 2.5 ml in assay buffer by ultrafiltration, loaded on a MonoQ 10/100 GL anion exchange column (1.0×10 cm) pre-equilibrated in assay buffer, and eluted with a linear 80-ml gradient of 0–1 M NaCl. Fractions of highest activity were concentrated to 2.5 ml in assay buffer by ultrafiltration, loaded on a MonoS 10/100 GL cation exchange column (1.0×10 cm) pre-equilibrated in assay buffer, and eluted with a linear 80-ml gradient of 0–1 M NaCl. Collected fractions were subjected to the liposome floatation assay to remove unbound proteins and analyzed by SDS-PAGE and Coomassie staining.

Mass spectrometry (S. Moes and P. Jenö)

Proteins were cut from SDS-polyacrylamide gels stained with either Coomassie or silver. After in-gel digestion with trypsin, peptides were separated by capillary liquid chromatography using a 300SB C-18 column (Agilent Technologies) and analyzed on an Orbitrap FT hybrid instrument (Thermo Finnigan). Proteins were identified using the SEQUEST software against the NCBI non-redundant database.

Immunodepletion of Amph1 and AP-1

To deplete cytosol (1 mg protein) of Amph1 and Amph2, Protein A-Sepharose beads (20 µl) were incubated with 8 µg anti-Amph1 (8) in 1 ml PBS overnight at 4°C. The washed beads were incubated with 1 mg cytosol in 1 ml PBS for 2 h at 4°C. After pelleting the beads, the supernatant was analyzed for Amph1, Amph2, and endophilin by immunoblotting.

Immunodepletion of AP-1 was performed similarly using 20 µg anti-γ-adaptin (100/3) to deplete 3.5 mg cytosol (Gregor Suri).

Cell culture and Immunofluorescence

Mouse cerebellar granule cells were isolated and cultured (C. Wentzel and Dr. H. Witte, Biozentrum) as previously described (Iijima et al., 2011) and fixed after 10 days of culture. HN10 and COS-1 cells were

grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine at 37°C in 5% and 7.5% CO_2, respectively. PC12 cells were grown DMEM supplemented with 10% Horse serum, 5% FCS, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine at 37°C in 7.5% CO_2. NIH3T3 cells were grown in DMEM supplemented with 10% newborn calf serum (NCS), 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine at 37°C in 5.0% CO_2.

Cells were transiently transfected using FuGENE HD one day after seeding them on poly-L-lysine coated glass coverslips. To induce differentiation, HN10 cells were cultured 1 day after transfection in serum-free high glucose DMEM with neuronal B-27 supplements (Brewer et al., 1993) and 6 µM retinoic acid. Transfected cells were grown on coverslips for 48 h and fixed with 3% paraformaldehyde (PFA) for 10 min at room temperature. To quench the reaction, fixed cells were incubated for 5 min in 50 mM NH_4Cl. Alternatively, HN10 cells were pre-permeabilized with 40 µg/ml digitonin in 110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$ for 5 min at 4°C to release the bulk of free cytosolic proteins prior to fixation. In other experiments, COS-1 cells were treated with 5 µg/ml brefeldin A (BFA) or 20 µM pitstop 2 for 15 min before fixation as above. For transferrin uptake assays, COS-1 cells were starved for 1 h in serum-free medium, before fluorescent-labeled transferrin was allowed to internalize for 45 min.

Fixed cells were washed in PBS and permeabilized with 0.1% Triton for 10 min. After blocking with 1% BSA in PBS for 15 min, cells were incubated for 2 h with primary antibodies in PBS containing 1% BSA, washed, and stained for 30 min with fluorescent secondary antibodies in PBS containing 1% BSA. After a 5-min staining with Draq5 or DAPI and several washes with PBS, coverslips were mounted in Mowiol 4-88. Antibodies which were used are described in the table above. Staining patterns were analyzed using a Point Scanning Confocal Zeiss LSM700 upright.

Co-immunoprecipitation

PC12 and NIH3T3 were transfected with Amph1-myc and Amph2-FLAG and cultured for 2 days as described above. Mouse cerebellar granule cells were isolated and cultured for 8 days as described above. To capture transient interactions, PC12 and NIH3T3 cells were incubated with 2 mM dithiobis[succinimidylproprionate] (DSP) in 100 mM Na-phosphate, pH 7.8, for 30 min at room temperature followed by addition of 50 mM Tris-HCl, pH 7.5, for 15 min to stop the reaction. For cross-

linking in cerebellar granule cells, 2 mM DSP in PBS was added for 10 min at room temperature, followed by the stop reaction with 50 mM Tris-HCl, pH 7.5, for 5 min. After washing, cells were lysed in 100 mM Na-phosphate, pH 8.0, 1% Triton, 2 mM PMSF for 1 h at 4°C. The cleared lysate was incubated with anti-γ-adaptin (88) or anti-giantin as well as with 1 mg/ml BSA overnight at 4°C. Antigen-antibody complexes were collected with protein A-Sepharose for 1 h and washed 4 times with lysis buffer and PBS. Washed beads were boiled in SDS-sample buffer and subjected to immunoblot analysis using anti-Amph1 (13), anti-Amph2 (N-19), and anti-γ-adaptin (88).

RNA Interference

PC12 and NIH3T3 cells were grown as described above. Cells were plated in 35 mm dishes and transfected with 20 nM ON-TARGETplus SMARTpool mouse clathrin heavy chain siRNA, ON-TARGETplus SMARTpool rat AMPH1 siRNA, or ON-TARGETplus non-targeting control pool siRNA (Dharmacon Thermo Scientific) using lipofectamine RNAiMAX (Invitrogen). After one day, NIH3T3 cells were transfected with Amph1-myc and Amph2-FLAG as described above and cultured for two more days. After three days, cells were harvested in lysis buffer (0.5% DOC, 1% Triton, 2 mM PMSF, 1 x PIC) for 1 h at 4°C. After clearing the lysate, 100 μg was supplemented with SDS-loading buffer and analyzed by immunoblotting using anti-clathrin, anti-tubulin, anti-Amph1 (13), and anti-Amph2 (N-19). Alternatively, cells were incubated with DSP cross-linker for co-immunoprecipitation or fixed with 3% PFA for immunofluorescence analysis as described above.

RESULTS

Interaction of Amphiphysins with AP-1 Clathrin Adaptors at the Membrane

Cytosolic factors contribute to membrane association of CCV-derived or cytosolic AP-1 to peptide-free liposomes (Gregor Suri)

To assay for AP-1 membrane association and stabilizing factors, liposomes made of a rich soybean lipid mixture were incubated at 37°C for 30 min with purified myristoylated ARF1 and GMP-PNP, as well as with mixed adaptors isolated from calf brain CCVs, and then floated on a sucrose step gradient. The top fraction containing the floated liposomes was analyzed for bound AP-1 by SDS-gel electrophoresis and immunoblotting using an antibody against the γ-subunit. As is shown in Figure 1A, coat-derived AP-1 was not bound to liposomes (lane 2) unless stabilized by cargo peptides (LY) coupled to the lipid membrane (lane 1; typically 30–50% of input). In contrast, when full calf brain cytosol was used as the source of AP-1, binding was equally efficient in the presence and absence of sorting peptides (lanes 3 and 4). To exclude that this is due to differences between coat-derived and free cytosolic adaptors, AP-1 was immunopurified from cytosol (Figure 1B). When tested in the floatation assay, cytosol-derived AP-1 associated to liposomes with sorting peptides, but not to those without (Figure 1C, lanes 1 and 2), just like coat-derived AP-1. Yet, AP-1-depleted cytosol enabled binding to peptide-free liposomes equally for both AP-1 preparations (lanes 4 and 5). AP-1 association to these liposomes is thus mediated by a cytosolic component and is not due to different states of AP-1 (e.g. phosphorylation (Ghosh and Kornfeld, 2003)).

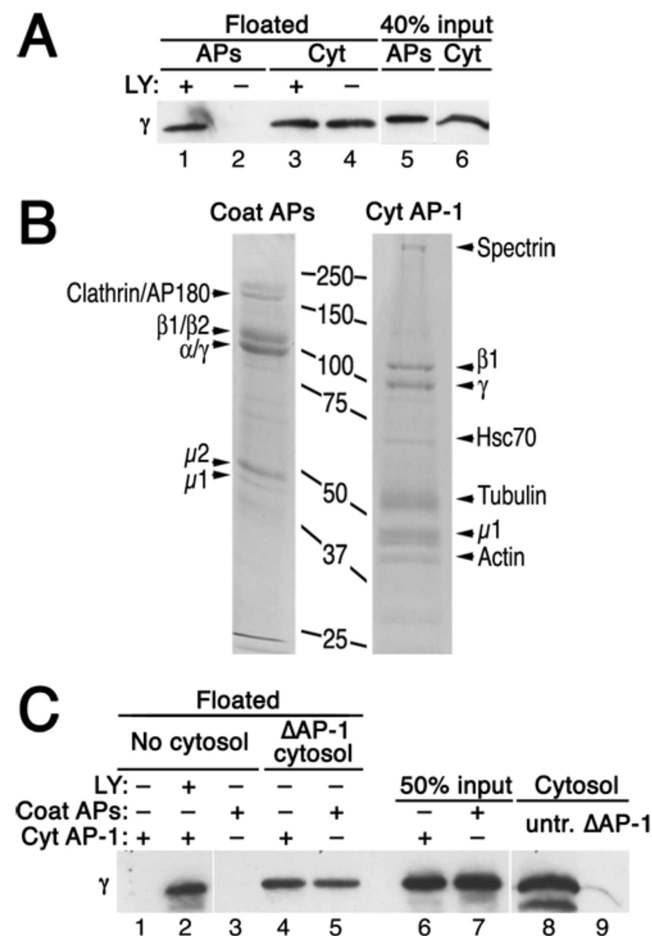

Figure 1: A cytosolic factor mediates ARF1-dependent AP-1 association with protein-free liposomes.

(**A**) Liposomes with or without lipid-anchored LY cargo peptides (corresponding to the cytoplasmic sequence of Lamp1 with a tyrosine-based AP-1 interaction motif) were incubated with ARF1, GMP-PNP, purified CCV-derived APs or bovine brain cytosol (Cyt) at 37°C for 30 min, loaded at the bottom of a sucrose gradient and centrifuged as described in *Materials and Methods*. The floated liposome fractions were collected and analyzed by immunoblotting for the γ-adaptin subunit of AP-1 (lanes 1–4). As standards, 40% of the input proteins were analyzed in parallel (lanes 5 and 6). (**B**) Coat-derived mixed APs (Coat APs) and immunopurified cytosolic AP-1 (Cyt AP-1) were separated by SDS-gel electrophoresis and stained with Coomassie-blue. Adaptor subunits and contaminating proteins identified by mass spectrometry are indicated. The positions of molecular weight standards are indicated in kDa. (**C**) Liposomes with or without lipid-anchored LY peptides were incubated with ARF1, GMP-PNP, and either cytosolic AP-1 or coat-derived APs in the presence or absence of cytosol depleted of AP-1 (ΔAP-1 cytosol). After floatation, the liposome fraction was analyzed by immunoblotting for γ-adaptin (lanes 1–5). As controls, 50% of the input proteins and aliquots of untreated and AP-1-depleted cytosol were analyzed in parallel (lanes 6–9). The fluorographs in each panel derive from the same blot and exposure.

Purification and identification of the cytosolic activity (Gregor Suri)

To purify the cytosolic factors, calf brain cytosol was first subjected to ammonium sulfate precipitation. Proteins precipitated up to 30% ammonium sulfate saturation (Figure 2A) were successively fractionated by hydrophobic interaction (B), anion exchange (MonoQ) (C), and cation exchange (MonoS) chromatography (D). Fractions were tested for AP-1 binding to liposomes after buffer exchange to assay conditions. The fractions with highest activity were used as the starting material for the next separation.

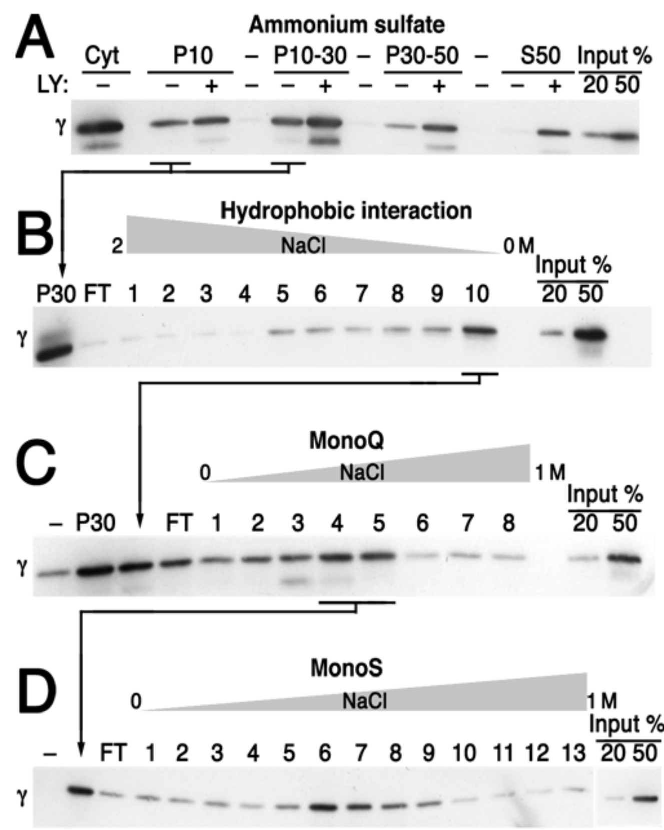

Figure 2: Purification of cytosolic factors supporting AP-1 membrane association.

Calf brain cytosol was sequentially mixed with saturated ammonium sulfate to 10%, 30%, and 50% (**A**). Upon centrifugation, the pellets of precipitated protein (P10, P10-30, P30-50) and the final supernatant (S50) were dialyzed to assay buffer and tested for AP-1 recovery on liposomes with or without LY cargo peptides and in comparison to full cytosol (Cyt) and buffer control (–). Proteins precipitated at 30% ammonium sulfate saturation (P30) were successively fractionated by hydrophobic interaction chromatography (**B**), anion exchange (MonoQ) chromatography (**C**), and cation exchange (MonoS) chromatography (**D**), in each case starting with the highest-activity fractions of the preceding separation (arrows). The NaCl gradients used are indicated. Starting material, flow-through (FT), buffer control (–), and the fractions were tested for AP-1 association to liposomes. The floated liposome fractions as well as 20% or 50% of the input APs were immunoblotted for γ-adaptin.

The fractions of the final MonoS chromatography were analyzed for their protein composition by SDS-gel electrophoresis and Coomassie staining (Figure 3, left). The strongest activity was present in fractions 6 and 7 whose major bands had apparent molecular weights of ~120, ~85, and ~40 kDa. When incubated with liposomes under assay conditions, all three proteins associated with the membranes, since they could be recovered with the floated liposomes (Figure 3, right). By mass spectrometry, they were identified as amphiphysin 1, amphiphysin 2, and endophilin A1, respectively.

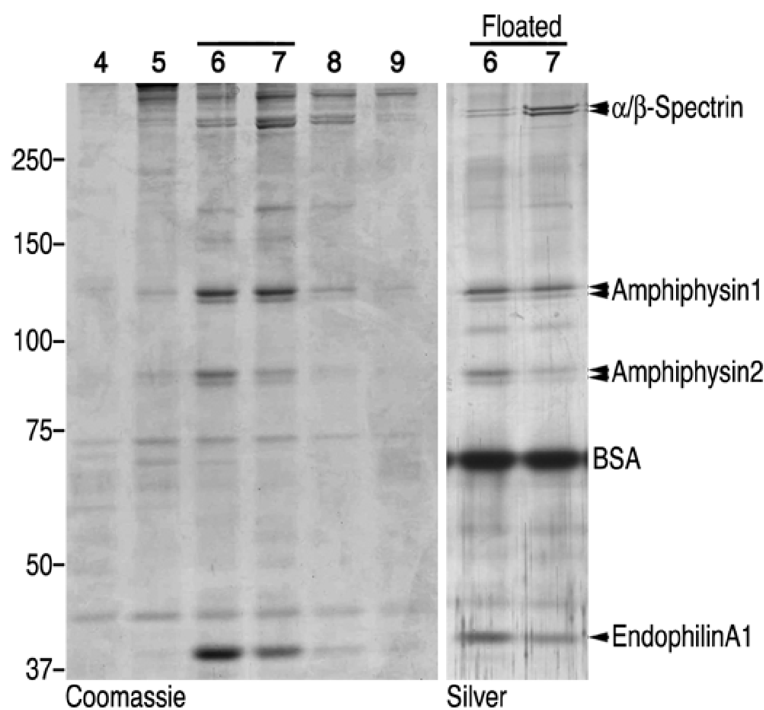

Figure 3: Identification of proteins in final active fractions.

Aliquots of the fractions 4–9 eluted in the final MonoS chromatography shown in Figure 2 were analyzed by SDS-gel electrophoresis and stained with Coomassie Brilliant Blue. In addition, liposomes were incubated with fractions 6 and 7, floated to the top of a sucrose gradient, collected, TCA-precipitated, separated by SDS-gel electrophoresis, and stained with silver. Bands were excised and analyzed by mass spectrometry. The major bands were identified as amphiphysin 1, amphiphysin 2, and endophilin A1. In addition, spectrin was detected, yet in a distribution not matching maximal activity (horizontal lines).

Amphiphysin 1, Amphiphysin 2, and Endophilin A1

All three proteins are known components of the AP-2/clathrin interactome at the plasma membrane (Schmid and McMahon, 2007). As illustrated in Figure 4, they all contain a C-terminal SH3 domain and an N-terminal amphipathic helix–BAR domain that mediates dimerization and forms a crescent-shaped, positively charged surface to bind and shape curved membranes (Frost et al., 2009). Across all domains, amphiphysin 1 and 2 share on average 50% amino acid identity. Unlike endophilin A1, they in addition include a large, unstructured middle part (M1 and M2 in Figure 4A), which comprises the binding sequences for endophilin, clathrin, and adaptors. Amphiphysin 1 contains an additional specific domain (M1') with partially duplicated M1 sequences. Amphiphysin 1 is expressed highest in neurons and concentrated at pre-synaptic terminals (Wigge et al., 1997a). Amphiphysin 2 is expressed in 10 different splice variants with different tissue and intracellular distributions (including muscle-specific and ubiquitous forms named BIN1), the longest splice variants being largely brain specific (Tsutsui et al., 1997; Wigge et al., 1997a). Both full-size amphiphysins were shown to bind clathrin heavy chain and the AP-2 α-appendage through distinct but partly overlapping sites in the CLAP domain located in their middle parts (Leprince et al., 1997; McMahon et al., 1997; Slepnev et al., 2000). The SH3 domains of all three proteins interact with dynamin and synaptojanin (David et al., 1996; McPherson et al., 1996; Ramjaun et al., 1997; Ringstad et al., 1999). The SH3 domain of endophilin furthermore binds to a proline-rich segment in the middle domains of amphiphysin 1 and 2 (Micheva et al., 1997b). Endophilin A1 is almost entirely specific to the brain (Ringstad et al., 1997). Interestingly, the peptide SIPWDLWEPT (including the distal clathrin-binding motif PWDLW) of amphiphysin 2 fused to GST was shown to bind *in vitro* to AP-1 and AP-2 from brain cytosol (Drake and Traub, 2001) and to the isolated γ-appendage of AP-1 (Bai et al., 2004). An interaction of full-length amphiphysin 1 or amphiphysin 2 with AP-1 had not been shown so far.

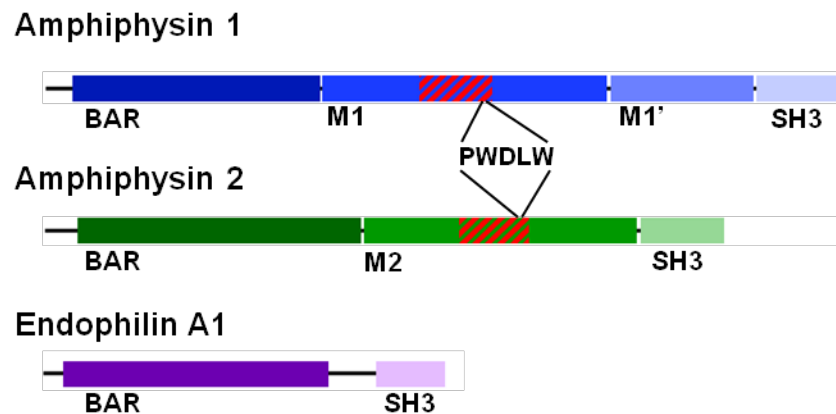

Figure 4: Amphiphysin 1, amphiphysin 2, and endophilin A1.

Domain organization of amphiphysin 1 and 2 and endophilin A1. All three proteins contain a N-terminal BAR domain and a C-terminal SH3 domain. Binding sites for clathrin and adaptor proteins (indicated in red) are located in the M1 and the M2 domains of amphiphysin 1 and amphiphysin 2, respectively. Amphiphysin 1 contains a additional downstream domain (M1') which has repeating sequences of M1.

Purification of Amphiphysin 1, Amphiphysin 2, and Endophilin A1

To confirm the identity of the protein(s) in the final fraction of Figure 3 that is responsible for AP-1–membrane association, amphiphysin 1 (Amph1) and amphiphysin 2 (Amph2) with a C-terminal His_6-tag, and GST-tagged endophilin A1 (Endo) were over-expressed in bacteria using IPTG. Purification of Amph2 as an example was performed as follows: After clearing the bacterial lysate by ultra-centrifugation (Figure 5A, lane 1), it was passed through a Ni-NTA column (lane 2). After washing with lysis buffer containing 50 mM imidazole (lane 3), proteins were eluted step-wise with 75-250 mM of imidazole (lanes 4-8). Usually, the peak concentration was found in the fraction with 150 mM imidazole. This fraction was further analyzed by immunoblotting to confirm that the lower running bands are degradation products rather than bacterial contamination (Figure 5B, lane 9).

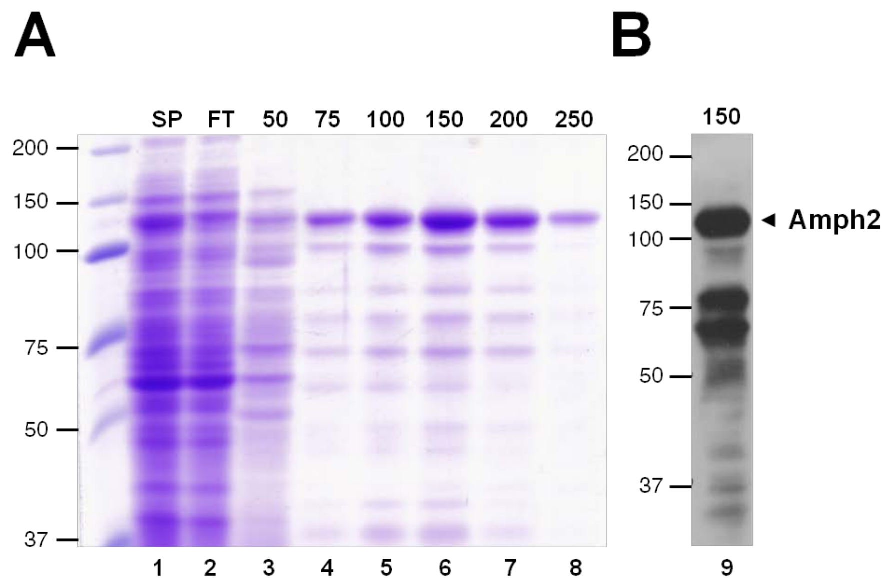

Figure 5: Purification procedure of bacterially expressed amphiphysin 2.

(**A**)Bacterially expressed Amph2 was purified using Ni-NTA chromatography. 10 µl aliquots of supernatant (SP) and flow through (FT) and 50 µl of washing with 50 mM imidazole (50) and of elution samples with 75 -250 mM imidazole (75, 100, 150, 200, and 250) were loaded on a 12.5% SDS-PAGE to monitor purification. (**B**) 5 µl of sample 150 was analyzed by immunoblot using αAmph2 (N-19). The positions of molecular weight markers (in kDa) are indicated.

Amph1-His_6 was purified according to the same protocol (Figure 6, lane 2). For GST-Endo, cleared lysate was incubated with glutathione beads before washing and elution with 40 mM glutathione (Figure 6, lane 8).

Mutant constructs of Amph1 and Amph2 where the SH3 domain is deleted (Amph ΔSH3) and where the clathrin and adaptor binding motif WDLW is mutated to ADLA (Amph WWAA) were generated, purified as described above, and analyzed by SDS-PAGE to check purity and degradation (Figure 6, lanes 4-7).

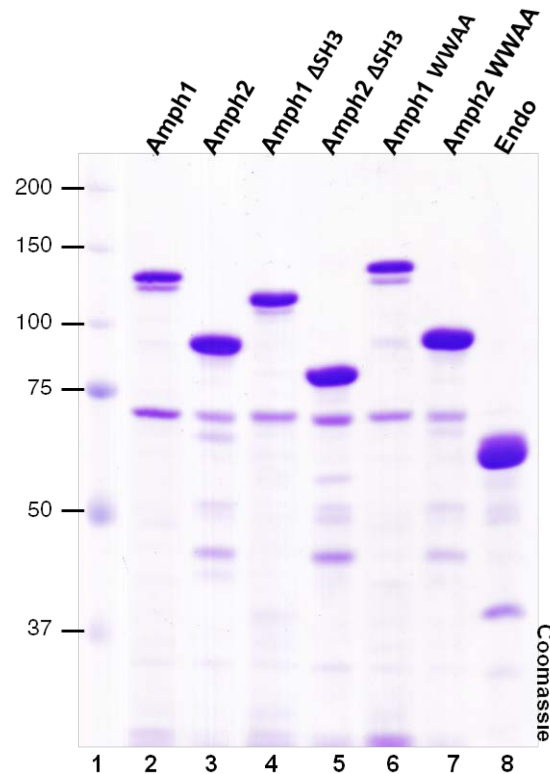

Figure 6: Purified amphiphysin 1 and 2 and endophilin A1 constructs.

The coding sequences of His_6-tagged Amph1 and Amph2, full-length as well as lacking the SH3 domain (ΔSH3) or mutated in the WDLW motif to ADLA (WWAA), were expressed in bacteria, purified by Ni-NTA chromatography, and 15 µg from the peak elution fraction was analyzed by SDS-gel electrophoresis and Coomassie staining. GST-tagged Endo was purified by glutathione chromatography. The positions of molecular weight markers (in kDa, lane 1) are indicated.

Purified Amphiphysins and Endophilin are present on floating liposomes

Purified proteins were tested in the floatation assay for their ability to bind to liposomal membranes. Liposomes were incubated with ARF1, GMP-PNP, mixed APs, and 30 µg of purified protein. After centrifugation on a sucrose gradient, floated liposomes were collected and bound proteins were analyzed by immunoblot and corresponding antibodies (Figure 7A, lanes 1, 3, and 5). As a comparison, 20% of the starting material was loaded (Figure 7A, lanes 2, 4, and 6). A significant fraction of each protein was recovered on the liposomes, indicating that the BAR domain is correctly folded and

dimerized. For the mutants, Amph1 ΔSH3 (Figure 7, lane 2), Amph2 ΔSH3 (lane 5), Amph1 WWAA (lane 3), and Amph2 WWAA (lane 6), the same experiment was performed, showing that all amphiphysin constructs bind to liposomes in the same range.

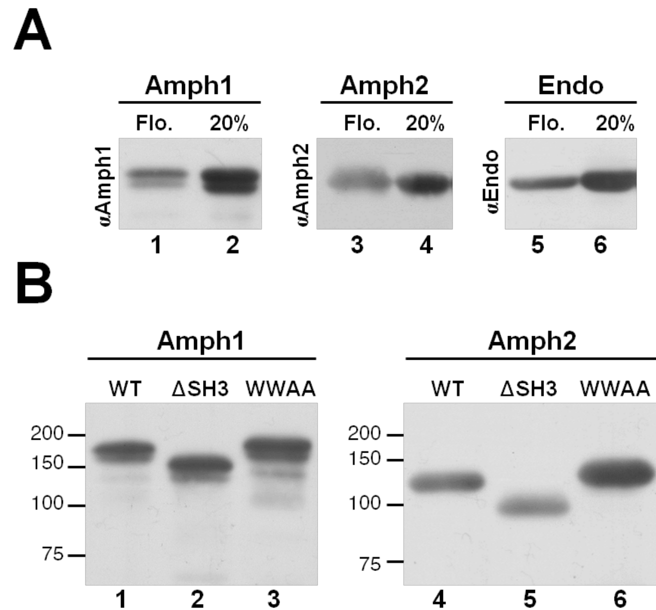

Figure 7: Presence of purified amphiphysin 1, amphiphysin2, and endophilin A1 on floated liposomes.

(**A**) Purified proteins (30 µg) were mixed with liposomes (0.5 µmol) as in an AP-1 binding assay and subjected to floatation gradient centrifugation. The floated liposomes were collected (Flo.) and analyzed by immunoblotting using antibodies against Amph1 (αAmph1), Amph2 (αAmph2) and Endo (αEndo) in comparison to 20% of the input proteins. (**B**) Mutant constructs of Amph1 (Amph1 ΔSH3 and Amph1 WWAA) and Amph2 (Amph2 ΔSH3 and Amph2 WWAA) were tested in the floatation assay as described in (A).

Amphiphysin 2 mediates AP-1 association with liposomal membranes, but not Amphiphysin 1 and Endophilin A1

To test the effect of amphiphysin and endophilin on AP-1 association with liposomal membranes, purified Amph2 was titrated (0.1–50 µg/assay) in the AP-1 floatation assay, revealing a dose-dependent activity above 2 µg/assay (Figure 8A, lanes 1–6).

Amph1 (Figure 8B, lanes 11 and 12) and Endo (lanes 14–17) showed no consistent membrane association of AP-1 even at the highest concentrations. This was somewhat unexpected in the case of Amph1, since it comprises an identical WDLW motif as Amph2, however in a different environment. For Endo, this result is consistent with what was expected, since it has no binding sites for adaptor proteins.

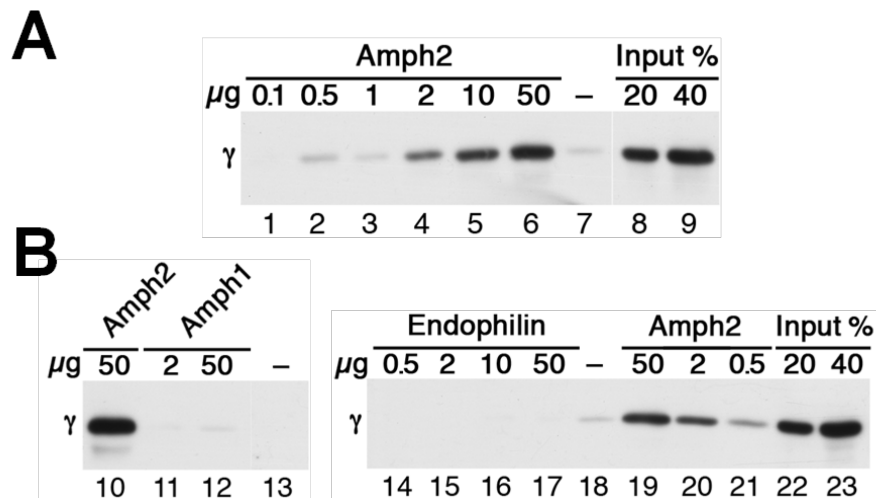

Figure 8: Bacterially produced amphiphysin 2 mediates AP-1 membrane binding.

Indicated amounts of purified wildtype Amph2 (**A**), Amph1, and Endo (**B**) were used in AP-1 floatation assays. The floated liposome fractions as well as 20% or 40% of the input APs were immunoblotted for γ-adaptin of AP-1. The fluorographs of lanes 1–9 derive from the same blot and exposure.

Amphiphysin 2-mediated liposome association of AP-1 depends on the Amphiphysin 2 WDLW motif in its middle domain

In addition to the wildtype proteins, the mutant Amph1 and Amph2 lacking the SH3 domain (ΔSH3) or mutated in the adaptor-binding motif (WWAA) were tested for their contribution to liposome association of AP-1.

Deletion of the SH3 domain had no effect on Amph2 activity and did not activate Amph1 (Figure 9, lanes 5–8). The latter rules out auto-inhibition of adaptor-binding by intramolecular interaction of the SH3 domain with the proline-rich segment in the middle domain as a cause for inactivity of full-length

Amph1, a regulatory mechanism shown to control clathrin binding (Farsad et al., 2003). In contrast, the WW→AA mutation in Amph2 strongly reduced AP-1 association to liposomes (Figure 9, lanes 9 and 10 vs. 1 and 2), confirming that the WDLW motif contributes to AP-1 binding.

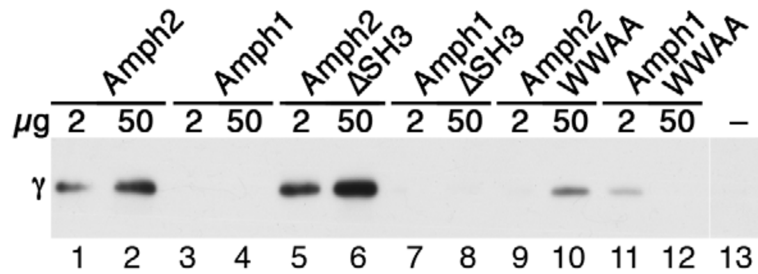

Figure 9: Amphiphysin 2-mediated liposome association of AP-1 depends on its WDLW motif.

Indicated amounts of purified wildtype Amph1, Amph2, and the Amph mutants were used in liposome floatation assays. The floated liposome fractions were immunoblotted for γ-adaptin of AP-1. The fluorograph derives from the same blot and exposure. The faint band in lane 11 was not consistently observed.

Combinations of purified proteins did not enhance AP-1 membrane association

Amphiphysin 1 and Amphiphysin 2 had been shown *in vivo* to exist as heterodimers (Wigge et al., 1997a). The fact that all three proteins were isolated together in our purification and the known interaction of endophilin with the amphiphysins suggests that they might form a functional complex. To test this, the three proteins were mixed in different combinations in the assay. The mixtures did not enhance AP-1 membrane association beyond that expected for the contribution of Amph2 in the mixture (Figure 10, lanes 4 -7). Accordingly, a ternary mixture with the WW→AA mutant of Amph2 showed no activity (lane 8). It is likely that the purified proteins already formed stable homodimers during their expression in bacteria and will not form heterodimers upon mixing.

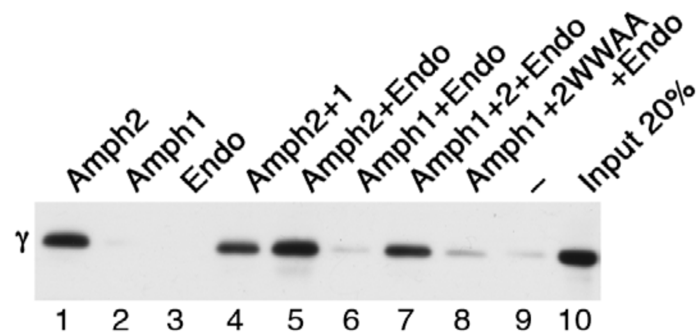

Figure 10: Combinations of purified proteins did not enhance AP-1 membrane association.

10 µg of each protein, individually and in combination, was analyzed in the floatation assay. The floated liposome fractions as well as 20% of the input APs were immunoblotted for γ-adaptin of AP-1. As a negative control (—), assay buffer was used instead of purified proteins.

Amphiphysin 1 and 2 heterodimers mediate AP-1 membrane binding

Immunodepletion of calf brain cytosol using an antibody directed against Amph1 depleted both Amphs, but not Endo (Figure 11A), confirming that essentially all of Amph2 is quite stably associated with Amph1 (Wigge et al., 1997a). Since dimerization via the BAR domain may not be readily reversible, His_6-tagged Amph1 and untagged Amph2 were co-expressed from a dual-expression plasmid in bacteria and purified by Ni-NTA chromatography as described above. Analysis of the purified fraction showed that all Amph2 is in heterodimers with Amph1-His_6 (Figure 11B, lane 1).

This preparation (Amph1/2) was tested in comparison to a mixture of corresponding amounts of separately purified Amph1 and Amph2 (Amph1+2; Figure 11B, lanes 2 and 3). The two samples showed equal AP-1 membrane binding activity as Amph2 alone (Figure 11C), confirming that the heterodimeric Amph1/2 is active for AP-1 association to liposomes at a level comparable to Amph2 homodimers.

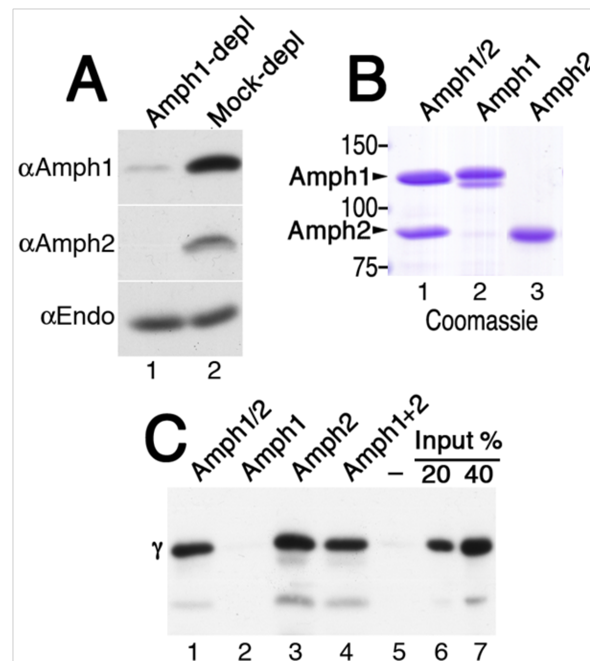

Figure 11: Amph1physin 1 and 2 heterodimers mediate AP-1 membrane binding.

(A) Calf brain cytosol immunodepleted for Amph1 and mock-depleted cytosol were subjected to SDS-gel electrophoresis and immunoblotting for Amph1, Amph2 and endophilin. Co-depletion of amphiphysin 1 and 2 indicates hetero-oligomerization. (**B**) Amph1 and Amph2 were co-expressed and co-purified. The resulting preparation containing Amph2 in complex with Amph1 was analyzed by SDS-gel electrophoresis and Coomassie staining (lane 1) with corresponding amounts of pure Amph1 (lane 2) and Amph2 (lane 3) produced separately. (**C**) The preparation containing Amph1/2 heterodimers, and matching amounts of Amph1 and Amph2 (as shown in panel B), as well as a mixture of the latter two (Amph1+2) were analyzed for AP-1 binding to liposomes. The floated liposome fractions as well as 20% or 40% of the input APs were immunoblotted for γ-adaptin.

Amphiphysin 2 also mediates binding of AP-2 to liposomal membranes

Since the amphiphysins are known interactors of AP-2, their ability to stabilize AP-2 on the liposomes was also tested by immunoblotting for α-adaptin in the floated fraction. Amph2 efficiently mediated AP-2 association with the liposomes (Figure 12, lane 1). Upon mutation of the WDLW motif, this was reduced to a lower level of activity that was also observed with Amph1 and Amph1WWAA (lanes 2–4). This basal WDLW-independent activity is most likely due to the AP-2 interaction sequences further upstream in the CLAP domain that have been identified by pull-down experiments using GST-fusion proteins (Ramjaun and McPherson, 1998; Slepnev et al., 2000) and that are not functional for AP-1.

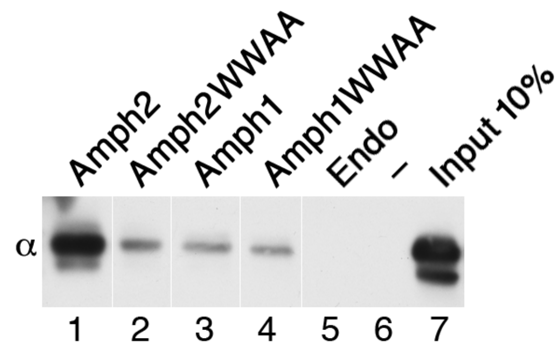

Figure 12: Amphiphysin 2 mediates AP-2 binding to liposomes.

10 µg of purified wildtype Amph1, Amph2, Endo, and the Amph mutants were analyzed in the floatation assay. The floated liposome fractions as well as 10% of the input APs were immunoblotted for α-adaptin of AP-2. The lanes of the fluorograph derive from the same blot and exposure.

Amphiphysin 1 middle domain M1 is not functional

It is surprising that Amph1 did not mediate AP-1 binding to membranes, even though it was functional in binding clathrin and AP-2 in GST-fusion pull-down experiments (Drake and Traub, 2001; Slepnev et al., 2000) and contains a WDLW motif as is necessary for Amph2's activity. We tested whether the additional middle domain sequence M1', which has no counterpart in Amph2, is responsible for this by deleting it in Amph1ΔM1'. Furthermore, we exchanged the middle domains M1 and M2 between Amph1 and Amph2 (schematically shown in Figure 13A).

Bacterially expressed and purified chimera proteins were tested for mediating AP-1 membrane binding (Figure 13B). Amph1 M1 was found to be inactive even in the context of the BAR and SH3 domains of Amph2 (lane 6). The functional middle domain M2 replacing M1 in Amph1 produced very little activity (lane 4), unless the repeat sequence M1' was deleted in addition (lane 5). Amph1 lacking M1' remained inactive (lane 3). These results indicate that the WDLW motif alone is not sufficient for AP-1 stabilization on the membrane and that the sequence context within the middle domain is important for the productive presentation of the interaction sequences.

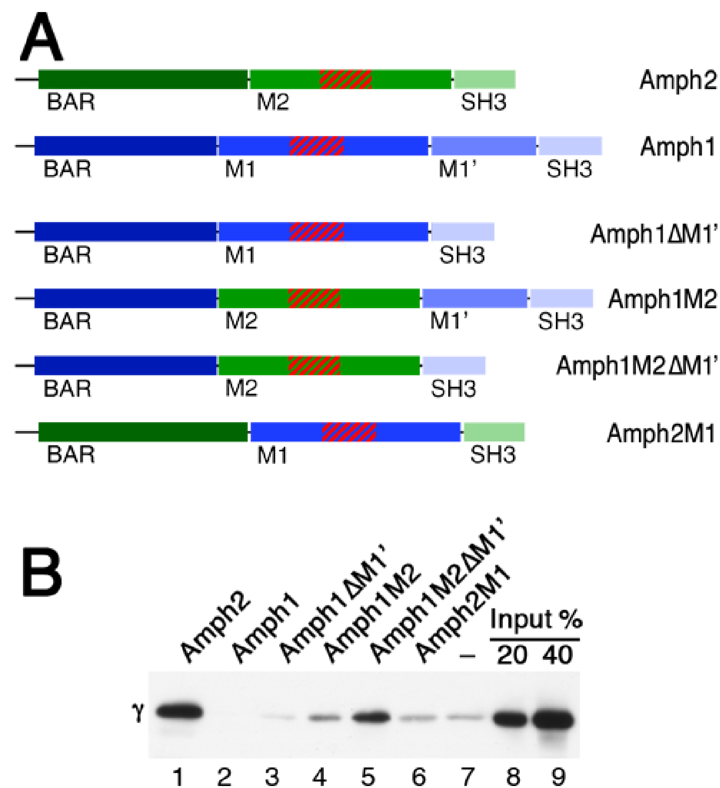

Figure 13: The middle domain M1 of Amphiphysin 1 is not functional even in the context of Amphiphysin 2.

(**A**) Wildtype and mutant Amphs in which the middle segments were deleted or exchanged (see Figure 4 for legend) were purified and 20 µg of each were analyzed for AP-1 binding to liposomes (**B**). The floated liposome fractions as well as 20% and 40% of the input APs were immunoblotted for γ-adaptin.

Amphiphysin 1 co-localizes with AP-1 at the TGN in primary neurons

To assess a participation of amphiphysin in AP-1/clathrin coats *in vivo*, primary cultures of mouse cerebellar granule cells were fixed after 8 days of proliferation and subjected to immunofluorescence staining of endogenous Amph1 and AP-1 (Figure 14). Amph1 was found in neurites, but also co-localized with AP-1 in its typical perinuclear accumulation corresponding to the position of the TGN.

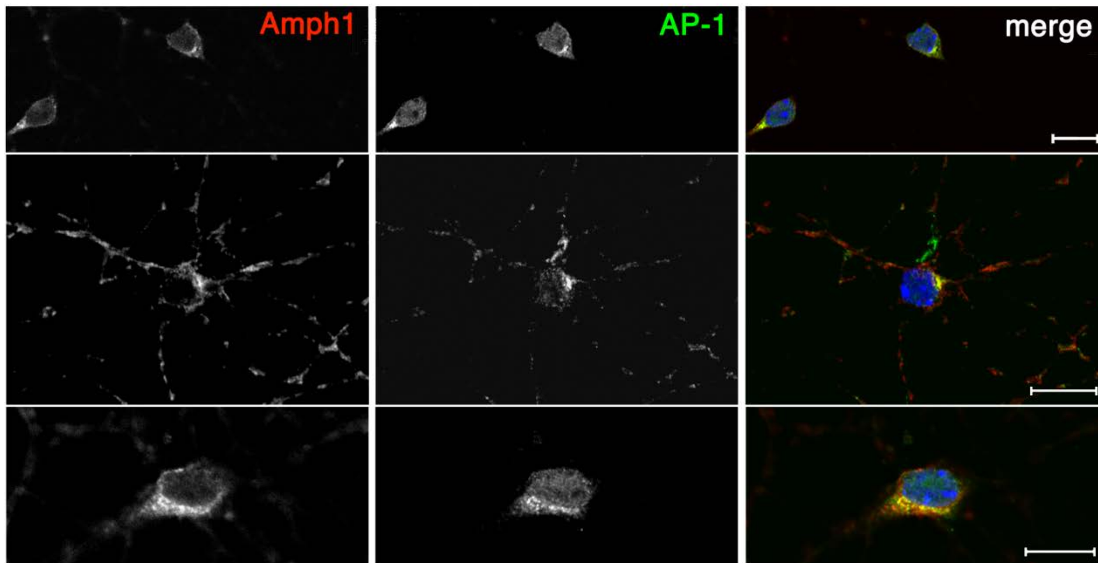

Figure 14: Amphiphysin 1 co-localizes with AP-1 at the TGN of primary cerebellar granule cells.

Primary cerebellar granule cells were stained for endogenous Amph1 and AP-1, which co-localized at the TGN. Nuclei were stained with Draq5 (blue). Bars, 10 µm.

Amphiphysin 1 and 2 co-transfect and co-localize with AP-1 at the TGN in transfected neuronal cells

In the neuronal HN10 cell line, the endogenous amphiphysin levels are too low to be detectable by immunofluorescence with available antibodies. For this reason, Amph1 and Amph2 tagged with myc and FLAG epitope tags, respectively, were expressed in HN10 cells and detected with a myc and FLAG antibody. Amph1-myc and Amph2-FLAG co-transfected in essentially all cells and were localizing to the cell surface as well as to the perinuclear region (Figure 15A). When Amph2 and endogenous AP-1 were stained, they co-localized in the perinuclear region where normally the TGN is localized (Figure 15B). Expressed endophilin, in contrast, did not localize to the TGN (Figure 15C).

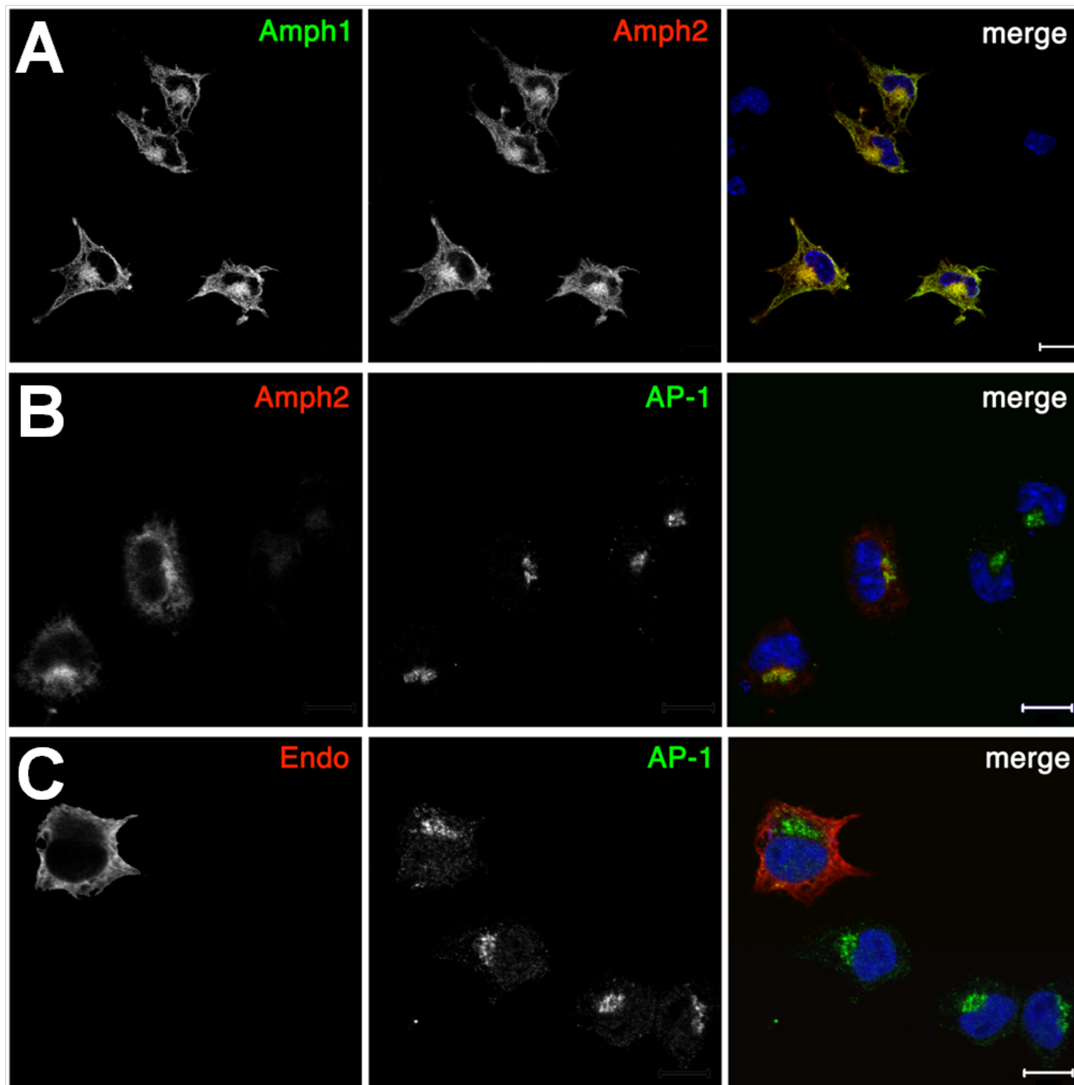

Figure 15: Amphiphysin 1 and 2 co-transfect and co-localize with AP-1 at the TGN in transfected neuronal cells.

HN10 cells were co-transfected with myc-tagged Amph1 and FLAG-tagged Amph2 and stained for Amph1-myc and Amph2-FLAG (**A**), or for Amph2-FLAG and AP-1 (**B**). In essentially all cells, both Amphs were expressed and co-localized in the perinuclear region together with AP-1. In contrast, transfected Endo-FLAG did not localize to the TGN (**C**). Bars, 10 µm. Nuclei were stained with Draq5 (blue).

Amphiphysin 1 and Amphiphysin 2 homodimers co-localize with AP-1 at the TGN region

To check the localization of Amph1 and Amph2 homodimers, Amph1 and Amph2, both tagged with a FLAG tag, were separately transfected into HN10 cells and attained with anti-FLAG and anti-AP-1. Both Amph1 (Figure 16A) and Amph2 (Figure 16B) were still found in the TGN region with AP-1, similar to the Amph1/2 double-transfected HN10 cells.

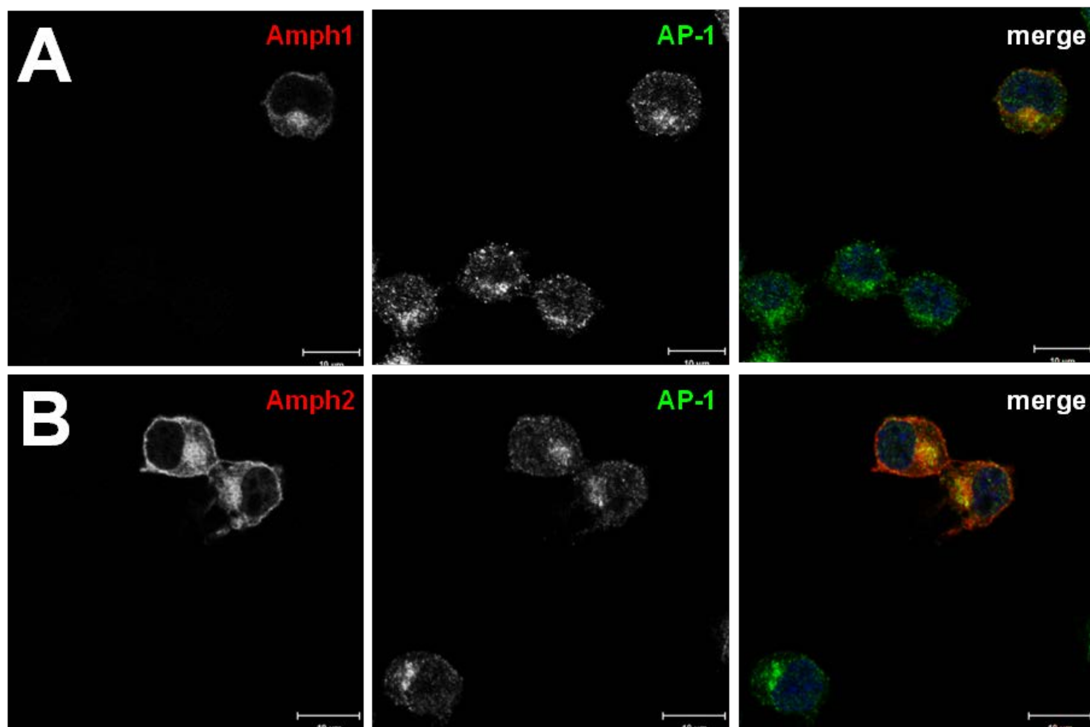

Figure 16: Amphiphysin 1 and Amphiphysin 2 homodimers localize to the TGN with AP-1.

HN10 cells were transfected with Amph1-FLAG or Amph2-FLAG and stained with anti-FLAG and anti-AP-1. As in cells expressing heterodimers, Amph1 (**A**) and Amph2 (**B**) homodimers localized in the perinuclear region with AP-1. Bars, 10 µm. Nuclei were stained with Draq5 (blue).

TGN localization of amphiphysins depends on the CLAP domain

Different mutants of amphiphysin were expressed in HN10 cells to check their localization. When Amph1 and Amph2 constructs lacking the CLAP domains (Amph ΔCLAP) were expressed, their perinuclear concentration and co-localization with AP-1 were lost (Figure 17A), demonstrating that TGN localization is due to the sequences involved in adaptor and clathrin binding. For Amph1 and Amph2 lacking the SH3 domain (Figure 17B), the localization was the same as for wildtype amphiphysin, indicating that amphiphysin recruitment does not depend on dynamin. In Amph1 and Amph2 where only the PWDLW motif was mutated (Figure 17C), the localization to the TGN was still observed, even though the staining appeared generally weaker and more diffuse than for the wildtype. This leads to the assumption that the PWDLW motif in amphiphysin is not the only motif for interaction with AP-1/clathrin coats.

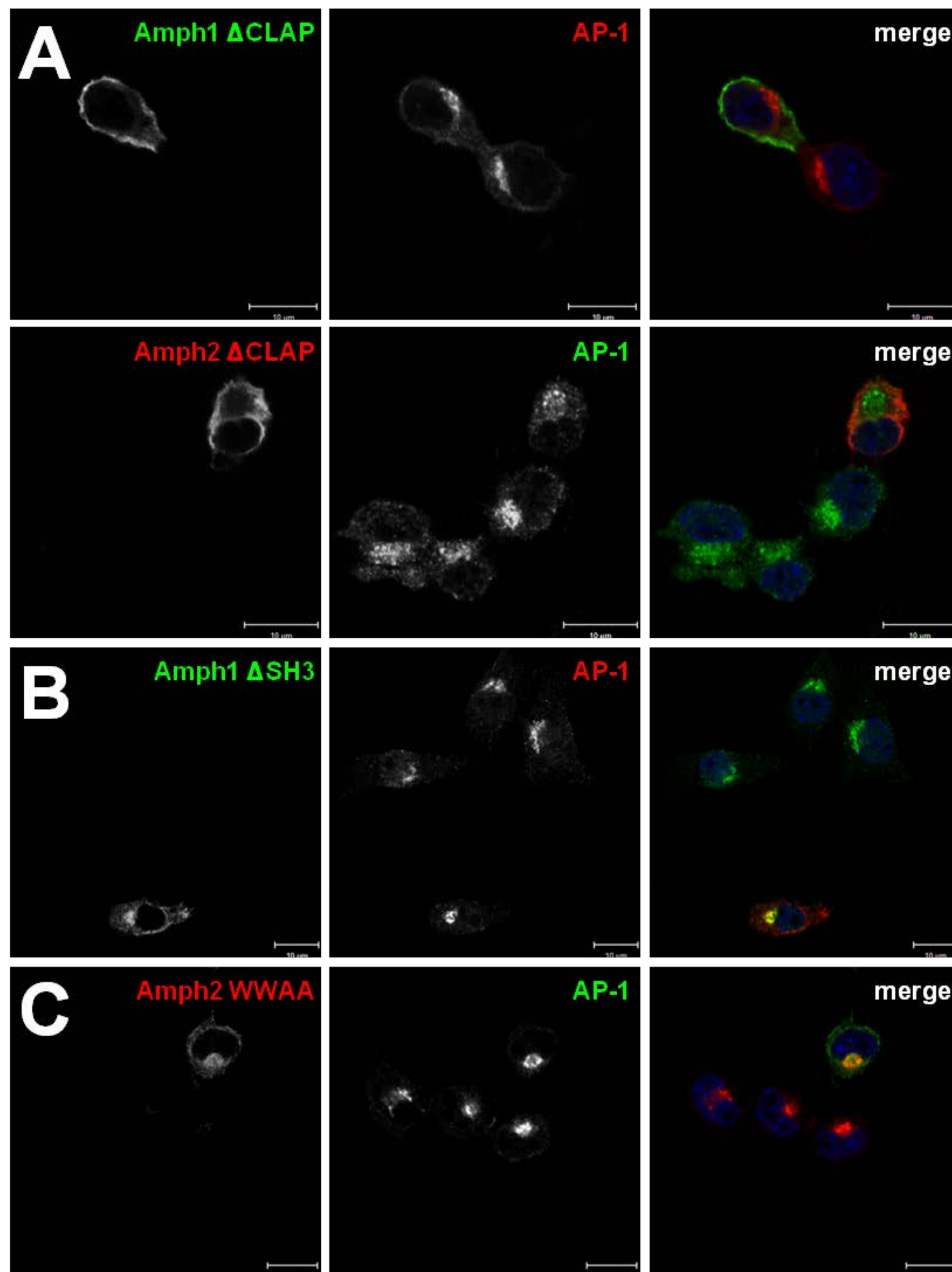

Figure 17: TGN localization of amphiphysin depends on the CLAP domain.

HN10 cells were co-transfected with Amph1 and Amph2 ΔCLAP, Amph1 and Amph2 ΔSH3, or Amph1 and Amph2 WWAA and stained with anti-FLAG or anti-myc and anti-AP-1. In cells expressing Amph1 and Amph2 ΔSH3 (**B**) and Amph1 and Amph2 WWAA (**C**), amphiphysin still localized to the TGN with AP-1, which was not the case for Amph1 and Amph2 ΔCLAP (**A**). Bars, 10 µm. Nuclei were stained with Draq5 (blue).

Amphiphysin localizes with clathrin and dynamin at the TGN

In cells co-expressing Amph1-myc and Amph2-FLAG and stained with anti-myc and anti-clathrin (Figure 18A) or anti-dynamin (Figure 18B), Amph1 and Amphi2 were found in the TGN region where also clathrin and dynamin were localizing. Furthermore, dynamin showed a strong punctate plasma membrane staining, that did not overlap with amphiphysins.

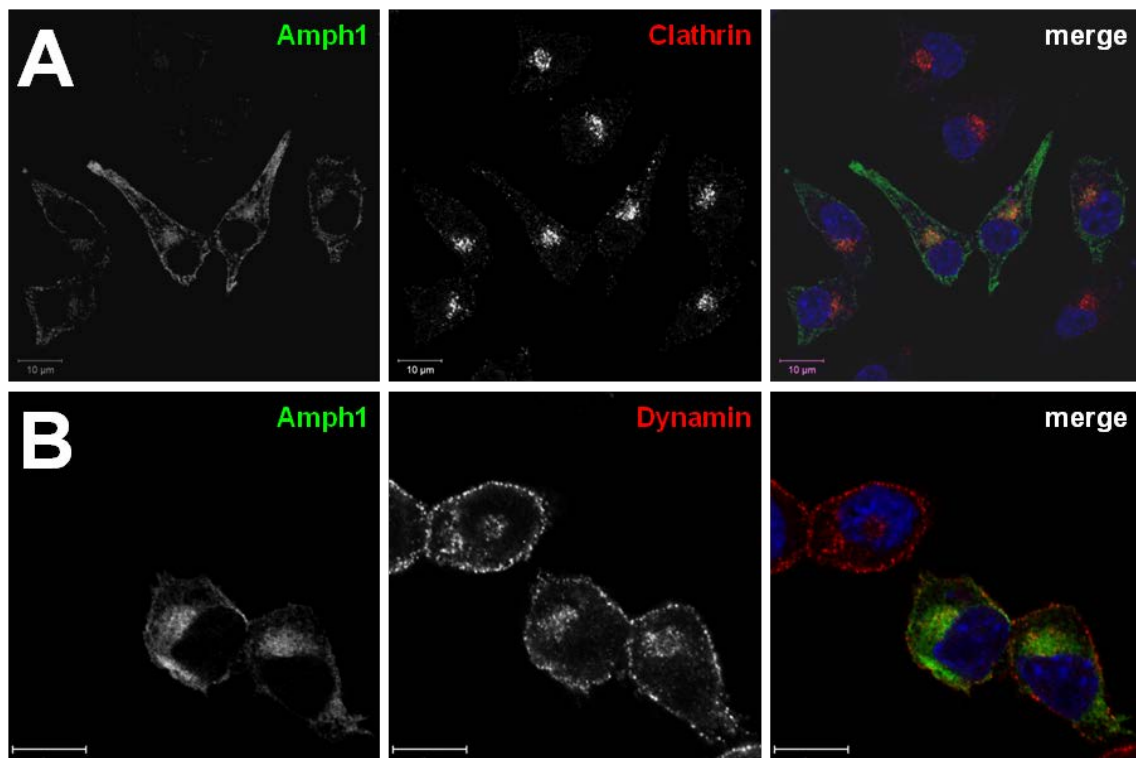

Figure 18: Amphiphysin co-localizes with dynamin and clathrin at the TGN.

HN10 cells were co-transfected with Amph1-myc and Amph2-FLAG and stained with anti-myc and anti-clathrin (**A**) or anti-dynamin (**B**). Amph1-myc and Amph2-FLAG co-localized in the TGN region with clathrin as well as with dynamin. Bars, 10 µm. Nuclei were stained with Draq5 (blue).

High expression levels of amphiphysin lead to its aggregation and interfere with TGN localization of AP-1

In cells with high Amph1/2 expression, no specific intracellular localization could be distinguished unless the bulk of cytosolic protein was released by permeabilization of the plasma membrane with digitonin before fixation and staining. In these cells, Amph1 and Amph2 were not concentrated at the Golgi, but aggregated in punctate structures (Figure 19, left panel) that did not coincide with giantin as a Golgi marker (Figure 19D), nor with endosomes or lysosomes (not shown). Amphiphysin aggregation has previously been reported in over-expressing cells (Farsad et al., 2003). Interestingly, the perinuclear localization of AP-1 at the TGN was drastically reduced (Figure 19A, middle panel), suggesting a dominant effect of high Amph1/2 concentrations on AP-1/clathrin coats at the TGN. This mis-localization of AP-1 was not observed in cells with Amph1/2 ΔCLAP aggregates (Figure 19B). Highly expressed endophilin also aggregated in cytoplasmic punctae, but did not affect the TGN localization of AP-1 (Figure 19C).

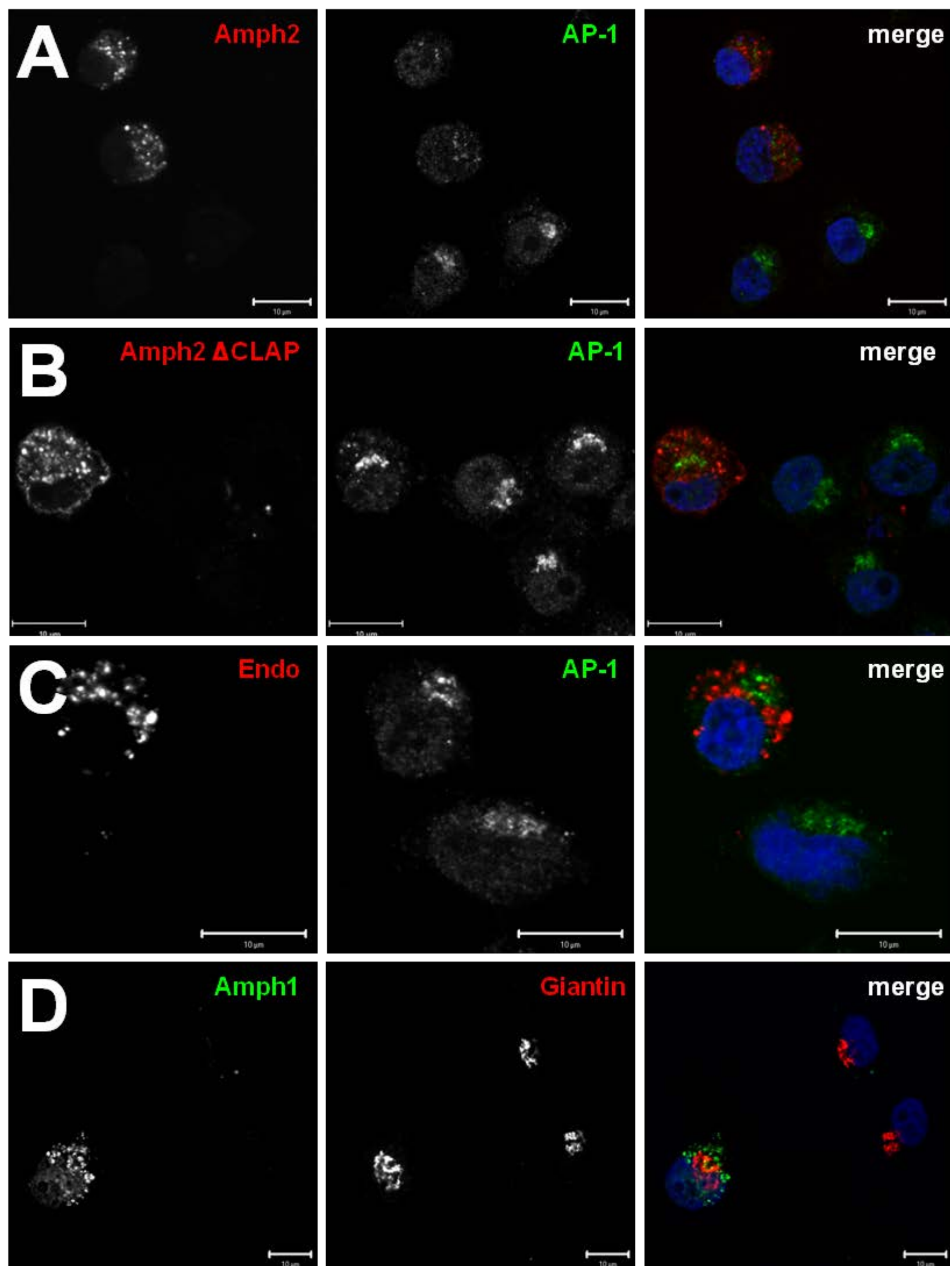

Figure 19: High expression levels of amphiphysin lead to aggregate formation and interfere with TGN localization of AP-1.

Cells expressing Amph1/2 were permeabilized with 0.04% digitonin prior to fixation, revealing Amph1/2 aggregations in highly over-expressing cells. These cells showed neither Amph1/2 nor AP-1 staining at the TGN (**A**), while Golgi staining for giantin was still normal (**D**). Highly expressing cells that produced aggregates of Amph1/2 ΔCLAP showed normal AP-1 staining at the TGN (**B**). Transfected Endo-FLAG also formed aggregates but did not disturb AP-1 distribution (**C**). Bars, 10 µm. Nuclei were stained with Draq5 (blue).

When 50 cells expressing Amph1/2, Amph1/2 ΔSH3, Amph1/2 ΔCLAP, or Amph1/2 WWAA were analyzed for their AP-1 localization (Figure 20), in over two third of the cells expressing wildtype and Amph1/2 ΔSH3, AP-1 disappeared from the TGN region. In Amph1/2 ΔCLAP expressing cells, almost all cells showed normal AP-1 localization, while in Amph1/2 WWAA, a slightly higher amount of cells showed aberrant AP-1 staining in comparison to Amph1/2 ΔCLAP expressing cells.

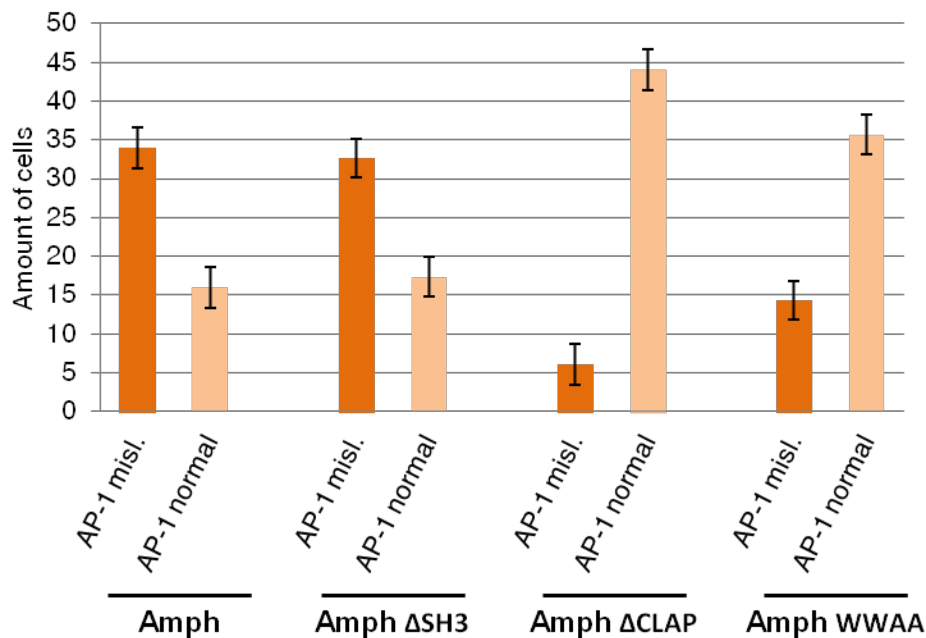

Figure 20: AP-1 mis-localization is specific for wildtype amphiphysin aggregates.

In three independent experiments, a number of fifty cells expressing wildtype Amph1/2, Amph1/2 ΔSH3, Amph1/2 ΔCLAP, or Amph1/2 WWAA were analyzed for their AP-1 localization.

Amphiphysin localizes to the TGN in an ARF1-dependent manner

Upon expression of wildtype Amph1 and Amph2 or Amph1 and Amph2 ΔCLAP in non-neuronal COS-1 cells, the same phenotypes as in HN10 cells were observed (Figure 21, A and B). Furthermore, treatment of cells with BFA, an inhibitor of guanine nucleotide exchange factors BIG1 and BIG2 of ARF1 that blocks the recruitment of AP-1 to the membrane (Ishizaki et al., 2008), caused both AP-1 and Amph2 to

dissociate from the TGN (Figure 21C). In contrast to this, the localization of TGN46, a transmembrane marker of this organelle, was unchanged (Figure 21D). These observations strongly support the notion that amphiphysins are localized to the TGN by interaction with AP-1/clathrin coat components.

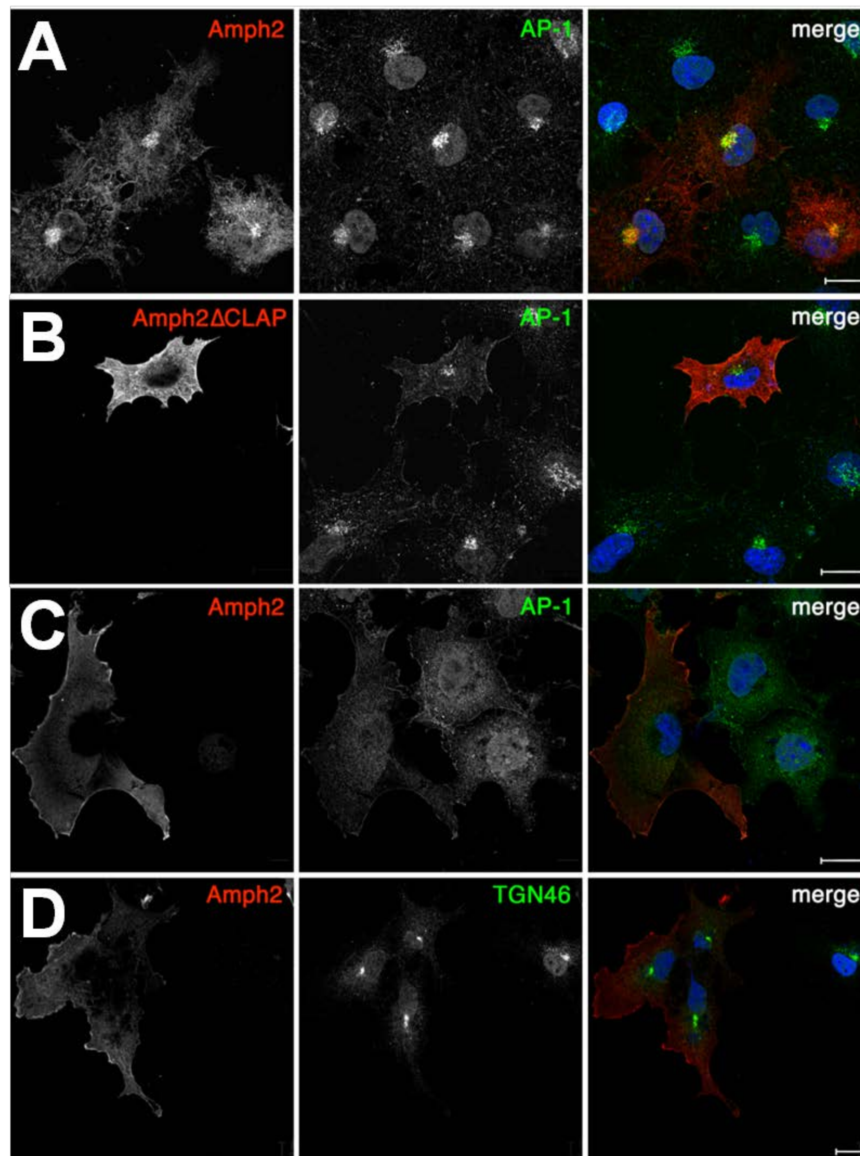

Figure 21: Amphiphysins and AP-1 disappear from the TGN in BFA-treated COS-1 cells.

Myc-tagged Amph1 and FLAG-tagged Amph2, wildtype (**A**) and ΔCLAP (**B**) were co-expressed in COS-1 cells and stained for FLAG and for endogenous AP-1. Furthermore, cells expressing wildtype myc-tagged Amph1 and FLAG-tagged Amph2 were incubated with 5 µg/ml BFA (**C** and **D**), which prevents ARF1-dependent recruitment of AP-1 to the TGN, before fixation and staining for Amph2 and AP-1 (**C**) or TGN-46 (**D**). Bars, 20 µm. Nuclei were stained with DAPI (blue).

Amphiphysin 1 and 2 can be cross-linked to AP-1 *in vivo*

To test a physical interaction between AP-1 and amphiphysin in neuronal cells, co-immunoprecipitation experiments were performed either using Amph1/2 transfected PC12 cells or freshly isolated primary cultures of cerebellar granule cells. Since amphiphysin and AP-1 interaction during vesicle formation may be very short-lived and only engage a small amount of amphiphysin present in the cell, a chemical crosslinker was used to capture transiently interacting partner proteins. Amph1/2 could not be detected with immunoprecipitated AP-1 without crosslinking, neither in transfected cells (Figure 22A, lane 2) nor in endogenously expressing neurons (Figure 22B, lane 5). However, when the intact cells were incubated with the bi-functional membrane-permeable crosslinker DSP before lysis and immunoprecipitation with anti-AP-1, both Amph1 and Amph2 were co-isolated (lanes 4 and 7). The interaction was specific, since no signal was obtained using an antibody against the unrelated Golgi protein giantin (lanes 3 and 6). This finding confirms the notion that in cells expressing amphiphysin 1 and 2 they are recruited to nascent AP-1/clathrin coats at the TGN.

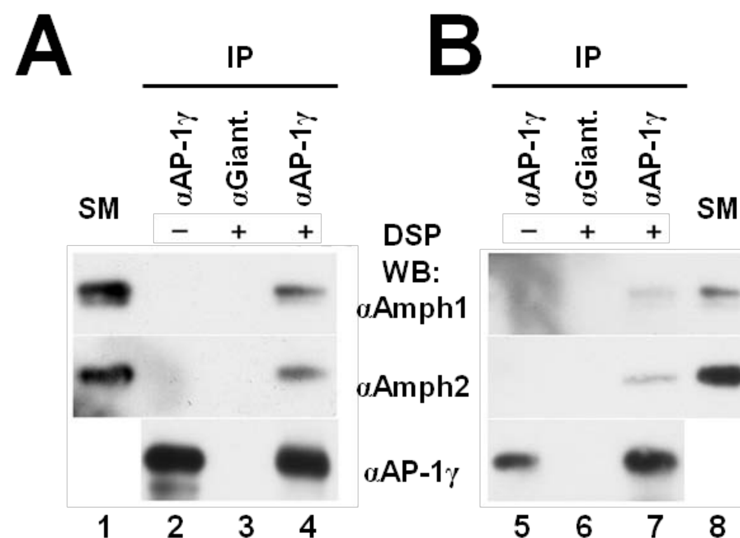

Figure 22: AP-1 and amphiphysin interact *in vivo*.

Amphi1- and Amphi2-transfected PC12 cells (**A**) and cerebellar granule cells (**B**) were treated with or without 2 mM DSP to crosslink interacting proteins. AP-1 or giantin were immunoprecipitated and samples were blotted for AP-1γ, Amph1, and Amph2. For comparison, an aliquot of the starting material (SM; 1%) was analyzed in parallel.

Amphiphysin and AP-1 interact independently of clathrin

To elucidate the effect of clathrin on the interaction of AP-1 and amphiphysin, clathrin was efficiently knocked down by siRNA in Amph1-myc and Amph2-FLAG transfected NIH3T3 cells (Figure 23A, lane 1 vs. 2) and co-immunoprecipitations using anti-AP-1 were performed. Both Amph1 and Amph2 could be co-isolated upon treatment with DSP cross-linker, however, the efficiency was the same whether clathrin was present or not (Figure 23B, lane 2 and 4).

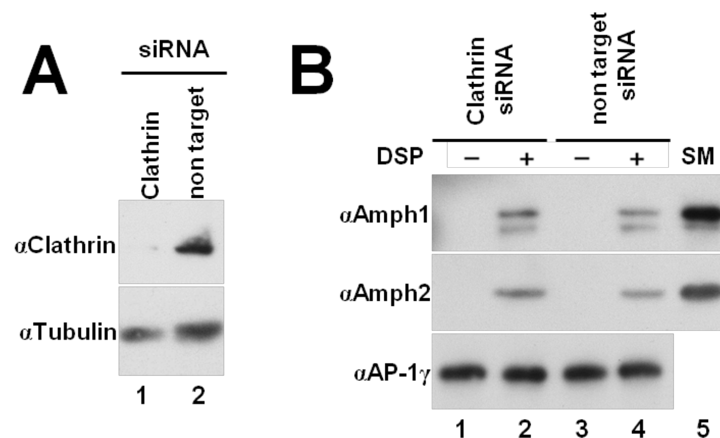

Figure 23: Cross-linking of AP-1 and amphiphysin is not influenced by clathrin.

(**A**) NIH3T3 cells were transfected with clathrin siRNA or non-targeting siRNA, harvested, and analyzed for the presence of clathrin. Anti-tubulin was used as a loading control in parallel. The fluorographs derive from the same blot and exposure. (**B**) Amphi1-myc and Amphi2-FLAG-transfected NIH3T3 cells which were clathrin siRNA- or non-targeting siRNA-treated were incubated with or without 2 mM DSP to crosslink interacting proteins. AP-1 was immunoprecipitated and samples were blotted for AP-1γ, Amph1, and Amph2. For comparison, an aliquot of the starting material (SM; 1%) was analyzed in parallel.

Another approach to test the influence of clathrin is to block the interaction of clathrin with amphiphysin by the addition of pitstop 2. This small molecule was shown to inhibit endocytosis of transferrin and EGF through its association with the clathrin terminal domain, competing with clathrin box ligands such as amphiphysin (von Kleist et al., 2011). In Cos-1 cells, the effect of pitstop 2 was demonstrated by monitoring the uptake of transferrin, which was blocked efficiently (Figure 24A vs. B). In Amphi1-myc and Amph2-FLAG transfected cells treated with pitstop 2, amphiphysin and AP-1 were still found in the

perinuclear region (Figure 24D), as was the case in untreated cells (Figure 24C). Taken together, these results show that the interaction of AP-1 and amphiphysin is not affected by clathrin.

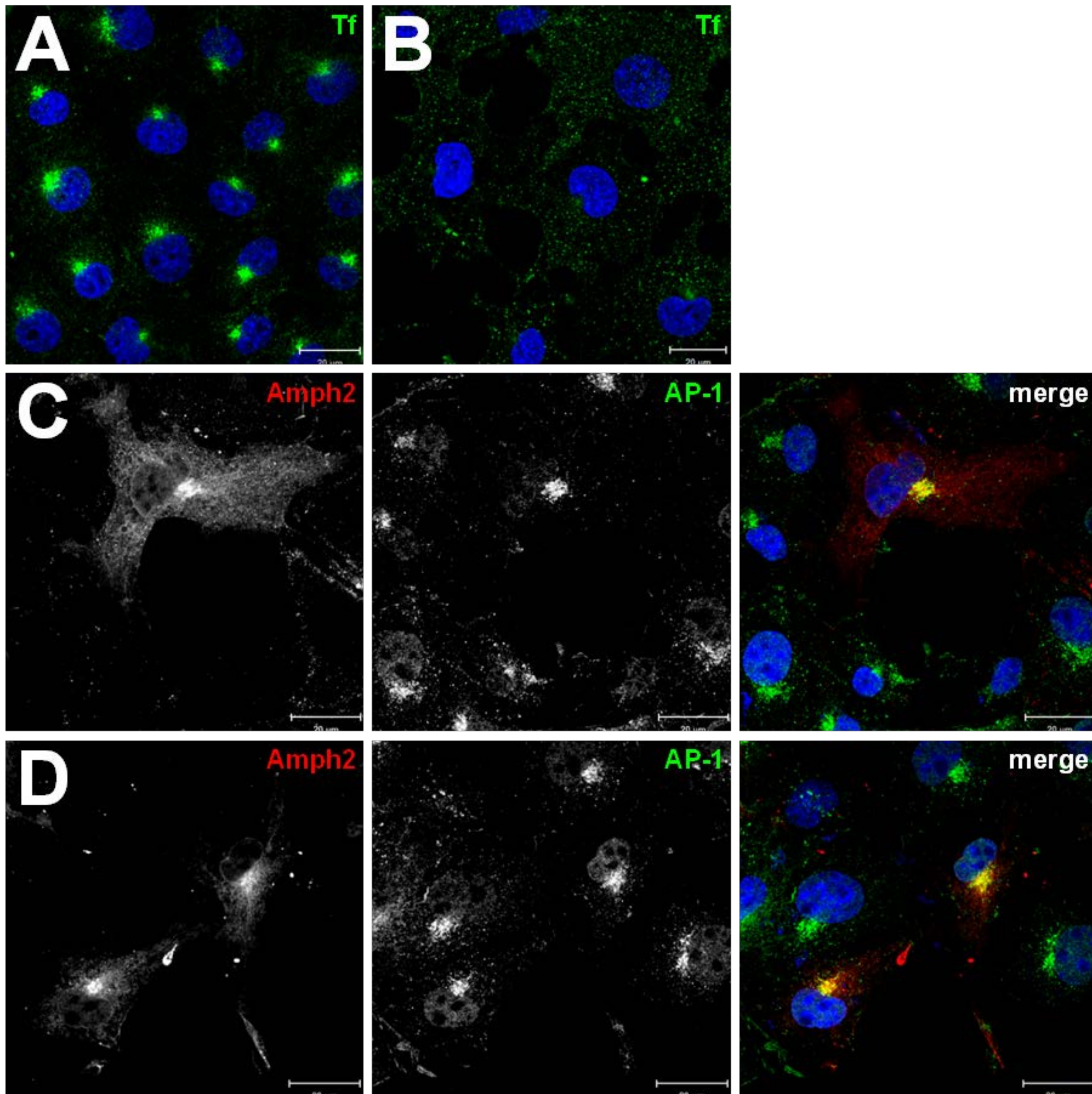

Figure 24: AP-1 and amphiphysin localize to the TGN in pitstop-treated cells.

Cos-1 cells were treated with 20 μM pitstop 2 in DMSO (**B**) or with DMSO only (**A**) for 15 min, before fluorescent transferrin was allowed to internalize for 45 min to monitor clathrin-mediated endocytosis. Cos-1 cells were transfected with Amphi1-myc and Amphi2-FLAG, treated with (**D**) or without (**C**) pitstop 2, and immunostained with anti-FLAG and anti-AP-1 to check co-localization. Bars, 20 μm. Nuclei were stained with DAPI (blue).

Amphiphysin is not essential for AP-1 localization to the TGN

Amphiphysin is a brain-specific protein and not essential for the formation of CCVs at the plasma membrane. In order to test the effect of amphiphysin depletion on AP-1 localization in neuronal cells, RNAi of amphiphysin in PC12 cells was performed. Amph1 knockdown (Figure 25A, lane 1) was not complete, however, the expression was strongly reduced when compared to cells transfected with non-targeting siRNA (lane 2). When AP-1 localization in untreated cells (Figure 25B) and cells treated with non-targeting siRNA (Figure 25D) was compared with Amph1 siRNA-treated cells (Figure 25C), no difference in AP-1 localization was observed. This result demonstrates that other factors besides amphiphysins are involved in targeting and stabilizing AP-1 on TGN membranes.

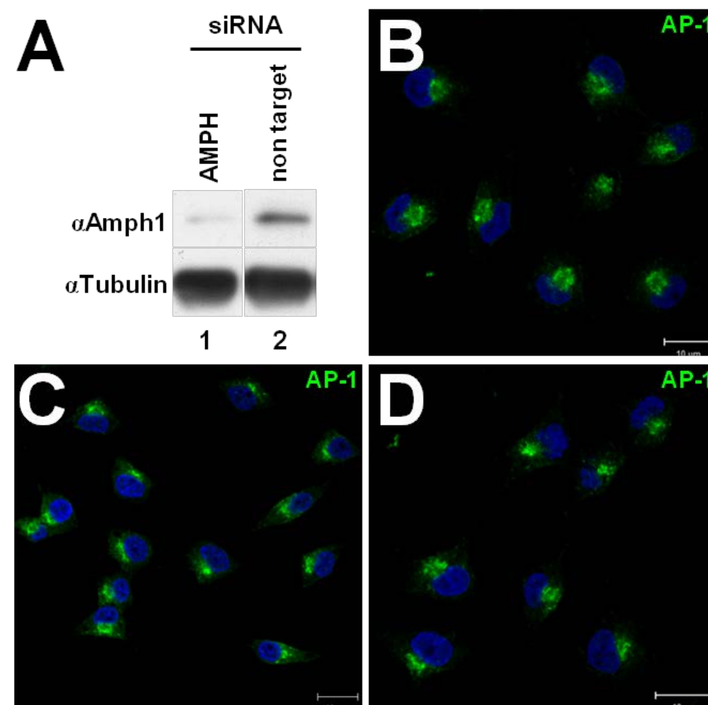

Figure 25: AP-1 still localizes to the TGN in cells with strongly reduced amphiphysin expression.

(**A**) PC12 cells were transfected with Amph1 siRNA (lane 1) or non-targeting siRNA (lane 2) and lysates were blotted for Amph1 and Amph2. Amph1 expression was strongly reduced in Amph1 siRNA-treated cells, while Amph2 was not detectable under both conditions. As a loading control, samples were analyzed with anti-tubulin in parallel. The fluorographs derive from the same blot and exposure. For immunofluorescence analysis, untreated PC12 cells (**B**) as well as transfected with Amph1 siRNA (**C**) and non-targeting siRNA (**D**) were fixed and immunostained with anti-AP-1 to check its TGN localization. Bars, 10 µm. Nuclei were stained with DAPI (blue).

Amphiphysins are not the only proteins stabilizing AP-1 on liposomal membranes

Amphiphysins are neuron-specific and not essential for AP-1 localization to the TGN (see Figure 25) leaving the possibility that there are other proteins with similar properties. Therefore, we tested Amph1- and Amph2-depleted brain cytosol (Figure 11A), mock depleted cytosol, and untreated cytosol in the floatation assay for AP-1 association with liposomes (Figure 26A). Amph1- and Amph2-depleted cytosol showed no consistent reduction of membrane-bound AP-1 (lane 1) in comparison to mock-depleted and untreated cytosol (lane 2 and 4). When cytosol of the non-neuronal HeLa (Figure 26B, lane 5) and Cos-1 (lane 6) cell lines were tested, their activity was similar to that of calf brain cytosol (lane 8). This leads to the conclusion that there are additional proteins that can stabilize AP-1 on liposomes and possibly also on the TGN and that amphiphysin is, as with AP-2/clathrin coats at the plasma membrane, only one component in a complex interaction network.

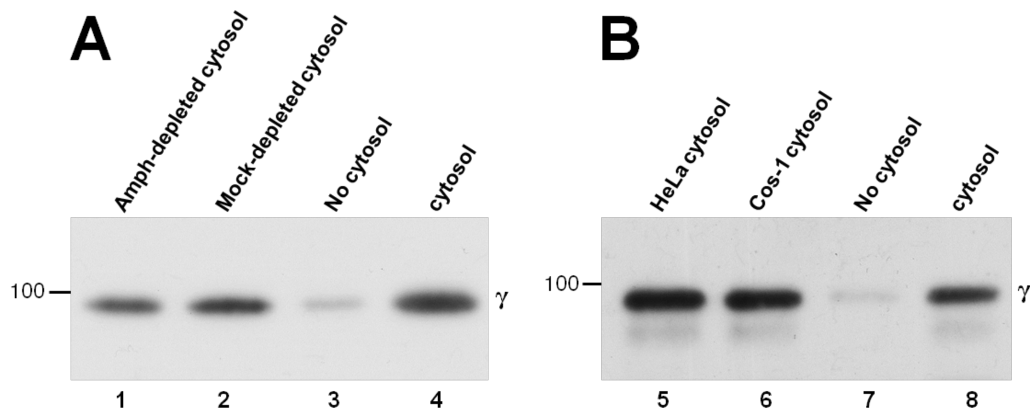

Figure 26: No reduced liposome binding of AP-1 derived from amphiphysin-depleted cytosol and non-neuronal cell cytosol.

200 μg of calf brain cytosol immunodepleted for Amph1 and Amph2 and mock-depleted cytosol (**A**) as well as 200 μg of HeLa and Cos-1 cytosol (**B**) were tested in the floatation assay and analyzed by immunoblotting using antibodies against AP-1 γ. As positive controls, 200 μg of untreated calf brain cytosol was used.

Discussion

In vitro reconstitution helps to define minimal mechanisms of physiological processes. In this manner it was shown that multiple interactions are required to recruit AP-1 adaptors to a liposomal membrane: interactions of AP-1 with specific lipids, active membrane-bound ARF1-GTP, and membrane-anchored cargo peptides (Crottet et al., 2002; Meyer et al., 2005; Zhu et al., 1999a). Cargo binding induces AP-1 to oligomerize and to enhance its association with ARF1-GTP (Lee et al., 2008a; Lee et al., 2008b; Meyer et al., 2005) producing a scaffold with multivalent membrane attachment. The observation that cytosolic proteins contribute to AP-1 association with liposomes in the absence of cargo in a lipid- and ARF1-dependent manner indicated the existence of additional factors that stabilize AP-1 on the membrane (Meyer et al., 2005; Zhu et al., 1999a). Purification of this activity from brain cytosol now yielded a fraction containing amphiphysin 1, amphiphysin 2, and endophilin A1. Using bacterially expressed and purified proteins, it is shown here that amphiphysin 2 – both alone and as a heterodimer with amphiphysin 1 – is responsible for this *in vitro* activity and mediates AP-1 stabilization on liposomal membranes.

Amphiphysins act at the plasma membrane in endocytosis

All three proteins are known to play a role in clathrin coat formation with AP-2 at the plasma membrane for endocytosis. The connectivity view of endocytic vesicle formation suggests the dynamic progression of a complex network of proteins which all interact with several binding partners. It was accepted that coat formation is initiated by FCHo1/2 (Henne et al., 2010), matures to an AP-2-centered network mediating cargo selection, then to a clathrin-dominated one at the time of coat assembly, and finally to one dominated by dynamin to bring about vesicle fission (McMahon and Boucrot, 2011; Schmid and McMahon, 2007). This model is supported by comparing CCV recruitment traces of different proteins using live-cell TIRF imaging, where FCHo1/2 was one of the first proteins at the site of CCV formation and its peak intensity was decreasing before dynamin-mediated scission of the vesicle (Taylor et al., 2011). In line with this is also the fact that FCHo1/2 has a F-BAR domain with a shallow concave face which can bind to relatively flat membranes (Henne et al., 2007). However, a recent study suggested that the coordinated arrival of AP-2 and clathrin is initiating pit formation, while FCHo1/2 is necessary for coat

assembly in a later stage (Cocucci et al., 2012). These contradicting results reveal the difficulty of defining the order of recruitment events and protein interactions in such a complex network. In any case, BAR domain proteins are involved during the entire process, shaping the membrane to increasing membrane curvature. The amphiphysins and endophilin appear to act in the late steps of AP-2/CCV formation. Analysis of the recruitment dynamics of fluorescent protein-tagged endocytic proteins showed the appearance of the amphiphysins and endophilin shortly before membrane fission after AP-2 and clathrin have assembled (Taylor et al., 2011). However, it is not known whether amphiphysins are recruited to the site of vesicle formation by the highly curved membrane itself or through interactions with coat proteins such as clathrin or AP-2.

At the membrane, they interact with dynamin via their SH3 domain, activating its GTPase for vesicle fission (David et al., 1996; Takei et al., 1999) and with the lipid phosphatase synaptojanin in preparation for uncoating (McPherson et al., 1996). Their N-BAR domains are highly curved, matching the highest membrane curvatures at deeply invaginated pits and the narrow neck before fission (Qualmann et al., 2011). It was shown that purified amphiphysin BAR domains were able to tubulate membranes *in vitro*, indicating that amphiphysin could also be involved in curvature generation (Takei et al., 1999). Interestingly, recent studies revealed another role for BAR domain proteins to not only promote membrane curvature, but also directly regulate fission. Theoretical analysis of biochemical models predicted that insertion of domains such as amphipathic α-helices into the membrane promotes membrane fission, whereas crescent-shaped BAR domains prevent membrane scission due to their scaffolding features (Boucrot et al., 2012). Indeed, over-expression of an endophilin A3 construct with a double N-terminal amphipathic α-helix generated more internal vesicles and less tubules in comparison to wildtype, while a construct where the α-helix was deleted produced mainly tubules and hardly any vesicles. Thus, proteins containing both amphipathic α-helices and BAR domains seem to fine-tune the balance between neck stabilization and vesicle scission.

A role for amphiphysins in CCV formation at the TGN

In contrast to the AP-2/clathrin coat formation machinery at the plasma membrane, the protein network associated with AP-1-dependent CCV formation at the TGN or endosomes has been characterized much less extensively. Our finding that Amph1/2 heterodimers support *in vitro* membrane association of AP-1 adaptors strongly suggests that these proteins are also involved in the process of AP-1/CCV formation.

By analogy, they are likely to perform a very similar, late function as with AP-2, i.e. binding and regulating factors for fission and uncoating (such as dynamin and synaptojanin) just before vesicle release. In fact, in similar assays as were used here, amphiphysins were also shown to stabilize AP-2 on liposomes. Di Paolo et al. (Di Paolo et al., 2002) showed that the association of cytosolic AP-2 and clathrin with liposomes was reduced when brain cytosol from Amph1 knockout mice (lacking both Amph1 and Amph2) was used. Similarly, purified Amph1 stimulated clathrin binding to liposomes (Farsad et al., 2003). As shown in Figure 12, Amph2, and to a lesser extent Amph1, mediated membrane association also of AP-2. *In vitro* membrane association reflects the mutual stabilization of coat components (adaptors and clathrin) and amphiphysins or other interactors on the membrane. This notion is emphasized by the network view of coat formation (Schmid and McMahon, 2007).

The function of amphiphysins in CCV formation both at the plasma membrane and the TGN mirrors the function of EPS15, which is a known accessory factor with AP-2 for endocytosis. EPS15 was recently shown to interact also with AP-1 at the TGN and to be involved in TGN exit of certain secretory proteins (Chi et al., 2008). Similarly, also p34 was shown to interact with both α- and γ-adaptin in a two-hybrid screen (Page et al., 1999).

Besides amphiphysin, also its main binding partner dynamin is proposed to be involved not only in CCV formation at the plasma membrane but also at the TGN. In fluorescence microscopy, dynamin was found associated with CCVs at both the plasma membrane and the TGN (Jones et al., 1998). In addition, VSVG accumulated in the Golgi in cells expressing mutant dynamin (Cao et al., 2000). However, since other groups reported no effect of mutant dynamin on vesicle formation at the TGN (Altschuler et al., 1998; Damke et al., 1994), this remains a matter of debate.

Amphiphysin is not only involved in endocytosis

Already in previous studies, amphiphysin was shown to be not only involved with endocytosis. In support of a role for amphiphysins at endosomes, amphiphysin 2 has previously been found to interact with SNX4 in a two-hybrid screen and co-immunoprecipitations (Leprince et al., 2003). Furthermore, both proteins co-localized on endosomal structures and to some extend on transferrin-containing vesicles. Similarly, AMPH-1, the only amphiphysin in *C. elegans*, was shown to regulate endocytic recycling cooperatively with RME-1 (Pant et al., 2009). Yet, AMPH-1 lacks a CLAP domain. In mammalian cells, knockdown of muscle BIN1, one of the isoforms of amphiphysin 2 lacking the CLAP domain, was found to

cause endosomal accumulation of transferrin as well as delayed recycling. Furthermore, BIN1 co-localized with EHD1 (the mammalian RME-1) on recycling endosomes (Pant et al., 2009). These findings indicate that amphiphysins may play a conserved role in the endocytic recycling pathway possibly even independently of a direct interaction with adaptors and clathrin. In the same line, over-expression of Amph2b (a brain specific splice variant lacking parts of the central insert domain including the WDLW motif (Ramjaun and McPherson, 1998)) in AtT-20 cells perturbed the constitutive TGN exit of somatostatin receptors (Sarret et al., 2004).

One motif in amphiphysin for adaptor and clathrin binding

In the middle domains of amphiphysin 1 and 2, partially overlapping interaction sites for AP-2 and clathrin have previously been identified (Ramjaun and McPherson, 1998; Slepnev et al., 2000; Slepnev et al., 1998). The PWDLW motif of amphiphysin 1 was first established as a binding site for clathrin, but not AP-2, using GST fusions with various middle domain fragments (Slepnev et al., 2000). Yet, the short peptides TLPWDLWTTS and SIPWDLWEPT derived from amphiphysin 1 and amphiphysin 2, respectively, fused to GST were found to pull down AP-1 and AP-2 in addition to clathrin from brain cytosol (Drake and Traub, 2001) and the amphiphysin 2 peptide SIPWDLWEPT to bind directly to the appendage domain of γ-adaptin (Bai et al., 2004). In this study we show that not only peptides, but full-length amphiphysin 2 interacts via its PWDLW motif with AP-1 at liposomal membranes (Figure 9).

Several proteins have been shown to contain WXXW/F motifs that mediate selective binding to either the α-appendage of AP-2 (e.g. synaptojanin, AAK1, GAK, NECAP1, connecdenn, and stonin) (Allaire et al., 2006; Jha et al., 2004; Ritter et al., 2003; Walther et al., 2004) or to the γ-appendage of AP-1 (NECAP1, separate from the motif recognizing α-adaptin, and GGA1) (Bai et al., 2004). The WDLW motifs of amphiphysin 1 and 2 thus seem exceptional in that they have the potential to interact with both adaptor complexes, as is uncovered when presenting them as short peptides.

Amphiphysin 1 middle domain is not functional

The liposome assay used here goes beyond a simple pull-down experiment, since it involves stabilization of an adaptor complex interacting with lipids and ARF1-GTP on a bilayer. It thus includes the specific arrangement of both AP-1 and the amphiphysin dimer on the membrane surface. The results demonstrate that full-length Amph2 interacts with AP-1 via its WDLW motif, either alone or as a

heterodimer with Amph1, whereas Amph1 alone does not. The domain swapping experiments (Figure 13) suggest that the WDLW motif in the Amph1 M1 environment is not functional and that in addition, the M1' repeat sequence may further reduce accessibility. An obvious explanation may be that the flanking sequences of Amph1, particularly the downstream sequence is very different from that in Amph2, fail to correctly present the WDLW motif to potential binding partners.

Also for the interaction with clathrin the binding motif flanking sequences of amphiphysin seem to play a role. In GST-pull down experiments with a mutant of Amph2 lacking 11 amino acids between its both clathrin binding motifs, LLDLD and PWDLW, interaction with clathrin was decreased, showing that appropriately spaced motifs are important for efficient binding (Drake and Traub, 2001).

Endophilin is not in a stable complex with amphiphysin

Purified endophilin had no activity in the liposome assay (Figure 8B) and over-expressed endophilin was not found in the TGN region with AP-1 (Figure 15C). Endophilin had been shown to interact *in vitro* with amphiphysin 1 and 2 via its SH3 domain (Micheva et al., 1997b), which may explain their co-purification from brain cytosol. Yet, endophilin was not co-depleted with Amph1 (Figure 11A), in agreement with a previous report (Micheva et al., 1997a), showing that they are not in a stable complex and may also act at separate locations within the cell. However, like amphiphysins and dynamin, also endophilin function was shown not to be restricted to clathrin-mediated endocytosis at the plasma membrane. A recent study demonstrated that endophilin not only interacted with the transmembrane protein retrolinkin, but was also recruited to endosomes through this interaction (Fu et al., 2011). Both proteins were required for the early endosome trafficking of the neurotrophin BDNF (brain-derived neurotrophic factor) in complex with its receptor TrkB, which regulates neuronal differentiation and development.

Non-essential proteins function in tissues with high activity

Importantly, amphiphysins and endophilins are not generally essential for AP-2/CCV formation, since amphiphysin 1, the CLAP domain-containing splice-variants of amphiphysin 2, and endophilin A1 are almost exclusively expressed in the brain (Ringstad et al., 1997; Tsutsui et al., 1997; Wigge et al., 1997a). They may thus perform an important enhancing function at sites of high transport demand such as in stimulated neurons. Amph1 knockout mice, which in parallel also lost Amph2 selectively in brain, were viable and largely normal, except for rare seizures and some learning deficits (Di Paolo et al., 2002).

Upon neuronal stimulation, defects in synaptic vesicle recycling could be detected, indicating a contribution of amphiphysins to the synaptic vesicle cycle under heavy load. In contrast to Amph1, loss of BIN1/Amph2 in mice resultedin perinatal lethality. However, in mouse embryo fibroblast gained from BIN1/Amph2 knockout mice, transferrin uptake was not affected and no impact on endocytosis could be observed (Muller et al., 2003). Perinatal lethality in these mice was due to a severe disorganization of myofibrils in ventricular cardiomyocytes, defining a critical role for BIN1 in cardiac muscle development. In line with these data is our finding that AP-1 localization to the TGN was unchanged in neuronal cells where amphiphysin was knocked down (Figure 24). Also endophilin A1 knockout mice had no phenotype; only a triple-knockout of all three endophilins (A1–3) caused postnatal lethality and the accumulation of AP-2/CCVs at synapses (Milosevic et al., 2011).

Consistent with an involvement of Amph1/2 in AP-1/clathrin coat formation, we found endogenous amphiphysin of cerebellar granule cell neurons as well as exogenously expressed Amph1/2 in the neuronal cell line HN10 and COS-1 fibroblasts to co-localize with AP-1 at the TGN (Figure 14 and 15). Surprising and in contradiction to the *in vitro* data is the observation that not only Amph2 homodimers, but also Amph1 homodimers were localizing to the TGN region (Figure 16). The localization required the CLAP domain, but did not involve the SH3 domain, indicating that amphiphysin localization to the TGN is not dependent on dynamin (Figure 17). When the cytosolic amphiphysin was released with digitonin, high-expressing cells contained amphiphysin aggregates, which interfered with AP-1 localization at the TGN in a CLAP domain-dependent manner (Figure 19). Aggregation of highly expressed amphiphysin was already reported previously (Farsad et al., 2003). Aggregates, formed upon over-expression of amphiphysin (mutated in the SH3 domain), contained clathrin and AP-2, however, in contradiction to our results, they did not interfere with AP-1 localization. We also found that the localization of both amphiphysin and AP-1 to the TGN was sensitive to BFA (Figure 21) indicating an ARF1-dependent recruitment to the site of vesicle formation. A role for amphiphysin in AP-1/clathrin coated vesicle formation was further confirmed by the finding that both endogenous and exogenously expressed amphiphysins could be cross-linked to AP-1 in intact cells (Figure 22). Importantly, when clathrin was knocked down, AP-1 and amphiphysin interacted together to the same extend as when clathrin was present. The same was also observed when amphiphysin interaction with clathrin was blocked by small molecule inhibition (Figure 23). These results show that amphiphysin and AP-1 interaction is direct and not via/influenced by clathrin.

Taken together, our results suggest that amphiphysin 1 and amphiphysin 2, two proteins which are involved in endocytosis, might also play a role in CCV formation at the TGN. In contrast to AP-2/clathrin coat formation, the detailed mechanism of AP-1/clathrin coat formation is still unknown. In this study, we could shed some light on the accessory proteins involved in this process and demonstrate that the machineries for CCV formation with AP-1 and AP-2 at different locations in the cell might share more components than previously anticipated.

A large part of my work is included in the manuscript:

Interaction of amphiphysins with AP-1 clathrin adaptors at the membrane

by

Sonja Huser, Gregor Suri, Pascal Crottet and Martin Spiess

It is currently under revision for publication in Biochemical Journal. The results section includes the initial experiments by Gregor Suri which started this project. They are marked by his name.

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Posters and Presentations

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