SNX18 is an SNX9 paralog that acts as a membrane tubulator in AP-1-positive endosomal trafficking

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Summary
SNX9, SNX18 and SNX30 constitute a separate subfamily of PX-BAR-containing sorting nexin (SNX) proteins. We show here that most tissues express all three paralogs, and immunoprecipitation and immunofluorescence experiments demonstrated that the SNX9-family proteins act as individual entities in cells. Their SH3 domains displayed a high selectivity for dynamin 2, and the PX-BAR units had the capacity to tubulate membranes when expressed in HeLa cells. As previously described for the PX-BAR domain of SNX9 (SNX9-PX-BAR), purified SNX18-PX-BAR caused liposome tubulation in vitro and had a binding preference for PtdIns(4,5)P_2. However, contrary to SNX9, which primarily acts in clathrin-mediated endocytosis at the plasma membrane, endogenous SNX18 localized to AP-1- and PACS1-positive endosomal structures, which were devoid of clathrin and resistant to Brefeldin A. Moreover, a γ-adaptin recognition motif was defined in a low-complexity region of SNX18, and a complex of endogenous SNX18 and AP-1 could be immunoprecipitated after Brefeldin A treatment. Overexpression of SNX18 sequestered AP-1 from peripheral endosomes and resulted in the formation of short SNX18-decorated tubes with distinct dynamin puncta. The results indicate that SNX9-family members make up discrete membrane-scission units together with dynamin, and suggest that SNX18 mediates budding of carriers for AP-1-positive endosomal trafficking.

Introduction
The molecular mechanisms implicated in endosomal sorting are currently attracting much attention and they have turned out to be more complex than first realized (Bonifacino and Rojas, 2006; Maxfield and McGraw, 2004). A number of different factors are required to support the sorting decisions that have to be made to ensure that different cargoes are taken to the appropriate destinations according to the needs of the cell. Transport intermediates can either be in the form of round vesicles or extended tubules, and some cells display an elaborate system of endocytic membrane networks that are thought to be involved in specific trafficking events. The dynamic organelles commonly referred to as early endosomes appear to be major sorting stations within the system, and can be functionally divided into sorting endosomes and recycling endosomes. Internalized material can either recycle back to the plasma membrane by one of several routes, be sorted to the trans-Golgi network (TGN) or end up in the lysosomal pathway leading to degradation of the cargo.

Membrane-binding and -modulating proteins are important for the mechanics of the endocytic system. Classical examples are the vesicular coat proteins, such as clathrin and its adaptor proteins (APs) (Edeling et al., 2006b; Robinson, 2004; Ungewickell and Hinrichsen, 2007). Clathrin acts as a vesicular stabilizing protein at several locations in the cell, and clathrin-dependent carrier formation requires membrane-specific APs for localization, coat assembly, accessory protein recruitment and cargo selection. There are four different kinds of APs, which operate at diverse cellular sites. For example, whereas AP-2 is mostly found at the plasma membrane participating in clathrin-dependent endocytosis, AP-1 is concentrated at the TGN to mediate clathrin-dependent trafficking of cargo to the endosomal system. AP-1 is also found on endosomal membranes and is proposed to take part in trafficking pathways for retrieval of cargo to the TGN. However, it is unclear how the same AP can work in trafficking pathways that go in opposite directions. An arrangement like this would probably require different procedures for carrier formation, to give unique identities of the transport intermediates. Such differences might be mediated by specific accessory proteins and membrane-modulating proteins. The advantage of using the same cargo-binding adaptor for anterograde and retrograde traffic is that efficient recycling systems can be created for proteins that shuttle between compartments, such as the mannose 6-phosphate receptor and furin.

Proteins that can bind and modulate specific membrane zones are important players in the process of vesicular- and tubular-carrier formation. Examples are proteins with phosphoinositide-binding PX (phox homology) and PH (pleckstrin homology) domains (Di Paolo and De Camilli, 2006; Lemmon, 2003), as well as proteins with membrane-bending function (Zimmerberg and Kozlov, 2006). A class of proteins with the latter property is the recently described BAR (Bin/Amphiphysin/Rvs)-domain proteins (Gallo and McMahon, 2005). The BAR domain has a crescent structure formed by dimerization, with the ability to shape the membrane into buds and tubules. The endocytic system appears to be especially rich in PX-, PH- and BAR-domain proteins. The large sorting nexin (SNX) family of PX-proteins (Seet and Hong, 2006; Worby and Dixon, 2002) consists of over 30 members in mammals. Most of...
the SNXs seem to function at various steps in endocytic trafficking. Certain members of the SNX family, such as SNX9, also contain BAR domains. This membrane-sculpting PX-BAR protein has its major function in clathrin-dependent endocytosis at the plasma membrane, where, through its SH3 domain, it recruits and activates the GTPase dynamin for vesicle scission (Lundmark and Carlsson, 2003; Lundmark and Carlsson, 2004; Pylpyenkon et al., 2007; Soulet et al., 2005; Yarar et al., 2008).

Vertebrate genomes express two paralogs to SNX9 that are essentially unstudied so far. One of them, SNX18, was found in proteomics screens for interaction partners to the poly-proline regions of Nos (Schulze and Mann, 2004) and Pax ligand (Thornhill et al., 2007). The other protein was detected as an ADAM15-binding SH3-protein in a phage-display assay, and was given the designation SNX30, using the consecutive number in the SNX series (Karkkainen et al., 2006). In a subsequent review (Seet and Hong, 2006), the same protein was unfortunately given the name SNX33, and the designation of this protein (originally hypothetical protein MGC32065) is currently mixed up in the sequence databases. We will, in the present article, refer to this protein as SNX30.

SNX9, SNX18 and SNX30 represent an apparent subfamily of vertebrate SNX proteins having the same domain structure (Fig. 1A). An SH3 domain at the N-terminus is followed by a low-complexity (LC) domain of variable length, which, in SNX9, harbors motifs for clathrin (PWSAW) and AP-2 (DPW) binding (Lundmark and Carlsson, 2003). These motifs are absent in SNX18 and SNX30, but other species-conserved sequence patches are present that are candidates as protein-specific interaction sites. The crystal structure of SNX9 (amino acids 214-595) (Pylpyenkon et al., 2007) showed that the PX-BAR region forms a dimerized superdomain structure, which is stabilized by large contact areas between the subdomains and by an additional domain called the Yoke (Y) domain. The latter domain is split into an N-terminal (YN) and a C-terminal (YC) part. At the sequence level, SNX18 and SNX30 are similar to SNX9 in these regions and probably adopt the same conformation. Membrane-tubulation experiments with truncated variants of the PX-BAR domain of SNX9 (SNX9-PX-BAR) showed that a short sequence (amino acids 201-213, suggested to form a membrane-penetrating amphipathic helix) immediately upstream of the determined crystal structure is essential for membrane-sculpting activity (Pylpyenkon et al., 2007). The amino acid properties of this stretch are conserved in SNX18 (amino acids 225-237) and SNX30 (amino acids 179-191), and these sequences therefore probably perform the same function. Comparison of the primary structures of the different domains (Fig. 1B) reveals that the ancestor of SNX18 and SNX30 branched off first from SNX9, and were then duplicated.

Because SNX9 has been shown to have important functions for the generation of vesicular carriers, it was of interest to investigate the expression and function of the two paralogs, SNX18 and SNX30. Here, we describe the general properties of SNX18 and SNX30, and focus on the subcellular localization and interaction partners of SNX18.

**Results**

The SNX9 protein family

Antibodies were raised against recombinant SNX18 and SNX30, and were used together with previously described anti-SNX9 antibodies (Lundmark and Carlsson, 2003) for immunoblotting analysis of the paralogs in different mouse tissues (Fig. 1C). Most tissues expressed all three proteins, but to a different extent. The highest amounts of SNX9 were found in the liver, lung, pancreas, spleen and testis, whereas SNX18 was mainly found in the heart, kidney, liver and lung, and SNX30 in the lung, pancreas and testis. The brain was almost devoid of all three proteins, instead expressing a high amount of the related protein amphiphysin 1.

HeLa cells were found to express all three family members, which could be separately immunoprecipitated from the cytosol (Fig. 2A). Immunoprecipitated bands were subjected to MALDI-TOF analysis (supplementary material Table S1) and the paralogs were identified as indicated. In the NCBI nucleotide database, a cDNA sequence of human SNX18 is deposited that has an unrelated C-terminus (accession BC117218). From the obtained peptide masses, we found no evidence for the presence of this isoform in HeLa cells. Instead, we could confirm the authenticity of the clone from which we derived our recombinant protein (accession BC060791), because one of the obtained peptides corresponded to the last part of the C-terminal α-helix in the BAR domain. It is presently unclear whether any of the three paralogs exist as isoforms at the protein level. SNX18 (71 kDa) and SNX30 (65 kDa) migrate on SDS-PAGE as expected from their theoretical Mr, whereas SNX9 migrates erroneously at a Mr higher than its theoretical weight (74 vs 67 kDa). Immunoprecipitates contained, in addition, dynamin (100 kDa) and aldolase (41 kDa) (arrows in Fig. 2A), as previously reported for cytosolic SNX9 (Lundmark and Carlsson, 2003; Lundmark and Carlsson, 2004), although the amounts that co-precipitated with SNX18 and SNX30 were lower. No other specific components that co-precipitated with the respective SNXs could be identified in this experiment.

BAR-domain proteins form a functional, membrane-deforming and -stabilizing unit by dimerization. In certain cases, such as amphiphysin 1 and amphiphysin 2 (Wigge et al., 1997), and SNX1 and SNX2 (Rojas et al., 2007), it has been suggested that the BAR...
domain might be formed through heterodimerization between related proteins. From immunoprecipitation experiments combined with mass spectrometry (Fig. 2A) and immunoblotting (Fig. 2A inset), we conclude that SNX9-family members do not exist as intra-family heterodimers in the cell. Moreover, immunofluorescence analysis of HeLa cells showed that endogenous SNX9 and SNX18, together with exogenously expressed full-length myc-tagged SNX30, localized differently (Fig. 2B). All three proteins were present in small punctate structures and short tubes throughout the cytoplasm, but no obvious colocalization could be detected. This finding argues for separate functions of the three family members. SNX9 was previously described to distribute between a cytosolic and a membrane-bound pool in the cell (Lundmark and Carlsson, 2003), and this was found to be the case also for SNX18 and SNX30. When an isolated membrane fraction from HeLa cells was separated according to density, SNX18 and SNX30 were found on slightly lighter membranes than SNX9 (Fig. 2C). SNX18-positive membranes were found at the same density as endosomal membranes, which were distinguished by the endosome-specific protein EEA1, but were absent from clathrin-containing fractions. However, all three proteins banded in the broad region in which dynamin 2 was distributed.

SNX9-family members function together with dynamin 2

To address whether binding to dynamin 2 is a common feature within the SNX9 protein family, the respective SH3 domain was purified as GST-fusion proteins and used in pull-down assays with cytosol from HeLa cells. We could indeed identify dynamin 2 as the major interacting protein for all three SH3 domains (Fig. 3A). The binding preference for the proline-rich domain (PRD) in dynamin 2 was tested by pull-downs with purified protein containing wild-type sequence or those with introduced mutations, covering all type I (RXXPXXP) and type II (PXXPXR) SH3-binding motifs in the PRD (Mayer, 2001). Fig. 3B shows that all three paralogs interact directly with the PRD, and the results obtained with mutated PRD showed that the binding is affected when type I motifs are modified (Fig. 3B, mutants 3 and 4). The binding preference for type I motifs was recently shown for SNX9 (Alto et al., 2007), and the same binding mode appeared to apply also for the other two proteins. This contrasts to that of the SH3 domain of amphiphysin 2 (Fig. 3B), which showed decreased binding to the type II mutants 1, 2 and 4, in accordance with earlier studies (Owen et al., 1998). As previously described, SNX9 localizes to dynamin-positive puncta in cells (Lundmark and Carlsson, 2003). Overexpression of SNX9 resulted in numerous such structures, in agreement with its role in activating dynamin at the neck of clathrin-coated vesicles (Fig. 3C, panel a with insets). In cells overexpressing SNX18, abundant semi-short tubes were detected. Interestingly, when co-stained for dynamin, these tubes were normally seen to start with, or end in, distinct puncta containing dynamin (Fig. 3C, panel b with insets). Overexpression of SNX30 resulted in a less obvious phenotype, which was intermediate to that seen for SNX9 and
SNX18 (data not shown, and see Fig. 2B). These contrasting effects seen upon overexpression of the different family members probably reflect differences in functions, such as the ability to activate dynamin fission.

The PX-BAR unit of SNX18 remodels membranes

The deletion of the SH3-domain and LC-region in SNX9 results in a protein with increased membrane-tubulating activity when overexpressed in cells, as previously shown (Pylypenko et al., 2007). This phenomenon is due to the activity of the membrane-sculpting PX and BAR domains, combined with the inability of this mutant to recruit dynamin and promote scission of membrane tubes into vesicles. When we assayed the effect of similar mutants in SNX18 and SNX30, we found that these proteins also generate extensive tubular networks in cells (Fig. 4A). We could detect a patchy overlap between SNX9- and SNX18-positive tubes when co-expressed, showing that these proteins assemble on curved surfaces of similar diameter (supplementary material Fig. S1A). To address whether the PX-BAR domain of SNX18 was sufficient to induce membrane deformation in vitro, we incubated purified protein together with liposomes generated from brain lipids. At a concentration of 20 μM, SNX18-PX-BAR induced extensive membrane tubulation in which the tubes frequently were entangled yielding a ‘ball of wool’ impression (Fig. 4B). The tubes were similar in diameter (approximately 20 nm, Fig. 4B inset) compared to those created by SNX9 (Pylypenko et al., 2007). So far, we have not been able to produce PX-BAR from SNX30 in a form that can be used for such experiments.

The lipid constitution is generally crucial for the ability of peripheral membrane proteins to interact with target membranes. PX domains bind preferentially to phosphorylated phosphoinositides, whereas BAR domains interact with charged membrane surfaces. The PX and BAR domains of SNX9 make up a superdomain that is perfectly adapted for both membrane-binding specificity and curvature generation. To further investigate the binding specificity of SNX18, we used the purified PX-BAR protein in a liposome co-sedimentation assay together with liposomes containing different phosphoinositides. In this assay, membrane binding was determined by the amount of protein detected in the pellet fraction. Interestingly, only phosphatidylinositol (4,5)-

Fig. 3. SNX9, SNX18 and SNX30 interact and colocalize with dynamin 2. (A) GST or GST-fusion proteins with SH3-domains from SNX9, SNX18 or SNX30 were incubated with (+) or without (-) cytosol from HeLa cells. After washings, bound protein was analyzed by SDS-PAGE and the gel was stained with Coomassie. The identity of dynamin 2 (Dyn2) was verified with immunoblotting. (B) The PRD of dynamin 2, containing wild-type (wt) or mutated (mut1–4) sequences, was purified and used in pull-downs with GST or GST-fusion proteins containing SH3-domains from SNX9, SNX18, SNX30 or amphiphysin 2 (Amph2), as described in the Materials and Methods. Bound protein, together with total protein (Input), was analyzed by SDS-PAGE and the gels were stained with Coomassie. The sequence of the C-terminal part of the PRD with indicated type I and type II SH3-binding motifs is shown below. Modified amino acids in mutated PRDs are highlighted. (C) Epifluorescent micrograph of HeLa cells expressing myc-tagged SNX9 (a) or myc-tagged SNX18 (b) and counterstained for dynamin 2 after wash-out of cytosolic components as described in the Materials and Methods. Magnifications of the boxed areas together with illustrations of the specific structures labeled with anti-myc and anti-dynamin-2 are shown to the right. Scale bar: 10 μm.
bisphosphate [PtdIns(4,5)P2]-containing liposomes and brain-derived liposomes [which are enriched in PtdIns(4,5)P2 lipids] yielded a substantial binding of the protein (Fig. 4C). This result implies that, although SNX9 also has a preference for PtdIns(4,5)P2, SNX18 is more specific than the rather promiscuous SNX9 (Lundmark and Carlsson, 2003; Pylypenko et al., 2007; Yarar et al., 2008). If SNX18 requires PtdIns(4,5)P2 for membrane binding and tubulation, it should be affected by the drug ionomycin, which reduces the levels of PtdIns(4,5)P2 by increasing the Ca2+ concentration (Varnai and Balla, 1998). Indeed, even very brief stimuli of HeLa cells with ionomycin led to a complete loss of tubular SNX18-PX-BAR (Fig. 4D). Treatment of the cells with the drug wortmannin, which reduces PtdIns(3)P levels (Vanhaesebroeck et al., 2001), had only a minor effect on the PX-BAR localization, which further supports the importance of PtdIns(4,5)P2 for the function of SNX18.

SNX18 interacts with AP-1 through the LC region
As shown above (Fig. 2B), the endogenous localization of SNX18 in HeLa cells was determined, using immunodetection analysis, as distinct small puncta and short tubes equally distributed throughout the cytoplasm. Antibodies generated against SNX30 did not result in reliable cell stainings, and this protein was not investigated further. We focused on identifying proteins that colocalized with the punctate stain observed for SNX18 and found a partial overlap with AP-1 (Fig. 5A). Because SNX9 is known to bind to the homologous AP-2 complex via motifs in the LC region, we speculated that the same region in SNX18 might bind to AP-1. Out of all of the domains, the LC region differs most between the paralogs, both in length and sequence (see Fig. 1A,B). The individual ear domains of AP complexes, which are the accessory-protein interaction units, were purified as GST-fusion proteins and assayed for their ability to bind the SNX18 LC region. Indeed, the LC-region interacted specifically with the γ1 ear domain but not with the companion ear domain of AP-1 (β1), nor with the analogous ear domains of AP-2, AP-3 or AP-4 (Fig. 5B). The previously described sequence motif recognized by the γ1 ear [W/F/Y]G[P/D/E][W/F/Y/L/M] (Mattera et al., 2004) is very similar to that of the close homolog γ2 ear and the GAE-domains of GGA proteins. Using purified proteins, we showed that the LC region of SNX18 interacted specifically with
γ1 and to a lesser extent with γ2, but insignificantly with the GGA adaptors (Fig. 5C). This suggested to us that a novel motif might be responsible for the specific interaction of SNX18 with AP-1.

A sequence patch is found in the LC regions of SNX9, SNX18 and SNX30 that is similar in sequence but located differently. The sequence consists of a stretch of negatively charged amino acids with two tryptophans positioned two (SNX30) or three (SNX9 and SNX18) residues apart. In SNX9, this patch was previously shown to be part of a second site for clathrin- and AP-2-binding (Lundmark and Carlsson, 2003), and was also found to possess a binding motif for the glycolytic enzyme aldolase (Lundmark and Carlsson, 2004). In SNX18, this region, which is highly conserved in vertebrates (Fig. 5D, top panel), is the most likely area for the interaction with AP-1. To test this, we mutated the individual tryptophans in this region to serines, which resulted in a major decrease in binding to the γ1 ear (Fig. 5D, lower panel). This shows that a region similar to the one in SNX9 that binds to AP-2 also binds SNX18 to AP-1, and is probably responsible for the localization of SNX18 to AP-1-positive structures in the cell.

SNX18 functions as part of an endosomal AP-1-positive budding machinery

The observed interaction between SNX18 and AP-1 proposed a common role for these proteins in transport-carrier formation at the TGN and/or endosomes. At the TGN, AP-1 is recruited by the small GTPase Arf1 to generate the budding of clathrin-coated vesicles (Gillingham and Munro, 2007). We could, however, not detect any colocalization of SNX18 with the distinct pool of AP-1 or Arf1 at the TGN (Fig. 5A and supplementary material Fig. S1B), nor could we detect any colocalization or interaction between SNX18 and clathrin (supplementary material Fig. S1C,D). Thus, the peripheral AP-1- and SNX18-positive puncta seen by immunofluorescence microscopy were more likely to be endosomal structures. In agreement with this, we found that treatment of cells with the drug Brefeldin A (BFA), which results in disruption of the Golgi due to inhibition of Arf loading (Gillingham and Munro, 2007), did not abolish the co-staining of SNX18 and AP-1. Instead, BFA treatment interestingly led to an increased colocalization of AP-1 and SNX18 in peripheral endosomal puncta (Fig. 6A). Counting the number of SNX18-positive puncta that colocalized with AP-1 in cells showed a BFA-dependent increase of 73% in the number of loci in which the proteins were found together (Fig. 6B). The effect of BFA could be further demonstrated by immunoprecipitation of the AP-1 complex. In contrast to untreated cells, which gave very little co-immunoprecipitation, endogenous SNX18 was significantly precipitated together with the AP-1 complex in immunoprecipitates from BFA-treated cells (Fig. 6C), confirming that the two proteins do indeed act as partners in the cell. Our results show that SNX18 is not part of the Arf1-, AP-1- and clathrin-dependent coat machinery at the TGN, but is rather participating in a budding machinery that is reliant on a distinct pool of endosomal AP-1 that is resistant to Brefeldin A. We next examined whether, and where, AP-1 was located on the short phenotypic tubes observed upon overexpression of full-length SNX18. To our surprise, the tubes were devoid of AP-1 and the punctate endosomal AP-1 stain was completely lost (Fig. 6D). The fact that AP-1 was still present on

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the TGN showed that only the pool of AP-1 that was associated with endosomes could be sequestered by overproduction of SNX18. In order to further characterize the nature of the membranes that were positive for both SNX18 and AP-1, we assayed to see whether SNX18 co-stained with marker proteins known to localize to membrane vesicles and dynamic regions of endosomes. SNX18 did not localize together with overexpressed Rab5, Rab7, Rab9 or Rab11 (specific proteins in the rab family of GTPases) (Pfeffer, 2003; Zerial and McBride, 2001) or epsinR (a previously identified membrane tubulator associated with AP-1) (Hirst et al., 2003; Kalthoff et al., 2002; Mills et al., 2003) (data not shown). However, we found that a substantial number of SNX18 puncta were positive for PACS1 (Fig. 6E), a protein known to function in retrograde trafficking from the endosomes together with AP-1 (Crump et al., 2001). Peripheral PACS1-positive puncta were also resistant to BFA (Wan et al., 1998) (and data not shown), in agreement with a common mechanistic basis. These data demonstrate that SNX18 localizes with PACS1 and a distinct pool of AP-1 recruited independently of Arf1. Previous data have suggested that AP-1 and PACS1 take part in specific carrier formation at the endosomes (Crump et al., 2001). It is possible that SNX18 participates in this process to create high membrane curvature and to recruit dynamin for tubular and/or vesicular scission.

Discussion

 Trafficking pathways that emanate from the early endosomal tubular network are thought to largely involve the budding and fusion of tubular-membrane carriers. The PX-BAR proteins, which all belong to the SNX family, have properties well suited for a function in this context. The PX-BAR superdomain structure possesses both lipid-binding specificity and membrane-sculpting function. Where present, additional domains and regions outside
this superdomain seem to mediate interactions with other proteins required for the sorting and/or tubulation process. Information on the roles of PX-BAR proteins in different endocytic trafficking pathways is rapidly accumulating. SNX1, SNX2, SNX5 and SNX6 are implicated in retromer function (Carlton et al., 2004; Griffin et al., 2005; Rojas et al., 2007; Wassmer et al., 2007), and SNX4 was recently suggested to participate in the transport of the transferrin receptor to the endocytic recycling compartment (Traer et al., 2007). SNX9 is a well-established accessory protein required for the fission of clathrin-coated endocytic vesicles, and we show here that SNX18 participates in membrane remodeling at endosomes together with AP-1 and PACS1. The functional role of SNX30 remains uncertain because of difficulties to localize the protein in cells and to identify specific interaction partners, but our data suggest that this protein might have a role as a tubulator in yet another membrane trafficking event. It can, however, not be entirely excluded that SNX18 and SNX30 are used as redundant proteins in the same pathway, because there is some tendency for reciprocal expression in different tissues, i.e. where SNX18 expression is high, SNX30 is low and vice versa (see Fig. 1C). However, their LC domains, which harbor putative protein-interacting motifs, are very dissimilar, which points to the possibility that they have distinct protein partners. Further studies on SNX18 and SNX30 will shed light on this issue.

A feature that distinguishes the SNX9-family proteins from other PX-BAR proteins is the presence of an SH3 domain. As previously found for SNX9 (Lundmark and Carlsson, 2003; Soulet et al., 2005), we show here that, in both SNX18 and SNX30, this domain interacts with dynamin. This interaction might be important for dynamin-mediated scission of the tubular extensions (Ramachandran and Schmid, 2008). We have demonstrated that all SNX9-family proteins bind to dynamin 2, but it is likely that they also interact with the homologous proteins dynamin 1 [as shown for SNX9 (Shin et al., 2007; Soulet et al., 2005)] and dynamin 3. However, dynamin 1 is restricted to the brain, in which all the SNX9-family members are minimally expressed. Interestingly, both SNX30 (see Fig. 1C) and dynamin 3 (Cao et al., 1998) are mainly expressed in the testis, which suggests that they might form a functional complex. It is possible that, in addition, the SH3 domains of SNX18 and SNX30 are used for interactions with actin polymerizing proteins such as N-WASP, as has been suggested for SNX9 (Badour et al., 2007; Shin et al., 2007; Yarar et al., 2007). Such interactions might be important for the proper growth and transport of the tubular carrier.

Without the presence of an SH3 domain, which consequently leads to the inability of dynamin to be recruited, SNX9-family proteins can no longer promote membrane scission. This results in the formation of long membrane tubes frequently starting or ending in distinct puncta (see Fig. 4A). Attempts to elucidate the membrane source of these tubes have so far been unsuccessful. This is due to the high curvature generated once the tube is formed, which might exclude potential marker proteins of the original organelle. SNX9-family proteins are very potent membrane binders. Similar to SNX9, SNX18 was shown here to require PtdIns(4,5)P2 for optimal binding. Because SNXs generally are known to bind PtdIns(3)P, the PtdIns(4,5)P2 specificity could be yet another defining feature that unites this subfamily. Our results also suggest that the PX-BAR unit is not alone responsible for the divergent localization of SNX9 and SNX18 (compare endogenous localization in Fig. 2B with expressed PX-BAR in supplementary material Fig. S1A). Full-length proteins have unique distributions despite the fact that the lipid-binding specificity and the geometry of the tubes formed are the same. Protein-protein interactions mediated by regions outside the PX-BAR domain are probably important for the correct localization, and the phosphoinositide specificity of the PX domain is crucial to augment the interaction with the target membrane (Pylypenko et al., 2007). In addition to the relatively high concentration of PtdIns(4,5)P2 at the plasma membrane, this phosphoinositide is also enriched at dynamic areas of both the Golgi, TGN and endosomes (Watt et al., 2002). The phosphoinositide preference of AP-1 was determined to PtdIns(4)P (which is enriched at the Golgi) as well as PtdIns(4,5)P2 (Crottet et al., 2002; Wang et al., 2003). As discussed above, however, we do not believe that SNX18 is normally acting at the Golgi, and because it is well-established that AP-1 is acting only on internal membranes, it is unlikely that SNX18 functions at the plasma membrane. PtdIns(4,5)P2-enriched loci on endosomes, created by locally activated lipid kinases, might serve as initiation sites for AP-1–SNX18 tubular budding.

The specific role of AP-1 in the TGN-endosome system is still uncertain, and several different pathways have been proposed to be dependent on this adaptor. It is, however, clear that a subpopulation of AP-1 resides on membrane structures outside the TGN, and two different endosome-to-TGN retrieval pathways have been proposed in which AP-1 participates: the epsinR pathway and the PACS1 pathway. EpsinR, together with AP-1 and clathrin, was shown to be involved in the retrieval of material from tubular endosomes to the TGN in a dynamin-dependent process (Saint-Pol et al., 2004). Because this pathway is BFA-sensitive and clathrin-dependent, we think that it is unlikely that SNX18 is involved here. In addition, we saw no colocalization of overexpressed epsinR and SNX18 in cells, as discussed above. The characteristics of SNX18 fit better with a role in PACS1-mediated retrieval function, because we indeed have detected a partial colocalization of the two proteins (see Fig. 6E). However, to our knowledge it has not yet been tested whether the PACS1 pathway is dependent on dynamin (as would be expected from a tubular process in which SNX18 participates), and further studies are required to more precisely define the role of PACS1 in endosomal trafficking and how SNX18 is related to this pathway.

The recruitment of accessory proteins by weak interactions through flexible ear domains makes up a mechanism by which AP complexes can provide directionality to the process of vesicular budding (Schmid and McMahon, 2007). Previous studies on motifs used for binding to the α, γ, β1 and β2 subunits have revealed both similarities and divergence in the consensus binding motifs. SNX9 uses cooperative binding to AP-2 through two motifs in the LC region. Both motifs use large hydrophobic residues to dock onto the ear domains (Lundmark and Carlsson, 2003). We have shown here that SNX18 possesses a similar motif in the LC region. This motif, however, does not mediate binding to the ears of AP-2 (see Fig. 5B). We were intrigued to find that the acidic sequence D[D/E]DWDD[D/E]WDD was involved in γ-ear binding, because the previously described consensus motif for this appendage is [W/F/Y][G/P/D/E] [W/F/Y/L/M] (Mattera et al., 2004), in which, instead of three, there are only two amino acids between the hydrophobic residues. This discrepancy could be due to motif degeneration, or might indicate the presence of a novel binding site on the γ ear. The latter option is appealing because two distinct binding sites have been identified on both the α- and β2-ear domains (Brett et al., 2002; Edeling et al., 2006a; Praefcke et al., 2004; Schmid et al., 2006). In addition, in both SNX9 and SNX18 we found that the sequences upstream of the binding sites are well
conserved and therefore likely to play an essential role in defining the binding specificity.

Because AP-1 has long been known to work together with clathrin for the formation of transport vesicles, it is intriguing that SNX18 was found not to colocalize with clathrin. In accordance with this finding, binding studies in vitro using the LC domain of SNX18 did not reveal any interaction with clathrin (see supplementary material Fig. S1C,D), as was previously found for the LC domain of SNX9 (Lundmark and Carlsson, 2003). It is therefore possible that clathrin is not involved in the formation of endosomal tubules that are positive for both AP-1 and SNX18. Potentially, clathrin might only work together with AP-1 to generate spherical vesicles of a uniform size (e.g. at the TGN), and that tubular extensions do not require (or are even inhibited by) a clathrin coat. If so, depending on what type of carrier is being made, AP-1 might function both with and without clathrin. This dual role has also been suggested for AP-3 (Lubben et al., 2007). In fact, in PACS1–AP-1-mediated endosomal sorting, it is not clear whether clathrin is indeed required. If clathrin does not participate in endosomal carrier formation, this might explain why PACS1 is not enriched in clathrin-coated-vesicle preparations (Lubben et al., 2007).

We have shown here that SNX18 is a membrane-sculpting protein with a function in AP-1-positive BFA-resistant endosomal trafficking. An unexpected finding was that BFA treatment of cells leads to an increased association between SNX18 and AP-1. It has been established that depletion of Arf activity in cells using BFA, or by using dominant-negative Arf constructs, results in the loss of peripheral membrane proteins, including both COPI and AP-1 coat machineries, at the Golgi (Gillingham and Munro, 2007). This has severe consequences for the integrity of membrane compartments, and results in the dispersion of the Golgi into the endoplasmic reticulum and sorting endosomes. No single Arf was shown to be responsible for this dramatic effect, but the combined depletion of Arfs using siRNA was found to replicate this phenotype (Volpicelli-Daley et al., 2005). We detected a BFA-stimulated complex formation between AP-1 and SNX18, which suggests that AP-1 in this case is recruited to membranes independently of Arf, or that a BFA-insensitive guanine nucleotide exchange factor (GEF) stimulates Arf and subsequently AP-1 to associate with endosomes. ARNO and cytohesin are members of the small GEFs that, in addition to the sec7 domain, have a PH domain. The GEF activity of these proteins is dramatically stimulated upon binding to PtdIns(4,5)P2-containing membranes (Casanova, 2007). It is possible that one of these GEFs triggers Arf to recruit AP-1 to endosomal membranes. However, because Arf at the TGN is locked in an unproductive BFA-GEF-Arf complex following BFA treatment, it is unlikely that the same Arf is responsible for the enhanced localization of AP-1 together with SNX18. Therefore, if an Arf protein is involved to promote SNX18-positive budding, it is likely that a BFA-insensitive PtdIns(4,5)P2-stimulated GEF activates a distinct Arf separate from the one activated at the TGN. There is, however, also a possibility that recruitment of different AP complexes to endocytic membranes (contrary to Golgi membranes) do not require a direct binding to Arf. These assembly processes might rely more on specific lipid interactions whereby Arf could influence membrane budding via the regulation of lipid-modifying kinases and phosphatases, as shown for Arf6 (Donaldson, 2003).

The metazoan phylogeny, requiring a gradually more sophisticated endosomal system for cargo sorting, calls for additional transport carriers and peripheral membrane proteins. Consequently, whereas yeast does not express any ortholog to SNX9, insects and worms possess a single version of the molecule, and by the appearance of vertebrates three paralogs emerged with distinct functions in membrane-tubulating processes. In addition to sequence homology and similarity in domain structure, there is also a functional basis to categorize SNX9, SNX18 and SNX30 into a distinct protein subfamily. We propose that, based on the described interactions, the SNX9-family members constitute independent membrane-scission units together with dynamin that are recruited to membrane trafficking events driven by AP complexes. SNX9-family proteins are perfectly equipped for this task via the membrane-tubulating capacity of the PX-BAR domain, and the ability to bind dynamin and promote its GTPase activity.

Materials and Methods
Antibodies
Rabbit (Lundmark and Carlsson, 2003) and chicken antibodies against SNX9, SNX18 and SNX30 were made by immunizing with full-length recombinant protein containing GST, or with the respective SH3 domain. Antibodies were subspotted on 96-well plates, purified using the respective SH3 domain immobilized on columns. Purchased antibodies were: mouse anti-Dyn2 Hudy (Upstate); rabbit anti-Dyn2, mouse anti-clathrin and anti-Arf1 (ABR); mouse anti-PACS1a and mouse anti-EEA1 (BD Transduction Laboratories); rabbit anti-tyr (Cell Signaling); mouse anti-tyr, mouse anti-AP3 (Oxford); rabbit anti-FLAG and HRP-conjugated anti-chicken and anti-mouse IgG (Sigma); mouse anti-aphymaphin 1 (Stress Gene), Alexa Fluor 488, 568, and 647-labeled antibodies (Molecular Probes); and HRP-conjugated anti-rabbit IgG (GE Healthcare).

Cloning and protein expression
IMAGE clones 30341956 (human SNX18) and 4869639 (human SNX30) were purchased from ATCC, amplified with appropriate primers and subcloned into the EcoRI-NorI (SNX18) or BamHI-Xhol (SNX30) of pGEX-6P-2 (GE Healthcare). Coding regions were subsequently inserted into a modified pCMV vector after a myc sequence (MEQKLI SEELI), using respective restriction enzymes. Regions were amplified by PCR (regions corresponding to amino acids 212-624 in SNX18 and 159-574 in SNX30) and subcloned into pGEX-6P-2 or myc-pCMV vector. SH3- and LC-domains were amplified by PCR (SH3 regions corresponding to amino acids 1-67 in SNX18 and 1-69 in SNX30, LC region corresponding to amino acids 67-218 in SNX18) and subcloned into pGEX-6P-2. Plasmid encoding the LC region was further modified by introducing a 6×His sequence at the C-terminus. SNX9 and AP-appendage constructs were as described previously (Lundmark and Carlsson, 2002; Lundmark and Carlson, 2003; Lundmark and Carlson, 2004). cDNA for AP-1 y2-subunit was purchased from Geneservice (AP1G2) and the appendage domain corresponding to amino acids 666-785 was amplified and expressed as GST-fusion protein. The PRD region of human dynamin 2 (amino acids 742-870) was amplified from a cDNA clone (kind gift from Mark McNiven, Mayo Clinic, Rochester, MN) and inserted into pGEX-6P-2. Mutagenesis was done using QuickChange (Stratagene) and all constructs were verified by sequencing. Plasmids encoding GST-aphymaphin-2 SH3 domain, GST-tyr car, and GST-GGA1 and GST-GGA2 GAE domains were kind gifts from Harvey McMahon (MRC Laboratory of Molecular Biology, Cambridge, UK). Proteins were expressed in BL21 plysS cells by IPTG induction and purified on glutathione-Sepharose columns (Lundmark and Carlson, 2005). Removal of GST was made by on-column cleavage with PreScission (GE Healthcare).

Extract preparation, immunoprecipitations and pull-downs
Mouse tissues were homogenized in KSHM buffer (100 mM potassium acetate, 85 mM sucrose, 1 mM magnesium acetate, 20 mM HEPES-KOH, pH 7.4) with protease corresponding to amino acids 666-785 was amplified and expressed as GST-fusion homogenizer. Tissue and cellular debris were pelleted by low-speed centrifugation and the protein concentration in the supernatant was measured with Coomassie Plus (Pierce). Extracts from brain and thymus were delipidated with acetone at -20°C overnight and the obtained protein precipitates were dissolved in sample buffer for SDS-PAGE.

Immunoprecipitations from detergent-lysed HeLa cells (1% NP40 in PBS with protease inhibitors), or from cytosol (see below), were performed with IgY antibodies coupled to Affi-Gel (Bio-Rad), or rabbit antibodies bound to Protein-A-agarose (Sigma), or with mouse antibodies precipitated with rabbit anti-mouse IgG antibodies (Dako) bound to Protein-A-agarose (Lundmark and Carlson, 2002; Lundmark and Carlson, 2005). Pull-downs using GST-fusion proteins bound to glutathione-Sepharose (GE Healthcare) were performed as described (Lundmark and Carlson, 2005). For the pull-down experiment with PRD, the concentration of PRD was adjusted for each GST-SH3 (10 μM) to achieve 20–30% binding for the wild-type
protein, and was 5 µM for SNX9, 10 µM for SNX18 and SNX30, and 20 µM for amphiphysin 2. Input shown corresponds to a 10-µM reaction.

Cell culture, transfections and microscopy
HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum. Cells were grown in six-well plates to 30-50% confluency prior to transfection with Lipofectamine 2000 (Invitrogen) mixed with 0.5-1 µg DNA and continuously grown for 16 hours before analysis. Cells for immunofluorescence were grown on coverslips and fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were washed in PBS prior to blocking and permeabilization in 2% goat serum, 0.1% saponin in PBS followed by incubations with primary and secondary antibodies for 1 hour each. Cells washed-out of cytosolic components before fixation were treated with blocking solution for 1 minute prior to PFA treatment. The cells were visualized using a Zeiss AxioImager Z1 system with AxioVision software and equipped with an Apotome device to allow for enhanced resolution and increased contrast in the focal plane for colocalization experiments.

Cells were treated at 37°C with medium containing 10 µM BFA for 30 minutes, 100 nM wortmannin for 30 minutes, or 10 µM tonicin for 5 minutes, before processing for microscopy. All drugs were from Sigma.

Cell fractionation
A protocol previously described (Lundmark and Carlsson, 2005) was used for cell fractionation. Briefly, HeLa cells were grown to approximately 75% confluency, washed with PBS and released by trypsinization. Cells were washed by centrifugation 3 times in PBS with 1 mM PMSF, and the final cell pellet was resuspended in one cell volume of KSHM containing protease inhibitors. Cells were frozen in liquid nitrogen, thawed in a 37°C water bath and centrifuged at 4°C at 350 g for 5 minutes. The supernatant, which contained the majority of SNX9, SNX18 and SNX30, consisted of cytosol, vesicles and membranes. Separation of the supernatant into a cytosol and a membrane fraction was made by centrifugation at 70,000 g for 30 minutes. Density equilibrium centrifugation of the membrane fraction was performed as described previously (Lundmark and Carlsson, 2003).

Liposome binding and tubulation
Generation of liposomes from synthetic lipids (Avanti polar lipids and Echelon) and total bovine brain lipids (Folch fraction I, Sigma) was performed as described previously (Peter et al., 2004). Liposomes with defined lipid composition contained 10% cholesterol, 55% phosphatidylcholine, 25% phosphatidylethanolamine and 10% phosphatidylserine (designated PS in Fig. 4C). Where indicated, 7.5% of different phosphatidyls inositols was included at the expense of phosphatidylserine. Liposome-binding assays for lipid specificity were performed essentially as described (Pylypenko et al., 2007). Briefly, purified SNX18-PX-BAR (0.7 µM) was incubated with liposomes (0.5 mg/ml in 200 mM NaCl, 25 mM HEPES-KOH, pH 7.4) for 5 minutes at room temperature, followed by centrifugation at 100,000 g for 30 minutes. Sample for electron microscopy was prepared as described (Pylypenko et al., 2007).


Supplementary Figure. (A) HeLa cells were co-transfected to express both FLAG-tagged PX-BAR from SNX9 and myc-tagged PX-BAR from SNX18. After incubation with appropriate antibodies, the cells were visualized by epifluorescence. The boxed area is magnified below. Scale bar is 10μm. Note the high degree of co-localization on the same tubes, which in higher magnification reveals a patchy distribution of the two proteins. (B) Endogenous SNX18 and Arf1 were visualized by immunofluorescence in HeLa cells. The magnifications of the boxed area show SNX18 and Arf1 separately and the merge image reveals minimal overlap between the stains. Scale bar is 10 μm. (C) HeLa cells were stained for endogenous SNX18 and clathrin. Insets show magnification of the boxed sections. SNX18 and clathrin positive puncta do not co-localize. Scale bars are 10 μm. (D) Pull-down experiment with GST and GST-fusion proteins with the LC-domains of SNX18 and SNX9. GST-proteins bound to beads were incubated with clathrin purified from K562 cytosol (Lundmark and Carlsson, 2003). Bound clathrin was analyzed by immunoblotting and densitometry analysis revealed that 20% of input bound to SNX9-LC, whereas no significant binding of clathrin could be detected for SNX18-LC and the GST control. The Coomassie-stained gel shows the GST-proteins. Bands below GST-LC are breakdown products of the fusion proteins.
### Table S1. MALDI-TOF analysis of SNX18 and SNX30. Immunoprecipitated SNX18 and SNX30 were separated by SDS-PAGE (see Fig. 2A), and the major specific bands were trypsinized and subjected to MALDI-TOF analysis. Obtained peptide masses (Submitted) were compared with masses from a theoretical trypsin digest of the respective protein (Matched) by using MS-Fit (http://prospector.ucsf.edu/).