Retromer and sorting nexins in development

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Retromer: conserved function from yeast to higher eukaryotes
4. The large family of sorting nexin proteins – regulation of trafficking and signaling
   4.1. Definition of sorting nexins
   4.2. The functions of sorting nexins
   4.3. Sorting nexins and retromer
   4.4. Role of sorting nexins in signaling
5. Role of retromer during development
   5.1. Retromer’s role in mouse embryogenesis
     5.1.1. Vps26
     5.1.2. Vps35
     5.1.3. Sorting nexins 1 and 2
     5.1.4. Other sorting nexins
     5.1.5. The cation-independent mannose 6-phosphate receptor
     5.1.6. The polymeric immunoglobulin receptor
   5.2. Retromer’s role in Wnt signaling
6. Retromer in disease: the importance of accurate intracellular protein sorting
7. Perspective
8. Acknowledgement
9. References

1. ABSTRACT

Trafficking and signaling processes involve common molecular components. The machinery that controls intracellular trafficking is vital in ensuring that signaling mechanisms take place correctly. An illustrative example of this relationship is the sustained signaling of endocytosed membrane receptors, such as receptor Tyr kinases and G-protein coupled receptors, after ligand-induced activation. An intriguing role in controlling the fate of these and other receptors at the endosome has been attributed to members of the sorting nexin protein family. The best characterized sorting nexins are subunits of a multimeric complex, termed retromer. It was first found in yeast that retromer mediates endosome-to-Golgi retrieval of receptors after they have delivered soluble hydrolase precursors into the vacuole, the organelle equivalent to the mammalian lysosome. Work in cultured mammalian cells later demonstrated that retromer performs an analogous function in higher eukaryotes. Data from genetically modified mice, and from a simpler organism such as the nematode Caenorhabditis elegans, has revealed that retromer performs an essential role during embryogenesis. This review will discuss implications of recent work on this subject.

2. INTRODUCTION

Subcellular organelles contain a distinct set of resident proteins required for the proper functioning of each compartment. The continuous exchange of proteins and lipids between intracellular compartments is largely mediated by transport vesicles, which are formed by the cooperative action of cytosolic protein coats (1). These coats shape the membrane into regions of high curvature and capture cargo proteins for their proper sorting (2-5). Protein sorting and trafficking operate under the regulation of well-conserved mechanisms. In a simple organism such as yeast, a pathway particularly important is protein delivery to the vacuole, the lytic compartment equivalent to the mammalian lysosome. Many Vacuolar protein sorting (Vps) proteins participate in this pathway (6, 7), and a small subset of these are the components of a multimeric protein complex, termed retromer, and composed of five Vps subunits: Vps35p, Vps26p, Vps29p, Vps5p and Vps17p (8, 9).

At the yeast late-Golgi, like in its functional equivalent in mammalian cells, the trans-Golgi network (TGN), specific receptors deliver soluble enzyme precursors to the vacuole. The dissociation of ligand from
Receptor takes place in the acidic environment of an endosomal prevacuolar compartment (PVC), from which the empty receptor must be recycled back to the Golgi for reuse. In yeast, retromer is an essential protein complex that performs endosome-to-Golgi retrograde transport of Vps10p, the receptor for the soluble vacuolar hydrolase precursor pro-carboxypeptidase Y (CPY) (8-11). The role of retromer is conserved in higher eukaryotes with Vps35, as the complex core (23, 24), and Vps29p, which provides cargo recognition (8, 9, 20-22). Retromer assembles in a similar way in higher eukaryotes, with Vps5p and Vps17p, which are in charge of membrane deformation (9, 18, 19), and a second subcomplex, consisting of Vps35p, Vps26p and Vps29p, which provides cargo recognition (8, 9, 20-22). Retromer assembles in a similar way in higher eukaryotes, with Vps35, as the complex core (23, 24), associating directly with cargo (12). Recent structural and binding studies have provided valuable information on the interaction sites among retromer subunits and on how these subunits integrate in the complex (24-26). Orthologs of the yeast VPS5, VPS26, VPS29 and VPS35 genes have been identified in several organisms, including humans (8, 18, 23, 27-29), and data from genetically modified mice sustain that retromer performs an essential role during mammalian embryogenesis (30-33). The findings indicate that these proteins act in the same pathway and provide evidence for the existence of a mammalian retromer structurally similar to the yeast retromer. While the mechanism of retromer function during development awaits further elucidation, recent data in the nematode C. elegans demonstrate the requirement of retromer to generate Wnt proteins competent for long-range signaling during establishment of neuronal polarity and migration (34, 35).

A major goal of this review is to emphasize the role of retromer and SNXs in controlling membrane trafficking, focusing on the implications in animal development.

3. RETROMER: CONSERVED FUNCTION FROM YEAST TO HIGHER EUKARYOTES

The vacuole of the budding yeast Saccharomyces cerevisiae is a central organelle in the physiology of this organism. Among its many roles, the vacuole functions in pH homeostasis and osmotic regulation, protein degradation, and as a storage organelle. To carry out all of these functions, it is essential that the vacuole contains its full complement of proteins, which reach the vacuole through different mechanisms, by pathways originated in the secretory or endocytic organelles, in the cytosol or by inheritance of vacuolar material by daughter cells during cell division. Genetic screens designed to identify components involved in each pathway showed that these pathways are intimately related. Around 60 genes required for proper trafficking to the yeast vacuole have been identified by genetic and biochemical approaches. A great deal of knowledge on these Vps pathways has been obtained from the use of VPS and PEP (peptidase-deficient) mutants displaying different degrees of alteration in trafficking to the vacuole. The VPS mutants were isolated as a collection of mutants that fail to sort pro-CPY or a CPY-invertase fusion protein to the vacuole, secreting the soluble hydrolase precursor because of a block in the vacuolar biogenesis pathway (36-38). The PEP mutants were isolated as being defective in CPY enzymatic activity (39). Subsequent complementation analysis revealed extensive overlap between these two mutant groups (40). These studies altogether demonstrated that the transport of vacuolar hydrolases from the late-Golgi is a key protein pathway to the yeast vacuole (reviewed in 41, 42).

Hydrolase precursors are sorted at the late-Golgi in yeast essentially in the same way as at the TGN in mammalian cells (Figure 1). In yeast, the late-Golgi type I transmembrane protein Vps10p is a major sorting receptor for pro-CPY (8-11). In mammalian cells, sorting is mediated by MPRs, through recognition of a mannose-6-phosphate tag added to the newly synthesized enzymes in the Golgi. There are two distinct MPRs in mammalian cells: the 46 kDa cation-dependent MPR (CD-MPR) and the 300 kDa CI-MPR. Both receptors are type I transmembrane proteins that share some sequence homology in their respective luminal domains (43). MPRs are sorted into clathrin-coated vesicles at the TGN through interactions with Golgi-associated, gamma-ear containing, ADP-ribosylation factor (ARF)-binding (GGA) proteins, which bind to acidic-cluster-dileucine signals in the cytoplasmic tails of the MPRs (44). Vps10p is also sorted at the yeast late-Golgi by clathrin and GGA proteins (45). While Vps10p shares no significant sequence homology with the MPRs, they both perform essentially an identical function in enzyme transport and, like the MPRs, Vps10p rapidly cycles between the late-Golgi and endosomes (10, 11, 46). These similarities emphasize that protein sorting and trafficking pathways are regulated by well-conserved mechanisms (see 16, 42, 47 for reviews).

Analysis of the collection of VPS mutants in Scott Emr's laboratory initially revealed that three mutants, VPS29, VPS30 and VPS35, had the same phenotypes as the VPS10 mutant. Thus, these gene products were likely candidates for mediating Vps10p trafficking. Subsequent experiments indicated that Vps29p, Vps30p and Vps35p are indeed required to retrieve Vps10p from endosomes to the Golgi, since loss of their function resulted in mislocalization of Vps10p to the yeast vacuole from where it could not return to the Golgi (8). Mislocalization of the
Sorting of hydrolase receptors in yeast and in mammalian cells by retromer. Vacuolar or lysosomal hydrolase precursors are sorted by specific receptors at the yeast late-Golgi, which is equivalent to the mammalian trans-Golgi network (TGN). In yeast, Vps10p is a major sorting receptor, while in mammalian cells hydrolases sorting is mediated by mannose-6-phosphate receptors (MPRs). Golgi-associated, gamma-ear containing, ADP-ribosylation factor (ARF)-binding (GGA) proteins are recruited at the late-Golgi / TGN membrane through interaction with ARF and then cooperate with the adaptor protein complex AP-1 to package Vps10p or MPRs into clathrin-coated carriers. Additional accessory proteins are recruited to function in vesicle assembly, release and delivery to the endosomal system (green arrows). Under the acidic environment of the yeast prevacuolar compartment (PVC), or in the mammalian endosome, the hydrolase precursors dissociate from their receptors and follow their fate to the yeast vacuole (Vac), or to the mammalian lysosome (Lys), where they are cleaved to produce their active forms. The empty receptors are then recycled back to the Golgi for reuse (red arrows), undergoing new rounds of hydrolases delivery. In yeast, the retromer complex, formed by the sorting nexins Vps5p / Vps17p and by the Vps35p-Vps26p-Vps29p subcomplex, retrieves Vps10p from endosome-to-Golgi. Retromer assembly and function are conserved throughout evolution, and thus it recycles in a similar manner one type of MPR, the cation-independent MPR (Cl-MPR). Additional budding / sorting mechanisms involved in Cl-MPR retrieval from early or late endosomes, as well as protein complexes described in yeast or in mammalian systems and performing vesicle tethering / fusion at the Golgi apparatus, are omitted for simplicity.

As observed in these early experiments, membrane stripping of retromer with salts partially disassembled retromer, which divided into two subcomplexes. One was composed of the SNXs Vps5p / Vps17p, and formed a stable dimer. The other subcomplex was composed of Vps29p / Vps35p (9). By size-exclusion chromatography, Vps26p eluted predominantly in later fractions, as a monomeric form interacting with Vps35p or with the other subunits in a more transient or more dynamic way (22). Interestingly, this division into subcomplexes mirrors the phenotypic differences between the respective mutant strains. Thus, yeast strains deleted for the \textit{VPS29} or \textit{VPS35} genes are class A mutants, which display morphologically normal vacuoles, whereas deletions of \textit{VPS5} or \textit{VPS17} produce class B mutants, with severely fragmented vacuoles. \textit{VPS26} mutants are class F mutants, and Vps29p were subsequently shown to interact with each other as part of a pentameric complex also including Vps5p, Vps17p and a fifth component, Vps26p (9). For its proposed function in endosome-to-Golgi retrieval, this complex was called “retromer”.

Figure 1. Sorting of hydrolase receptors in yeast and in mammalian cells by retromer. Vacuolar or lysosomal hydrolase precursors are sorted by specific receptors at the yeast late-Golgi, which is equivalent to the mammalian trans-Golgi network (TGN). In yeast, Vps10p is a major sorting receptor, while in mammalian cells hydrolases sorting is mediated by mannose-6-phosphate receptors (MPRs). Golgi-associated, gamma-ear containing, ADP-ribosylation factor (ARF)-binding (GGA) proteins are recruited at the late-Golgi / TGN membrane through interaction with ARF and then cooperate with the adaptor protein complex AP-1 to package Vps10p or MPRs into clathrin-coated carriers. Additional accessory proteins are recruited to function in vesicle assembly, release and delivery to the endosomal system (green arrows). Under the acidic environment of the yeast prevacuolar compartment (PVC), or in the mammalian endosome, the hydrolase precursors dissociate from their receptors and follow their fate to the yeast vacuole (Vac), or to the mammalian lysosome (Lys), where they are cleaved to produce their active forms. The empty receptors are then recycled back to the Golgi for reuse (red arrows), undergoing new rounds of hydrolases delivery. In yeast, the retromer complex, formed by the sorting nexins Vps5p / Vps17p and by the Vps35p-Vps26p-Vps29p subcomplex, retrieves Vps10p from endosome-to-Golgi. Retromer assembly and function are conserved throughout evolution, and thus it recycles in a similar manner one type of MPR, the cation-independent MPR (Cl-MPR). Additional budding / sorting mechanisms involved in Cl-MPR retrieval from early or late endosomes, as well as protein complexes described in yeast or in mammalian systems and performing vesicle tethering / fusion at the Golgi apparatus, are omitted for simplicity.

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Figure 2. Working model for retromer assembly and functioning. Assembly of retromer involves the cooperation of two subcomplexes. One consists of two sorting nexins (SNXs). In yeast, these are Vps5p and Vps17p. In mammalian cells, one is SNX1, the Vps5p ortholog, which could work as a homodimer or heterodimerize with the closely related SNX2, or with a different SNX, such as SNX5 or SNX6 (see Table 1). SNXs dimerize through their C-terminal coiled-coil regions forming a functional banana shaped Bin/Amphiphysin/Rvs (BAR) domain. Both BAR and Phox homology (PX) domains preferentially target the SNXs dimer to tubular endosome microdomains rich in phosphatidylinositol 3-phosphate (PI 3-P), or other 3-phosphoinositides (PIs), hence the requirement of phosphoinositide 3-kinase (PI3K) for proper membrane association of SNXs and for retromer function, which are affected by PI3K inhibitors (inh). It is thought that the SNXs act as a scaffold for the correct membrane association of the Vps35-Vps26-Vps29 subcomplex, although the latter subcomplex probably assembles and associates independently to the membrane. Vps35p serves as the platform for the formation of this subcomplex, provides cargo specificity and associates directly with cargo proteins (referred to here as ‘sorted receptor’). Vps26 may undergo an arrestin-like conformational change, allowing its interaction with a hypothetical transmembrane protein. This would promote or stabilize the association of Vps35 to the membrane, thus affecting Vps35 interaction with the receptor’s cytosolic tail. Vps29, which may also associate with the N-terminal SNX region, seems to have a catalytic role, either upon Vps35, as proposed in yeast, or upon the receptor itself, as suggested from in vitro studies. This activity could serve to promote clustering of the subcomplex or even the receptor’s retrieval process itself.

Remarkably, the mentioned Vps30p was not found to be part of retromer. However, its role in endosome-to-Golgi retrieval was later characterized. It was shown that Vps30p forms a complex with Vps38p, a protein unique in yeast (49). Vps30p / Vps38p bind to a second complex formed by Vps34p, the only phosphoinositide 3-kinase (PI3K) in yeast (50), and its activator Vps15p. This interaction promotes the production of phosphatidylinositol 3-phosphate (PI 3-P). Studies in both yeast and mammalian cells have shown that membrane PI 3-P is recognized by specific phosphoinositide binding motifs found in proteins acting as downstream effectors in the regulation of membrane trafficking (51-54). In this way, spatially restricted synthesis of PI 3-P could concentrate PI 3-P effectors at the membrane, thereby allowing the effector proteins to interact with specific membrane-bound molecules via other protein-protein interaction domains. As members of the SNX family of proteins, Vps5p and Vps17p contain a conserved phagocyte NADPH oxidase or Phox homology (PX) domain that binds to PI 3-P (55) (see Section 4). PI 3-
Retromer in development

P was indeed found required for proper membrane localization of Vps5p / Vps17p (56). As a requisite for efficient sorting function, this interaction likely provides the site for proper membrane location of the Vps35p-Vps26p-Vps29p subcomplex (19, 56). However, a role of PI 3-P or other 3-phosphoinositides on SNXs recruitment remains a matter of controversy (57; reviewed in 58-60), because deletion of Vps34p, or deletion of the PX domain of either Vps5p or Vps17p, only caused a modest effect on Vps5p membrane association (19).

The current view on 3-phosphoinositides requirement for retromer function mainly derives from studies in higher eukaryotes (Figure 2). Thus, we have observed redistribution to cytosol of SNXs 1 and 2 (the mammalian Vps5p orthologs) upon PI3K inhibition, leaving the subunits of the Vps35p-Vps26p-Vps29p subcomplex largely unaffected (61). This effect, however, seems to be more or less striking depending on the PI3K inhibitor used, and while even Vps26p disassociated in some cases (12, 62), SNX1 appeared unaffected in others, with SNX2 concentrating on enlarged early endosomes formed by effect of the inhibitor (63). Nevertheless, colocalization of the two retromer subcomplexes (61) and between each subcomplex and cargo (61, 64) greatly decreases upon PI3K inhibition. Therefore, more than simply a redistribution of SNXs to the cytosol, it seems clear that the primary role of PI3K on SNXs is to determine their proper membrane location, which is affected by depletion of PI 3-P from membranes. Under these circumstances, the Vps35p-Vps26p-Vps29p subcomplex, although efficiently recruited to the membrane, becomes unable to cluster cargo for its efficient sorting from appropriate locations that must be defined by the SNXs subcomplex (61).

Recent findings provide evidence that assembly of each retromer subcomplex is regulated independently. Thus, SNX1, or the closely related SNX2, have not been found in co-immunoprecipitated Vps35p-Vps26p-Vps29p subcomplexes (14, 61, 62). In agreement with these data, SNX1 was not detected in pull-down assays of mammalian Vps29p (24). Moreover, antisense depletion of both SNX1 and SNX2 did not prevent targeting of Vps26 to membranes (65), although it did in other cases (62), perhaps depending on the endogenous levels of SNXs. In a reciprocal way, knocking down Vps35p, Vps26p or Vps29p (12-14, 62, 66) depletes the whole Vps35p-Vps26p-Vps29p subcomplex but it does not affect the levels of SNX1 or SNX2. In spite of these negative data, it has been demonstrated, by immunoprecipitations performed with exogenously expressed proteins, and by yeast-two hybrid assays suitable for detecting weak interactions, that SNX1 (23, 62, 63) as well as SNX2 (62) physically interact with the Vps35p-Vps26p-Vps29p subcomplex. This suggests that the association between these subcomplexes is transient and dynamic. Alternatively, it is possible that different SNX family members associate more strongly than SNX1 / SNX2 with the Vps35p-Vps26p-Vps29p subcomplex in some systems. In this regard, a very recent work demonstrates that SNX5 and SNX6 may constitute functional equivalents of the yeast Vps17p and therefore they emerge as potential components of the mammalian retromer (67) (see Section 4).

From the current data in yeast and in mammalian cells, a general model of possible retromer functioning can be envisaged (Figure 2). By this model, SNXs are preferentially targeted, via their Bin/Amphiphysin/Rvs (BAR) and PX domains, to tubular endosome microdomains rich in 3-phosphoinositides (57, 68). SNXs would thus act as a scaffold for association of the Vps35p-Vps26p-Vps29p subcomplex, which then selects cargo for sorting based on the geometric efficiency of these tubular elements (68). Correct membrane association of the Vps35p-Vps26p-Vps29p subcomplex requires of SNXs (61, 62), which does not exclude that interactions between the two subcomplexes already take place in the cytoplasm (23). Vps29p appears important to mediate the interaction between these subcomplexes, at least in the yeast retromer (22). Vps26p structure reveals an arrestin fold (26). Similarly as arrestins binding to G-protein coupled receptors (GPCR) to regulate receptor endocytosis and signaling (69), Vps26p may undergo a conformational change, allowing its interaction with a putative multipass transmembrane protein. Thus, Vps26p appears involved in targeting Vps35p to the membrane (26). Once at the membrane, Vps35p associates directly with cargo (12, 21). Vps29p structure has also been revealed and it has a phosphoesterase fold with no apparent enzymatic activity (24, 25). Recent in vitro data suggest, however, that receptor dephosphorylation by Vps29p may be involved in the endosome-to-Golgi retrieval process (70).

The similarity in the sequences and interactions among orthologs of retromer subunits quickly suggested that retromer function was conserved in higher eukaryotes (23, 71-73). Indeed, it was found that retromer mediates endosome-to-Golgi sorting of the mammalian CI-MPR (12, 13, 15, 16). The study of retromer role in various model organisms has shown, however, that retromer plays additional roles in protein sorting along the endocytic pathway. These include the following. First, a retromer-like complex in the enteric protozoan parasite Entamoeba histolytica, composed of Vps35, Vps26 and Vps29, regulates transport to phagosomes of cysteine proteases by interaction with the small GTPase Rab7A and probably an as-yet-unidentified protease receptor(s) (74). It is important to point out that this microorganism lacks the CI-MPR, as well as the tail-interacting protein of 47 kD (TIP47) and Rab9 (74, 75), both of which have also been involved in late-endosomal retrieval of the CI-MPR (76). Secondly, Vps35, as part of an analogous retromer complex in plants, interacts with the vacuolar sorting receptor (VSR) (64), a molecule similar to Vps10p and the mammalian Vps10-domain protein sortilin (10, 77). While Vps35 and VSR colocalized on multivesicular bodies (MVB), functional evidence for a role of the plant retromer in VSR sorting was not provided in this study (64). Nevertheless, Arabidopsis thaliana Vps29 was found to function in the VSR retrograde transport needed for sorting of storage protein precursors during seed maturation (78). In addition, another report demonstrates the role of A. thaliana SNX1 in endosomal trafficking of the phytohormone auxin, implicating retromer in growth and developmental processes in plants (79). Third, we have shown that the Vps35p-Vps26p-Vps29p subcomplex associates with the pIgR
in a post-endocytic step and promotes transcytosis of pIgR and its ligand pIgA in polarized epithelial cells (14). Fourth, Vps26B, a Vps26 paralog, was shown to interact with Vps35 and integrate in the retromer complex (80). Unlike the original Vps26 (referred to as Vps26A in this report), Vps26B does not associate with endosomes, and instead is targeted to the plasma membrane, becoming concentrated at actin-rich lamellipodia, which intriguingly also contain some Vps26A and Vps35 (80). The possibility that this cell surface pool of retromer is playing a role in cell migration and/or adhesion clearly extends retromer functions beyond its originally proposed action in the endosomal system. Finally, a role of retromer has been proposed for regulating endosome-to-Golgi traffic of endosomal system. These are domain-specific, proteins participating in MPR trafficking were identified (29). Soon after, it was demonstrated that receptors is well-conserved from yeast to man (12, 13, 15, 35), provide strong evidence that retromer performs an essential role during embryonic development that is independent of MPR trafficking (33). These aspects will be discussed in detail in Section 6.

Most of the reports discussed above were based on data from cell culture systems. Data from genetically modified mice (30-33), and more recent publications using transgenic and deletion mutant strains of C. elegans (34, 35), provide strong evidence that retromer performs an essential role during embryonic development that is independent of MPR trafficking (33). These aspects will be discussed in detail in Section 5.

4. THE LARGE FAMILY OF SORTING NEXIN PROTEINS – REGULATION OF TRAFFICKING AND SIGNALING

Receptor sorting at the endosomal compartment is mediated by interactions of the receptor’s cytosolic domain with peripheral membrane proteins or protein complexes. As we have discussed in the previous section, a remarkable example of these sorting events is performed by the retromer complex. Given the many potential trafficking itineraries through the complex endosomal pathway (16, 86), it has been proposed that receptor’s fate is governed by its ability to interact with, or to avoid, a number of these endosomal sorting complexes. In an attempt to understand further endosomal retrieval of hydrolases receptors in higher eukaryotes, adaptor complexes and adaptor-like proteins participating in MPR trafficking were identified and studied. Initially, the findings pointed to important mechanistic differences in endosome-to-Golgi transport between yeast and mammals (47, 76). Alternatively, they suggested the existence of evolutionarily conserved components of the MPR retrieval machinery that remained to be identified. Soon after, it was demonstrated that retromer function in endosome retrieval of hydrolase receptors is well-conserved from yeast to man (12, 13, 15, 16). Of the five Vps protein subunits composing the retromer complex, mammalian orthologs for the yeast Vps5p, Vps26p, Vps29p and Vps35p have been identified (8, 18, 23, 27-29). Except in fungi, no ortholog of the SNX protein Vps17p has been found in higher eukaryotes (23, 87). Thus, both SNX1 and SNX2, as Vps5p orthologs, can assemble as homodimers or heterodimers (62), although it is possible that a different SNX heterodimerizes with SNX1 or SNX2 in the retromer (23, 88, 89), as proposed recently for SNX5 and SNX6 (67).

The screening for protein interactions has proven an excellent way of identifying components with a functional relevance. Thus, the numerous screenings performed to identify proteins associated with the cytosolic tail of endosomal receptors, or with other proteins involved in controlling membrane traffic and differentiation, has led to an increasing understanding, and at the same time complication, of the role played by SNXs in higher eukaryotes (90-105). In view of their involvement in protein sorting events, the SNX family of proteins has attracted considerable attention as potential candidates for determining protein itinerary in different cell types and development conditions. It is important to remark that their role in controlling protein export from endosomes potentially contributes to modulate signaling, perhaps after activated receptors have been internalized (59, 106-108). The essential role of SNXs in development has been revealed by gene-deletion experiments in mice, demonstrating the genetic interaction of SNXs 1 and 2 with retromer (33). The implication of SNXs in diverse trafficking pathways has been further illustrated by the recent description of the SNX13 knockout phenotype in mouse, which is also embryonic lethal (109).

4.1. Definition of sorting nexins

The reader is referred to references 58-60 for comprehensive reviews on this large protein family, which currently includes 10 members in yeast (59) and 30 in humans (110), plus other 17 PX domain-containing human proteins of related structure albeit diverse functions (60). The SNXs are defined in part by the presence of a PX domain belonging to a subgroup of the PX domain superfamily and with at least 50 % similarity to the PX domain of SNX1 (111). The SNXs are oligomeric proteins found distributed between membranes and cytosol, and the different family members contain a variety of protein-protein and protein-lipid interaction domains in addition to their PX domain. One is the BAR domain, present in one third of the SNXs. By this definition, it appears that several members included in the family are not involved in cargo-sorting, and instead they have a role in oxidative processes, cytoskeleton arrangement or protein translocation. Therefore, they are not expected to be related to any process controlled by retromer, or even to any other membrane trafficking associated mechanism, and thus some proteins may have been erroneously included in the SNX group. Thus, it has been suggested that the criteria for defining a protein as a SNX require revision and that only those with a BAR domain and a few others should actually belong to this group (59).
Figure 3. Functions of sorting nexins in yeast and in mammalian cells. The image shows major trafficking pathways where SNX family members have been involved in yeast or in a nonpolarized mammalian cell. Newly synthesized proteins are first sorted in the late-Golgi / trans-Golgi network (TGN) for delivery to the plasma membrane (dark green arrows). Proteins, such as a transmembrane receptor bound to its ligand (green trapezoid), can be endocytosed from the plasma membrane (orange arrows) via clathrin coated pits (CCP) and become incorporated into endocytic vesicles (end. ves.). From the post-Golgi endosome (PGE) / early-sorting endosome, a possible pathway involves ligand dissociation and receptor recycling back to the cell surface (yellow arrows) by recycling vesicles or tubules (recycl. ves.). Alternatively, endocytosed receptors and / or ligands can be degraded in the vacuole (Vac) / lysosomes (Lys; purple arrows) by the action of hydrolases (red trapezoids). In their precursor form, the hydrolases are also delivered by specific receptors to the endosomal system (light green arrows). These receptors are then recycled back to the Golgi for new rounds of enzyme delivery (red arrows), either from the PGE / early-sorting endosome or from the prevacuolar compartment (PVC) / late endosome-multivesicular body (MVB). This pathway is also followed by other integral membrane proteins that are retrieved to the Golgi. According to studies in yeast, the group of Snx4p/41p/42p (SNX4 orthologs) mediates retrieval from the PGE independently of retromer, while retromer and associated SNXs mediate retrieval from the PVC; Snx4p can also associate with retromer at the PVC. It is not known whether this applies to mammalian cells, where SNX1 and SNX2 are the Vps5p orthologs, while there is no ortholog for Vps17p, and SNX5, SNX6 or another SNX work in place of Vps17p in retromer action. Grd19p and Mvp1p are, respectively, the SNX3 and SNX8 orthologs and their possible integration in retromer for endosome-to-Golgi retrograde transport has not been reported. The SNXs color code matches that of the pathway where they have been implicated. A question mark is placed where the evidence is not solid.

The PX domain acts as phosphoinositide-binding motif that contributes to the localization of SNXs at membrane domains enriched in 3-phosphoinositides, such as PI 3-P (57). Since PI 3-P is found throughout membranes of the endocytic-lysosomal pathway (51), it seems plausible that other components, or other sequences apart from the PX domain, determine the precise localization of individual SNXs. Interestingly, it was observed that the PX domains of certain SNXs do not share some key residues, i.e. Arg58 and Arg105, that are essential for PI 3-P binding in p40phox, the prototype of PX domain proteins (58), and that are conserved in SNX1 (57). One example is SNX5, which binds to other phosphoinositide lipids, and hence to specific membrane subdomains, may help to explain the various physiological roles of the SNX family members. In addition, membrane association of SNXs may be further strengthened by stabilization from protein-protein interactions through adjacent domains and by the BAR domain itself. The BAR domain, located at the C-terminus in some SNXs, is a dimerization and membrane binding module. As sensor of membrane curvature, it provides an explanation for how membrane geometry can regulate protein localization (114). The localization of SNXs to tubular elements of the sorting endosome containing PI 3-P is consistent with their BAR domain functioning as a binding-module and curvature-sensor. Thus, the SNXs would function as a coincidence detector in a way that, although several proteins associate with endosomes through PI 3-P, the SNXs becomes preferentially enriched in endosomal microdomains defined by high membrane curvature and the presence of PI 3-P or other 3-phosphoinositides (68). The ability to sense membrane curvature may contribute to retrieval of the CI-MPR or other transmembrane proteins into elongated membrane and budding vesicles. This hypothesis of coincidence detection can probably be applied to any BAR domain containing SNX, including SNX9 in its cooperation with dynamin for clathrin-coated vesicle budding at the plasma membrane (105, 115). Thus, while the BAR domain targets SNX homo or heterodimers to the membranes, the PX domain is necessary for proper membrane / endosomal localization (116). How both the PX and BAR domains are coordinated for SNX membrane targeting is not yet clear.

4.2. The functions of sorting nexins

In early studies, it was found that none of the members of the growing SNX family contained putative signal peptides or transmembrane domains (111), although an N-terminal short region of hydrophobic residues now suggests the presence of a transmembrane domain in three SNXs (60). It was also found that many SNXs have 1-3 stretches of 21 or more aminoacids within their C-terminal region that can form coiled-coil structures (111), later predicted to form BAR domains (65). Coiled-coil domains, as potential sites for protein-protein interactions, have been established as crucial for the function of other membrane transport proteins, such as syntaxins (117). Given their strong predisposition to form coiled-coil mediated protein complexes, SNXs emerged as possible candidates to control a number of membrane trafficking pathways or events. The findings showing that they self-associate forming homodimers or heterodimers (23, 118), and their differential interaction with transmembrane receptors in mammalian cells (88, 90), further increased the interest of these proteins as putative regulators of protein transport performing specific sorting roles depending on cell type and development stage (Figure 3; Table 1).
Retromer in development

<table>
<thead>
<tr>
<th>Sorting nexin</th>
<th>Pathway</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNX1</td>
<td>Endosome-to-Golgi retrieval of CI-MPR (retromer)</td>
<td>23, 62, 67, 68</td>
</tr>
<tr>
<td></td>
<td>Downregulation of EGFR and other Tyr kinase receptors</td>
<td>63, 88, 90, 98, 134</td>
</tr>
<tr>
<td></td>
<td>Downregulation of the GPCR PAR-1 (independent of retromer)</td>
<td>66, 120</td>
</tr>
<tr>
<td></td>
<td>Transferrin receptor pathway</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Macropinosome maturation</td>
<td>228</td>
</tr>
<tr>
<td>SNX2</td>
<td>Endosome-to-Golgi retrieval of CI-MPR (retromer)</td>
<td>62, 65</td>
</tr>
<tr>
<td></td>
<td>Downregulation of EGFR and other Tyr kinase receptors</td>
<td>63, 88</td>
</tr>
<tr>
<td>Vps5p</td>
<td>Endosome-to-Golgi retrieval of Vps10p, Kex2p, DPAP A and Pep12p at the PVC (retromer)</td>
<td>8, 9, 18, 19, 29, 132</td>
</tr>
<tr>
<td>Vps17p</td>
<td>Endosome-to-Golgi retrieval of Vps10p (retromer)</td>
<td>9, 19</td>
</tr>
<tr>
<td>SNX3</td>
<td>Endosome recycling</td>
<td>121, 141</td>
</tr>
<tr>
<td>Grd19p</td>
<td>Endosome-to-Golgi retrieval of Kex2p, DPAP A and Pep12p, but not Vps10p, at the PVC (retromer?)</td>
<td>132, 229</td>
</tr>
<tr>
<td>SNX4</td>
<td>Endosome vesicles fate</td>
<td>97</td>
</tr>
<tr>
<td>Snx4p/41p/42p</td>
<td>Retrieval of Snx1p at the PGE (independent of retromer)</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm-to-vacuole pathway at pre-autophagosomes</td>
<td>133</td>
</tr>
<tr>
<td>SNX5</td>
<td>Endosome-to-Golgi retrieval of CI-MPR (retromer)</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>EGFR downregulation</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>EGFR signaling at the plasma membrane</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Macropinosome maturation</td>
<td>228</td>
</tr>
<tr>
<td>SNX6</td>
<td>Endosome-to-Golgi retrieval of CI-MPR (retromer)</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Trafficking of TGF-β family of receptor Ser-Thr kinases</td>
<td>119</td>
</tr>
<tr>
<td>Mvp1p</td>
<td>Endosome-to-Golgi retrieval of Vps10p and Kex2p at the PVC (retromer?)</td>
<td>230, 231</td>
</tr>
<tr>
<td></td>
<td>Endosome-to-plasma membrane recycling of the GSE complex at the PVC (independent of retromer?)</td>
<td>17, 232, 233</td>
</tr>
<tr>
<td>SNX9</td>
<td>EGFR downregulation</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Regulation of dynamin assembly and clathrin-mediated endocytosis</td>
<td>105, 115</td>
</tr>
<tr>
<td></td>
<td>Trafficking and signaling of insulin receptors</td>
<td>234</td>
</tr>
<tr>
<td>SNX10</td>
<td>Endosome homeostasis</td>
<td>142</td>
</tr>
<tr>
<td>SNX13</td>
<td>EGFR downregulation</td>
<td>122, 123</td>
</tr>
<tr>
<td>SNX15</td>
<td>Association to retromer?</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>Protein sorting to the MVB by interaction with the ESCRT-III complex?</td>
<td>235</td>
</tr>
<tr>
<td>SNX16</td>
<td>EGFR downregulation</td>
<td>124, 125</td>
</tr>
<tr>
<td></td>
<td>PI 3-P signaling effector in endosome-to-cytosol export of viral nucleocapsids</td>
<td>126</td>
</tr>
<tr>
<td>SNX17</td>
<td>Endocytosis and/or recycling of LDLR family members (LDLR, VLDLR, ApoER2 and LRP)</td>
<td>96, 100, 129</td>
</tr>
<tr>
<td></td>
<td>Endocytosis and degradative pathway of P-selectin</td>
<td>130, 131</td>
</tr>
</tbody>
</table>

Abbreviations: ApoER2: ApoE receptor 2; CI-MPR: cation-independent mannose 6-phosphate receptor; DPAP A: dipeptidyl aminopeptidase A; EGFR: EGFR receptor; ESCRT: endosomal sorting complex required for transport; GPCR: G-protein coupled receptor; Grd: Golgi retention defective; GSE: GTPase-containing complex for Gap1p (general amino-acid permease) sorting in the endosomes; Kex2p: killer expression-2; LDLR: LDL receptor; LRP: LDLR-related protein; MVB: multivesicular body; Mvp1p: Vps1p-interacting protein; PAR-1: protease activated receptor-1; Pep12p: syntaxin, formerly Vps6p; PGE: post-Golgi endosome; PI 3-P: phosphatidylinositol 3-phosphate; PVC: prevacuolar compartment; Snc1p: suppressor of the full allele of CAD-1; SNX: sorting nexin; TGF-β: transforming growth factor-β; VLDLR: VLDL receptor; Vps: Vacular protein sorting; Yif1p: Yip1p (yeast Golgi integral membrane protein)-interacting factor

Thus far, the retromer subunit which identification in mammals has led to a deeper investigation is SNX1, the Vps5p ortholog (18, 29). SNX1 was first shown to interact with the cytosolic domain of the EGF receptor (EGFR) (90). Overexpression of SNX1 increased the receptor’s turnover, leading to the suggestion that SNX1 is required for endosome-to-lysosome transport and downregulation of the EGFR and probably other receptor Tyr kinases (90). Further work in the field extended this intriguing role of SNX1 throughout the SNX family of proteins and it was proposed that they might modulate signals sent by receptors, perhaps after they have been internalized, which suggests that SNXs could act as truly signaling platforms (59). In this regard, SNX1, SNX1A (a presumed splice variant of SNX1), SNX2 and, to a lesser extent, SNX4 were reported to interact with the receptor Tyr kinases for EGF, the platelet-derived growth factor and insulin (88). Another example is SNX6, which interacts with the transforming growth factor-β (TGF-β) family of receptor Ser-Thr kinases and interferes with their signaling pathways (119). Moreover, SNX1 also associates with the protease activated receptor-1 (PAR-1), a GPCR for the coagulant protease thrombin (120). Here, overexpression of the C-terminal domain of SNX1, which includes its coiled-coil domains, specifically inhibited agonist-induced degradation of PAR-1, thus involving SNX1 in PAR-1 downregulation (120). Other SNXs involved in EGFR downregulation include SNX3 (121), SNX5 (113), SNX9 (95), SNX13 (122, 123), and SNX16 (124, 125). The whole picture becomes complicated when observing at the multiple effects described on EGFR trafficking upon overexpression of wild-type or mutant forms of these SNXs. For instance, in one study SNX5 overexpression inhibited EGFR degradation (113), while in another report it ensured that the receptor becomes quickly available at the cell surface (112). EGFR degradation was also inhibited in cells overexpressing SNX3 (121), and ligand-dependent degradation of EGFR was delayed in transfected SNX13 cells (122). On the contrary, EGFR degradation was accelerated in SNX16 overexpressing cells (125), although only the SNX16 coiled-coil deletion mutant, and not the wild-type form, had such effect, as reported by a different group (124). Finally, while overexpression of SNX9 alone had no effect on EGFR degradation, EGFR degradation was promoted upon co-expression of a cooperative SNX9 partner, the activated Cad42-associated kinase-2 (95). In
summary, while it is clear that SNX1 and other SNXs are involved in endosomal sorting of signaling receptors, how this process takes place remains to be elucidated. A possible mechanism could be based on SNXs acting as 3'-phosphoinositide effectors in processes of endosome dynamics. This has been shown for SNX16, which appears required for back fusion of internal vesicles in late endosomes, a process utilized by certain viral nucleocapsids to move from endosomes to cytosol (126, 127).

To the above discussion, one could add the studies on the well-characterized SNX17, to which a number of interacting partners and roles have been ascribed. For such promiscuity are probably responsible a truncated band 4.1-ezrin-radixin-moesin (FERM) domain, a feature unique in the SNX family (except for the uncharacterized SNX31), and a C-terminus lacking the BAR or coiled-coil domains, which maintain the protein in oligomeric form (60, 128). SNX17 roles include binding and modulation of endocytosis and/or recycling of LDL receptor (LDLR) family members, such as the receptors for LDL and VLDL, the ApoE receptor 2 (ApoER2) and the LDLR-related protein (LRP) (96, 100, 129). SNX17 also interacts with the endothelial cell receptor P-selectin (responsible of leukocyte recruitment at the sites of inflammation) to regulate its internalization and degradation (130, 131). Finally, SNX17 has been shown to associate with krev1 interaction trapped 1, a protein involved in integrin signaling during cell adhesion and pathogenesis of an inherited vascular disorder known as cerebral cavernous malformation (128).

Studies in yeast, taking advantage of the relatively easy genetic manipulation of this simple eukaryote, have provided a simpler picture of the SNXs role in membrane trafficking. In general, SNXs in yeast participate in the recycling of certain membrane proteins, preventing them from reaching the vacuole. While some SNXs act, in concert with retromer, in the PVC, others operate independently in the so-called post-Golgi endosome (PGE) (132). A slight complexity worth mentioning here is the presence of the Snx4p group members (Snx4p/41p/42p) on specific perivascular structures called pre-autophagosomes, organelles distinct albeit probably derived from endosomes (132); in pre-autophagosomes, the Snx4p group plays a role in the cytosol-to-vacuole pathway, induced under nutrient starvation (133). In addition, a recent study found yeast Snx4p associated with retromer at the PVC (85), although previous evidence indicated that it functions in a retromer independent way at the PGE (132).

In contrast to the apparent simplicity found in the yeast model, mammalian SNXs have been suggested to function in internalization, pro-degradative sorting, endosomal recycling, or simply in endosomal sorting (58-60), as discussed above (Figure 3; Table 1). Since most of the evidence for SNXs function in mammalian cells derives from overexpression experiments, and antisense approaches have often given contradictory results, some of the roles proposed for members of this large family remain under debate. To illustrate this point, it is worth mentioning that antisense knockdown of SNX1 had no effect on EGFR degradation (63, 68) or on transferrin receptor trafficking (68). This contrasts with findings from previous studies utilizing overexpression approaches in which SNX1 (90, 134) or other SNXs (95, 113, 121, 122, 124, 125) were implicated in EGFR downregulation. According to others, however, overexpression of SNX1, SNX2, SNX3, or SNX4 did not increase the turnover of EGFR or insulin receptor (88). These contradictory results may be due to the disruption of a protein complex by excess amounts of one of its subunits upon overexpression, or to the titration of other interacting proteins by the overproduced component. Therefore, they may reflect the common difficulty of finding out whether overexpression has a positive or a dominant-negative effect. These discrepancies have lead to the suggestion that the apparent differences in SNXs functions between yeast and mammalian cells could be accounted for by differences in experimental approaches rather than by the notion that these proteins really display more varied properties in higher eukaryotes (135).

Does the interaction with SNXs determine receptor segregation into endosome tubules? It was once proposed that recycling is the default pathway for internalized membrane proteins. According to this idea, endosomal sorting involves repeated fusion of endocytosed vesicles with the endosomal compartment, which simultaneously exports components for recycling and accumulates material for degradation. This process leads to a greater accumulation in the compartment’s lumen of ligands directed to the lysosome than receptor-ligand complexes destined for recycling, which remain at the membrane and, given the larger surface-to-volume ratio in endosome tubules, they accumulate relatively more in these. Such iterative fractionation process would provide a simple and efficient mechanism for recycling proteins and lipids back to the cell surface. Therefore, it was suggested that membrane proteins require specific signals to be routed to lysosomes, rather than to recycle (136). Indeed, biochemical analysis of various endocytic receptors has shown that these were nonselectively incorporated into endosome tubules for recycling or transcytosis, although receptor segregation was evident in the MVB en route to lysosomes (137). In this nonselective model, the geometry of the endocytic compartment, as well as its fusion and budding properties, must have a major influence on controlling efficiencies and rates of sorting membrane components from the luminal fluid phase (138). This scheme represented an alternative to the model of active segregation of receptors into endosome tubules, from which receptors could either be included or excluded (139). Here, alterations in the phospholipid composition, or changes in the physical properties of membranes caused by lipid modifications, membrane associated proteins or the cytoskeleton, were mechanisms proposed to explain such receptor segregation (139). Thus, it was later suggested that several proteins containing lipid-binding domains might function in organizing functional regions of endosome membranes; these include proteins containing phospholipid-binding domains, such as the FYVE motif, or components of cholesterol and sphingolipid-rich raft
microdomains, but also the SNXs, acting through their PX domains (140). The observations that overexpression of SNX3 (121, 141), SNX10 (142) or SNX15 (143) alters endosome morphology and perturbs the endocytic pathway in general is clearly suggestive that members of this family could perform both specific and general roles along membrane networks. Thus, a consensual feature of SNXs function seems to be the regulation of membrane trafficking for endosomal protein export (Figure 3; Table 1).

4.3. Sorting nexins and retromer

SNX1, and the closely related SNX2, are the mammalian orthologs of yeast Vsp5p. In the absence of a Vps17p ortholog, it was initially proposed that SNX2 or another SNX plays the role of Vps17p in higher eukaryotes (23). Given the high sequence similarities among several of the SNX members, in particular in their common PX domains (58-60), and their somehow analogous role in controlling receptors sorting, it is plausible that some other SNX interacts with the Vps35-Vps26-Vps29 subcomplex to perform specific functions in mammalian cells. Indeed, a recent study provides evidence that SNX5 and SNX6 are potential retromer subunits. In a loss-of-function screen, each one of the 30 SNX members was antisense targeted. Affected retromer function was determined by looking at the relative percentage of cells with CI-MPR dispersed in peripheral early endosomes, as opposite to TGN localized. Suppression of either SNX5 or SNX6 gave the strongest phenotype, far more dramatic than that observed by SNX1 depletion and, interestingly, SNX2 depletion did not produce significant changes (67). Further morphological and biochemical analysis showed colocalization of both SNX5 and SNX6 with SNX1 on early endosomes, but only SNX6 physically interacted with SNX1 (67), in apparent contrast to the association of SNX5 with SNX1 reported by others (113). In addition, because depletion of SNX5, but not of SNX6 or SNX1, induced fragmentation of the Golgi complex, the authors proposed that the alteration in CI-MPR distribution seen upon SNX5 depletion could be a consequence of a more general defect in endosome and Golgi trafficking (67).

The study discussed above did not provide data on interaction between SNX5 or SNX6 and the Vps35-Vps26-Vps29 subcomplex, as seen for SNX1 (23, 62, 63) as well as for SNX2 (62). However, it is noteworthy to point out that a link between retromer and SNXs 1 and 2 is not evident in all studies. Various reports have in fact suggested an independent regulation of retromer and SNX1 or SNX2. In analogy to the link between SNX1 and the EGFR (90), SNX1 also plays a role in agonist-induced PAR1 degradation independently of the Vps35-Vps26-Vps29 subcomplex (66). A different localization of SNX1 and SNX2 has also been reported, with SNX2 localizing mostly at early endosomes, while SNX1 was found associated to small vesicles distributed throughout the cytoplasm and devoid of the early endosome marker EEA1 (63). In contrast, another study found extensive colocalization of SNX1 and SNX2 on tubular elements of the early endosomes (65). Contrary to SNX1 (68), SNX2 was found not essential to mediate endosome-to-TGN transport of the CI-MPR (65), which again suggested that a different SNX heterodimerizes with SNX1 in mammalian cells (89, 143). Nonetheless, a partial colocalization of SNX2 with subunits of the Vps35-Vps26-Vps29 subcomplex appears clear in various studies (12, 61, 62, 65). Moreover, antisense depletion of both SNX1 and SNX2 was needed to affect markedly levels and intracellular distribution of the CI-MPR (62). Further evidence showing the relevance of SNX2 in retromer comes from genetic studies in mice. Mice lacking SNX1 or SNX2 are viable and fertile, whereas embryos deficient in both proteins arrest at midgestation. The observed phenotypes indicate that these two proteins act redundantly, but they perform a necessary function in mouse development (32). Interestingly, the phenotype of Snxl−/− Snx2−/− embryos is very similar to that of embryos lacking Vps26, a protein that in the initial studies in mice development was named Hβ58 (30, 31). These genetic experiments in mice suggest that SNX1 / SNX2 and Vps26 function in the same pathway. Considering the high similarity between these SNXs, the presence of both proteins may provide an evolutionary advantage in mammals. Recent data also indicate that SNX2 is surprisingly more critical than SNX1 for retromer function during development, although SNX1 participates in some retromer activity (33). Intriguingly, the localization and stability of CI-MPR was unaltered in mouse embryonic fibroblasts (MEF) (33), contrary to what others found in SNX1 or Vps26-antisense depleted HeLa cells (12, 13, 68) or in undifferentiated embryonic stem (ES) cells depleted for Vps26 (13). Finally, Snxl−/− embryos have recently been generated. While their embryonic lethal phenotype overall resembles that of the Snxl−/− Snx2−/− embryos, differences in organelle ultrastructure and in the distribution of endosome-lysosomal markers were seen in cells of extraembryonic tissue. This study supports the idea that SNXs play different roles in membrane traffic (109), without excluding their interaction with retromer. (These results will we discussed in more detail in Section 5.)

4.4. Role of sorting nexins in signaling

Retromer retrieves the CI-MPR from endosomes back to the Golgi in mammalian cells (12, 13, 15, 16). Retromer also associates with plgR (14), which transports plgA across polarized epithelial cells by a mechanism coupled to a signaling pathway (144). Manipulation of Vps35 levels, along with morphological evidence, showed that retromer plays a role in a post-endocytic compartment, promoting transcytosis of plgR and its ligand IgA, thus preventing plgR-plgA degradation (14). The accumulated evidence sustains that retromer retrieves receptors from an endosomal compartment, thus preventing their degradation. This “rescue” mechanism carried out by retromer seems a very efficient system by which certain receptors could prevent their mis-sorting to lysosomes and degradation. Several reports discussed above implicate SNXs in controlling traffic of receptors involved in cell proliferation processes. Hyperactive receptor signaling leads to deregulation of cell growth control and to alteration of developmental programs, with the consequent initiation of malignancy. Their intracellular pathways, towards degradation or recycling, therefore play a key role in
processes of cell growth control, as proliferation or apoptosis, and thus in important human diseases, including cancer (see 107, 108, 145 for reviews). Considering the interactions described, retromer and SNXs might regulate the cell response to signaling factors and thus become implicated in mechanisms of cell proliferation.

Taking the picture as a whole, the contribution of retromer and SNXs to the control of protein trafficking should not be considered separately. By controlling receptor sorting, retromer and SNXs necessarily play an important role in maintaining the cellular equilibrium. Therefore, one could speculate that a high activity of retromer / SNXs contributes towards blocking downregulation, and therefore degradation, of signaling receptors in situations where the cell must keep a proliferative state. This presumably takes place, in a generalized way, in early stages of the embryogenesis. Likewise, excessively active retromer / SNXs may indirectly induce the cell to uncontrolled proliferation by preventing that proliferative signals are turned off. The overall effect could largely be analogous to the overgrowth and tumors observed in animal models and in humans by mutations in class E VPS orthologs (146, 147). In this latter case, however, the impairment is instead in the machinery required for receptor downregulation, i.e., in components of the endosomal sorting complex required for transport (ESCRT) machinery. With these considerations, it would be interesting to test the possibility that molecules interconnecting retromer / SNXs with signaling pathways control mechanisms of cellular proliferation. Hypothetically, this link might lead to the finding of potential therapeutic targets towards which drugs could be directed to treat diseases affecting cell proliferation or development.

5. ROLE OF RETROMER DURING DEVELOPMENT

The phenotype analysis of null mutations, as well as knockdowns obtained by antisense approaches, has established the key role of various Vps proteins during embryogenesis in mice (148-150), C. elegans (151), and Drosophila (147, 152). These studies emphasize the importance of the lysosomal pathways in development of higher eukaryotes. The first studies of mouse orthologs of retromer subunits supported that the essential components of this pathway are highly conserved in all eukaryotic cells (28, 30, 31). Undoubtedly, these early studies also emphasized the importance of controlling membrane trafficking in particular cell lineages during initial development stages. This section will cover the implications of recent work in genetically modified mice and in the worm C. elegans establishing an essential function of retromer during embryogenesis apparently unrelated to its conserved role in CI-MPR sorting.

5.1. Retromer’s role in mouse embryogenesis

5.1.1. Vps26

The first indication of an ortholog of a yeast retromer subunit in higher eukaryotes comes from a study of the phenotype caused by an insertionional mutation affecting early development of the mouse embryo (30, 153). This mutation affected the chromosome 10 at the Hb58 locus, which gave name to the gene, later shown to be equivalent to the yeast PEP8 gene (27) and now also called VPS26 (23). We will use throughout the following paragraphs the Vps nomenclature derived from research in yeast, except when specific references either to the mouse Hb58 locus or to the yeast PEP8 gene are required.

Vps26-/- embryos developed normally through the late egg cylinder stage (embryonic day E6.5), but by the gastrulation stage (E7-7.5), they began to display a growth retardation of the embryonic ectoderm, the tissue giving rise to the epidermis, associated epidermal structures, the brain and the nervous system. These embryos survived for ~ two more days, but never progressed, in size or complexity, further than a normal E8.5 embryo (30). Interestingly, it was possible to derive permanent ES cell lines with normal growth and differentiation from Vps26-/- blastocysts, which indicates that Vps26 is not required for cell proliferation in vitro (154). Considering also the extensive growth and differentiation seen in vivo in homozygous mutant embryos, these authors concluded that Vps26 does not perform a housekeeping role but rather serves in a more specialized function (30). The widespread expression of Vps26 mRNA throughout various development stages of the wild-type embryo and in many differentiated tissues of fetal and adult mice suggested that Vps26 does not function solely during early embryogenesis. Instead, Vps26 appears to be redundant with other gene products (from zygotic or maternal origin) at early stages, but unique and essential after the egg cylinder stage (31). Very high mRNA levels in the chorioallantoic placenta (31), along with a failure of the mutant mouse to develop this organ (30), suggested a role of Vps26 in placental formation, which was later pursued by other researchers. Thus, it was proposed that Vps26 (or at least a protein with similar sequence to the Hb58 product) may function in both maternal and fetal tissues during placental formation and maturation in pregnant rats (155). Vps26 distribution in erythroid cells of the developing fetal vasculature suggested a role in hematopoietic development, which is a key process to bring the maternal and fetal systems into close proximity and ensure embryo survival. However, the strong nuclear staining observed in those analysis questions the specificity of the antibody used (155). A similar morphological study performed in a viviparous reptile confirmed the role of Vps26 in chorioallantoic placental formation and development (156). Interestingly, strong staining was found here at the subapical region in some epithelial cells of the uterine epithelium (156), but how the conclusions from these studies in animal models could fit with the data on Vps26 and retromer gathered from research in yeast was not clear.

At the time when the first observations in Vps26 knockout mice were made, the VPS gene products were being characterized in yeast (38, 48, 157) and their corresponding homologs were only beginning to be identified in higher eukaryotes (158), although research on mutant strains of the equivalent VPL (Vacuolar protein

3835
Retromer in development

localization) (37), VPT (Vacuolar protein targeting) (36) and PEP genes had a somewhat older history (39, 159). Those that had described the PEP mutants were actually those that first found a role of Pep8p / Vps26p in protein delivery to the yeast vacuole (27). They examined the processing of three soluble vacuolar proteases, CPY and the proteases A and B, in PEP8 deletion mutants, and found defects in enzyme processing, which were only striking for CPY. Based on localization data, it was suggested that Pep8p has a role at a postsorting stage in protein delivery to the yeast vacuole (27). This was later confirmed when Vps26 was found to be part of retromer and thus involved in endosome-to-Golgi retrieval (9). In analogy with the ES cells isolated from Vps26-/- blastocysts (154), disruption of the PEP8 gene in yeast did not affect growth or viability. In addition, the yeast mutant was shown that VPS26 deleted cells, as other class F VPS mutants, display a large vacuole and fragmented vacuolar-like structures around (48). The reason for these different interpretations is unknown.

The relatively mild defects in protein processing to the vacuole described for the PEP8 / VPS26 yeast mutants (9, 27) are in striking contrast with the embryonic lethality seen in mice harboring the Hb58 mutation (30, 31). It was surprising, and at the same time paradoxical, to find that while major effects in these mutant mice were found in the embryonic ectoderm, Vps26 expression is much lower in this cell lineage than in extraembryonic lineages, such as the visceral endoderm / yolk-sac lineage, which contained the highest mRNA levels although they were morphologically unaffected by the mutation (30, 31). These authors speculated that Vps26 would behave as a secreted protein of which normal expression in the extraembryonic tissue may be required for proper development of the embryonic ectoderm, but this hypothesis was not tested directly (31). Since Vps26 lacks a recognizable signal sequence, an alternative and more interesting explanation was that Vps26 would instead have a role at an early step of synthesis, transport or secretion of other molecule(s) – nutrient or growth factor – thus indirectly affecting the ability of the visceral endoderm to support embryonic growth (31). Indeed, it has become clear that while the visceral endoderm, and the yolk-sac as a whole, have a role in nutrient uptake and transport, they also have an active role in guiding early embryo development (reviewed in 160-162). This idea still remained untested after the function of Vps26p in yeast lysosomal enzyme processing (27) and its incorporation in the retromer were defined (9). Intriguingly, Vps26p appears to have a relatively minor role in stabilization of the complex (22). However, in agreement with the studies on the mouse Hb58 mutation, it has recently been observed that SNX2 (but not SNX1) is also highly expressed in the yolk-sac of embryos at midgestation (33). A possible explanation to these observations in mouse models can now be found in recent studies on developing nematodes, as it will be discussed later in this section.

5.1.2. Vps35

Another retromer subunit found implicated in mammalian embryogenesis is Vps35. To identify genes expressed during early mouse embryogenesis, cDNA libraries were constructed from unfertilized eggs and embryos until the blastocyst stage (163). Based on its high expression levels in unfertilized egg and preimplantation embryo, a novel maternal transcript was named maternal-embryonic 3 (MEM3) and turned out to be the Vps35p mammalian ortholog (28). MEM3 mRNA levels decreased in the 2-cell embryo and increased again in the 8-cell embryo, decreasing again during embryo postimplantation and remaining low in adult tissues (28). From this expression profile, and the already reported role of this protein in yeast intracellular sorting (164), it was suggested that MEM3 follows the expression pattern of a housekeeping gene (28), which contradicted the interpretation previously made for the Hb58 gene product (30, 31).

5.1.3. Sorting nexins 1 and 2

As the extensive SNX family was beginning to be explored in higher eukaryotes, their role in vivo was addressed in mice lacking those SNX members initially proposed to form part of a putative mammalian retromer (23). SNXs 1 and 2, as Vps5p orthologs, were proposed to assemble as homo or heterodimers in a conserved mammalian retromer (see Sections 3 and 4) and mice that lack either one of these proteins were generated. While Snx1-/- and Snx2-/- mice were viable and fertile, and displayed no obvious phenotype, Snx1+/- Snx2-/- embryos arrested at midgestation, indicating that SNX1 and SNX2 are functionally redundant and perform an essential role in embryogenesis (32). The phenotype of these double-knockout embryos was very similar to that of embryos lacking Vps26 (30, 31), showing growth retardation by E7.5 and arresting between E9.5 and E11.5. They also displayed a disproportionate growth of extraembryonic structures, such as the yolk-sac, in comparison to the embryonic ectoderm (32). Ultrastructural analysis showed an accumulation of apical electron dense structures of unknown nature in visceral endoderm cells of Snx1-/- Snx2-/- embryos at E9.5. These morphological alterations supported the hypothesis that alterations in nutrient transport to the developing embryo could be the cause of the fatal phenotype, as proposed previously for Vps26-/- embryos (31). They also contrasted with an apparently normal endosome-to-lysosome pathway in primary embryonic fibroblasts, as seen by staining of endocytosed molecules. The latter also implies that SNX1 and SNX2 appear not essential for this pathway, at least in some cells (32).

To address if absence of SNXs 1 and 2 in embryonic tissues also contributes to lethality, tetraploid aggregation rescue experiments were carried out. This system is based on aggregation of ES cells with developmentally compromised tetraploid embryos. In the chimeric embryos, the tetraploid component is selected against in all lineages where ES cells properly differentiate. ES cells will thus form the embryo proper, while the tetraploid component only contributes to the extraembryonic tissues, allowing the production of mice entirely derived from ES cells (165, 166). By this technique, it was possible to generate diploid / tetraploid...
chimeras formed by $\text{Snx1}^{-/-} \text{Snx2}^{-/-}$ embryos with partially wild-type extraembryonic tissues. Interestingly, these mutant embryos were much larger than the regular double-knockouts, although they still arrested at E12.5. While defects in extraembryonic tissues were partially corrected by the presence of wild-type cells, the prevailed lethality indicates that defects are also present in embryonic tissues (32). Nevertheless, it is possible that wild-type SNX1 and SNX2 in extraembryonic tissues were insufficient to fulfill the proposed supportive role to the developing embryo, since the yolk-sac mesoderm and the amnion are completely ES-derived and thus composed by SNX1 and SNX2 depleted cells (165).

These genetic analyses in mice suggest that SNX1, SNX2 and Vps26 act in the same pathway and provide in vivo evidence of a mammalian retromer structurally similar to the yeast retromer. To address a possible link with the EGFR pathway, in which both SNXs have been implicated (63, 90), $\text{Snx1}^{-/-} \text{Snx2}^{-/-}$ mice were crossed with mice wearing the EGFR waved-2 mutation ($\text{EGFR}^{\text{w}2}$), a Val-to-Gly substitution at residue 743 which nearly abolishes its Tyr kinase activity (167) and causes impaired maternal lactation without affecting newborn viability (168). While genetic interactions have previously been found between the waved-2 mutation and other gene products of the EGFR signaling pathway (169), no alterations in phenotype or viability were observed here in $\text{Snx1}^{-/-} \text{EGFR}^{\text{w}2} \text{Snx2}^{-/-}$ or $\text{Snx2}^{-/-} \text{EGFR}^{\text{w}2} \text{Snx1}^{-/-}$ double homozygous (32). In principle, these data seem to exclude an interaction between SNXs 1 or 2 with the EGFR pathway during development. However, the relatively mild phenotype caused by this hypomorphic mutation may be appropriate to identify components required for EGFR signaling (169), but unsuitable to study molecules involved in subsequent downregulation events.

The first evidence that SNX1 and SNX2 are not genetically equivalent comes from the observation that, whereas $\text{Snx1}^{-/-} \text{Snx2}^{-/-}$ embryos reached term in expected numbers, 40 % of $\text{Snx1}^{-/-} \text{Snx2}^{-/-}$ embryos died, and those that survived showed clear signs of growth retardation continuing into later life, since remained 10-20 % smaller than their $\text{Snx1}^{-/-} \text{Snx2}^{-/-}$ littermates (32). These differences were further studied in knockouts for SNX1, SNX2 and Vps26. By RT-PCR, it was shown that, while SNX1 levels were similar in the extraembryonic yolk-sac as in the developing embryo, SNX2 and Vps26 levels were proportionally more abundant in the yolk-sac (33). This finding supports the previously observed high expression of Vps26 in the visceral endoderm (31). These data also emphasize a stronger relevance of SNX2 vs. SNX1 in the retromer complex. The observation that $\text{Snx1}^{-/-} \text{Snx2}^{-/-} \text{Vps26}^{-/-}$ embryos displayed 100 % lethality, whereas $\text{Snx1}^{-/-} \text{Snx2}^{-/-} \text{Vps26}^{-/-}$ embryos were born in expected ratios, is explained by differences in the rate of the receptor’s trafficking pathways, which are responsible for its diverse subcellular localization among Golgi, endosomes and the cell surface, as previously reported (see 43, 47, 170 for reviews on MPRs distribution and traffic). The reason for these discrepancies described in cultured HeLa cells is unknown, although probably both the CI-MPR distribution and levels are altered upon affecting retromer’s function, as observed upon combined depletion of SNX1 and SNX2 (62). In either case, interaction and localization data convincingly demonstrate the retromer’s role in endosome-to-Golgi retrieval of the CI-MPR (15, 16).

CI-MPR localization was also investigated in the mouse models. Evidence for CI-MPR mislocalization or instability in MEF isolated from single or double-knockout embryos for the SNXs 1 and 2 or from $\text{Vps26}^{-/-}$ embryos could not be provided (33). The discrepancy of these observations with the previous data showing altered distribution of the CI-MPR in ES cells derived from undifferentiated $\text{Vps26}^{-/-}$ embryos (13) was explained by the authors as owed to differences in cell type and degree of differentiation. It was also reminded that one cannot exclude a possible effect on other linked genes by the insertional mutation technique used to initially obtain the mice with the $\text{H{	extbeta}38}$ mutation, from which those ES cells
were derived (30, 153). On the contrary, the \( V_{\text{ps}26}^- \) MEFs were derived from embryos carrying a targeted mutation of the gene (33). Further immunocytochemical analysis in SNXs 1 and 2 double-knockout embryos and in \( V_{\text{ps}26}^- \) embryos showed that CI-MPR was also found correctly localized in the perinuclear area of yolk-sac endoderm cells (33), where \( V_{\text{ps}26} \) normally displays a high expression (31). These negative data suggest that altered trafficking of the CI-MPR is not responsible for the lethal phenotype in retromer-depleted embryos. Finally, concerning the more critical role in retromer activity during embryogenesis proposed for SNX2 vs. SNX1, one may speculate that SNX2 may have become specialized in retromer assembly or stability, whereas SNX1 in interactions with certain cargo proteins. That way, SNX2 gene deletion may have a more striking effect in vivo. On the contrary, SNX1 antisense depletion in cells might particularly affect certain pathway regulated by retromer and not necessarily essential for development or survival. Thus, depending on the level of SNXs depletion by antisense, and on the assay used to measure the effect, alterations on e.g. CI-MPR trafficking in cultured cells could seem more (62) or less striking (65, 67).

5.1.4. Other sorting nexins

Other SNXs recently proposed to be retromer subunits are SNX5 and SNX6 (67), for which knockout mice have not yet been reported. Additional importance on the role of SNXs in embryo development has been provided by a recent study on SNX13. As in the knockouts for retromer subunits, targeted disruption of the mouse SNX13 gene resulted in embryonic lethality, further demonstrating the importance of regulating endocytic trafficking in early stages of development (109). Besides the PX domain, the SNX13 sequence includes a regulator of G protein signaling (RGS) domain. Hence, SNX13 is also known as RGS-FX1 and it belongs to both the SNX and the RGS protein families (60). SNX13 was involved in lysosomal degradation of the EGFR (122), functioning as a GTPase activating protein for the heterotrimeric G protein. Ga complicated with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a component of the ESCRT machinery (123).

Similar to \( \text{Snx}1^{-/-} \) \( \text{Snx}2^{-/-} \) and \( V_{\text{ps}26}^- \) embryos, \( \text{Snx}13^{-/-} \) embryos arrest at midgestation, showing growth retardation and, in particular, displaying abnormalities in the yolk-sac endoderm (109). However, ultrastructural analysis of visceral endoderm cells suggests that different intracellular pathways are affected. \( \text{Snx}13^{-/-} \) embryos lacked the large apical electron dense structures negative for both endosome and lysosome markers that were seen in cells from \( \text{Snx}1^{-/-} \) \( \text{Snx}2^{-/-} \) embryos (109). Instead, abundant and elaborate dense tubules resembling an enlarged recycling endosome system (16, 86) were seen. Autophagic vacuoles were also observed, and this was interpreted as an effect of cell stress by nutrient shortage, probably caused by deficient nutrient uptake and further lysosome processing (109). These observations agree with the hypothesis that nutrients' processing in the yolk-sac endoderm is needed to supply the embryo with the resultant products (160), as suggested to be affected also in the \( V_{\text{ps}26} \) and \( \text{Snx}1/2 \) knockouts (30-33). Thus, knockouts for \( \text{Snx}1/2 \) and \( \text{Snx}13 \) develop comparable defects that largely result in the same phenotype. However, this study implies that SNX13 acts at different intracellular compartments than \( \text{Snx}1 \) and \( \text{Snx}2 \) in the extraembryonic tissue, and probably through interactions with molecules not involving the retromer.

The recent studies in mice knockout for various retromer subunits (33) and this newer report on SNX13 knockouts (109) suggest that more evidence on the participation of these proteins in embryonic development is expected to emerge shortly. Another recent example in this context is the involvement of SNX5 in hematopoiesis and blood vessel formation in zebrafish early development (171). Intriguingly, this is reminiscent of a role proposed for \( V_{\text{ps}26} \) in controlling hematopoietic development in rat placenta (155). Overall, these approaches shed light on the mechanism(s) of retromer and SNXs functioning during development.

5.1.5. The cation-independent mannose 6-phosphate receptor

The studies on retromer-depleted embryos suggest that altered trafficking of the CI-MPR is not responsible for their lethal phenotype (33). If an increased turnover of the CI-MPR was the cause, the \( CI-MPR^- \) embryos would then be expected to share a similar phenotype as retromer-depleted embryos, which is not the case, nor is the case of humans suffering I-cell disease (ICD). These individuals are defective in the phosphotransferase that generates the mannose-6-phosphate tags on lysosomal enzymes and still survive through early adolescence (172-174). The CI-MPR was however reported to be essential for late embryonic development and growth regulation, although the phenotype of \( CI-MPR^- \) embryos clearly differs with that of knockouts for retromer subunits (175). \( CI-MPR^- \) homozygosis is generally lethal at birth, and mutants display increased size. This phenotype was explained by receptor interactions with the insulin-like growth factor II (IGF2), hence the receptor is also known as IGF2/MPR (43, 170). While the role of CI-MPR in adult tissues would primarily be to maintain enzyme levels in the lysosome, in the embryo, however, the receptor is mainly involved in internalizing IGF2 in excess, thus inhibiting growth; an impairment of this function is reported to be the primary cause of death in \( CI-MPR^- \) embryos (175, 176). Therefore, the complex embryonic phenotype displayed by the IGF2/MPR knockout mice could result from this multifaceted activity. Absence of CI-MPR would lead to high serum concentrations of active proliferative ligands such as IGF2 (175, 176), accumulation of inactive ligands such as latent TGF-\( \beta \) (176) and proiferin (177), as well as impaired growth-inhibitory effects by retinoids (178) (see 47, 179 for a complete list of CI-MPR ligands, containing or not a mannose 6-phosphate residue). Finally, it should be kept in mind that similar defects in steady-state levels of lysosomal enzymes are present in the CD-MPR null mice, with no obvious effect in viability (180, 181), making very unlikely that affected CI-MPR sorting is responsible of the fatal phenotype in retromer-depleted embryos.
Retromer in development

5.1.6. The polymeric immunoglobulin receptor

Retromer also associates with the plgR and promotes transcytosis of plgR and its ligand plgA in polarized epithelial cells. This interaction prevents recycling or degradation of plgR-plgA in a somehow analogous way as Cl-MPR trafficking is regulated by retromer (14). Mice knockout for plgR have also been reported (182, 183). While these mice lacked active epithelial and hepatic plgA transport, they were born in expected ratios and therefore disruption of the plgR gene is not lethal. Complete absence of plgR in humans has not been identified (184), although it is clear that the receptor is not essential neither for development nor for the health of mice kept in a conventional laboratory facility. Although IgA secretion appears already operative in early stages of the embryogenesis (185), from these studies in plgR-deleted mice one can conclude that the essential role of retromer during embryogenesis is unrelated to plgR-plgA trafficking.

5.2. Retromer’s role in Wnt signaling

We have seen that the retromer’s role, while evolutionary conserved from yeast, may vary considerably in different cell types or developmental stages. Importantly, the key participation of retromer during embryogenesis has remained unsolved until very recently. Two independent studies in the worm C. elegans have come up with a possible explanation. With the use of transgenic and deletion mutant strains, they demonstrate the requirement of retromer to produce Wnt proteins efficient for long-range signaling in the establishment of anteroposterior (A/P) neuronal polarity (34, 35).

The Wnt signaling pathway (wingless in Drosophila) is highly conserved throughout evolution and extensively used during animal development, from the simplest Cnidaria to humans. Activation of Wnt signal transduction pathways upon ligand binding can regulate multiple aspects during embryogenesis, including cell proliferation, migration, fate specification, polarity, differentiation and axon outgrowth. In addition, an overactivation of Wnt signaling by mutation is a major factor of oncogenesis in various human tissues (186). An understanding of the Wnt action mechanisms has emerged from several experimental systems, including genetics in Drosophila and C. elegans, biochemistry in cell culture models and ectopic gene expression in Xenopus embryos (see 187-191 for reviews).

Secreted Wnt proteins can form a concentration gradient during embryonic development and thus function as morphogens. They then interact with different cell surface receptors located on the target cells, particularly with the seven-transmembrane Frizzled and Ryk/Derailed Tyr kinases (191). Among Wnt proteins, the Egg-laying defective family member EGL-20 expressed by a group of cells located at the posterior end of the nematode C. elegans during early larval development. EGL-20 controls long-range migrations of the neuroblasts VL and QR and their descendants along the A/P body axis (192). Other well studied neurons in C. elegans are a set of six touch-receptor or mechanosensory neurons that mediate touch sensitivity. These are the PLML, PLMR, and PVM neurons, present in the posterior region of the worm, and the ALML, ALMR, and AVM neurons, located anteriorly; all of them send processes in anterior direction (193). Recently, it has been shown that Wnt and Frizzled proteins control the development and organization of the mechanosensory neuron processes along the A/P body axis, playing a role in axonal growth cone migration by orienting polarity of the developing neurons (35, 194, 195). In one of these studies, the worms were mutated with ethylmethane sulfonate and screened for neurons with possible defects, which were visualized by expression of a GFP-tagged reporter gene (35). Remarkably, a mutation in the VPS35 gene, involving a deletion that probably eliminated most or all protein function, caused polarity defects as those seen in Wnt and Frizzled mutants. In particular, the polarity of ALM and PML neurons was frequently altered in VPS35 mutants. The mutants were slightly small and uncoordinated and most died during embryogenesis, although heterozygous animals appeared like wild-types (35). VPS26 and VPS29 deletion mutants were also analyzed to understand these alterations within the retromer context. Interestingly, VPS26 mutants displayed a comparable phenotype, but no alterations were seen in the VPS29 mutants, and thus Vps29 appears less essential for retromer function in Wnt signaling (35). This observation does not fully contradict previous data obtained in yeast, where Vps35p and Vps26p can still interact, albeit at reduced levels, in VPS29 deleted cells (22), with Vps35p, but not Vps26p, becoming substantially less stable (24). It does not agree however with antisense experiments in mammalian cells, where Vps29 knockdown depletes the whole Vps35p-Vps26p-Vps29 retromer subcomplex (62). Effects in C. elegans mechanosensory neurons caused by mutations in SNXs were not reported in this study. Instead, these authors analyzed effects of mutations in subunits of the Vps35-Vps26-Vps29 subcomplex on PVM neuron migration (35). Resembling EGL-20 mutants, PVM polarity was reversed in mutants for either subunit. Intriguingly, EGL-20 intracellular levels were unaffected in retromer mutants and the study underlined that the polarity defects in retromer mutants were often less severe than in Wnt-null mutants. In summary, this study demonstrates the requirement of retromer for establishing A/P neuronal polarity in C. elegans (35).

Comparable results were obtained in a contemporary study, which demonstrated a role of retromer in EGL-20 producing cells for long-range Wnt signaling along the C. elegans A/P body axis (34). In a high-throughput genome-wide screen, C. elegans genes were antisense targeted (196) and the worms analyzed for Q cell migration. By this technique, the authors found the Vps35p ortholog to be required for posterior location of the QL descendant neuroblasts, for EGL-20 dependent anterior migration of the QR descendants and the hermaphrodite specific neurons (HSN), and for the correct polarity of the posterior epidermal seam cell V5, which becomes a neuroblast. The phenotype of VPS35 deletion mutants was also seen strikingly similar to that of EGL-20 mutants (34). Vps35 was found involved in a critical step in EGL-20 expressing cells that allows EGL-20 to spread anteriorly.
Here, embryonic lethality did not greatly increase in VPS35 mutants. The same largely applied to VPS26, VPS29 and VPS5 mutants (34), contrasting with the high embryonic death rate of the corresponding mutants in the study discussed previously (35). VPS29 mutants had also a milder phenotype than VPS35 mutants, but polarity of Q cell migration was clearly affected. Interestingly, loss of VPS5 had no effect by itself, but VPS5 antisense depletion in VPS26 or VPS35 mutants enhanced their defects and caused lethality (34). An important aspect to remark is that this study provides evidence that the essential function of retromer during development is conserved in Wnt-producing cells of vertebrates. Thus, Vps35 antisense knockdown in Xenopus embryos or in cultured human cells also impaired Wnt signaling, albeit not Wnt secretion (34).

The evidence presented in these studies indicates that retromer does not participate in formation and secretion of active Wnt proteins, but rather in a specific mechanism ensuring production of Wnt proteins competent for long-range signaling. Therefore, rather than a housekeeping role, a specific function of retromer in embryogenesis has now been uncovered. A possible model to explain the critical role of retromer in Wnt producing cells was proposed as follows (Figure 4). Wnt proteins undergo post-translational modifications to become fully active morphogens at both short and long-range distances during animal development. In particular, Wnt proteins are palmitoylated at the endoplasmic reticulum by the O-acyltransferase porcupine (Porc), a multipass transmembrane protein that also stimulates Wnt N-glycosylation and targets them to the ER membrane (dark green arrows). Probably at the trans-Golgi network (TGN), Wnt proteins interact with another multipass transmembrane protein, Wntless / Evenness interrupted (Wls/Evi), also named Sprinter (Srt). By an unknown mechanism, that may involve an additional post-translational modification, Wls/Evi ensures Wnt secretion. The partition of Wnt proteins into vesicles targeted to cholesterol-sphingolipid rich raft domains is also important for Wnt secretion (raft microdomains are only represented at the plasma membrane). In addition, a putative Wnt receptor (Wnt rec?) may deliver Wnt proteins to the endosomal system (pink arrow), where they could associate with lipoprotein particles (LP). Alternatively, this receptor may transport enzymes or other proteins involved in Wnt processing / secretion (not represented). Wnt proteins are thus sorted out of default secretion (dark green arrows) and are incorporated into specialized secretory vesicles (light green arrows). Lipoproteins could then be the vehicles facilitating Wnt extracellular transport and transfer to target cells at long distances; this will ensure long-range signaling. In retromer depleted mutants, the retrieval mechanism that returns a putative Wnt receptor back to the TGN (red arrow) would not be operative, and Wnt proteins would instead be secreted via the default pathway, becoming only suitable for short-range signaling. Wnt retention at the secretory pathway, or at the cell surface for subsequent Wnt internalization (orange arrow) and recycling, may also help to sustain signaling.

What is the receptor for Wnt proteins sorted by retromer in Wnt producing cells? While many components required for reception and processing of the Wnt signal in target cells have been identified and characterized, the
process of Wnt secretion is considered a “black box”. Thus, apart from Porc/MOM-1 role in Wnt posttranslational modifications and the subsequent association of Wnt to lipoproteins, very little is known on the regulation of synthesis, secretion and release of the Wnt signal out of the cell. However, a piece of information on the process of Wnt secretion has been recently provided by independent studies (201-204). By using different approaches of genetic screening in Drosophila, these research groups identified a new component specifically controlling Wnt secretion, which was named Wntless (Wls) (201). Evenness interrupted (Evi) (202) or Sprinter (Srt) (204). We will refer to here as Wls/Evi, since this is how it simultaneously appeared in the literature (203). Wls/Evi is a multipass transmembrane protein evolutionary and functionally conserved from worms to humans. Absence of Wls/Evi led to abnormal Wnt folding and retention in cells, without affecting Hh secretion or expression of Hh downstream target genes (201, 202, 204). Although no obvious enzymatic activity can be inferred from its sequence, Wls/Evi might be an enzyme that specifically modifies Wnt proteins, similarly to Porc/MOM-1, but acting subsequently in the pathway. Alternatively, Wls/Evi could be part of a specialized machinery for regulating Wnt secretion, ensuring proper intracellular transport of Wnt proteins to the cell surface. It probably acts at the TGN or in specialized secretory vesicles en route to the subapical cell surface of polarized epithelial cells and plays a role in packaging, localizing or fusing Wnt-containing vesicles with the plasma membrane (201, 202, 204). Related to this idea, earlier findings demonstrated that a significant proportion of Wnt proteins are found in recycling endocytic vesicles, by which they are sent back to the surface for secretion (205). This provides additional support for the existence of a transmembrane protein functioning as a putative receptor that allows Wnt proteins to follow a privileged pathway to the cell surface. Whether Wls/Evi is specifically sorted by retromer remains to be tested, although from what we know it seems unlikely, since contrary to the observations in Wls/Evi mutants, Wnt secretion is not affected in retromer mutants (34, 35). At any rate, the function proposed, almost fifteen years ago, for the Hh58 gene product, of regulating synthesis, transport or secretion of some other molecule required for proper embryo development seemed a clever anticipation of retromer’s essential role during embryogenesis (31).

6. RETROMER IN DISEASE: THE IMPORTANCE OF ACCURATE INTRACELLULAR PROTEIN SORTING

Many human disorders arise from defects in intracellular trafficking. Over the years, researchers have studied how defects in the ability to sort or transport molecules to their appropriate cellular destinations can greatly affect cell functionality and therefore cause disease (206-210). Organ-specific diseases can arise by alterations in the sorting signals of individual proteins, or in the trafficking machinery delivering these proteins to their proper destinations. Some of the well-known diseases resulting from altered trafficking or cell polarity include cystic fibrosis, Wilson disease (copper toxicity) or familial hypercholesterolemia. In polarized epithelial cells, for instance, altered localization of molecules can dramatically affect barrier and transport functions, thereby causing disease. In these cells, basolateral sorting, endocytosis, recycling and transcytosis of cell-surface molecules are key processes for maintaining epithelial polarity, and pathological conditions can arise because of the inappropriate removal or insertion of itinerary proteins (209). Similarly significant is tight junction dysfunction, which deregulates cell polarity and causes kidney disease (209, 211).

Given their diversity and complexity, a particular interest has been given to defects in delivery of lysosomal proteins, most of them enzymes, which cause LSD. Because of the variety of undegraded substrates that accumulate in lysosomes, patients with LSD display multiple and complex phenotypes that include, among others, psychomotor retardation, mental / intellectual retardation / deterioration and enlarged internal organs, such as liver and spleen (see 173, 174 for recent reviews on the biology and pathology of lysosomal disorders). A relatively large subgroup of LSDs is caused by the accumulation of lipids in late endosomes or lysosomes. Among these diseases, a well studied is the Niemann-Pick (NP) syndrome, caused by accumulation of constituents of lipid raft microdomains and for which three forms have been described. NP types A and B are characterized by sphingomyelin accumulation, whereas NP type C is caused by cholesterol accumulation (208). Two interesting aspects must be mentioned for the type C form, a rare autosomal recessive neurovisceral disorder caused by mutations in one of two genetic loci, NPC1 or NPC2 (mutations in NPC1 account for 95% of the cases). First, the cholesterol accumulation in degradative compartiments of the endocytic pathway is apparently due to a failure in the mechanism responsible for redistribution of cholesterol taken up by LDL endocytosis, in contrast to most of the LSDs, which are caused by defective metabolic enzymes. Since both NPC1 and NPC2 may participate in delivering LDL-cholesterol from late endosomes to proper cellular sites, retrieval mechanisms at the endosome appear affected in this pathology (reviewed in 210). Secondly, there is a mouse model exhibiting a clinical phenotype very similar to that seen in the human disease (208, 210). More recently, a model in Drosophila has also been created by mutating NPC1a, the fly NPC1-like gene, which intriguingly encodes a transmembrane protein related to the Hh receptor Patched (212). This is also an example that animal models involving altered trafficking mechanisms could reveal new links between signaling pathways that may help to deal with diseases.

Retromer is involved in CI-MPR retrieval (15, 16), and this receptor ensures arrival of enzymes to lysosomes (43, 47, 181). Although alterations in retromer function as a cause of LSD have not been described thus far, a relationship cannot be excluded. An aspect that has increased the difficulty of understanding the variability of LSD phenotypes is that cells in certain tissues have developed effective compensatory mechanisms to maintain their lysosomal function. In hepatocytes of CI-MPR (172)
Retromer in development

or CD-MPR (180) knockout mice, newly synthesized enzymes are secreted, which prevents them of reaching the lysosome. However, cells can still acquire the lysosomal enzymes via secretion-recapture or direct cell-to-cell transfer mechanisms (172, 174). Alternative, MPR-independent mechanisms for transport of newly synthesized enzymes to the lysosomes are likely to exist also in patients with ICD. While fibroblasts from these patients have indeed a marked deficiency in lysosomal enzymes, enzyme levels are normal in most tissues, including liver, spleen, kidney and brain (172). Interestingly, the phenotype of mice triple-deficient for CI-MPR, CD-MPR and IGF2 that survive resembles that of ICD patients, presenting dwarfism, facial dysmorphism, elevated activities of lysosomal enzymes in serum and abnormal lysosomal storage in connective tissue cells (213). A parallelism between deficiencies in the CI-MPR and in retromer is clearly not obvious in view of the animal models, and from the current evidence, we conclude that the essential role of retromer during development is unrelated to its conserved role in CI-MPR sorting, as discussed in Section 5 (33). Thus, rather than having a general function in controlling membrane trafficking, retromer specifically sorts certain transmembrane proteins at the TGN-endosome interface.

As advanced at the end of Section 3, a link between retromer and disease was recently described for two neurological disorders, one of them very well known. First, retromer was proposed to regulate endosome-to-TGN traffic of BACE (81, 82), the enzyme that cleaves β-APP, leading to the release of the neurotoxic Aβ peptide and progression of AD (83). In the model proposed, retromer dysfunction, suggested to take place by reduction of retromer levels, would increase the concentration of BACE in the endosome. Since BACE activity is maximal in the acidic environment of the endosome, Aβ will be produced and subsequently secreted in large quantities (81, 82). An interaction between BACE and retromer might be explained via a Vps10-domain containing protein, increasing the relevance of this aspect in the context of this review, because retromer depletion by antisense results in mistrafficking to the endosome of the Vps10p homolog sortilin (13). Sortilin is believed to have a similar trafficking itinerary as the CI-MPR and to be engaged in protein sorting at the TGN (77). Importantly, the brain appears to be the organ with the highest expression of this family of Vps10-domain containing proteins. Another receptor of particular interest in this family is SorLA (LR11), a multiligand hybrid receptor containing 11 LDLR class A repeats and a Vps10p/sortilin domain (214, 215). Among other brain regions, SorLA is abundant in the hippocampal formation and particularly in the entorhinal cortex (215, 216), the subregion of the hippocampus that appears most vulnerable to AD (82). In a microarray screening, it was found a loss of SorLA expression in lymphoblasts derived from patients with AD and in histologically normal-appearing neurons of AD brains (217). Indeed, subsequent research confirmed the interaction of SorLA with APP (218) and BACE (219). These findings also support a possible relationship between retromer and trafficking of LDLR family members in neurons. Related to these aspects, the retromer subunit Vps26 also emerged as a positional candidate gene involved in AD. Thus, the human VPS26 gene was located at locus 10q21.1 (220), and more recently it was found that this chromosome 10 region contains a quantitative trait locus displaying susceptibility to late-onset AD (221, 222). Although a recent study did not find an association of single nucleotide polymorphisms of the VPS26 gene with sporadic forms of AD (223), these data do not exclude a possible functional involvement of Vps26 in AD pathogenesis.

Even more recently, retromer has been involved in another neurological disorder. This is the case of INCL, a LSD also known as BD, for which are responsible mutations in the human CLN3 gene, that codifies for an endosome/lysosomal protein of elusive function (224). BD is an autosomal recessive disease leading to progressive neurodegeneration and premature death in humans. A characteristic of BD and related diseases is the abnormal accumulation of autofluorescence storage material in the lysosome (225, 226). This altered membrane trafficking has been studied in yeast, in which two BD related proteins have been identified. One is Btn1p, the CLN3 ortholog, and the other is Btn2p, which is upregulated upon Btn1p deletion and has a role in cell growth and homeostasis. This model of BD in yeast (84) has encouraged further research. Thus, a recent study shows a direct interaction between Btn2p and Vps26p in a complex also including Snx4p and regulating endosome-to-Golgi retrieval of Yif1p (Yip1p-interacting factor; Yip1p: yeast Golgi integral membrane protein) (85), a component of the endoplasmic reticulum-Golgi fusion machinery (227) that needs to be moved to the Golgi. This study also supports that retromer subunits can assemble with other sorting components and thereby transport other molecules apart from Vps10p, although an association between Snx4p and retromer in yeast contradicts previous data (132). Nonetheless, the possible implications of these data in BD are worth being considered for future studies. While the functional link between retromer and neurodegeneration remains to be confirmed, these examples emphasize that alterations in the endosome-to-Golgi pathway can lead to serious human disorders.

7. Perspective

In the relatively short period of eight years since the word “retromer” first appeared in the scientific literature (9), over half of the reports on retromer are found in the last two years. Similarly, since the role of SNX1 was first described ten years ago (90), the number of SNX family members quickly grew up and their involvement in membrane trafficking and signaling pathways has grown increasingly. The mouse Hb38 locus, which disruption blocks early embryonic development, was described fifteen years ago (30). New evidence of retromer and sorting nexins controlling development processes is now accumulating from studies in model organisms. These studies also provide new data on how alterations in the strict control of trafficking pathways can lead to disease. With no doubt, there is more soon to come on the essential function of these proteins in development and their relevance in human disease.

3842
Retromer in development

8. ACKNOWLEDGEMENT

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9. REFERENCES

Retromer in development


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Retromer in development


Retromer in development


Abbreviations: A/P: anteroposterior; AD: Alzheimer disease; AML, ALMR, AVM, PLML, PLMR, and PVM: touch-receptor / mechanosensory neurons; ApoER2: ApoE receptor 2; APP: amyloid precursor protein; ARF: ADP-ribosylation factor; ARFβ: amyloid-β; BACE: β-site APP cleaving enzyme; BAR: Bin/Amphiphysin/Rvs; BD: Batten disease; Btc: BD related protein; CD-MPR: cation-dependent mannose 6-phosphate receptor; CI-MPR: cation-independent mannose 6-phosphate receptor; CLN3: ceroid-lipofuscinosis neuronal-3; CPY: carboxypeptidase Y; E: embryonic day; EGFR: EGF receptor; EGL: Egg-laying defective; ES: embryonic stem; ESCRT: endosomal sorting complex required for transport; Evi: Evenness interrupted; FERM: band 4.1-erzidin-moesin; GGA: gamma-ear containing, ARF-binding; GPCR: G-protein coupled receptor; Hh: Hedgehog; HSN: hermaphroditic specific neurons; ICD: I-cell disease; IGF2: insulin-like growth factor II; JNCL: juvenile neuronal ceroid lipofuscinoses; LDLR: LDL receptor; LRP: LDLR-related protein; LST: lysosomal storage diseases; MEF: mouse embryonic fibroblasts; MEM3: maternal-embryonic 3; MVB: multivesicular body; NP: Niemann-Pick; PAR-1: protease regulated tyrosine kinase substrate; ICD: I-cell disease; IGF2: insulin-like growth factor II; JNCL: juvenile neuronal ceroid lipofuscinoses; LDLR: LDL receptor; LRP: LDLR-related protein; LSD: lysosomal storage diseases; MEF: mouse embryonic fibroblasts; MEM3: maternal-embryonic 3; MVB: multivesicular body; NP: Niemann-Pick; PAR-1: protease activated receptor-1; Pep (PEP): peptidase-deficient; PGE: post-Golgi endosome; Pl 3-P: phosphatidylinositol 3-phosphate; PI3K: phosphoinositide 3-kinase; plgA: polymeric immunoglobulin A; plgR: polymeric.
Retromer in development


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