PROPPINs and membrane fission in the endo-lysosomal system

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Abstract

PROPPINs constitute a conserved protein family with multiple members being expressed in many eukaryotes. PROPPINs have mainly been investigated for their role in autophagy, where they cooperate with several core factors for autophagosome formation. Recently, novel functions of these proteins on endo-lysosomal compartments have emerged. PROPPINs support the division of these organelles and the formation of tubulo-vesicular cargo carriers that mediate protein exit from them, such as those generated by the Retromer coat. In both cases, PROPPINs provide membrane fission activity. Integrating information from yeast and human cells this review summarizes the most important molecular features that allow these proteins to facilitate membrane fission and thus provide a critical element to endo-lysosomal protein traffic.

Introduction

Cells constantly internalize parts of their plasma membrane during endocytosis, for example for receptor downregulation, feeding, or pathogen clearance. During this process, the plasma membrane pinches off endocytic vesicles. They fuse with other early endocytic vesicles and early endosomes to constitute a first compartment where endocytosed material arrives [1]. At these early endosomes, the fates of the different constituents diverge. They can be either recycled back to the plasma membrane, targeted for degradation by lysosomal compartments, or be sent towards the Golgi [2] (Fig. 1). Proteins and lipids are sorted into these different pathways by tubulo-vesicular carriers, which are generated by protein coats that deform the membrane and select and accumulate cargo. Some of these coats are formed by large protein complexes, which can have tubular structures, such as the sorting nexin-based coats Retromer, ESCPE-1, or Commander [3], or the AP1 or ACAP-based coats [4–6]. Others can be more spherical, such as clathrin, COP1 or caveolin coats [7–9]. While there have been recent advances in deciphering the mechanism of recruitment of these protein coats and the formation of tubular carriers, the machinery that drives the fission of these tubules from the endosomal membrane is poorly understood. Recent observations revealed that PROPPIN proteins (β-propellers that bind phosphoinositides [10]) promote membrane fission on endosomal compartments [11–14].

PROPPINs are conserved proteins that bind PI(3)P, PI(5)P and PI(3,5)P_2 [15–17]. There are three homologues in yeast (Atg18, Atg21 and Hsv2), and mammals (WIPI1, WIPI2, WIPI3/WDR45B and WIPI4/WDR45) express four PROPPIN genes in multiple splice variants [15,18,19]. PROPPINs function in PI3P and PI5P-dependent processes, such as autophagy,

and in PI(3,5)P_2-dependent processes, such as membrane fission on endo-lysosomal compartments. The function of PROPPINs in autophagy was discovered by genetic screening in yeast [20,21]. Different PROPPIN isoforms support various forms of selective and non-selective autophagy through links to core components of the autophagic machinery. Atg21 and WIPI2 support the elongation of the phagophore by recruiting Atg16, and with it the Atg8/LC3 lipidation machinery Atg12-Atg5, which mediates lipidation of Atg8, a key component coating the phagophore [15,22,23]. Atg18 forms a complex with the lipid transfer protein Atg2 and the lipid scramblase Atg9 at ER phagophore contacts, which may facilitate the flow of lipid that is required to allow the phagophore to expand [24–29]. Atg18 also mediates retrieval of Atg9 from the phagophore towards the Golgi. These and other roles of PROPPINs in autophagy have been elaborated in comprehensive reviews [30–33], which provide a more complete picture of these activities.

Autophagic and endo-lysosomal functions of PROPPINs might be interrelated at some level because the endosomal system is a significant source of lipids for autophagosome biogenesis - membrane contacts between phagophores and endosomes hold these two organelles in proximity and thus link two sites of PROPPIN activity [34,35]. However, as described below, PROPPINs appear to rely on distinct molecular features for supporting membrane fission and autophagy. Here, we focus on the membrane fission function of PROPPINs on endo-lysosomal compartments and on their role as effectors of PI(3,5)P_2.

PI(3,5)P_2 and membrane fission in the endo-lysosomal system

PI(3,5)P_2 is a minor phospholipid on endo-lysosomal membranes [36]. It is synthesized from PI3P by the lipid kinases Fab1 in yeast and PIKfyve in mammalian cells [37–43]. PIKfyve can also generate PI(5)P by directly phosphorylating PI [38,44,45].

Fab1 and PIKfyve knockout cells show enlarged endo-lysosomal compartments. Since the structure of these organelles depends on an equilibrium of fusion and fission activities [46–52], this phenotype could result from impaired fission and/or enhanced fusion. Since absence of Fab1 or PIKfyve activity does not enhance fusion of these compartments [53,54], these phenotypes support a requirement of PI(3,5)P_2 for fission. This requirement becomes directly apparent in the rapid fission of the yeast vacuole/lysosome, which can be triggered in vivo and in vitro [55–58], and in the fission of endo-lysosomes that undergo kiss-and-run, i.e. transient fusion followed by immediate re-fission [51,53,59–61].

Apart from regulating the size and fission of endo-lysosomal compartments, PI(3,5)P_2 also supports the exit of proteins from them. Cargo proteins are collected by tubulo-vesicular endosomal carriers, which must undergo membrane fission to detach from the organelle and migrate to their target compartments [62]. In line with this, PI(3,5)P_2 is required for endosome to TGN trafficking of the M6PR, Shiga toxin, or Sortilin, for the transfer of EGFR for lysosomal degradation [45,63,64], and the recycling of integrins towards the plasma membrane [65].

Structure of PROPPINS

PROPPINs bind membranes via two lipid binding sites (Fig. 2). These sites recognize the phosphoinositides PI(3)P, PI(5)P and PI(3,5)P_2 [11,18,25,66–68]. Atg18 illustrates that these

lipid binding sites can display different specificities, with lipid binding site 2 showing preference for PI(3,5)P_2 [68]. Autophagy requires PI(3)P and PI(5)P [44]. PI(3,5)P_2 does not play a major role in this process [36,44,69] and inactivation of the PI-5-kinase PIKfyve can even enhance autophagy [64]. The capacity of PROPPINS to bind PI(3,5)P_2 may therefore relate to a role in the endo-lysosomal system that is independent of autophagy. In line with this, substitutions in lipid binding site 2 of WIPI1 impair the exit of protein cargo from endosomes but do not affect the contribution of this protein to autophagy [14]. Simultaneous substitutions in both lipid binding sites reduce or abolish membrane binding and impair both autophagic and endosomal functions [14,16,18,55,70,71], demonstrating that these lipid binding sites make a major contribution to the recruitment of PROPPINs to membranes.

Besides their two lipid binding sites, PROPPINs also carry a disordered and relatively hydrophobic loop on blade 6 of their β-propeller structure, i.e. between the two lipid binding sites. A part of this loop folds into an amphipathic α-helix when it is brought in contact with a lipid bilayer, but the loop itself does not suffice for binding to the membrane [11]. The amphipathic character of the α-helix is a conserved feature of PROPPINs. Its role was probed by swapping 2-4 amino acids in the amphipathic helix sequence to make the helix lose its amphipathic character while maintaining its overall content of hydrophobic amino acids. Such loop mutants sustain the autophagic functions of Atg18 and WIPI1 but fail to support fission of the lysosome-like vacuoles of yeast (for Atg18) and the formation of human endosomal transport carriers (for WIPI1) [11,14], underlining the functional importance of this conserved loop on endo-lysosomal compartments.

Atg18 can self-assemble into different oligomeric structures in solution and on membranes. In solution they form a helical assembly that has been analyzed by cryo-electron microscopy [72]. It is composed of Atg18 tetramers forming a lozenge cylindrical lattice. The physiological significance of this assembly is not clear. However, since the lattice sterically hinders the lipid binding sites of Atg18 from interacting with a bilayer it is assumed to represent a soluble and membrane-inactive form of Atg18. On membranes, Atg18 forms dimers that can engage bilayers and tether opposing membranes at a distance of 80 Å. Based on structure predictions, this tethering activity was proposed to enhance lipid transfer during autophagy by providing a platform recruiting the lipid transfer protein Atg2 [72]. In line with this, in vitro lipid transfer experiments showed that Atg18 and WIPI1 or WIPI4 increase the lipid transfer activity of their binding partner Atg2 [27,73]. The presence of membranes also induces the formation of higher order oligomers of Atg18 [11]. In contrast to the oligomers formed in solution, membrane-triggered oligomers form at much lower concentrations. The structure of these membrane-bound oligomers is unknown. Their formation depends on PI(3,5)P_2 rather than on PI3P, which links them more to endosomal than to autophagic functions of Atg18.

PROPPINs as membrane fission proteins and PI(3,5)P_2 effectors

PI(3,5)P_2 is required for vacuole fragmentation in yeast, for the division of endo-lysosomes, and for the formation of endosomal carriers, which are all processes depending on membrane fission [10,18,57,74]. That mutations inactivating the respective PROPPINs reproduce many of these defects made them good candidates for representing PI(3,5)P_2 effector proteins in these processes. In further support of this notion, substitutions in the two lipid binding sites can have differential impact. Site 2 affects the binding of Atg18 to

$PI(3,5)P_2$-containing membranes more strongly than equivalent substitutions in site 1, which suggested a preference of site 2 for $PI(3,5)P_2$ [68]. Introducing such site 2 substitutions into human WIPI1 impaired the $PI(3,5)P_2$-dependent endosomal cargo exit and led to the accumulation of long endosomal tubules, whereas PI3P-dependent autophagy was unaffected [14]. The link between PROPPINs, $PI(3,5)P_2$, and membrane fission could be firmly established by in vitro experiments with Atg18 and synthetic liposomes. Purified Atg18 suffices to drive fission of giant unilamellar vesicles (GUVs). This in vitro fission activity depends on $PI(3,5)P_2$ and the amphipathic helix in the loop on blade 6 [11]. By contrast, PI3P supports only efficient membrane binding of Atg18, but not fission. Also $PI(4,5)P_2$ could not promote fission activity, demonstrating isomer specificity. This indicates that Atg18 is a $PI(3,5)P_2$ effector protein.

The amphipathic nature of the helix and the fact that its formation is triggered by contact with the membrane suggests a mechanism of action for Atg18. Shallow insertion of amphipathic helices modifies spontaneous membrane curvature and can significantly perturb bilayer structure, particularly if the density of inserted helices is high [75,76]. Interestingly, $PI(3,5)P_2$, but not PI3P, drives Atg18 oligomerization on membranes in vitro [11,77]. Therefore, it was proposed that $PI(3,5)P_2$-triggered oligomerization of Atg18 could be a means to reach a high local density of membrane-inserting amphipathic helixes, thus generating high curvature and bilayer destabilization that favors membrane fission [11].

Links to endosomal coat proteins: The CROP complex

Searches for Atg18 interactors in yeast were performed by Bio-ID and by SILAC approaches [12,13]. Both studies identified the endosomal Retromer complex as a major interactor. Retromer consists of three subunits, Vps26, Vps29, and Vps35, and cooperates with sorting nexins such as Vps5 and Vps17 to form tubular membrane coats that export cargo proteins from endosomes and transfer them to the plasma membrane or the Golgi [78]. Purified Retromer binds Atg18 with a K_d of 50 nM, forming a novel complex that was termed CROP [12]. Atg18 competes for Retromer binding with the sorting nexins Vps5 and Vps17. This competition is also evident at the functional level by epistasis experiments. Yeast lacking Vps5 or Vps17 show hyper-fragmented vacuoles, suggesting that the responsible fission machinery may be overactivated. This hyper-fragmentation phenotype coincides with increased formation of CROP. Deleting either subunit of CROP rescues the hyper-fragmentation phenotype, genetically linking CROP to the fission of vacuoles in vivo [12].

CROP is conserved and can be detected in human cells as an association of the Atg18 homolog WIPI1 with Retromer. In both yeast and human cells, CROP stability depends on an intact LFSTSL motif in blade 2 of the β-propeller of the PROPPIN subunit [12]. This motif overlaps with the binding site for Atg2. Substitutions in this motif - T57 in yeast or S69 in human cells - labilize the respective CROP complexes, abolish the in vivo fission activity of CROP on yeast vacuoles, and lead to an enlargement of endosomes and the accumulation of long endosomal tubules in human HK2 cells. This latter phenotype suggests that tubular endosomal carriers may form but cannot detach when CROP cannot assemble [12,14]. Substitutions destabilizing CROP in human cells phenocopy deletion or inactivation of WIPI1 not only morphologically, but also by interfering with exit of multiple protein cargos from endosomes, such as Transferrin receptor, GLUT-1, EGF receptor, and Shiga toxin. This

supports the notion that PROPPINs act in endo-lysosomal protein traffic and vacuole fission mainly through CROP.

CROP contains the entire Retromer complex. Retromer can oligomerize through homotypic Vps26-Vps26 or Vps35-Vps35 interactions of its subunits [79–82]. This allows Retromer to form a network that interconnects and stabilizes the protein layer that sorting nexins from on endosomes to scaffold their membranes into an endosomal carrier. In this way Retromer contributes driving force to the scaffolding of the membrane and accelerates coat formation [83]. Given that Atg18 competes with the sorting nexins for binding Retromer it is plausible that it might take the place of a sorting nexin on a coated membrane tubule (Fig. 3). Although Atg18 and sorting nexins compete globally for Retromer binding, the oligomeric nature of Retromer could allow for simultaneous recruitment of Atg18 and sorting nexins into a Retromer-coated tubule, using distinct Vps26 subunits in the network. It was hence proposed that multiple Atg18 could integrate into a Retromer tubule. Integration could be favored at the end of the tubule when the coat seizes to grow, and new sorting nexins competing with Atg18 integration may no longer be recruited [12]. This could concentrate multiple Atg18 subunits and thereby promote fission at the end of a tubule. While this is an attractive model, experimental evidence for integration of PROPPINs into Retromer coats is still missing.

Regulation of PROPPIN activity

PROPPIN recruitment and oligomerization can be controlled through the abundance of their lipid ligands PI3P, PI5P and $PI(3,5)P_2$. At least one of these lipids must be present to allow PROPPINs to bind the membrane [16,18,19,44,68,84]. Direct regulation through lipid abundance may underlie the activation of vacuole fission in yeast. This reaction is stimulated by hypertonic shock, which triggers a rapid, transient increase in $PI(3,5)P_2$. If this increase is prevented, for example through ablation of Fab1 activity, fission is impaired [40,41,43,85]. Hyperactive *fab1* mutants show a corresponding constitutive hyper-fragmentation of the vacuolar compartment [86]. In yeast, Atg18 is not only an effector of $PI(3,5)P_2$ but also feeds back on the synthesis of this lipid. Genetic ablation of Atg18 leads to constitutively increased levels of $PI(3,5)P_2$ [18]. While elevated $PI(3,5)P_2$ is usually associated with highly fragmented vacuoles, lack of Atg18 prevents the development of this fragmented phenotype, despite the elevated $PI(3,5)P_2$ content of *atg18* knockout cells. This is another support for the role of Atg18 as a $PI(3,5)P_2$ effector [55]. However, it should be kept in mind that $PI(3,5)P_2$ also activates further targets that are needed for vacuole fission. It promotes V-ATPase assembly, which stimulates vacuoles fission and impairs the opposing reaction of vacuole fusion [46,47,49,87,88], and it activates the vacuolar two-pore Ca^{2+} channel Yvc1 [47,57,89].

Additional regulation of PROPPINs can be achieved by phosphorylation. Overexpression of Atg18 in the yeast *Pichia pastoris* leads to a majority of the Atg18 pool being phosphorylated [90]. This phosphorylation occurs in the amphipathic α-helix of the CD loop on blade 6 that is critical for the fission function of Atg18 [11]. Phosphorylation in this loop prevents membrane binding of Atg18 and the phosphorylation and dephosphorylation of the loop coincides with the activation and inactivation of vacuole fission [90].

The endo-lysosomal fission activity of PROPPINs could also be regulated at the level of the CROP complex. Several PROPPINs, such as Atg18 and WIPI1, carry an LFSTSL motif, which is present in blade 2 of their β-propeller structure and required for the formation of

CROP [12]. While the grouping of serine and threonine residues in this motif is suggestive of a potential target for phosphorylation, available high-throughput data has so far not yielded any indications for a phosphosite in this motif. By contrast, PROPPINs carry numerous other sites that are modified by phosphorylation or ubiquitylation (see e.g. www.phosphosite.org). Targeted work will be required to identify the proteins targeting these sites and to probe their impact on endosomal membrane traffic, autophagy, and the known interactors of PROPPINs in these processes.

Perspectives

1) PROPPINs have important functions in two distinct cellular processes of great physiological relevance: autophagy and the formation of endosomal carriers that sort proteins between endosomes, the plasma membrane, and the Golgi.

2) Although it appears that PROPPINS support endosomal protein sorting and autophagy using distinct molecular features these two processes are not completely disconnected. Phagophores and endosomal compartments have physical contacts, PROPPINs interact with core machinery of both compartments, and endosomes may serve as a source of lipids for autophagosome formation.

3) It will hence be interesting to see to which degree the endosomal and autophagic functions of PROPPINs are intertwined. To this end, we need to understand how PROPPINs link to endosomal coats and trafficking factors in the CROP complex, and how their recruitment into CROP or onto phagophores is regulated. To this end, structural studies of the complexes and detailed analyses of their interaction with the membrane will be crucial.

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Figures:

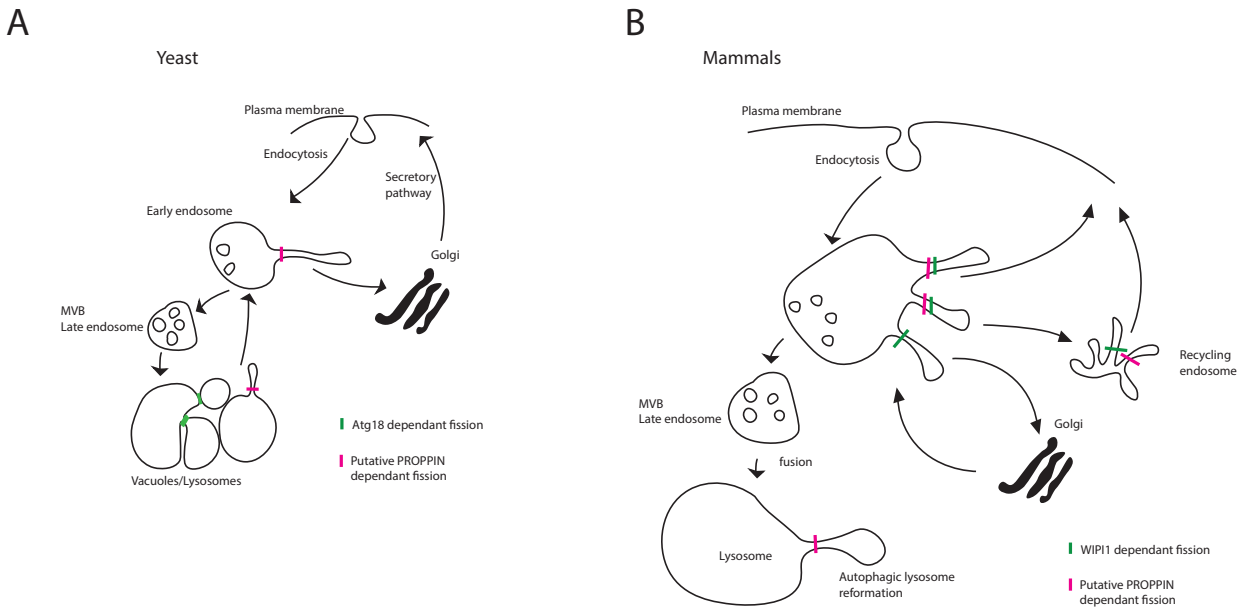

Figure 1: Schematic view of the endocytic pathway (Yeast vs mammals)

Schematic view of the endocytic pathway for yeast (A) and mammalian (B) cells. The sites were PROPPINs are known to act are highlighted in green (Atg18 for yeast and WIPI1 for mammals). Sites were PROPPINs likely participate in fission are marked in magenta.

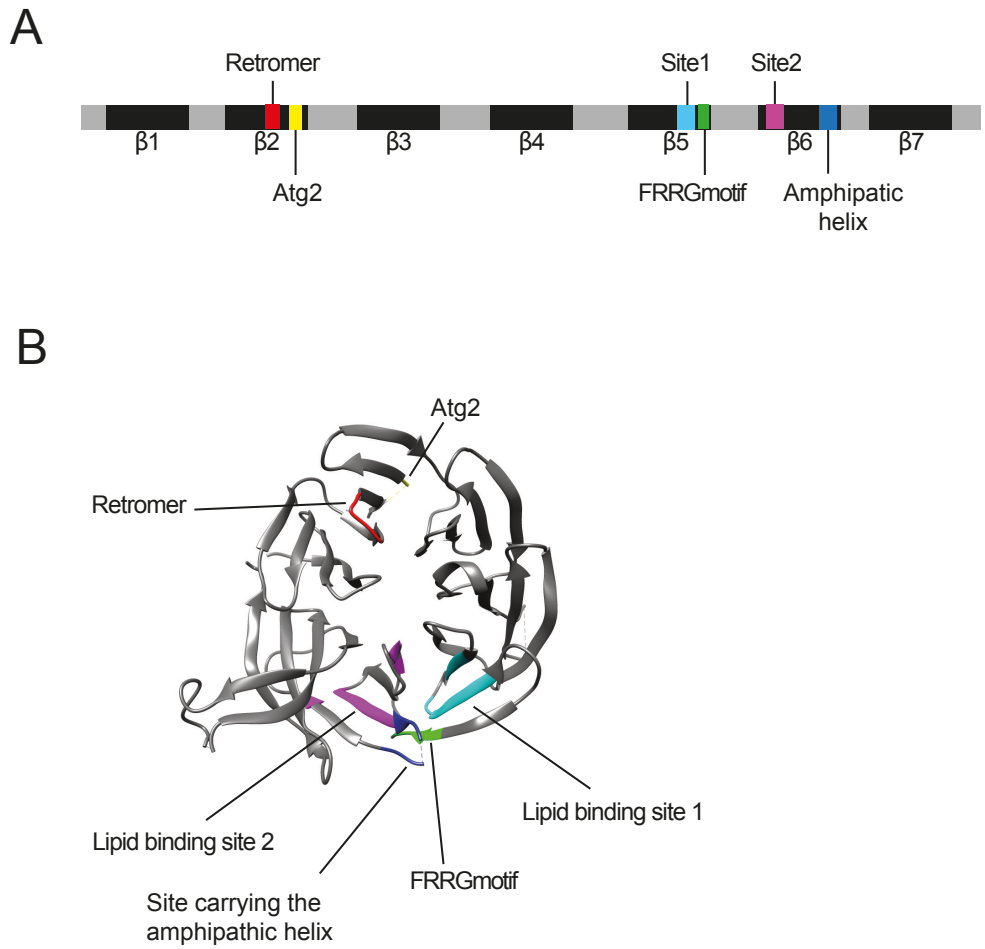

Figure 2: Structural features of PROPPINS

General structure of PROPPINs showing the 7 blades of the β-propeller in (A) linear or (B) in a 3D structure model (based on structure PDB:8AFW, Mann et al, 2023). Critical structural features and the following sites relevant for interaction with known ligands are highlighted: Regions relevant to the binding of Retromer (red) and Atg2 (yellow); lipid binding site1 (cyan); FRRG motif (green); lipid binding site2 (magenta); site carrying the loop with the amphipathic helix (blue, the loop itself is not shown).

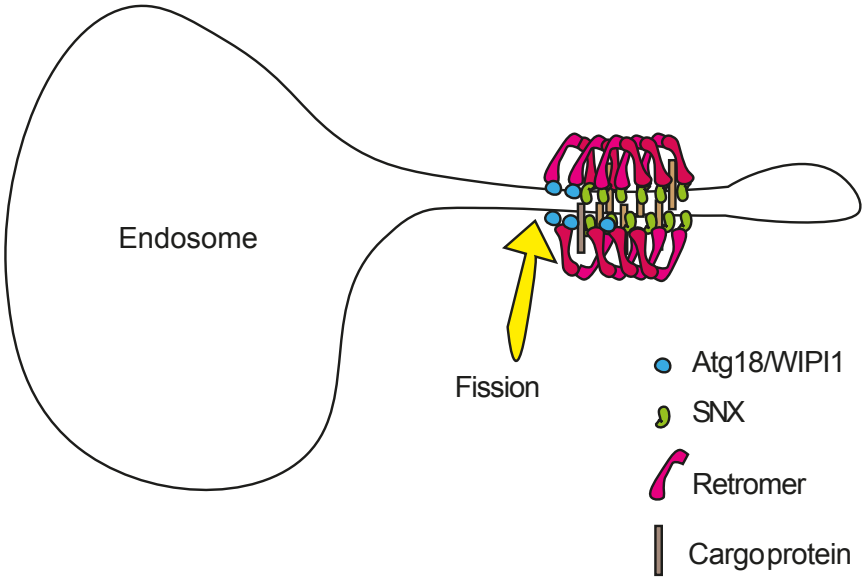

Figure 3: Suggested co-integration of PROPPIN into Retromer-coated endosomal tubules

Putative model for PROPPIN/CROP mediated fission on endosomal tubules (A). Retromer dependent clustering of PROPPINs leads to an accumulation of amphipathic helixes in the membrane resulting in membrane destabilization and fission.