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Review

Retromer-mediated endosomal protein sorting: The role of unstructured domains

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ABSTRACT

The retromer complex is a key element of the endosomal protein sorting machinery that is conserved through evolution and has been shown to play a role in diseases such as Alzheimer's disease and Parkinson's disease. Through sorting various membrane proteins (cargo), the function of retromer complex has been linked to physiological processes such as lysosome biogenesis, autophagy, down regulation of signalling receptors and cell spreading. The cargo-selective trimer of retromer recognises membrane proteins and sorts them into two distinct pathways; endosome-to-Golgi retrieval and endosome-to-cell surface recycling and additionally the cargo-selective trimer functions as a hub to recruit accessory proteins to endosomes where they may regulate and/or facilitate retromer-mediated endosomal proteins sorting. Unstructured domains present in cargo proteins or accessory factors play key roles in both these aspects of retromer function and will be discussed in this review.

1. Introduction

In eukaryotic cells endosomes serve as vital sorting stations at the crossroads of the secretory and endocytic pathways. A membrane protein arriving at an endosome via either pathway can then be sorted for delivery to three distinct destinations: the plasma membrane, the Golgi and the lysosome. Membrane proteins that traverse the endocytic system and undergo endosomal protein sorting control a variety of key physiological processes including: lysosome biogenesis, nutrient uptake, growth factor receptor signalling, autophagy and other specialised mechanisms such as the response to infection and synaptic transmission (for review see [1]). It is not surprising therefore that increasingly genes linked to many diseases, including neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, are being shown to operate in endosomal protein sorting [2].

2. The retromer complex

Due to the ubiquitous nature of endosomal protein sorting, much of the machinery that functions to drive endosomal protein sorting is conserved throughout the eukaryotic kingdom. One of the key elements of the endosomal protein sorting machinery is the retromer complex (reviewed in [3]). Initially, retromer was identified in the simple eukaryote, *Saccharomyces cerevisiae* and was shown to be a heteropentameric protein complex associated with endosomes and required for endosome-to-Golgi retrieval of receptors (e.g. the Vps10 protein) that mediate delivery of vacuolar hydrolases to the yeast vacuole. The five proteins that comprise yeast retromer are all encoded by vacuolar protein sorting (VPS) genes and are: Vps5p, Vps17p, Vps26p, Vps29p and Vps35p. Phenotypic and biochemical analyses enabled retromer to be functionally dissected into two subcomplexes: a cargo-selective trimer of Vps35p, Vps29p and Vps26p and a membrane-bending dimer of Vps5p and Vps17p [4]. The constituent proteins of retromer are conserved throughout eukaryotic life and in mammals the VPS35, VPS29 and VPS26 proteins assemble to form a stable heterotrimeric complex that selects cargo (i.e. membrane proteins) at endosomes [5–7]. The yeast Vps5 and Vps17 proteins are members of the sorting nexin (SNX) family of proteins and both contain a p40 phox-homology (PX) domain that binds to phosphatidyl inositol 3-phosphate (PI3P) – a lipid enriched in endosomal membranes [8]. In humans, the homologues of Vps5p are SNX1 and SNX2 whilst SNX5 and SNX6 function as orthologs of Vps17p although sequence homology is limited. In mammals SNX1 and SNX2 both form heterodimers with either SNX5 or SNX6 (reviewed in [9]).

The proteins that comprise the cargo-selective trimer of retromer (VPS35, VPS29 and VPS26) are all largely globular. VPS29 is a mixed α-helical and β-sheet protein that is structurally related

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to the PPP family of protein phosphatases [10]. VPS26 is all β-sheet and is structurally similar to the arrestin family of proteins [11,12]. VPS35 is comprised of many α-helical HEAT repeats arranged in a solenoidal configuration that produces what is believed to be a flexible rod like conformation with VPS29 bound at the C-terminus and VPS26 at the N-terminus [13]. The SNX dimer proteins both contain a globular PX domain in the middle of the protein and a region of coiled-coils in the C-terminal half of the SNX protein that conforms to a Bin-Amphiphysin/Rvs (BAR) domain and is required for tubule formation [14]. In the yeast Vps5 protein, the PX domain is preceded by a substantial (~250 amino acids) region of unstructured sequence that mediates the association with the cargo-selective trimer of Vps35p, Vps29p and Vps26p [15]. However, in mammals the association between the cargo-selective trimer and the SNX dimer is much weaker and the Vps5p homologues, SNX1 and SNX2, do not possess such a large N-terminal unstructured domain [16].

One important distinction between the retromer complex as identified in yeast and retromer that functions in higher eukaryotes such as humans is that the association between the two subcomplexes: the cargo-selective trimer of VPS35, VPS29 and VPS26 and the membrane-bending SNX dimer (i.e. SNX1 + 5/6 or SNX2 + 5/6) is much weaker and more transient in higher eukaryotes than the relatively stable heteropentamer that exists in yeast [17]. Thus, although the components that make up retromer are conserved from yeast to humans, the way the five proteins assemble and associate with each other is somewhat distinct. Another key difference is that in yeast, retromer functions in endosome-to-Golgi retrieval with relatively few accessory proteins. In mammals the situation is quite different and it is now known that mammalian retromer operates in endosomal protein sorting with a number of accessory proteins including: TBC1D5, a GTPase activating protein (GAP) for a small GTPase of the rab family; EHD1, an ATPase that contains a Eps15-homology (EH) domain and is required for stabilising tubules generated by the SNX dimer; and the WASH complex, a protein complex that facilitates endosomal protein sorting by stimulating production of filamentous (F-) actin on endosomal membranes [18]. None of these proteins are present in yeast and therefore not required for efficient endosome-to-Golgi retrieval. It is possible that the absence of so many retromer-accessory proteins in yeast reflects a simplified role for retromer, namely that of sorting cargo (membrane) proteins into the endosome-to-Golgi retrieval pathway whereas in mammals, retromer functions in two pathways: endosome-to-Golgi retrieval and endosome-to-cell surface recycling. The ability to sort into two pathways may require the function of retromer-accessory proteins such as the WASH complex and thus the accessory proteins that associate with retromer in mammalian cells provide additional layers of complexity and regulation to the process of endosomal protein sorting in higher eukaryotes.

Endosomal protein sorting is a highly dynamic process linked with endosomal maturation where endosomes are in a constant state of flux being mobile structures that can move along microtubules whilst undergoing membrane remodelling through the action of membrane-bending proteins such as the SNX dimers. Additionally, as endosomes move down microtubules towards the perinuclear region of the cell where the microtubule organising centre (MTOC) is located, they become increasingly acidic. The involvement of retromer in endosomal protein sorting dictates that retromer must be able to operate in an equally dynamic fashion and this is where the role of unstructured protein domains could be especially important. Indeed, there are two key activities where unstructured domains play a vital role in retromer-mediated endosomal protein sorting; namely interactions between retromer and accessory proteins and the recognition of cargo (membrane) proteins by retromer. These aspects of retromer function are discussed in detail below (see Figs. 1 and 2).

3. The retromer accessory proteins

1. *TBC1D5:* Using native immunoprecipitation, the TBC1D5 protein was shown to associate with the retromer cargo-selective trimer and subsequent studies revealed that TBC1D5 binds directly to VPS29 [19,20]. The TBC1D5 protein is a member of the Tre-Bub-Cdc16 family of Rab GTPase activating proteins (GAP). Overexpression of TBC1D5 negatively regulates recruitment of the retromer cargo-selective complex and has an opposite effect to the small GTPase Rab7a that is required for recruitment of the cargo-selective trimer [19]. Thus TBC1D5 is likely to be acting as a GAP for Rab7a. The TBC1D5 protein comprises a globular TBC domain in the N-terminal half of the protein along with a region of predicted coiled-coils in the C-terminal half. There are unstructured regions at either end of TBC1D5 that each contain an LC3-interacting region (LIR) that is reported to mediate the association between TBC1D5 and VPS29 [21,22]. Presently it is not known how the VPS29-interacting motifs in the unstructured regions of TBC1D5 contribute to the association of TBC1D5 with the retromer cargo-selective trimer but it seems likely that the combination of the globular VPS29 with the unstructured regions of TBC1D5 enables the two proteins to dynamically arrange themselves such that TBC1D5 can function as a GAP for Rab7a – which has been shown to bind to VPS35 at the N-terminal region [23] (see Table 1).

2. *VARP:* The VARP protein has recently been identified as a retromer-associated accessory protein that, like TBC1D5, requires retromer for its membrane association and also binds to VPS29 [24]. Indeed a mutation in VPS29 that blocks TBC1D5 binding also prevents VARP from binding suggesting that TBC1D5 and VARP may compete for binding to VPS29 [20,24]. VARP is a guanine nucleotide exchange factor (GEF) for Rab21, a small GTPase that is required for endosome-to-cell surface recycling [25] and VARP has been shown to be required for the proper localisation of the Glut-1 protein, a glucose transporter that traffics between the plasma membrane and endosomes [24]. Additionally, VARP is important for trafficking membrane proteins involved in pigmentation from endosomes to the melanosome [26]. VARP comprises several different domains including a VPS9 Rab GEF domain and several Ankyrin repeats. Although VARP binds to VPS29, the mechanism of interaction appears to be different to that of TBC1D5 as VARP employs a conserved region rich in cysteine residues that coordinates a Zn^{2+} ion to form a 'knuckle' that provides the binding interface for VPS29. Presently it is not known what contribution, if any, unstructured domains play in the VARP-VPS29 association. Interestingly, a homologue of VARP, Vrl1p, has recently been found to associate with yeast retromer [27] although the mechanism of association is likely to be somewhat different due to absence of cysteine 'knuckles' in Vrl1p.

3. *EHD1:* The EHD1 protein associates with the retromer cargo-selective trimer although presently it is not known how the association is mediated [28,29]. The physiological role of EHD1 in endosomal protein sorting remains to be defined but it has been reported that EHD1 is required for stabilising the membrane tubules generated by the SNX dimer [28] and structural studies of EHD2, a closely related paralog of EHD1, have shown that EHD proteins can form ring like oligomers around highly curved membranes such as tubules. This property could suggest that EHD proteins serve as 'pinchases' required for tubule scission or possibly as a form of scaffolding to stabilise nascent membrane tubules [30]. It is currently not known what role, if any, unstructured domains play in EHD1 function with retromer.

4. *The WASH complex:* As its name suggests, the WASH complex is not one protein but a complex consisting of five individual subunits. The WASH complex is named after one of its components, the WASH1 protein, a homologue of the WASP and SCAR actin

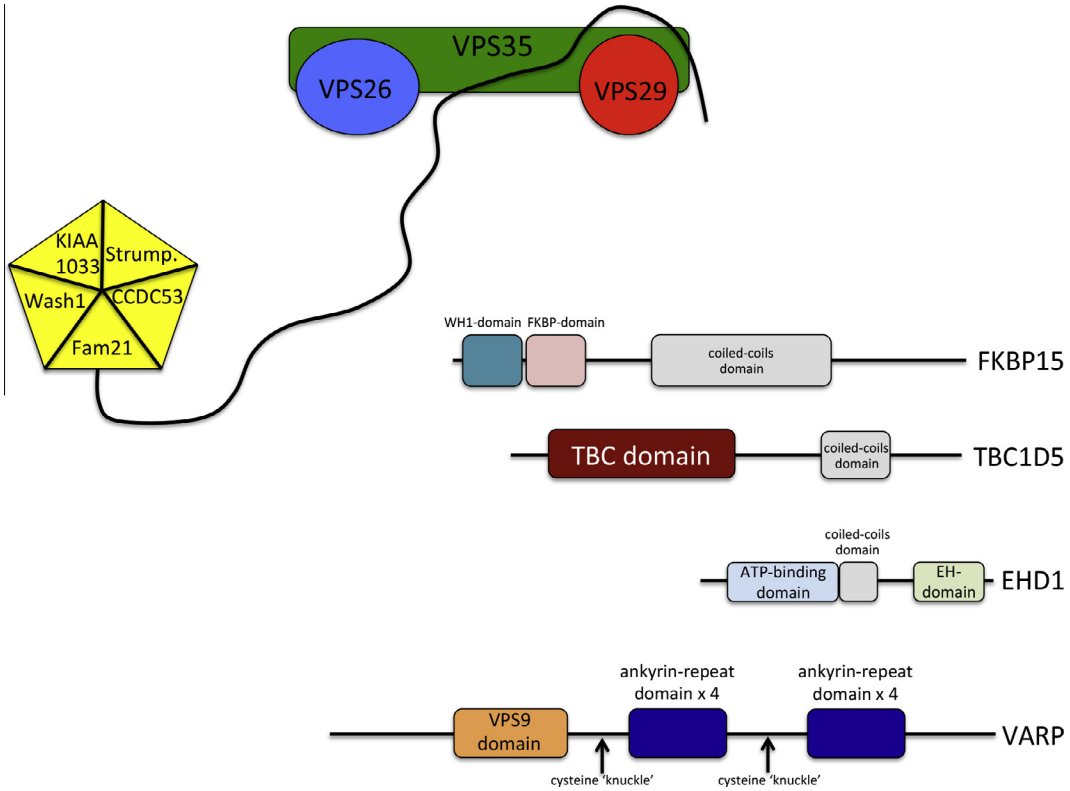

Fig. 1. The retromer cargo-selective trimer and its accessory proteins. Schematic diagram of the retromer cargo-selective trimer comprising VPS35, VPS29 and VPS26. Some of the accessory proteins and their respective functional domains are shown in schematic form. A key retromer accessory is the WASH complex that mediates formation of F-actin on endosomes. The WASH complex associates with the retromer cargo-selective trimer through the long unstructured 'tail' domain of FAM21 binding to VPS35.

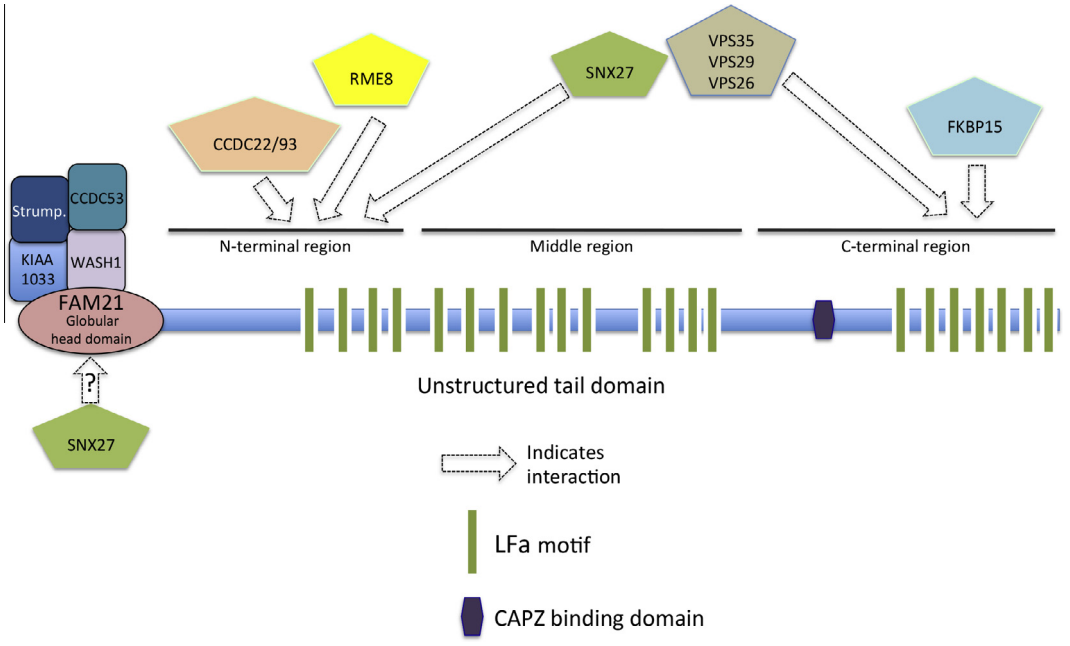

Fig. 2. The interactions of the FAM21 protein. In addition to binding to VPS35 via numerous LFa motifs, the unstructured 'tail' domain of FAM21 also associates with a number of proteins involved in endosomal protein sorting including RME-8, SNX27, FKBP15 and the dimer of CCDC22, CCDC93. The regions of the FAM21 tail that mediate binding to the respective associated proteins are indicated by arrows but in most cases have not been precisely defined.

nucleating promoting factor proteins [31]. The WASH complex mediates the production of F-actin on endosomes by stimulating the Arp2/3 complex. The precise role(s) of actin on endosomes is being intensively researched but it is now known that many membrane proteins such as Glut-1, integrins and G-protein coupled receptors including the β2-adrenergic receptor depend on the WASH complex for their proper localisation (reviewed in [18]). Through its ability to promote the formation of branched

Table 1

Protein	Mutation	Effect on structure/ interactions	Naturally occurring?	Disease causing?	Refs.
VPS35	L108P	Predicted to affect helical repeat Abolishes binding to VPS26	No	No	[60]
	D260N	Alters charge on helix Impairs binding to WASH complex	Yes	Causes Parkinson's disease	[41]
	H675R	Unknown effect on structure Abolishes binding to VPS29	No	No	[40]
VPS29	V90D	Unknown effect on structure Impairs binding to VPS35	No	No	[10]
	I91S	Unknown effect on structure Abolishes binding to VPS35	No	No	[10]
	L152E	Unknown effect on structure Abolishes binding to Vps5p (yeast) or TBC1D5 and VARP (mammals)	No	No	[10] [24]
Strumpellin	I226T N471D L619F V620A V626F G696A	Unknown effect on structure Does not affect WASH complex assembly	Yes	Causes hereditary spastic paraplegia	*

* See http://www.uniprot.org/uniprot/Q12768 for references describing mutations in Strumpellin.

actin networks on endosomes, the WASH complex might act to create actin-stabilised micro-domains that restrict the retromer cargo proteins to discrete endosomal regions, resulting in the accumulation of cargo at sites of tubule formation. Alternatively, the actin-stabilised micro-domains could function as signalling platforms, where localised signalling events are regulated.

Interestingly, studies have also shown that an inhibition of the WASH complex can result in the accumulation of endosomal tubules indicating that the WASH complex can regulate and control the fission of endosomes, potentially through the association with dynamin-2 [31,32]. The WASH1 protein has also been reported to interact with microtubules and could, through as yet unidentified mechanisms, facilitate endosomal protein sorting through microtubule-based mechanisms [33]. Along with WASH1, the WASH complex also comprises CCDC53, KIAA1033, Strumpellin and FAM21 forming a ~500kDa heteropentamer [31]. The KIAA1033 and Strumpellin proteins are both globular proteins whilst CCDC53 and WASH1 contain both globular and unstructured domains. A combination of sequence, biochemical, and structural data has shown that the WASH complex is structurally similar to the WAVE complex, a complex that promotes the formation of branched actin patches at the plasma membrane [34]. Of the 5 WASH complex proteins, it is perhaps FAM21 that is most interesting. This is because FAM21 comprises a globular N-terminal 'head' domain of ~200 amino acids followed by a hydrophilic and unstructured 'tail' domain of ~1100 amino acids [35]. The FAM21 head domain interacts with KIAA1033 and WASH1 as part of the core of the WASH complex – an assembly of globular proteins – whereas the unstructured FAM21 tail is free to undergo multiple interactions and associations [36]. These are discussed in the sections below.

4. FAM21

FAM21 is the largest and least conserved members of the WASH complex sharing relatively little similarity with its counterpart in the WAVE complex [34]. Crucially, the WASH complex is recruited to the endosomes via the interaction between the retromer complex and FAM21 [36]. The ~1100 amino acid unstructured tail of FAM21 has many interesting features including a series of 21 repeats termed the LFa motifs that broadly conform to a consensus of: L-F-acidic$_{3-10}$-L-F [37]. Additionally the FAM21 tail contains a conserved capping protein interacting (CPI) motif that mediates interactions with the CAPZa and CAPZb proteins to regulate actin-capping activity [38]. The acidic nature of the FAM21 tail is enhanced by phosphorylation at multiple sites, the functional significance of this is, however, currently unknown.

4.1. Protein interactions between the unstructured tail of FAM21 and VPS35

The LFa repeats spaced along the unstructured tail of FAM21 are involved in the interaction with the retromer complex protein VPS35 and it is this interaction that facilitates the recruitment of the WASH complex to endosomes. The multivalency of the FAM21 repeats could have a crucial role in allowing FAM21 to detect the density of retromer on membranes, enabling coordination of WASH complex recruitment, and consequent actin polymerisation only where there is a sufficient accumulation of retromer on the endosomes [37].

The activity of the WASH complex has been shown to be important for the endosome-to-cell surface cycling of integrin proteins and therefore required for cell-spreading and possibly tumour metastasis [39]. Intriguingly, a significant cell-spreading defect was observed in cells over-expressing full-length FAM21-tail. This could be due to dominant-negative effects exerted by the full-length tail competing for binding to retromer and therefore displacing the endogenous WASH complex from endosomes [36].

By dividing the unstructured tail of FAM21 into 3 approximately equal fragments, studies have shown that LFa repeats at the very C-terminal end of the tail are most important for the interaction with VPS35 [37,40]. Given that the identity of the repeats throughout the tail are more or less similar it is intriguing as to why it would be only the repeats located within the very C-terminal end of FAM21 that have such a crucial role in the interaction. Interestingly, in vivo, the FAM21–VPS35 interaction can only occur when VPS35 is in a complex with VPS29 [40]. Biochemical and structural studies have revealed the VPS35 and its C-terminal fragment that binds to VPS29 are both poorly stable

when expressed in isolation and depend on scaffolding by VPS29 for structural stability [13,16].

Alternatively it is possible that VPS29 binding to VPS35 confers a conformation on VPS35 that greatly favours the binding of the WASH complex via the LFa repeats in the FAM21 tail.

Disruption of the VPS35–FAM21 tail interaction may play a causal role in Parkinson's disease (PD). A VPS35 mutation (D620N) that causes an autosomal dominant form of PD is able to interact normally with VPS29, but only weakly with FAM21 [41,42]. The reduced retromer-WASH complex association results in impaired recruitment of the WASH complex to endosomes and adversely affects endosomal protein sorting of membrane proteins including the glucose transporter Glut-1 and the ATG9a protein that is required for autophagy.

4.2. FAM21 and RME-8

Among other proteins shown to associate with the WASH complex by binding to the FAM21 tail is RME-8 [43]. The RME-8 protein is a member of the DNAJ-domain containing family and is also known as DNAJC13 and has been shown to also interact with the retromer protein SNX1 [44]. Native immunoprecipitation experiments have indicated that RME-8 can interact with several elements of the tail domain with its strongest binding preference being for the full-length FAM21 tail.

Profound endosome tubulation is observed when the WASH complex is depleted, and, intriguingly, this phenotype is observed to an even greater degree when RME-8 expression is abolished [31,45,43]. An excess of membrane tubulation may arise from altered kinetics of the association and dissociation of SNX1 from the membrane. FAM21, by being able to interact or mediate interactions with both sub-complexes of the retromer, has a crucial role to play in both endosomal sorting and endosome tubulation. Along with VPS35, a mutation in the DNAJC13 gene that encodes RME-8 has recently been shown to cause autosomal dominant PD – further cementing the importance of retromer-mediated endosomal protein sorting in the pathogenesis of PD [46,47].

5. Interactions between FAM21 and other proteins

Interestingly, by biochemically dissecting FAM21 into its head- and tail-domains interactions that were not observed when studies were carried out with full-length FAM21 have been revealed [36]. An example is the FAM21 tail-FKBP15 interaction, the physiological implication of which is currently unclear, although it is known that this interaction is required for the localisation of FKBP15 to the endosome. A similar phenomenon was observed for the globular head region of FAM21 as well; this globular region was shown to interact with WASH1 and SNX1 and 2, but these interactions were not observed with the full-length FAM21 [35].

The tail of FAM21 is also implicated in interactions with the CCDC22 and CCDC93 proteins [36,43]. CCDC93 can interact with a component of the exocyst complex, a complex involved in exocytosis, cell migration and cell growth. Recently it has been shown that CCDC93 and CCDC22 form a complex that is required for the endosome-to-cell surface trafficking of the ATP7A copper transporter but it seems likely that other membrane proteins will require the function of CCDC93 and CCDC22 for their proper localisation and it is note-worthy that mutations in the CCDC22 gene are associated with X-linked mental retardation [48–50].

Another protein that is linked with retromer and is implicated in the trafficking from endosomes to the plasma membrane is the phox-homology domain-containing protein SNX27 [51,52]. SNX27, like SNX1 and 2, contains a PI3P-binding domain but not the membrane-bending BAR domain, a PDZ domain and a FERM-like domain. The PDZ domain is required for binding to the retromer complex and enables SNX27 to function as an adaptor protein by being able to bind PDZ ligand-containing cargoes that are destined for transport to the cell surface via the recycling pathway [52–54]. The FERM domain of SNX27 is implicated in the interaction with FAM21 and therefore the WASH complex. Studies appear to indicate that both the head and tail regions of FAM21 play a role in this interaction. The functional implication of this interaction requires further work.

6. Cargo recognition by retromer

There is, as yet, much to be learned regarding how retromer recognises cargo (membrane) proteins for sorting into either the endosome-to-Golgi retrieval pathway, or the endosome-to-cell surface recycling route. It is generally believed that much of the cargo-recognition activity is mediated by the cargo-selective trimer of VPS35-VPS29-VPS26 with both biochemical and genetic evidence supporting a role for VPS35 and VPS26 in recognising cargo proteins [6,54,55]. As these proteins are both globular, the importance of unstructured domains in the process of cargo recognition by retromer is through the cytoplasmic tail regions of membrane proteins that are recognised as cargo by retromer. One of the best studied retromer cargo proteins in mammalian cells is the cation-independent mannose 6-phosphate receptor (CIMPR) [6,7]. This is a type-I membrane protein that binds the lysosomal hydrolases carrying a mannose 6-phosphate tag in the Golgi/trans-Golgi network (TGN). The receptor and its ligand then traffic to an endosome where the lower pH of the endosome lumen triggers the release of the hydrolase by the receptor that is then transported back to the Golgi/TGN by retromer.

The cytoplasmic tail of the CIMPR is ~160 amino acids long and predicted to be unstructured. Studies using reporter constructs comprising the lumenal and transmembrane domain of CD8 fused to the cytoplasmic tail of the CIMPR revealed that a conserved hydrophobic motif of Trp-Leu-Met (WLM) in the CIMPR tail is necessary for retromer-mediated endosome-to-Golgi retrieval of a CD8-CIMPR reporter [56]. Another protein that is dependent on retromer for its localisation is Sortilin – a type-I membrane protein related to yeast Vps10p. In the cytoplasmic tail of sortilin is a motif – Phe-Leu-Val (FLM) – that is necessary for its endosome-to-Golgi retrieval by retromer whilst the DMT1-II iron transporter utilises a Tyr-Leu-Leu (YLL) sequence in its cytoplasmic tail for retromer-mediated sorting [57]. Thus retromer appears to recognise short aromatic-containing hydrophobic motifs in the cytoplasmic tail of cargo proteins but how retromer recognises these motifs has yet to be determined. It seems likely that VPS26 plays an important role in cargo-recognition and in vitro experiments have shown that recombinant VPS26 can bind to the cytoplasmic tail of SorL1 (also known as SorLA), a type-I membrane protein that, like Sortilin, is related to Vps10p. In the cytoplasmic tail of SorL1, a sequence, Phe-Ala-Asn-Ser-His-Tyr (FANSHY) is required for SorL1 to bind to VPS26 [55].

Additionally, it is now known that retromer accessory proteins, for example, SNX27 and the CCDC93–CCDC22 dimer can themselves function in cargo-selection. For SNX27, cargo-selection occurs through the FERM domain of SNX27 recognising motifs (usually Asn-Pro-x-Tyr – NPxY) present in the cytoplasmic tails of specific cargo proteins such as the β-adrenergic receptor [58]. Although cytoplasmic tails of membrane proteins are often quite short and unstructured, it is possible that reversible palmitoylation can restrict the freedom of movement of the cytoplasmic tail to favour an association with protein sorting machinery such as retromer. For both the CIMPR and Sortilin, palmitoylation on cysteine residues has been shown to facilitate the endosome-to-Golgi

retrieval of those proteins [59]. However, reversible palmitoylation cannot be a universally employed regulatory mechanism for retromer-mediated endosome-to-Golgi retrieval as other retromer cargo proteins, e.g. SorL1, do not have the requisite cysteine residues in their cytoplasmic tail and thus cannot be a substrate for palmitoylation.

7. Summary

The retromer complex is a key element of the endosomal protein sorting machinery being involved in sorting into two distinct pathways; endosome-to-Golgi retrieval and endosome-to-cell surface recycling. Retromer also operates as a hub to recruit additional proteins and protein complexes to endosomes and unstructured domains, such as the long FAM21 tail, play a key role in these interactions. Cargo recognition by retromer involves short conserved motifs present in the unstructured cytoplasmic tails of membrane proteins although the precise mechanisms that govern cargo-selection remain to be elucidated.

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