Endosomal retrieval of cargo: retromer is not alone

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Abstract

Endosomes are major protein sorting stations in cells. Endosomal localized multi-protein complexes sort integral proteins, including signaling receptors, nutrient transporters, adhesion molecules and lysosomal hydrolase receptors, for lysosomal degradation or conversely for retrieval and subsequent recycling to various membrane compartments. Correct endosomal sorting of these proteins is essential for maintaining cellular homeostasis, with defects in endosomal sorting implicated in various human pathologies including neurodegenerative disorders. Retromer, an ancient multi-protein complex, is essential for the retrieval and recycling of hundreds of transmembrane proteins. Whilst retromer is a major player in endosomal retrieval and recycling, several studies have recently identified retrieval mechanisms that are independent of retromer. Here we review endosomal retrieval complexes, with a focus on recently discovered retromer-independent mechanisms.

Endosomal sorting of cargo is achieved by multi-protein complexes

The endocytic pathway consists of a series of membrane trafficking steps which act together to regulate cell surface levels of lipids and integral proteins (referred to as cargo). Following endocytosis cargo enter the endosomal network and are sorted for one of two fates; degradation as mature endosomes fuse with lysosomes or retrieval from this fate. Following retrieval, cargo are recycled back to the plasma membrane, the TGN (*trans*-Golgi Network) or to other organelles such as melanosomes (**Figure 1**) [1, 2]. Thus endosomal sorting of cargo for degradation or retrieval and recycling tightly regulates the composition of the cell surface and hence the ability of the cell to sense and respond to its environment. While endocytosis is a key entry point into the endosomal network, cargo such as the lysosomal hydrolase receptors CI-MPR (cation-independent mannose 6-phosphate receptor) and sortilin undergo iterative rounds of trafficking between the biosynthetic pathway and endosomes, in order to deliver lysosomal hydrolases to the endosomal lumen (**Figure 1**) [2]. Therefore, endosomal retrieval and recycling is essential not only for plasma membrane homeostasis but also for maintaining lysosomal health [3]. Consistent with this, defective endosomal sorting is associated with many human diseases, including Parkinson's disease [4, 5].

Endosomal sorting requires a series of spatially and temporally regulated multi-protein complexes to facilitate cargo enrichment and membrane deformation to generate cargo-enriched budding profiles that undergo scission to generate cargo-enriched transport carriers [6]. Whilst ESCRT (endosomal sorting complexes required for transport) complexes specifically recognize ubiquitinated cargo and sort these into **ILVs** (**intra-luminal vesicles; see Glossary**) for lysosomal degradation [7], it was long thought that retrieval and recycling of cargo was achieved through **geometric-**

based principles and bulk membrane flow [8]. However, many studies have established that endosomal retrieval and recycling is achieved by sequence-dependent sorting of cargo by endosomal localized multi-protein machineries and branched actin [9, 10]. One of the best characterized endosomal retrieval complexes is the evolutionary conserved retromer complex [2, 11]. In human cultured cells, retromer regulates cell surface levels of over 100 integral plasma membrane proteins as well as mediating recycling of cargo from the endosome to the TGN and to lysosomal-related organelles [1, 2, 12]. However, not all cargo require retromer for their retrieval and recycling [13-16]. Recently retriever and the CCC complex have been identified as additional evolutionary conserved endosomal complexes [14, 17]. Importantly, together these complexes provide a mechanism for retromer-independent endosomal sorting of additional cargo [14]. The WASH complex, which promotes endosomal branched actin polymerization, plays essential roles in both retromer-dependent and retromer-independent pathways, demonstrating that the WASH complex is fundamental for endosomal cargo sorting [14, 17-19]. Here, we review the recent advancements in our understanding of endosomal cargo recycling, with a focus on the first step of this process - retrieval of cargo from a lysosomal degradative fate (**Figure 1**).

Lysosomal degradation vs retrieval and recycling

Once cargo enter the endosomal network, a major fate decision is made; are cargo targeted for lysosomal degradation or will they be retrieved and subsequently recycled back to the relevant membrane compartment [2]? Integral membrane cargo to be degraded are marked through lysine-63 (K-63)-linked ubiquitination [20]. The

evolutionary conserved ESCRT complexes sequentially localize to the cytosolic face of the endosome and drive cargo enrichment, membrane deformation, and vesicle scission to generate cargo-enriched ILVs. Upon fusion of late endosomes with lysosomes, cargo within the ILVs are degraded (**Figure 1**) [7]. Whilst ubiquitination is a key marker of cargo to be degraded, it should be noted that ubiquitin-independent cargo incorporation into ILVs has been reported. ALIX (apoptosis-linked gene 2-interacting protein X) interacts with sequence-specific motifs within cargo and acts as an adaptor for the ESCRT pathway, bypassing the requirement of ubiquitination [21].

Interestingly, upon suppression or knock-out of retromer, retriever, the WASH or CCC complexes (see subsequent discussions), cargo are degraded in lysosomes suggesting that in the absence of these sorting complexes, cargo degradation may be the default pathway [12, 14]. Conversely, suppression or deletion of proteins involved in membrane remodelling, **carrier scission** and **carrier transport** and fusion often results in the accumulation of cargo in endosomal or vesicular compartments. For example, depletion of retromer results in lysosomal degradation of the glucose transporter GLUT1, whereas suppression of SNX-BARs, which are important for carrier formation, results in endosomal accumulation of the transporter [12]. Retromer, retriever, the CCC and WASH complexes will therefore be referred to as retrieval complexes because they are required for retrieval of cargo away from lysosomal degradation.

Retrieval and degradative functions of the endosome are spatially segregated

The antagonistic roles of ESCRT and retrieval complexes in cargo sorting are spatially segregated on the cytosolic leaflet of the endosome (**Figure 1**). ESCRT and clathrin

co-localize in endosomal sub-domains which are distinct from sub-domains containing endosomal retrieval complexes such as retriever, retromer and the WASH complex [14, 22, 23]. The formation and maintenance of these distinct subdomains may spatially segregate cargo into either degradative or retrieval sub-domains based upon recognition of ubiquitinated cargo by ESCRT or sequence-specific recognition of cargo by retrieval complexes. Whilst the precise mechanism by which cargo leak into the lysosomal pathway in the absence of retrieval complexes is not established, it could be speculated that cargo are no longer spatially segregated away from ESCRT sub-domains and instead become incorporated into ILVs. The dependence of this process on cargo ubiquitin status or recognition by ALIX remains to be investigated.

The WASH complex organises endosomal retrieval sub-domains

Flat clathrin lattices are proposed to scaffold the ESCRT subdomain whereas the formation of endosomal retrieval sub-domains, in which cargo and their respective retrieval complexes are enriched, is driven by branched actin polymerization by the **Arp2/3** complex [10, 22-25]. WASH1 (WASHC1) is an endosomally localized nucleation promoting factor for Arp2/3 and it exists as part of the pentameric WASH complex along with (new nomenclature for the subunits are shown in brackets): FAM21A/B/C (WASHC2A/B/C), CCDC53 (WASHC3), SWIP also known as KIAA1033 (WASHC4) and strumpellin also known as KIAA0196 (WASHC5) (**Figure 1**) [26, 27]. The WASH complex is of ancient origin but has not been retained by Fungi [28]. Depolymerization of endosomal actin causes distinct WASH labelled retrieval subdomains to merge indicating that endosomal actin is important for the formation and maintenance of retrieval subdomains [23]. However WASH labelled subdomains

remain spatially distinct to degradative sub-domains upon actin depolymerization [23]. Depletion of the WASH interactor RME-8 (receptor-mediated endocytosis-8, also known as DNAJC13) or the **sorting nexin** SNX1 (see later discussion) however results in the mixing of degradative and retrieval subdomains in *Caenorhabditis elegans* and HeLa cells, a process speculated to occur due to defects in removing clathrin from the retrieval subdomain [22, 29].

Moreover the WASH complex also organizes the retrieval sub-domain by acting as an endosomal scaffold for the recruitment of additional multi-protein retrieval complexes and regulators of endosomal sorting to the retrieval sub-domain. The long unstructured tail of FAM21 interacts with and recruits the CCC complex along with retriever, to the endosomal membrane (see subsequent discussions) [14, 17]. Moreover, the tail of FAM21 interacts with FKBP15 (FK506-binding protein 15) and mediates its localization to endosomes, although a functional role for FKBP15 is yet to be reported [30-32].

Strikingly, in fibroblasts derived from WASH knock-out mice and melanocytes from strumpellin knock-out mice, the endosomal and lysosomal networks become collapsed to the peri-nuclear region suggesting that the WASH complex is also important for the spatial organization of the endo-lysosomal system although the underlying mechanisms remain unknown [33, 34].

The WASH complex is essential for the retrieval and recycling of many cargo

Consistent with the notion that the WASH complex is essential for retrieval sub-domain formation and maintenance as well as endosomal positioning, the WASH complex is necessary for endosomal retrieval and recycling of many cargo to both the TGN and the plasma membrane, including: CI-MPR [35], the retromer-independent cargo $\alpha_5\beta_1$

integrin [14, 16, 36], GLUT1 [34], TCR (T cell receptor) [37], TfnR (transferrin receptor) [26], β2-adrenergic receptor [10, 38] and LDLR (low-density lipoprotein receptor) [18]. In *Dictyostelium discoideum*, the WASH complex is required for recycling of phagocytic receptors from phagosomes to the plasma membrane [39]. Moreover, WASH and its ability to polymerize actin was required for the removal of **v-ATPase** from lysosomal membranes, to allow lysosome neutralization prior to exocytosis of indigestible material [40]. The role for WASH in recycling of the v-ATPase was also demonstrated in WASH knock-out *Drosophila melanogaster* [41]. In addition hemocytes from WASH knock-out *Drosophila* display defects in cell spreading and cell migration, indicative of impaired integrin recycling [41].

Besides the role of the WASH complex and actin in endosomal organization, it was reported that depletion of components of the WASH complex resulted in elongated endosomal recycling tubules, suggesting that the WASH complex is required for tubule scission [26, 35]. However WASH knock-out MEFs (mouse embryonic fibroblasts) did not display this phenotype [34]. Endosomal actin also helps to generate and stabilize endosomal tubules into which cargo are sorted in a sequence-dependent manner [10]. Interestingly, β2-adrenergic receptors that lack a PDZbm (**PDZ binding motif**), required for retrieval and recycling, but instead harbor an actin-binding domain can recycle normally, indicating that interactions between endosomal actin and cargo may be sufficient for recycling [10].

Recruitment of the WASH complex to endosomes

Retromer directly binds to the multiple **LFa** (leucine-phenylalanine-a series of acidic residues) repeats in the carboxy-terminal tail of FAM21 and this was reported to be

sufficient and necessary for endosomal recruitment of the WASH complex [31, 32, 42, 43]. However, in a retromer knock-out HeLa cell line, a significant proportion of FAM21 remains associated with the endosomal network, establishing that in human cells the endosomal association of the WASH complex is mediated through both retromer-dependent and retromer-independent mechanisms [14]. This agrees with work in *Dictyostelium* describing the retromer-independent recruitment of the WASH complex [39]. Importantly, retromer-independent recruitment of the WASH complex may explain how endosomal retrieval of some cargo requires the WASH complex but occurs independently of retromer (see subsequent discussions) [14].

The mechanism of retromer-independent recruitment of the WASH complex to endosomal membranes remains poorly characterized. However, purified WASH complex displayed the ability to associate with liposomes and the carboxy-terminal tail of FAM21 was shown to bind to PI3P and $PI(3,5)P_2$, phosphoinositides found enriched on the cytosolic leaflet of early and late endosomes, suggesting that intrinsic lipid-association of the WASH complex may be important for retromer-independent recruitment to endosomal membranes [26, 27, 44]. In addition, interactions between the WASH complex and endosomally localized proteins including the sorting nexin SNX27 (see subsequent discussion) and RME-8 may also underlie retromer-independent recruitment of the WASH complex [12, 29].

Retromer

Retromer is an ancient endosomal sorting complex, conserved in all eukaryotes [45]. Retromer was first discovered in the budding yeast *Saccharomyces cerevisiae,* where retromer exists as a pentameric complex, composed of a dimer of Vps5p and Vps17p

and a trimer of Vps26p, Vps35p and Vps29p [11, 46]. In mammals the genes encoding retromer have undergone duplication and divergence [45]. SNX1 and SNX2 are orthologues of the yeast *Vps5* gene, whilst SNX5, SNX6 and SNX32 are orthologues of *Vps17* [45]. These proteins contain a **BAR domain** (Bin/amphiphysin/Rvs) which can sense and induce curvature, generating endosomal tubules for cargo recycling [47, 48]. SNX1 or SNX2 dimerize with SNX5, SNX6 or SNX32 [49]. In mammals VPS26 exists as three paralogues: VPS26A, VPS26B and DSCR3 [45]. DSCR3 is not a sub-unit of retromer and instead incorporates into the retriever complex (see subsequent discussion) [14]. It is important to note that whilst retromer is highly evolutionary conserved, mammalian retromer is not an obligatory pentameric complex. The mammalian VPS26, VPS35 and VPS29 proteins exist as a hetero-trimer which does not form a stable complex in solution with the SNX-BAR dimer [50]. When discussing mammalian retromer, the membrane deformation SNX-BAR hetero-dimer will subsequently be referred to as the 'retromer linked SNX-BAR' complex whereas the VPS26:VPS35:VPS29 hetero-trimer will be referred to simply as 'retromer' [13].

Retromer was originally identified as being required for endosome to TGN retrograde transport of multiple cargo including Vps10p, a receptor in *S. cerevisiae* required for delivery of hydrolases such as carboxypeptidase Y, from the Golgi to the vacuole (the yeast equivalent of the lysosome) and its mammalian homologues sorLA and sortilin, the lysosomal hydrolase receptor CI-MPR and the mammalian iron transporter DMT1-II [11, 46, 51-57]. Retromer engages cargo containing a ØX(L/M) (where Ø represents an aromatic amino acid and x represents any amino acid) motif in their cytosolic tail and VPS26 directly binds a FANSHY motif within the cytoplasmic tail of sorLA [52, 57-59].

Whilst some cargo have been reported to directly bind to retromer, it was discovered that a 'cargo adaptor', SNX3, could directly engage cargo such as the yeast iron transporter Fet3p-Ftr1p and Wntless whilst simultaneously associating with retromer for retrieval of these cargo away from degradation and for their recycling back to the TGN [59-62]. In addition, retromer can retrieve and recycle cargo back to the plasma membrane and this often occurs through association with a distinct retromer cargo adaptor, SNX27, which recognises a PDZbm within the cytosolic domain of integral proteins [12, 38, 63, 64].

Retromer is suggested to function by directly engaging specific sequences in the cytosolic tails of cargo through its VPS26:VPS35:VPS29 hetero-trimer or through association with SNX3 or SNX27 [38, 59, 60]. Cargo are enriched and corralled, with the aid of WASH-dependent actin polymerization, into endosomal tubules generated by the retromer-linked SNX-BAR hetero-dimers for transport to target membranes [1]. Recently super-resolution imaging established that retromer cargo destined for the plasma membrane and the TGN are sorted into the same tubular carriers, suggesting that recycling to either membrane occurs downstream of retromer [65].

Organization and structure of retromer

VPS35 forms an extended central scaffold with VPS26 and VPS29 independently engaging VPS35 at the amino- and carboxy-terminals respectively (**Figure 2 A**) [50, 59, 66]. VPS35 is composed of 33 helices, which form 16 pairs of antiparallel α-helices, a domain structure referred to as a HEAT (Huntington/EF3/PP2A/TOR1) repeat. These HEAT repeats form an extended α-helical solenoid structure which is slightly curved [59, 66]. At the carboxy-terminus of VPS35, the solenoid structure of VPS35

wraps itself around VPS29 which forms a fold similar to that found in phosphoesterases, although no phosphoesterase activity of VPS29 is observed *in vitro* (**Figure 2 A**) [66, 67]. VPS26A and VPS26B adopt arrestin-like folds, which consist of two beta-sandwich domains connected by a flexible linker and a polar core (**Figure 2 A**) [68, 69].

SNX3 binds to the VPS26-VPS35 interface through its amino terminal flexible region and its **PX domain**. The binding of retromer to SNX3 causes a conformational change in VPS26 which opens up a hydrophobic, cargo-binding pocket in the interface between the carboxy-terminal lobe of VPS26 and the PX domain of SNX3 [59]. SNX27 directly binds to VPS26 through SNX27's PDZ (Postsynaptic density 95 – Disc large – ZO1) domain [12, 70]. The **PDZ domain** of SNX27 also mediates the interaction with cargo, although through a distinct binding site within the PDZ domain [70, 71].SNX27's affinity for cargo is increased when SNX27 is bound to VPS26 thus coupling cargo recognition with retromer association [70].

Not all cargo are retrieved away from lysosomal degradation by retromer

The ability of retromer to directly interact with cargo and via cargo adaptors such as SNX3 and SNX27 increases the repertoire of cargo retromer can retrieve and recycle in a sequence-dependent manner, thus increasing the plethora of cellular functions for which retromer mediated retrieval and recycling is required [72]. Quantitative proteomic analysis of plasma membrane integral proteins under retromer or SNX27 suppression indicated that retromer is required for the endosome to plasma membrane retrieval and recycling of over one hundred cargo including proteins involved in cell adhesion, ion transport and amino acid transport [12]. It is therefore not surprising that

mutations in retromer and retromer-associated proteins have been associated with various human diseases including Parkinson's disease (see [3, 73] for recent reviews).

Yet, even multiple SNX-retromer complexes are unlikely to account for the sequence-dependent retrieval and recycling of all cargo transiting through the endosomal system, suggesting that additional retrieval and recycling pathways exist. Consistent with this, two papers have recently reported that the retromer linked SNX-BAR proteins (SNX1/SNX2:SNX5/SNX6/SNX32) can bind to cargo such as CI-MPR and IGF1R (insulin-like growth factor 1 receptor) and facilitate their retrieval and recycling back to the TGN or the plasma membrane respectively, independently of the retromer VPS26:VPS35:VPS29 trimer [13, 15]. Further support for the existence of retromer-independent retrieval pathways came from studies on the SNX27-related sorting nexin, SNX17. SNX17 and SNX27 both contain a **FERM** (4.1/ezrin/radixin/moesin) **domain** but SNX17 lacks the amino-terminal PDZ domain found in SNX27 required for binding to PDZbm in cargo and for interactions with retromer [63, 74, 75]. The FERM domain of SNX17 mediates the binding to NPx(Y/F) motifs within the cytosolic tails of cargo such as LRP1 (Low density lipoprotein receptor-related protein 1), LDLR and $\alpha_5\beta_1$ integrin and upon suppression of SNX17, these cargo are degraded in lysosomes [16, 76-78]. A global proteomic approach in HeLa cells revealed that SNX17 regulates the plasma membrane levels of over 200 cargo [14]. Consistent with the inability of SNX17 to engage retromer, the recycling of the majority of these cargo were not affected by SNX27 or retromer suppression [12, 14, 16]. SNX17-dependent, retromer-independent retrieval of cargo instead relies upon the recently discovered ancient endosomal retrieval complex, retriever [14].

Retriever is a 'retromer-like' retrieval complex

Retriever is a hetero-trimer consisting of DSCR3, C16orf62 (chromosome 16 open reading frame 62) and the retromer sub-unit VPS29 (**Figure 2 A**). Suppression or knock-out of retriever sub-units or perturbations in the ability of retriever to interact with SNX17 results in the lysosomal degradation of SNX17 cargo such as $\alpha_5\beta_1$ integrin. Importantly, knock-out of VPS35, the core component of retromer, does not give this phenotype demonstrating that retriever-dependent sorting of cargo is independent of retromer [14].

Retriever shares several similarities with retromer, for example, both complexes are ubiquitously expressed and are found in the last common eukaryotic ancestor [28, 45]. However, DSCR3 and C16orf62 have been selectively lost, along with the WASH complex and the CCC complex (see later discussion) in all Fungi [14, 28, 45] (**Figure 2 B**) In addition, in HeLa cells the estimated protein copy number of retromer sub-units is much higher than the retriever sub-units DSCR3 and C16orf62 (**Figure 2 B**) [79].

Retriever and retromer share similarities in their structural composition. The most obvious similarity is that VPS29 is a sub-unit in both retromer and retriever. DSCR3, as previously discussed, is a known paralogue of VPS26 [45, 80]. Furthermore, although C16orf62 and VPS35 share little residue conservation, C16orf62 is predicted to contain HEAT repeats, similar to those found in VPS35. Therefore retriever and retromer are hetero-trimers which contain a VPS29 subunit, a protein with an arrestin-like fold (either DSCR3 or VPS26A/B respectively) and a protein containing a series of HEAT-repeats (C16orf62 or VPS35 respectively) (**Figure 2 A**) [14].

Immuno-precipitations in DSCR3 knock-out HeLa cells indicate that SNX17 may interact with retriever via DSCR3, although evidence of a direct interaction is currently lacking [14]. This is reminiscent of SNX27 engagement with the equivalent subunit of retromer, VPS26 [12, 70]. SNX31, which is expressed mainly in the urinary tract, is closely related to SNX17 and is also important for the recycling of various integrins [81]. Both SNX17 and SNX31 possess the conserved carboxy-terminal motif that is necessary and sufficient to bind to retriever, indicating that SNX31 may play an important role in retrieving cell-type specific cargo [14]. It has yet to be established whether retriever can bind to cargo directly, as has been reported for retromer [52, 59].

Retriever and retromer undertake distinct cargo retrieval itineraries but are localized to the same endosomal retrieval sub-domain [14, 17]. Retromer does not contain any membrane binding domains and instead relies upon interactions with SNX3 and the late endosomal/lysosomal Rab GTPase Rab7 for membrane association to early and early-to-late transitioning endosomes (**Figure 3**) [82-84]. Like retromer, retriever is not predicted to bind membranes and therefore retriever also relies upon protein: protein interactions for its recruitment to endosomes. In contrast to retromer, the recruitment of retriever to endosomal sub-domains does not depend upon SNX3 and Rab7 but through interactions with an additional endosomal sorting complex, the CCC complex (**Figure 3**) [14].

The CCC complex

The CCC complex colocalizes with retromer, retriever and the WASH complex on endosomes and is required for the endosomal retrieval and recycling of LDLR, Notch2

and ATP7A to the plasma membrane [14, 17, 18, 85]. The CCC complex comprises of a heterodimer of CCDC22 (coiled-coil domain-containing protein 22) and CCDC93 (coiled-coil domain-containing protein 93) and the association of COMMD (copper metabolism MURR1 domain–containing) proteins [17, 86].

The COMMDs are a family of 10 members (named COMMD 1-10), largely conserved throughout evolution [87, 88]. Whilst vertebrate genomes contain all 10 COMMD genes, *Drosophila* only have 5 COMMD genes (COMMD 2, 3, 4, 5 and 10), *C*. *elegans* only encodes COMMD4 whereas *Dicytostelium* possesses all 10 genes [87-89]. The defining characteristic of COMMDs is the presence of a unique carboxy-terminal COMM domain. The COMM domain is responsible for homo- and hetero-dimerization of COMMD proteins and there is evidence for preferential dimers existing in cells [85, 87]. In addition, the COMM domain has been shown to be essential for interactions between COMMD1 and the CCC complex subunits CCDC22 and CCDC93, a mechanism which probably underlies how every COMMD protein has the ability to associate with CCDC22 and CCDC93 [17, 86]. It should be noted that whilst the CCC complex has recently been implicated in endosomal retrieval and recycling of cargo, roles for these proteins, especially the COMMDs, in other cellular functions such as the hypoxia response and NF-κB activation have been reported (see [89, 90] for recent reviews on COMMD1 function).

The retriever-CCC-WASH complex pathway

A full mechanistic understanding of how the CCC complex engages cargo and promotes its retrieval from degradation for subsequent recycling has not been achieved. However, like retromer, components of the CCC complex have been shown

to directly interact with the tail of FAM21, an interaction which is necessary for CCC complex localization [17, 31]. In agreement with the WASH complex being important for the function of the CCC complex, *Wash1* knock-out MEFs display endosomal accumulation of LDLR and increased degradation of the receptor, which can be rescued through treatment with **bafilomycin**, which prevents lysosomal acidification and thus pH-dependent degradation [18]. Furthermore, the reintroduction of a WASH mutant, that cannot activate Arp2/3, into *Wash1* knock-out MEFS did not rescue LDLR from lysosomal degradation, suggesting that branched actin polymerization is essential for CCC complex-dependent recycling of cargo [18].

Several proteomics studies have identified that the CCC complex interacts with components of retriever (**see Box 1**) [17, 28, 91, 92]. Moreover, components of the CCC complex were also identified as interactors of SNX17 [14]. SNX17 interactions with the CCC complex are dependent upon retriever, suggesting that SNX17 interacts indirectly with the CCC complex through retriever [14]. Furthermore, suppression of CCDC22 or CCDC93 results in the lysosomal degradation of $\alpha_5\beta_1$ integrin, indicating that SNX17, retriever and the CCC complex are functionally linked.

A model for how these complexes function together in retrieval of $\alpha_5\beta_1$ integrin was proposed when it was observed that suppression of the CCC complex results in the loss of endosomal association of retriever [14, 17]. Extending this further, knock-out of FAM21, which recruits the CCC complex to endosomes, also results in the loss of endosomal association of retriever and consequently lysosomal degradation of $\alpha_5\beta_1$ integrin [14]. It has previously been shown that the WASH complex is essential for the retrieval and recycling of $\alpha_5\beta_1$ integrin (or their homologues) in invasive ovarian cancer cells, mouse fibroblast cells, *Dictyostelium* and *Drosophila* [36, 39, 41, 93]. Similarly, the retrieval of LFA-1 (lymphocyte function-associated antigen 1) and TCR (T cell

receptor), two other SNX17-dependent cargo, from lysosomal degradation in T cells, also depends upon the WASH complex [37, 94]. However, until recently the mechanism linking SNX17 and the WASH complex, in a retromer-independent manner, had not been described. The current model suggests that the WASH complex is recruited to endosomes in retromer-dependent and retromer-independent mechanisms, where it recruits the CCC complex through interactions with FAM21. The CCC complex then recruits retriever to the retrieval sub-domain of endosomes where it can engage SNX17 and its bound cargo, driving retrieval of cargo away from degradation (**Figure 3**). Evolutionary conservation analysis infers that the WASH, CCC and retriever complexes co-evolved, consistent with the notion that these complexes depend on each other for localization and function [14, 28, 89].

Whilst this model suggests that the CCC complex is forming a bridge between retriever and the WASH complex it must be stressed that it is highly unlikely that this is the only function of the CCC complex. Indeed it has been proposed that the COMMDs themselves may act as cargo-binding adaptors [18, 85]. Whilst many of the COMMD proteins are ubiquitously expressed in tissues, some COMMDs such as COMMD9 have preferential expression in myeloid cells and the central nervous system [88]. Moreover, it has recently been shown that the CCC complex containing COMMD9 dimerized to COMMD5 or COMMD10 was involved in the retrieval and recycling of Notch2 to the plasma membrane [85], whereas COMMD1 is involved in ATP7A and LDLR recycling [17, 18]. This raises the possibility that distinct CCC complexes containing different COMMD hetero-dimers exist, analogous to the SNX-retromers [2]. Consistent with this idea, COMMD9 and COMMD1 knock-out mice, whilst both embryonic lethal, display different phenotypes indicating that they are not completely redundant with one another [85, 95].

Furthermore, the COMM domain of COMMDs shares structural homology with the lipid binding **pleckstrin homology (PH) domains** [89, 96]. Recombinant COMMD1 was found to bind to liposomes containing $PI(4,5)P_2$, an interaction which required the COMM domain and promoted COMMD1 oligomerisation [96]. It is unknown whether the proposed lipid-binding ability of COMMDs plays a role in endosomal retrieval and recycling.

Concluding remarks and future perspectives

At the time of writing, four major, evolutionary conserved, endosomal retrieval multi-protein complexes (retromer, retriever, WASH and the CCC complexes) are thought to drive retrieval of cargo in a sequence-dependent manner away from a lysosomal degradative fate. These complexes are summarized in **Table 1**. The identification of retromer-independent pathways has expanded our knowledge of sequence-dependent cargo retrieval and recycling, with retriever likely to be responsible for retrieval and recycling of at least an additional 200 integral proteins back to the plasma membrane [14]. As yet unidentified cargo adaptors could feed into the retriever-CCC-WASH pathway and these complexes may directly engage sequence motifs in cargo, accounting for additional cargo retrieval events.

The importance of endosomal retrieval complexes in maintaining cellular homeostasis is highlighted by patients carrying mutations in genes encoding for these retrieval complexes (**Table 2**). Interestingly, mutations in CCC and WASH complex subunits present very similar patient phenotypes, such as intellectual disability, suggesting a shared mechanism of pathology (**Table 2**). Moreover, homozygous knock-out mouse models of COMMD1, COMMD6, COMMD9, COMMD10, WASH1, strumpellin are

434 embryonic lethal [34, 85, 95, 97, 98] and a point mutation in SNX17 in mice causes
435 congenital abnormalities such as an atrioventricular septal heart defect and duplex
436 kidneys [99, 100], demonstrating that these complexes play critical roles in
437 developmental processes.

438 Whilst the discovery of retromer-independent endosomal retrieval pathways have
439 allowed a greater understanding of the molecular mechanisms of endosomal sorting,
440 many outstanding questions remain (**see Outstanding Questions**). Future research
441 investigating the molecular mechanisms of endosomal cargo retrieval and recycling
442 and how they are regulated will answer these important questions.

443

Glossary

Arp2/3 (Actin-related protein 2/3) complex: A complex composed of seven subunits which nucleates branched actin polymerisation. However, Arp2/3 itself is not intrinsically active and requires activation by nucleation promoting factors such as the WASH complex.

Bafilomycin: An inhibitor of the vacuolar-H^+-ATPase, preventing acidification of lysosomes.

BAR domain: BAR domains are a protein dimerization interface that dimerise to form a rigid banana-shaped structure. BAR domains are membrane curvature sensing domains and can also induce membrane curvature and tubulation of liposomes.

Carrier scission: Detachment of the nascent cargo enriched carrier from the donor membrane.

Carrier transport: Transport of the cargo enriched carrier from the donor to the acceptor membrane. Usually occurs via microtubule and actin based motors.

FERM domain (4.1/ezrin/radixin/moesin): A domain with a cloverleaf structure and binds to NPx(Y/F) motifs within proteins.

Geometric based principles: The recycling tubules display a high surface area to low volume ratio, thus transmembrane proteins entering the endosome are much more likely to be present in the endosomal tubules than the vesicular portion of the endosome which has a high volume to low surface area ratio. Therefore, unless cargo are specifically recognized by ESCRT, they are likely to be recycled back to the plasma membrane or the TGN based upon bulk membrane recycling.

Intra-luminal vesicles (ILVs): Vesicles formed from inward-budding of the endosomal membrane.

LFa motifs: Leucine-Phenylalanine-a series of acidic residues. Multiple motifs found in the tail of FAM21 that mediate binding to VPS35.

PDZ binding motif: Protein:protein interaction motif found in the cytosolic tails of transmembrane cargo, usually at the extreme carboxy-termini. The consensus sequence of a class I PDZbm is [T/S]-x-ϕ, where x denotes any amino acid and ϕ represents an amino acid with a bulky hydrophobic group.

PDZ domain (Postsynaptic density 95 – Disc large – ZO1): PDZ domains interact with PDZ binding motifs (PDZbm).

Pleckstrin homology (PH) domain: A phosphoinositide binding domain, interacts with $PI(3,4)P_2$ and $PI(3,4,5)P_3$ and various other phosphoinositide species.

PX domain (Phox homology): A phosphoinositide binding domain, interacting primarily with PI3P.

Sorting nexin (SNX): Sorting nexins are a family of proteins defined by the presence of a PX domain with over 50% sequence similarity to the first member of the family, SNX1. Some members of this protein family also contain extra functional domains such as PDZ and FERM domains.

v-ATPase: vacuolar H^+-ATPase. Proton pump that utilises ATP hydrolysis to pump protons from the cytosol into the lumen of endosomes and lysosomes, generating and maintaining an acidic pH in the lumen of these organelles.

BOX 1:

The COMMander complex

Genome-wide human interaction networks and phylogenetic analyses have suggested the presence of a large, ancient, multi-protein complex named 'COMMander' because of the presence of a large number of COMMD proteins [28, 92, 101, 102]. The current evidence for COMMander has been recently reviewed, suggesting that that the core COMMander complex consists of 14 proteins: CCDC22, CCDC93, COMMD 1-10, C16orf62 and DSCR3 [89]. It could be speculated that COMMander is a super-complex, composed of retriever and the CCC complex. Whilst retriever can be purified from insect cells as a stable hetero-trimer, the CCC complex is required for retriever localization, protein stability and function [14, 103]. Moreover, their shared evolution, along with the WASH complex, suggests a fundamental role for the assembly in cellular function [28]. Further biochemical evidence will be needed to clarify the organization, definition and functional role of COMMander.

Table 1 Summary of endosomal retrieval complexes

	Retromer	Retriever	CCC complex	WASH complex
Subunits	• VPS26A/B • VPS35 • VPS29	• DSCR3 (VPS26C) • C16orf62 (VPS35L) • VPS29	• CCDC22 • CCDC93 • COMMD 1-10	• WASH (WASHC1) • FAM21A/C (WASHC2A/C) • CCDC53 (WASHC3) • SWIP (WASHC4, KIAA1033) • Strumpellin (WASHC5, KIAA0196)
Recruitment to endosomes	• SNX3 • Rab7	• CCC complex	• WASH complex	• Retromer • Unknown retromer-independent mechanism • Intrinsic lipid binding?
Direct cargo binding	• VPS26 binds to FANSHY motifs • Øx(L/M) (where Ø represents any hydrophobic amino acid and x represents any amino acid) motif	• Unknown	• Unknown • Cargo possibly binding to COMMDs	• Unknown • Cargo may bind actin
Cargo adaptors	• SNX3 • SNX27	• SNX17 • SNX31	• COMMDs?	

C16of62; Chromosome 16 open reading frame 62, DSCR3; Downs syndrome critical region 3, VPS35L; VPS35-like, CCDC; coiled-coil domain containing, WASH; Wiskott-Aldrich syndrome protein and SCAR homologue, SWIP; Strumpellin and WASH

interacting protein, CCC complex; CCDC22, CCDC93 and COMMD, SNX; sorting nexin.

Table 2 Human diseases associated with mutations in the retriever-CCC-WASH pathway

Protein	Mutation	Disease	Examples of phenotypes	References
CCDC22	p.T17A	X-linked intellectual disability	• Intellectual disability • Ventricular and atrial septal defects • Craniofacial dysmorphisms • Hypercholesterolemia	[18, 86, 104]
	p.Y557C	X-linked intellectual disability with symptoms of Ritscher/Schinzel syndrome	• Intellectual disability • Dandy-walker malformations • Camptodactyly • Ventricular septal defects	[105]
Strumpellin	p.I226T	Hereditary spastic paraplegia (SPG8)	• Lower limb weakness • Spasticity	[106]
	p.N471D			[107]
	p.R583S			[108]
	p.S591P			[109]
	p.L619F			[107]
	p.V620A			[110, 111]
	p.V626F			[107]
	p.G696A			[112]
	p.E713K			[113]
	g.ex11-15del			[108]
	c.3335+2T>A c.3335+4C>A	Ritscher–Schinzel/3C syndrome	• Intellectual disability • Hypertelorism	[114]

	c.3335+8A>G		• Atrial and ventricular septal defects vary within the patient cohort	
SWIP	p.P1019R	Autosomal recessive intellectual disease	• Intellectual disability • Short stature • Severe delays in motor development	[115]

CCDC; coiled-coil domain containing, SWIP; Strumpellin and WASH interacting protein

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Figure 1 Overview of endosomal retrieval and recycling of cargo

Cargo from the biosynthetic pathway (e.g. CI-MPR) or the plasma membrane (e.g β2 adrenergic receptor, $\alpha_5\beta_1$ integrin, EGFR) enter the endosomal system where they are either sorted for degradation or are retrieved away from a degradative fate and recycled to the plasma membrane, to the TGN or to other organelles such as melanosomes. Retrieval complexes such as retriever, retromer, the CCC and WASH complexes localize to retrieval sub-domains, which are spatially distinct from the degradative sub-domain in which ESCRT complexes reside. Cargo destined for lysosomal degradation, such as activated EGFR, are recognized by the ESCRT complexes which package the cargo into intraluminal vesicles (ILVs) that bud into the endosomal lumen and eventually pinch off (represented as black circles inside the endosome). As endosomes mature, the number of ILVs in the lumen increases and eventually late endosomes fuse with lysosomes and cargo is degraded. Hydrolase receptors, such as CI-MPR, deliver hydrolases from the TGN to the endosome. Upon delivery to endosomes, the change in pH results in the dissociation of the hydrolase from the receptor into the endosomal lumen. The hydrolase is ultimately delivered to the lysosome upon fusion of endosomes and lysosomes. The hydrolase receptor and other cargo destined for recycling are conversely retrieved away from lysosomal degradation through the sequence-dependent recognition of cytosolic facing motifs by cargo retrieval complexes. After retrieval, cargo are recycled back to various membrane compartments.

C16of62; Chromosome 16 open reading frame 62, DSCR3; Downs syndrome critical region 3, VPS35L; VPS35-like, CCDC; coiled-coil domain containing, WASH; Wiskott-Aldrich syndrome protein and SCAR homologue, SWIP; Strumpellin and WASH interacting protein, CCC complex; CCDC22, CCDC93 and COMMD, SNX; sorting nexin, ESCRT ; endosomal sorting complex required for transport, EGFR; epidermal growth factor receptor, CI-MPR; cation independent mannose-6 phosphate receptor, TGN; trans-Golgi network, ILVs; intraluminal vesicles

Figure 2 Retriever is predicted to be a retromer-like complex

A) The mammalian retromer hetero-trimer consists of VPS26A/B, VPS35 and VPS29. VPS35 forms an extended central scaffold with VPS26 and VPS29 independently engaging VPS35 at the amino- and carboxy-terminals respectively. Retriever is a hetero-trimer consisting of DSCR3 (VPS26C), C16orf62 (VPS35L) and VPS29, with predicted structural similarity with retromer.

B) Estimated protein copy numbers of retrieval complexes in *S. cerevisiae* and HeLa cells. Subunits of retriever, the CCC complex and the WASH complex are absent in the yeast *S. cerevisiae*. Interestingly, the retriever subunits C16orf62 and DSCR3 are expressed at much lower levels than the equivalent VPS35 and VPS26A/B subunits in retromer. Copy number data from [79].

The predicted structures of retromer and retriever, adapted from [116].

C16of62; Chromosome 16 open reading frame 62, DSCR3; Downs syndrome critical region 3, VPS35L; VPS35-like, CCDC; coiled-coil domain containing, WASH; Wiskott-Aldrich syndrome protein and SCAR homologue, SWIP;

Strumpellin and WASH interacting protein, CCC complex; CCDC22, CCDC93 and COMMD

Figure 3 The endosomal retrieval sub-domain

'Degradative' sub-domains containing ESCRT complexes and 'retrieval' sub-domains containing retriever, retromer and the WASH and CCC complexes are spatially segregated on endosomal membranes. 1) The pentameric WASH complex (individual subunits are not shown for simplicity) activates Arp 2/3, which polymerizes branched actin on the endosomal membrane. The WASH complex is recruited to endosomal membranes through interactions of the FAM21 tail (represented as a black wavy line) with retromer and also through unidentified retromer-independent mechanisms. 2) Retromer is recruited to the endosomal membrane through interactions with SNX3 and GTP-bound Rab7a. Sequence motifs within cytosolic domains of cargo are recognized by retromer, either directly via a ϕx(L/M) motif (where ϕ represents an aromatic residue and x represents any residue) or indirectly through the cargo adaptors SNX3 and SNX27, the latter of which binds to cargo containing a PDZbm. VPS29 can also bind to regulatory factors such as TBC1D5, a Rab7 GAP and VARP. 3) The tail of FAM21 can interact with and recruit the CCC complex to endosomal membranes. The CCC complex interacts with and recruits retriever to the retrieval subdomain. Precise interactions between the CCC complex and retriever have not been elucidated. 4) SNX17 associates with endosomal membranes through its PX domain and engages cargo, such as $\alpha_5\beta_1$ integrin, containing NPxY or NPxF motifs. SNX17 is a cargo adaptor for retriever, associating with retriever through its flexible carboxy-terminal tail. Retriever may be able to directly engage cargo. Other sequence-dependent cargo entry points into this pathway could occur via the CCC complex. Adapted from [14].

878 C16of62; Chromosome 16 open reading frame 62, DSCR3; Downs syndrome critical
879 region 3, VPS35L; VPS35-like, CCDC; coiled-coil domain containing, WASH; Wiskott-
880 Aldrich syndrome protein and SCAR homologue, SWIP; Strumpellin and WASH
881 interacting protein, CCC complex; CCDC22, CCDC93 and COMMD, SNX; sorting
882 nexin, ESCRT; endosomal sorting complex required for transport, TBC1D5; TBC1
883 domain family member 5, PDZbm; PDZ binding motif, GAP: GTPase-activating
884 protein, VARP: VPS9-ankyrin-repeat protein.

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Outstanding questions

- **What is the precise role of retromer in endosome-to-TGN transport?**
- **Is retriever also involved in endosome-to-TGN trafficking of cargo and if so, which and how many?**
- **Can retriever bind to cargo directly?**
- **Are retromer and retriever cargo corralled into the same cargo carriers?**
- **What complexes act downstream of retriever and the CCC complex to mediate cargo carrier formation, scission and transport?**
- **How is sequence-dependent cargo retrieval and recycling co-ordinated with RAB switching to allow for pathway progression?**
- **Is there redundancy in cargo retrieval and is this a point of regulation and/or therapeutic intervention?**
- **What are the regulators of endosomal retrieval complexes?**
- **Are there other undiscovered multi-protein complexes which regulate endosomal retrieval of cargo?**

Highlights

- Endosomal cargo are retrieved from degradation by multi-protein complexes prior to recycling
- Retromer, retriever, the CCC and WASH complexes are required for sequence-specific retrieval of cargo
- Retromer is a key orchestrator of endosomal sorting but retromer-independent pathways exist
- Retriever is a retromer-like complex that functions independently of retromer
- Retriever requires the CCC and WASH complexes for endosomal localisation and function
- Mutations in these multi-protein complexes are increasingly associated with human pathologies including neurodegeneration and developmental disorders.

Glossary:

Arp2/3 (Actin-related protein 2/3) complex: A complex composed of seven subunits which nucleates branched actin polymerisation. However, Arp2/3 itself is not intrinsically active and requires activation by nucleation promoting factors such as the WASH complex.

Bafilomycin: An inhibitor of the vacuolar-H^+-ATPase, preventing acidification of lysosomes.

BAR domain: BAR domains are a protein dimerization interface that dimerise to form a rigid banana-shaped structure. BAR domains are membrane curvature sensing domains and can also induce membrane curvature and tubulation of liposomes.

Cargo: Transmembrane proteins, and their associated proteins and lipids, that are trafficked through the endosomal network.

Carrier scission: Detachment of the nascent cargo enriched carrier from the donor membrane.

Carrier transport: Transport of the cargo enriched carrier from the donor to the acceptor membrane. Usually occurs via microtubule and actin based motors.

FERM domain (4.1/ezrin/radixin/moesin): A domain with a cloverleaf structure and binds to NPx(Y/F) motifs within proteins.

Geometric based principles: The recycling tubules display a high surface area to low volume ratio, thus transmembrane proteins entering the endosome are much more likely to be present in the endosomal tubules than the vesicular portion of the endosome which has a high volume to low surface area ratio. Therefore, unless cargo are specifically recognized by ESCRT, they are likely to be recycled back to the plasma membrane or the TGN based upon bulk membrane recycling.

Intra-luminal vesicles (ILVs): Vesicles formed from inward-budding of the endosomal membrane.

LFa motifs: Leucine-Phenylalanine-a series of acidic residues. Multiple motifs found in the tail of FAM21 that mediate binding to VPS35.

PDZ binding motif: Protein:protein interaction motif found in the cytosolic tails of transmembrane cargo, usually at the extreme carboxy-termini. The consensus sequence of a class I PDZbm is [T/S]-x-ϕ, where x denotes any amino acid and ϕ represents an amino acid with a bulky hydrophobic group.

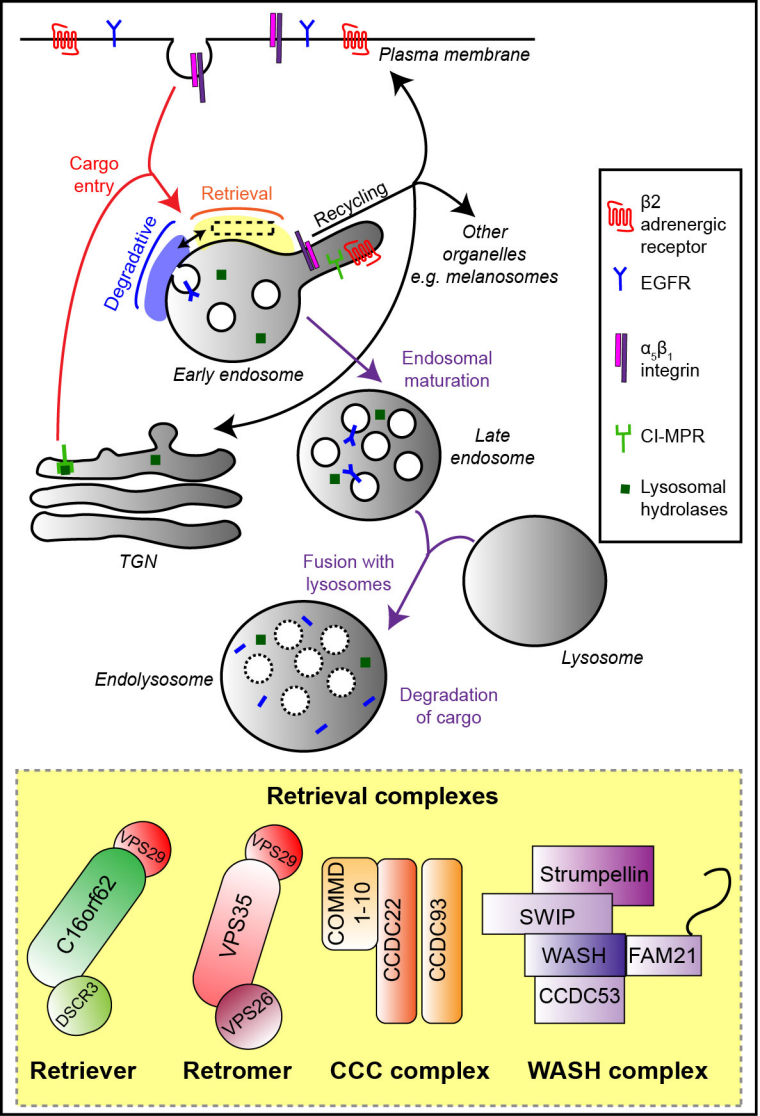
PDZ domain (Postsynaptic density 95 – Disc large – ZO1): PDZ domains interact with PDZ binding motifs (PDZbm).

Pleckstrin homology (PH) domain: A phosphoinositide binding domain, interacts with PI(3,4)P_2 and PI(3,4,5)P_3 and various other phosphoinositide species.

PX domain (Phox homology): A phosphoinositide binding domain, interacting primarily with PI3P.

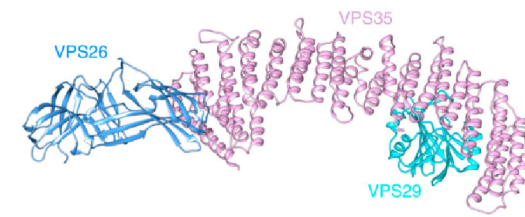
Sorting nexin (SNX): Sorting nexins are a family of proteins defined by the presence of a PX domain with over 50% sequence similarity to the first member of the family, SNX1. Some members of this protein family also contain extra functional domains such as PDZ and FERM domains.

v-ATPase: vacuolar H^+-ATPase. Proton pump that utilises ATP hydrolysis to pump protons from the cytosol into the lumen of endosomes and lysosomes, generating and maintaining an acidic pH in the lumen of these organelles.

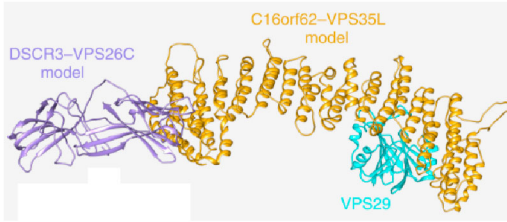

Plasma membrane
Cargo entry
Retrieval
Recycling
Degradative
Other organelles e.g. melanosomes
Early endosome
Endosomal maturation
Late endosome
TGN
Fusion with lysosomes
Lysosome
Endolysosome
Degradation of cargo
β2 adrenergic receptor
EGFR
α5β1 integrin
CI-MPR
Lysosomal hydrolases
Retrieval complexes
VPS29
C16orf62
DSCR3
VPS29
VPS35
VPS26
COMMD 1-10
CCDC22
CCDC93
Strumpellin
SWIP
WASH
FAM21
CCDC53
Retriever
Retromer
CCC complex
WASH complex

A

Retromer

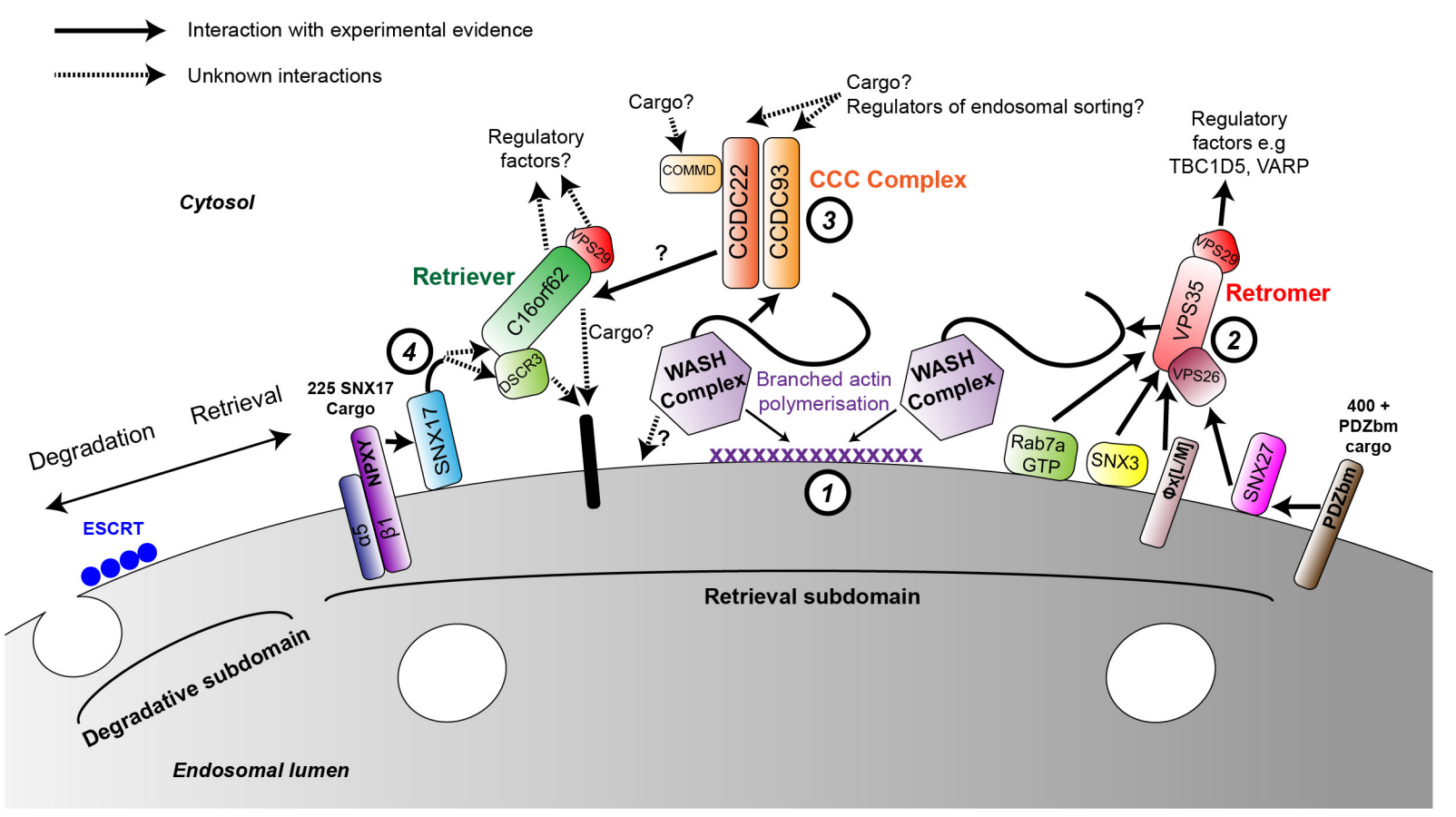


Retriever



B

Complex	Protein	Copy Number in *S. cerevisiae*	Copy Number in HeLa cells
Retromer	VPS26A	1140.8	826147.4
	VPS26B	0.0	76397.8
	VPS35	571.3	780462.6
Retromer/Retriever	VPS29	4009.1	1424734.0
Retriever	C16orf62 (VPS35L)	0.0	286.3
	DSCR3 (VPS26C)	0.0	2751.5
CCC complex	CCDC93	0.0	33942.3
	CCDC22	0.0	55222.3
	COMMD1	0.0	83103.2
WASH complex	WASH1 (WASHC1)	0.0	43936.1
	FAM21A (WASHC2A)	0.0	58981.4
	FAM21B (WASHC2B)	0.0	558.1
	FAM21C (WASHC2C)	0.0	1135.1
	CCDC53 (WASHC3)	0.0	99617.0
	SWIP (WASHC4)	0.0	65245.8
	Strumpellin (WASHC5)	0.0	141599.6


Interaction with experimental evidence
Unknown interactions
Cytosol
Regulatory factors?
Cargo?
Cargo?
Regulators of endosomal sorting?
COMMD
CCDC22
CCDC93
CCC Complex
3
Regulatory factors e.g TBC1D5, VARP
VPS29
Retriever
C16orf62
DSCR3
?
Cargo?
4
WASH Complex
Branched actin polymerisation
WASH Complex
VPS35
Retromer
2
VPS26
225 SNX17 Cargo
SNX17
NPxY
gp
β1
Degradation
Retrieval
ESCRT
xxxxxxxxxxxxxxx
1
Rab7a GTP
SNX3
ΦxΦLMη
SNX27
PDZbm
400 + PDZbm cargo
Retrieval subdomain
Degradative subdomain
Endosomal lumen