To degrade or not to degrade: mechanisms and significance of endocytic recycling

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ABSTRACT:

Newly endocytosed integral cell surface proteins are typically either directed for degradation or subject to recycling back to the plasma membrane. The sorting of integral cell surface proteins, including signalling receptors, nutrient transporters, ion channels, adhesion molecules and polarity markers, within the endo-lysosomal network for recycling is increasingly recognised as an essential feature in regulating the complexities of cell, tissue and organism-level physiology. Historically, endocytic recycling has been regarded as a relatively passive process, where the majority of internalized integral proteins are recycled via an unspecific sequence-independent “bulk membrane flow” pathway. Recent work has increasingly challenged this view. The discovery of sequence-specific sorting motifs and the identification of cargo adaptors and associated coat complexes has begun to uncover the highly orchestrated nature of endosomal cargo recycling, thereby providing new insight into the function and (patho)physiology of this process.

[H1] INTRODUCTION:

The human genome encodes for between 5,500 and 7,500 integral membrane proteins[1]. These proteins are essential for regulating a wide array of core cell functions that include cell signalling, nutrient sensing and transport, cell adhesion, establishment and

maintenance of cell polarity and cell migration. The abundance and spatial organization of cell surface integral proteins relies on two central pathways: the secretory pathway and the endo-lysosomal network (Figure 1). Through the integration of these pathways the steady-state plasma membrane integral membrane proteome is established and is actively remodelled in response to changing physiological demand. Disruption of this balance is increasingly associated with diseases especially those linked with ageing and neurodegeneration[2].

The entry into the endo-lysosomal network begins with the endocytosis of cell surface integral membrane proteins together with their associated proteins and lipids through clathrin-dependent and clathrin-independent mechanisms, as well as through phagocytosis and pinocytosis[3,4] (integral proteins and their associated proteins and lipids are referred to as 'cargo'). The newly formed and cargo-enriched peripheral endocytic carriers undergo homotypic fusion to form the early endosome, which then matures becoming a late endosome. The early and late endosomes essentially serve to sort cargo between two fate decisions: cargo is either sorted for degradation within the lysosome or it is retrieved from entering this fate (of note, some sorting may occur prior to reaching the early endosome either during the process of endocytosis or through sorting at a pre-early endosome compartment)[5-8]. If subject to retrieval, cargo is packaged into tubulo-vesicular transport carriers for recycling to the plasma membrane or the secretory pathway (Figure 1).

While endocytosis from the cell surface and the machinery controlling the degradative pathway are extensively characterized, the mechanistic basis of retrieval and recycling processes remains poorly understood. The discovery of endocytic cargo adaptors and regulated coat complexes has begun to shed some light onto this important part of the ubiquitous endo-lysosomal network. In this Review we will discuss two key questions related to cargo retrieval and recycling. First, we will explore the molecular events that define the degradative versus retrieval fates decisions. For those cargoes that are retrieved, we will discuss how they are packaged into tubulo-vesicular transport carriers for subsequent recycling.

[H1] CARGO FATE DECISIONS AT THE ENDOSOME

Sorting of cargo between the degradative and recycling fates is inherently linked with the complex morphology of the early endosome (Figure 1). This organelle comprises a

central vacuole (approx. 100 – 500 nm in diameter) that is associated with cytosolic facing tubular membrane extensions (approx. 20 to 50 nm in diameter) and inwardly budding intraluminal vesicles **[G]** (ILVs) (approx. 40 to 60 nm in diameter)[9]. Cargoes destined for lysosomal degradation are sorted into the ILVs[10]. Multiple rounds of cargo sorting and ILV biogenesis occur as the early endosome matures into a late endosome (globular vacuoles 250 – 1000 nm in diameter)[9]. Late endosomes are typified by the appearance of numerous ILVs and are often referred to as multivesicular bodies (MVBs) or multivesicular endosomes (MVEs)[9,10]. For the majority of late endosomes their fate is to fuse with the lysosome, generating an endo-lysosome compartment that provides a controlled acidic environment for the degradation of the cargo-loaded ILVs[11]. In many cell types, particularly in immune cells, a sub-population of cholesterol-enriched late endosomes avoids lysosome fusion and instead fuses with the plasma membrane to release the contents of ILVs as extracellular vesicles known as exosomes[12].

In parallel, within the same early and late endosomes, cargo destined for recycling first needs to avoid inclusion into ILVs: a process that is termed cargo retrieval[13]. Once retrieved, these cargoes are sorted and enriched into the cytosol-facing tubular membrane extensions, which give rise to tubulo-vesicular transport carriers that move cargo back to the cell surface[14,15]. Two kinetic routes classically define endosomal recycling: a fast recycling pathway where cargo is targeted directly back to the plasma membrane and a slow recycling pathway where the cargo first transits through the endocytic recycling compartment before being delivered to the cell surface[16,17]. There is mounting evidence that cargo can be recycled from early and late endosomes and from the endocytic recycling compartment by further transiting through the *trans*-Golgi network (TGN)[18-22]. Why so many recycling routes? In polarised cells the diversity of recycling ensures robustness of plasma membrane protein delivery, which is necessary to establish and maintain plasma membrane polarity, whereas in other cell types the recycling route taken by signalling receptors affects their intracellular residence time and thereby can influence their signalling outputs.

[H1] THE DEGRADATIVE FATE DECISION

Key to initiating the fate decision between degradation and cargo retrieval is the presence of cargo and an early endosome specific phospholipid, phosphatidylinositol 3-monophosphate (PtdIns(3)P)[23]. Cargo destined for lysosomal degradation, such as the activated epidermal growth factor receptor (EGFR), first undergoes ubiquitylation on

lysine residues present within their intracellular cytosolic domain(s)[24]. This modification serves to direct the internalised cargo for degradation and is generally of the mono-ubiquitylation type but may also include Lys63-linked poly-ubiquitylation[24]. The presence of the ubiquitin modification is detected by a series of multi-protein complexes belonging to endosomal sorting complexes required for transport **[G]** (ESCRT) family: ESCRT-0, ESCRT-I and ESCRT-II refs. [25-27] (Figure 2). ESCRT-0 is a heterodimer of HRS and STAM. At steady-state ESCRT-0 resides on the early endosome, which is mediated by HRS binding to PtdIns(3)P. HRS and STAM each bind multiple ubiquitin-containing cargoes with low micromolar affinity for ubiquitin. Ubiquitylated cargoes that arrive at the early endosome are recognised by ESCRT-0 and become clustered as a result of the non-stoichiometric binding of ESCRT-0 components to ubiquitin and the ability of ESCRT-0 to self-associate into larger complexes[25-27]. Together this leads to the establishment of a degradative subdomain, the formation of which may be further stabilised through HRS recruiting clathrin[28-31]. Components of ESCRT-I (TSG101 and UBAP1) and ESCRT-II (VPS36) also bind ubiquitin with low affinity and may help to further enrich ubiquitylated cargo at degradative subdomains[25-27].

A distinct late acting ESCRT complex, ESCRT-III, all components of which lack ubiquitin binding, is recruited to the degradative subdomain through sensing the density of ESCRT-II ref [32]. ESCRT-III components, including SNF7, assemble into oligomeric fragments thereby corralling and restricting the lateral diffusion of captured cargoes on the endosomal membrane[32]. The ESCRT-III (and ESCRT-0) mediated recruitment of deubiquitylating enzymes leads to deubiquitylation of the corralled cargoes and the reuse of the ubiquitin moiety. Other ESCRT-III components control and regulate self-assembly of SNF7 into a flat spiral lattice that surrounds the corralled cargo and undergoes transition to a three-dimensional spring. This is considered to be essential in generating the required membrane tension for the generation of the inwardly budding profile of forming ILVs[32]. ESCRT-0, ESCRT-I and ESCRT-II dissociate from the maturing budding profile prior to scission — which occurs through an incompletely understood process that requires an ESCRT-III accessory protein, the AAA ATPase VPS4 — to form an isolated, cargo-enriched ILV[32]. The ESCRT machinery therefore constitutes a highly orchestrated system for co-ordinating the selection and enrichment of cargoes destined for degradation with the biogenesis of the ILVs to allow their controlled delivery to the lysosome (Figure 2).

Although ubiquitylation is the major tag that destinies cargo for ESCRT-driven incorporation into ILVs, ubiquitin-independent routes for targeting cargo to ILVs have

also been identified. For example, the protease-activated receptor-1 (PAR1) and the $P2Y_1$ purinergic receptor rely on cargo binding to the ESCRT accessory protein ALIX[33,34]. Endosomes also display poorly characterised ESCRT-independent mechanisms for the biogenesis of ILVs[35,36].

When considering the fate decisions between cargo degradation and cargo retrieval a fundamentally important question concerns the frequency of ILV biogenesis and its relationship with the maturation state of the early and late endosome. In yeast, in the absence of ubiquitylated cargo, ILVs fail to form even in the presence of the complete ESCRT machinery[37,38]. This failure points to an essential role for sensing the presence of ubiquitylated cargo in the process of ILV biogenesis. It is tempting to speculate that the frequency of ILV biogenesis may be greatest at the newly formed early endosome where the density of ubiquitylated cargo is high, in contrast to the late endosome where the density of ubiquitylated cargo is low (owing to the fact that most of the ubiquitylated cargo has already been sorted into ILVs). As we will discuss below, a consequence of removing the early endosome associated machinery that governs the fate decision towards retrieval is the missorting of internalised cargo into the lysosomal degradative pathway. If such an initial burst of ILV biogenesis were to occur (to date there is no direct evidence of this in yeast or mammals), the retrieval of cargo destined for recycling at this early stage of the endosomal network may be necessary to prevent cargo leakage into the degradative route. For cargoes that arrive later in the endo-lysosomal maturation pathway, for example those coming directly from the secretory pathway, there may be a reduced need for active cargo retrieval simply because the frequency of ILV biogenesis is lower within a more mature endosome.

[H1] AVOIDING THE DEGRADATIVE FATE

The ESCRT-mediated degradative sorting of endocytosed cargo conforms with the central dogma of intracellular membrane trafficking: proteinaceous coat complexes co-ordinate the recognition of sorting signals with membrane remodelling, leading to the biogenesis of cargo-enriched transport carriers. The early models describing endocytic cargo retrieval and recycling did not conform to this dogma. Based on the observation that the prototypical recycling cargo, the transferrin receptor (TfnR), was recycled in the absence of its entire intracellular cytosolic facing ‘tail’ domain[39], it was concluded that recycling was the default pathway in the absence of any specific sorting signal. The majority of cargoes were considered therefore to undergo recycling to the cell surface by

following the bulk membrane flow, a model that was termed 'sequence-independent, geometric-based sorting'[40,41].

At the heart of geometric-based sorting is evidence that tubular extensions enriched in recycling cargo emerge from the vacuolar membrane of the early endosome[42]. These tubules undergo fission to form cargo-enriched tubulo-vesicular transport carriers. Given the high membrane-to-volume ratio of tubules compared with the vacuolar portion of the endosome, the repeated formation of tubules would generate a bulk membrane flow away from the vacuolar portion of the endosome and the associated lysosome degradative fate. The tubular geometry also restricts the amount of luminal content - for example nutrients that have been absorbed through endocytosis and lysosomal hydrolases that have been delivered from the biosynthetic pathway - leaving the endosome through tubular recycling carriers. Any integral membrane protein with high lateral mobility that is not restricted through capture and corralling into the degradative sub-domain, can in principle follow the bulk membrane flow and be retrieved and recycled[14].

Although the recycling of some cargoes may well conform to this model, two broad observations have established that for numerous cargoes their recycling involves sequence-dependent mechanisms. First, a number of cargoes contain linear peptide sequences present within their cytosolic tails, so-called sorting motifs **[G]**, that are essential for their recycling (Table 1)[43]. Indeed, a critical reevaluation of TfnR recycling established that its intracellular cytosolic domain contains one or more sorting motifs[44,45]. Second, the identification of various cargo adaptors that recognise the sorting motifs, and the characterisation of membrane remodelling complexes have provided molecular insight into the process of sequence-dependent cargo sorting and the biogenesis of cargo-enriched tubulo-vesicular transport carriers[46-51] (Supplementary Table S1). This step forward is perhaps best illustrated by the appreciation of the role of two ancient and highly conserved heterotrimeric protein complexes, retromer and retriever as mediators of retrieval and recycling[52,53] (Figure 3).

[H1] RECOGNITION OF CARGO FOR RETRIEVAL

Retromer was identified in yeast through its ability to sort cargo from endosomes to the Golgi[13,54]. In metazoans however, the principle role of retromer is in retrieving cargo from the degradative fate prior to transporting the retrieved cargo back to the cell surface,

although retromer-dependent recycling to *trans*-Golgi network has also been demonstrated[49,55-61] (Box 1). For most cargoes that require retromer for their retrieval, perturbation of retromer leads to their missorting towards the lysosome for degradation[52]. Why recycling cargo enters the degradative fate remains unclear but it may reflect a high rate of ILV biogenesis early in the endo-lysosomal network as discussed above. Alternatively, it may be a consequence of aberrant cargo ubiquitylation when retrieval is not initiated. Either way, this missorting phenotype is not restricted to retromer[53]. For numerous endocytosed cargoes a common theme therefore, is that in the absence of active sequence-dependent retrieval, their default route is lysosomal degradation.

[H2] Endosomal recruitment and cargo recognition by retromer.

Retromer is a stable heterotrimer of VPS35, VPS29 and VPS26 (two paralogs, VPS26A and VPS26B, are expressed in humans) (Figure 3a). VPS35 forms an extended α-helical solenoid that associates with VPS26A or VPS26B and VPS29 at its amino-terminal and carboxy-terminal ends respectively[62,63]. Retromer is a complex composed of peripheral membrane proteins that, at steady state, is enriched on the cytosolic face of the early and late endosome. The association with the late endosome is mediated through binding to RAB7-GTP[64,65] whereas association with the early endosome is governed through interaction with a sorting nexin (SNX) family **[G]** member SNX3, which binds PtdIns(3)P[66,67]. The interaction of retromer with SNX3 is essential for retromer to bind to certain cargoes[63]. In the case of the divalent cation transporter DMT1-II, which requires retromer for its endosomal sorting[68] a hydrophobic QPELYLL sorting motif present within its cytosolic tail directly binds to an interface region between SNX3 and the VPS26 retromer subunit[63]. Association of cargo with retromer further enhances retromer-endosome association[67]. Because hydrophobic motifs are present in the tails of other retromer cargoes (for example, the cation-independent mannose 6-phosphate receptor (CI-MPR), sortilin, Wntless and the TfnR[51,63,69]), SNX3-assisted recognition of such hydrophobic motifs may be a common route by which retromer bind its cargo in a sequence-dependent manner.

Further sequence-dependent cargo recognition is mediated indirectly through the association of retromer with the cargo adaptor, SNX27[48,49,57]. SNX27 contains two distinct domains that govern sequence-dependent cargo recognition. An amino-terminal PDZ (PSD95-Dlg-ZO1) domain binds to cargo proteins containing a carboxy-terminal

type I PDZ domain-binding sorting motif, whereas a carboxy-terminal FERM (band 4.1-ezrin-radixin-moesin)-like domain binds to cargoes with ΦxNPxY or ΦxNxxY as a sorting motif[70-74] (where 'Φ' is a hydrophobic residues and 'x' is any residue) (Table 1). Association of SNX27 with the early endosome is mediated through cargo recognition and the binding to PtdIns(3)P[72,73]. Engagement of SNX27 with retromer occurs through a direct association of its PDZ domain with the VPS26 subunit[75]. This increases the affinity of the SNX27 PDZ domain for PDZ-binding motifs by at least an order of magnitude and may serve to promote sequence-dependent cargo recognition[75].

Evidence of the importance of SNX27–retromer in cargo retrieval initially came from studying the β2-adrenergic receptor, which contains a PDZ-binding motif recognised by SNX27[48,49]. Under conditions of SNX27 suppression (or retromer suppression) internalised β2-adrenergic receptor is missorted for lysosomal degradation and displays a reduced rate of transport to the cell surface[48,49]. Data from subsequent work, including a global proteomic analysis[57] and a detailed biochemical analysis[74], has established that, in humans, more than 400 cargo proteins require SNX27–retromer for their retrieval and recycling. These cargoes include signalling receptors, regulators of synaptic activity and neuronal health, as well as numerous transporters for amino acids, nutrients and metal ions[48,49,57,70,76,77].

[H2] Retriever-dependent cargo retrieval.

New insights into additional retrieval pathways were obtained by studying another adaptor for recycling cargoes, SNX17. An unbiased global proteomic analysis has identified over 220 integral proteins that require SNX17 for their steady-state cell surface expression[53]. Within this cargo cohort integral membrane proteins required for cell adhesion, maintenance of lipid homeostasis, transport of nutrients, and receptor signalling are particularly enriched[53]. Some of the established SNX17 cargoes include P-selectin, LRP1, Stabilin-1, APP, β1-integrins, all of which undergo lysosome mediated degradation in the absence of SNX17 [refs.78-83]. Similar to SNX27, SNX17 is associated with the early endosome through binding to cargo and to PtdIns(3)P[72]. Cargo selection is mediated through the FERM-like domain of SNX17 that binds to ΦxNPxY or ΦxNxxY sequence motifs embedded within cargo tails[73]. SNX17 lacks the PDZ domain found in SNX27 and does not associate with cargoes containing the PDZ-binding motif nor does it bind retromer[53].

To regulate the retrieval and recycling of one of its cargoes, β1-integrin, SNX17 associates with retriever: a stable heterotrimeric 'retromer-like' complex composed of

C16orf62 (chromosome 16 open-reading frame 62), DSCR3 (Down's syndrome critical region 3), and the retromer component VPS29 ref 53 (Figure 3b). The carboxy-terminal region of C16orf62 is predicted to possess an α-helical HEAT-repeat solenoid similar to that observed in VPS35 and DSCR3 has structural homology to the arrestin-like fold of VPS26 refs. 84,85. C16orf62 and DSCR3 have therefore been renamed VPS35L and VPS26C respectively[53]. SNX17 associates with retriever via a conserved carboxy-terminal tail sequence that may engage the VPS26C component of retriever - an interaction conceptually equivalent to the binding of SNX27 to the VPS26 component of retromer[75]. A detailed structural analysis of retriever is currently lacking, but the apparent conservation in the architecture of retromer and retriever suggests a level of conservation in their underlying mechanism of action.

[H2] The CCC complex in sequence-dependent cargo recognition.

SNX17–retriever-mediated retrieval of β1-integrin requires the CCC complex, a heterodimeric assembly of coiled-coil domain-containing protein 22 (CCDC22) and CCDC93 to which one or more of the COMM domain-containing (COMMD) proteins associate[86] (Figure 3b). Retriever and the CCC complex may assemble to form a super-complex dubbed 'COMMander', although a detailed biochemical reconstitution of this putative complex has yet to be achieved[87,88]. Like for retriever, suppression or knockout of the CCC complex leads to the missorting of internalised β1-integrin into the lysosome for degradation[53].

The COMMD family of proteins, of which there are ten in humans[89], are predicted to consist of two modular domains connected through a flexible linker: a variable amino-terminal domain composed of packed α-helices[90] and the characteristic carboxy-terminal COMM domain. For COMMD1, the COMM domain binds to phosphoinositides[91], drives COMMD1 homo-dimerisation and hetero-dimerisation with other COMMD proteins, and is required for association with CCDC22 and CCDC93[86,92]. The CCC complexes may therefore come in different 'flavours' depending upon the composition of COMMD protein dimers. A CCC complex containing COMMD9 dimerised with COMMD5 or COMMD10 is involved in the endosomal retrieval and recycling of Notch2 ref. 93, whereas a CCC complex containing COMMD1 regulates the recycling of the copper transporter ATP7A[86] and the LDL receptor[94]. There is evidence that the COMMD1-dependent sorting of the LDL receptor is mediated through the COMM domain recognising an NPxY sorting motif in the cytosolic tail of the receptor[94]. This suggests that COMMDs may function as cargo adaptors. If so, the CCC complexes may provide further insight into

the evolutionary conserved mechanisms for sequence-dependent inclusion of cargo into the retrieval subdomain. In addition, the COMMD proteins are linked to cellular processes that do not immediately fit with a role in endosomal cargo retrieval and recycling such as the regulation of NF-κB and hypoxia-induced transcription[95]. Studying the CCC complexes may reveal some unexpected interfaces between endosomal cargo sorting and these, and other, cellular and physiological processes.

[H1] REGULATION OF CARGO FATE DECISIONS

Besides acting to terminate signalling, the endocytic internalization of a receptor serves to fine-tune its spatial and temporal signalling outputs[96]. For example, there is now direct evidence that the canonical β2-adrenergic receptor is present in its active form in the early endosome, locally producing cAMP signals, thereby challenging the long held dogma that G-protein coupled receptor signalling takes place exclusively at the plasma membrane[97]. As we have discussed, the activated internalised β2-adrenergic receptor is delivered to the early endosome where it associates with the SNX27–retromer for retrieval and recycling[48,49]. It is from the SNX27–retromer-rich and actin-rich (see below) tubular profiles that endosome associated β2-adrenergic receptor signals intracellularly[96]. Interestingly, cAMP-dependent protein kinase A mediates phosphorylation of the cytoplasmic tail of β2-adrenergic receptor, which results in switching the recycling route from constitutive bulk flow to the sequence-dependent route[98]. This generates an endosome segregated production of cAMP that is necessary to establish the full set of cAMP mediated transcriptional responses[99].

Whereas this cAMP-dependent protein kinase mediated phosphorylation of the β2-adrenergic receptor occurs outside of the PDZ binding motif required for SNX27 binding, phosphorylation of the β2-adrenergic receptor mediated by G protein-coupled receptor kinase 5 (GRK5) occurs within the canonical PDZ binding motif at Ser 411[ref. 46]. This phosphorylation disrupts binding to SNX27 and leads to enhanced lysosomal turnover[46,74]. By contrast, phosphorylation of either Thr408 or Ser407 within the PDZ-binding motif by GRK5 markedly enhance binding of β2-adrenergic receptor to SNX27 by providing negative charges that engage a conserved arginine residue within the binding pocket of the SNX27 PDZ domain[74]. Also GRK2 is known to phosphorylate both Ser411 as well as Ser407 upon β2-adrenergic receptor activation, but it remains to be investigated whether this determines the recycling rates of this receptor[103]. Thus, differential phosphorylation of PDZ binding motifs, by distinct kinases, potentially serves to “fine tune” the balance between the recycling and degradation of the β2-adrenergic

receptor. A phosphorylation-dependent increase of SNX27 affinity to the PDZ motif has also been observed for NMDA and AMPA type glutamate receptors[74], suggesting that these and other cargoes could use such a phosphorylation switch to promote SNX27–retromer-dependent retrieval and recycling.

A role of phosphorylation in regulating the retrieval and recycling versus ligand-induced degradation has also been described for EGFR[104]. In the absence of EGF, the multi-domain adaptor protein intersectin-s recycles the inactive EGFR by linking it to the RAB13 guanine nucleotide exchange factor DENND2B (DENN/MADD domain containing 2B) and a RAB13 dependent recycling route. Upon EGF induced EGFR activation this recycling is disrupted by protein kinase D-dependent phosphorylation of intersectin-s, which dissociates from DENND2B and switches the EGFR from the retrieval and recycling fate to a degradative fate[104] (it is presently unclear how this recycling pathway relates to retromer and retriever-mediated recycling). Importantly, the EGFR can also be activated by transforming growth factor-α (TGF-α). Whereas EGF activation induces a fast receptor degradation and a transient mitogen-activated protein kinase (MAPK) activation, TGF-α leads to sustained MAPK activation and retrieval and recycling of the receptor. Through a sophisticated proteomic screen, phosphorylation of endosomal RAB7 looks to prime the EGFR for degradation following EGF stimulation, whereas recruitment of Rab coupling protein (RCP) to the EGFR following TGF-α stimulation leads to retrieval and recycling[105]. Thus, differential signalling of the EGFR modifies the trafficking machinery, at least in part through phosphorylation of multiple targets, to induce distinct receptor fate decisions and distinct cellular responses. Teasing apart how these events relate to retromer and retriever-mediated sorting will be an important undertaking.

The retrieval machinery itself is subject to phosphorylation[100]. As shown in yeast, phosphorylation–dephosphorylation reactions act as a gating mechanism for cargo recruitment by retromer, whereby phosphorylation of retromer subunit Vps26 determines its affinity for cargo, which is negatively regulated by CDC25 protein phosphatase[101]. Similarly, association of the retromer subunit VPS35 with the protein phosphatase 1 regulator subunit 14C terminates parathyroid hormone (PTH) signalling through the PTH1 receptor in mouse osteoblasts[102] (possibly by switching the fate from recycling to degradation). Defining how signalling modulates these phosphorylation switches and gating mechanisms to promote or inhibit cargo retrieval and recycling will be a major area of research, which will likely broaden to the analysis of other post-translational modifications such as ubiquitylation and sumoylation.

The studies of β2-adrenergic receptor and the EGFR have provided first insights into how phosphorylation can influence fate decisions between retrieval and recycling versus degradation and thereby influence receptor deactivation and can modulate signalling outputs. This regulatory power of cargo fate decisions could have much wider implications. There is now evidence showing that the SNX27–retromer retrieval subdomain acts to restrict receptor signalling through parathyroid hormone receptor[106,107]. Furthermore, retromer has been implicated in regulating the endosome retention time of internalised type-I interferon α/β receptor, thereby controlling type-I interferon induced JAK–STAT signalling and downstream transcription outputs[108]. Overall, while much remains to be learned, the early and late endosome associated machineries that determine the degradation versus retrieval and recycling fates should be considered as points of regulation, mediated in part by phosphorylation, that serve as intracellular platforms that regulate the signalling strength and duration of cell-surface receptors.

[H1] TUBULAR TRANSPORT CARRIER FORMATION

The identification of retromer, retriever, the CCC complex and their associated cargo adaptors has provided the foundation to describe the endosomal retrieval and recycling of hundreds of functionally diverse cargo proteins. The ancient origin and evolutionary conservation of these machineries have renewed interest in endosomal cargo retrieval and recycling in the development and physiology of multicellular organisms (Box 2). In addition, the study of these machineries is providing exciting new insight into the molecular aetiology of human disease (Box 3).

One of many outstanding basic mechanistic questions concerns how cargo retrieval is coupled to the biogenesis of tubulo-vesicular transport carriers. Retromer, retriever, and the CCC complex co-localise with the same early and late endosomes, forming retrieval sub-domains[53]. Importantly, these retrieval subdomains are spatially segregated from the ESCRT decorated degradative subdomains[53,109,110] (Figure 3c and 3d). Fate decisions between cargo degradation and cargo retrieval are therefore considered to be orchestrated through differential cargo partitioning between ESCRT degradative subdomains and the opposing retrieval subdomains.

[H2] The role of actin polymerisation in endosomal retrieval.

Arrays of functional sub-domains have been identified on the limiting membrane of endosomal vacuoles[e.g. 28-31,53,109-112]. For the organisation of the retromer-decorated retrieval sub-domain an important element is the polymerisation of branched actin networks mediated by the actin-related protein-2/3 (ARP2/3) complex on the cytosolic face of endosomes[113,114]. Prominent in regulating endosomal actin polymerisation is the Wiskott-Aldrich syndrome and SCAR homologue **[G]** (WASH) complex[115-117], which is a pentameric assembly of FAM21 (also known as WASHC2), CCDC53 (WASHC3), SWIP (WASHC4), Strumpellin (WASHC5) and WASH (WASHC1) that stimulates the ARP2/3 complex[115-119] (Figure 3). Like other ARP2/3 regulators, the activity of WASH is precisely regulated, in this case through poly-ubiquitylation mediated by the MAGEL2-USP7-TRIM27 (MUST) complex[120,121]. Loss of WASH in mammalian cells results in pleiotropic effects that include collapse of the endo-lysosomal network into the juxta-nuclear region, elongation of endosome-associated tubular profiles and defects in the retrieval and recycling of numerous cargoes, many of which require retromer, retriever and/or the CCC complex[122]. How defects in endosome morphological and endo-lysosomal network organisation translate into effects on cargo sorting remains to be fully understood.

The WASH complex is an assembly of peripheral membrane proteins. Its association with the membrane of endosomes is highly dynamic and may be mediated, in part, by association with phospholipids[115]. When associated with an endosome, the WASH complex recruits both retriever and the CCC complex to the retrieval subdomain. Accordingly, the retrieval of SNX17–retriever and CCC complex-dependent cargoes relies on the WASH complex[53,86]. A large population of the WASH complex is recruited to endosomes through a distinct retromer-dependent mechanism that is mediated by a series of L-F-acidic (Leucine and Phenylalanine followed by an acidic amino acid) repeats in the carboxy-tail of FAM21 that bind to VPS35[119,123]. Importantly, FAM21 binding to VPS35 is non-stoichiometric, with one FAM21 molecule associating with multiple copies of retromer[123]. This mode of WASH-endosome association could provide a means of sensing the concentration of retromer and hence the density of cargo, thereby allowing the coordination of branched actin polymerisation with the enrichment of cargo during the nucleation of the forming retrieval subdomain[123]. The localised and timed formation of branched actin likely serves to restrict the lateral mobility of captured cargo and to prevent their leakage into the ILV pathway. In addition, it was shown that including an acting binding domain in a β2-adrenergic receptor mutant devoid of its PDZ binding motif was sufficient to rescue normal receptor retrieval and recycling[47,124]. The ability of cargo to bind to filamentous endosome-associated actin may therefore

constitute an additional mechanism for cargo selection into the actin-enriched retrieval subdomain, independently of the sorting motifs.

[H2] Tubule formation from the retrieval sub-domain.

The timed and spatially restricted polymerisation of branched actin may also support membrane remodelling to produce an isolated cargo-enriched tubular transport carrier (Figure 3d; see also further below). Consistent with this, live cell imaging of the internalised β2-adrenergic receptor has revealed that the receptor is recycled via tubular profiles that form from the actin-rich retrieval subdomain[47-49,51,99].

The best-studied orchestrators of endosomal tubule formation are, however, the BAR (Bin/Amphiphysin/Rvs) domain **[G]** containing proteins PACSIN1 (*a.k.a.* syndapin-1) and PACSIN2 (*a.k.a.* syndapin-II)[(125-127)], ACAP1[44,128,129] and the SNX-BAR family[130-134] (see also Supplementary Table S1 for a list of membrane-remodelling protein and complexes currently implicated in endosomal recycling). BAR domains are α-helical coiled coils that dimerise to form a banana-shaped structure whose concave surface is lined with positively charged residues that mediate electrostatic interactions with the negatively charged membrane surface[135] (Figure 4). Membrane binding by BAR domains is tightly controlled[126,136]. Once associated with the membrane, the rigid nature of the BAR domain binding surface senses and/or imposes positive membrane curvature[137]. BAR domain dimers further associate through tip-to-tip and lateral contacts to partially coat the membrane with a helical assembly that drives and/or stabilises membrane tubule formation (*in vitro* experiments show that for efficient tubule formation membranes only need to be 30-40% coated by the BAR domain thereby leaving room for cargo)[137].

The defining member of the SNX-BAR family is SNX1, which forms a heterodimer with another family member SNX5 (or closely related proteins SNX6 and SNX6B)[130,132]. The presence of a PtdIns(3)P-binding PX domain in SNX1 drives recruitment of the heterodimer to endosomes harbouring retromer or retriever complexes[130,138]. From here, SNX1-SNX5 drives and/or stabilises the formation of tubular profiles that originate from the retrieval subdomain[124] - it remains unclear whether SNX-BARs initiate tubule formation or whether they merely stabilize membrane curvature and tubulation generated by other means, for example actin polymerization. In yeast, Vps5 and Vps17 (the orthologues of SNX1 and SNX5) form a stable pentameric complex with the retromer[55]. However, in higher metazoans such a complex cannot be biochemically

isolated (Box 1). Instead, the WASH complex accessory protein, RME-8 (also known as DNAJC13), may co-ordinate the function of the WASH complex with the membrane remodelling SNX1-SNX5 complex[109,139,140]. RME-8 also has an additional, perhaps related role, in maintaining the segregation of the degradative and retrieval subdomains[109,141], although precisely how this segregation is achieved remains unclear.

The simultaneous analysis of two retromer-dependent recycling cargoes, β2-adrenergic receptor and Wntless, has established that upon their endocytosis these cargoes enter the same retromer decorated early endosome[51]. Interestingly, they exit this compartment through shared tubule-vesicular transport carriers even though their final steady-state destinations are distinct: β2-adrenergic receptor is enriched at the cell surface, whereas Wntless resides at the *trans*-Golgi network[51]. Thus, an additional level of sorting, downstream of the degradative versus retrieval fate decision and the exit of retrieved cargo from the endosomal vacuole, must operate in order to achieve cargo specific distribution. These molecular events remain to be defined.

Further levels of complexity of how cargoes exit the endosome for recycling arise simply from the number of distinct endosome associated tubules and the relative lack of information as to the cargos that they recycle[142]. Dimers of other members of the SNX-BAR family, including SNX4-SNX4, SNX8-SNX8, SNX18-SNX18, SNX4-SNX7 and SNX4-SNX30 also associate with the retromer and retriever labelled endosome where they drive the formation of distinct recycling tubules[131,134,143-145]. Furthermore, the Eps15 homology domain (EHD) family **[G]** of proteins (the founding member being the *C. elegans* protein RME-[ref. 146]) can associate with MICAL-L1 and the F-BAR **[G]** proteins PASCIN1 and PACSIN2 to form endosome tubules, although the function of these complexes may be linked more with tubule scission than tubule biogenesis (see below)[125,146-150]. Perturbation of EHD1 can affect retromer function[151,152], but equally there is evidence that EHD1 and retromer function separately from distinct endosome populations[153,154]. In addition, ACAP1 has been shown to form a tubulating coat complex with clathrin[155]. The BLOC-1–KIF13A–annexin A2 assembly[156,157] and a RAB10–EHBP-1 complex[158] are also able to generate endosome-associated tubules. Understanding the relative localisations of these complexes and the relative cargo preferences of the tubules that they generate (we would argue this needs to be achieved through a global scale analysis of cargo sorting) is now a major question, as is their relationship with the clathrin-coated vesicular transport carriers that have also been established to regulate endosomal cargo recycling (Supplementary Table S1).

Following nucleation, the recycling tubules mature, which involves elongation, stabilisation and ultimately scission and requires a network of accessory proteins that include cytoskeletal motors and regulators of actin polymerisation, local modifiers of the phospholipid environment and scission machinery (Figure 4, Supplementary Table S1). How all of these components are co-ordinated to deliver the mechanical forces and the alteration of biophysical properties of the lipid bilayer that are required for tubule scission is largely unknown[159]. Somewhat unexpectedly, the constriction and scission of a population of recycling tubules is spatially and temporal controlled through close membrane contacts (membrane contact sites) of endosomes with the endoplasmic reticulum (ER)[160]. In this context, a dynamic ER tubule is considered to "attack" the site at which a future scission event is likely to occur[160]. Evidence that ER–endosome contacts are formed through association of the SNX-BAR tubulating protein SNX2 with the ER resident VAP proteins (VAP-A and VAP-B) and by a CHMP1B–IST1 complex – which resides on SNX1 decorated tubules – with the ER resident protein spastin, are consistent with a role for the ER in endosomal tubule scission[161,162]. Perturbing these membrane contacts affects the overall efficiency of endosomal tubule scission by altering the local endosomal phosphoinositide environment, regulating the actin polymerising activity of the WASH complex, and through the activity of spastin in severing a subset of microtubules[161,162]. An additional role for the cytoskeleton in endosomal tubule scission comes from the association of endosomal tubules with microtubule motors dynein and kinesin, which are required for efficient tubule scission[131,132,156,157,163,164].

It would be highly informative to define how these scission sites relate to the endosomal localisation of EHD proteins. These are endosome associated dynamin-like ATPases that assemble into membrane-bound oligomeric rings that through an ATPases activity have the potential to mediate mechano-chemical membrane scission[165,166]. EHDs associate with the F-BAR domain containing PACSINs which themselves bind to the ARP2/3 regulator neural Wiskott-Aldrich syndrome protein (N-WASP)[167]. The curvature sensing properties of the F-BAR domain may therefore focus the assembly of EHDs oligomeric rings to the neck of forming tubules. Coupling the properties of the EHD proteins with forces applied to the membrane from a localised burst of branched actin formation through N-WASP activation and the pulling forces generated by association of the tubule with microtubule motors could bring about membrane scission. The scission may be further influenced by the build-up of tension between the phospholipids and the

rigid EHD and/or BAR domain scaffold on the rapidly growing membrane tubule, which, by perturbing lipid flow, leads eventually to friction-dependent scission[168].

Finally, and to return to the question of sequence-dependent cargo retrieval and recycling, recent data suggests that membrane tubulating complexes can directly associate with sorting motifs on cargo. For example, ACAP1 recognises the TfnR, β1-integrin and GLUT4[44,169,170], and SNX5 binds to the CI-MPR[60,61]. In each case the association is required for cargo recycling. Sequence-dependent endosomal cargo retrieval and recycling is, therefore, not restricted to a single layer of adaptor mediated cargo recognition, but actually encompasses a multilayered system of recognition. This complexity almost certainly reflects the need to retrieve and recycle thousands of internalised cargoes. It also provides the necessary plasticity to allow for tuning the fates and recycling itineraries of cargoes, especially internalised signalling receptors.

[H1] CONCLUSIONS AND PERSPECTIVES

The last few years have seen a rapid expansion in our appreciation of the molecular events that orchestrate the endocytic retrieval and recycling of internalised cargo proteins. In particular, this has refocused attention on the active role that cargoes play in sequence-dependent endocytic recycling that contrasts sharply with the historical view of cargoes being passively recycled through bulk membrane flow. Identifying additional molecular players that define this process in even greater detail is clearly an important goal, as is the building of a detailed road map for describing how distinct machineries communicate along the entire length of a given endocytic retrieval and recycling pathway: from sorting and carrier biogenesis at the endosome to carrier transport and fusion at the cell surface (either by direct transport to the cell surface or via indirect routes that transit the endocytic recycling compartment and/or the *trans*-Golgi network). The vexing question of exactly how many retrieval and recycling pathways are present within a typical cell type as well as more specialised cell types remains open, as does the issue of pathway integration and pathway redundancy. Another important question to address is the mechanisms of cross-talk between the retrieval and degradative pathways and how these can be tuned to determine cargo fate. Downstream responses from various signalling receptors have been demonstrated to rely on such decisions, whereby coordination between retrieval and recycling and degradation is important to regulate the rates of receptor deactivation and the propensity for intracellular signalling (from the endosome compartment). Evidence that post-translational modifications of

cargo sorting motifs as well as the sorting machinery themselves can control cargo fate decisions and the itineraries of cargo retrieval and recycling, raises the vital question of how the cellular state influences cargo sorting to allow active remodelling of cell function. This will be an important consideration moving forward.

With an increased molecular understanding has come renewed interest in endosomal cargo retrieval and recycling during development and in the physiology of multicellular organisms, and it is certain that new discoveries are to be made in this arena. For human biology, the increased mechanistic understanding has provided exciting new insight into the role of endosomal cargo retrieval and recycling in the pathology of neurodegenerative diseases and in host-pathogen interactions (Box 3). Achieving a thorough appreciation of the cellular consequences of the deregulation of endosomal sorting in specific disease contexts will be an essential and rewarding undertaking and is very likely to provide new possibilities for rational treatment of various human disorders.

BOX 1: Retromer's role in retrograde endosome-to-TGN transport.

Retromer was identified in yeast as a pentameric protein complex comprising a Vps26-Vps35-Vps29 heterotrimer and a Vps5-Vps17 heterodimer[54,55]. In metazoans, including humans, the equivalent proteins do not assemble into a biochemically stable pentameric complex. With the exception of yeast, the term retromer is therefore used to refer to the VPS26-VPS35-VPS29 heterotrimeric complex, whereas retromer-associated SNX (sorting nexin)-BAR (Bin/Amphiphysin/Rvs) proteins are equivalents of the Vps5-Vps17 heterodimer.

In yeast, main role of retromer is to regulate endosome-to-Golgi retrograde transport[54,55]. Initially, it has been considered that mammalian retromer functions in the equivalent retrograde transport pathway, as demonstrated by retromer-dependency of retrograde transport of cation-independent mannose-6-phosphate receptor (CI-MPR)[58,59]. Recently, independent studies have indicated that the human retromer (the VPS26-VPS35-VPS29 heterotrimer) is dispensable for the retrograde transport of the CI-MPR[60,61], and that retromer-associated SNX-BAR proteins, SNX1 or SNX2, in complex with SNX5 or SNX6 are the dominant cargo selective elements that promote retrograde transport of the CI-MPR. Importantly, SNX5 and SNX6 directly bind to a hydrophobic W-L-M sorting motif present in the cytosolic tail of CI-MPR, previously identified as being required for sequence-dependent endosome-to-TGN transport[69].

So, what role does retromer play in the endosomal sorting of the CI-MPR? In cells with CRISPR/Cas9 deletion of retromer, the delivery of endocytosed CI-MPR to the TGN is faster, consistent with a regulatory role of retromer in tis process[61]. Retromer together with TBC1D5 (GTPase activating protein of RAB7) has been reported to act as a master regulator of the localization and activity of RAB7 [ref. 171]. RAB7 is required for the retrograde transport of CI-MPR and likely regulates the entire retrograde pathway, the molecular basis of which is presently unclear. Upon loss of retromer or TBC1D5, RAB7 is hyperactivated and accumulates on endosomes leading to increased trafficking of the autophagic transmembrane protein ATG9A to the TGN. The faster TGN delivery of the CI-MPR in retromer deficient cells might therefore reflect the deregulated activity of RAB7.

A crystal structure of retromer (specifically the VPS26 and VPS35 components) together with sorting nexin-3 (SNX3) and the cytosolic tail domain of the retromer cargo divalent metal transporter 1 (DMT1-II) has revealed the presence of a cargo-binding pocket at the interface between SNX3 and VPS26[ref 63]. As this pocket binds to a hydrophobic sorting motifs[63,68], it is likely that it also engages the hydrophobic W-L-M sorting motif in the CI-MPR. Why would retromer engage the CI-MPR, given that it does not directly regulate the retrograde transport? One attractive idea builds on the proposed role of retromer as a check-point for timing the fusion between the late endosome and the lysosome[172,173]. This fusion is regulated by vesicle-associated membrane protein 7 (VAMP7), an R-SNARE that is trapped in a fusogenic inactive conformation through the binding to ankyrin repeat domain-containing protein 27 (VARP, also known as ANKRD27)[172]. The inhibition of VAMP7 is dependent on the local enrichment of VARP on the late endosome. VARP is recruited to the late endosome through a direct binding to the VPS29 subunit of retromer[172,173]. The potential ability of retromer to bind to the CI-MPR may therefore provide a means to sense the local density of the cargo on the late endosome, which through a VARP-VAMP7 relay prevents pre-mature fusion of the late endosome with the lysosome. Decrease in retrograde transport density resulting from SNX-BAR driven retrograde transport would lead to a reduction in retromer association with the endosome, decrease in the local concentration of VARP and the release of the VAMP7 fusogenic activity. This would ensure that the late endosome only undergo fusion with the lysosome when all of the cargo destined for recycling have been retrieved.

BOX 2: Functional role of cargo retrieval and recycling across multicellular organisms

The ancient origin and conservation of the cargo retrieval machinery, including retromer, retriever, Wiskott-Aldrich syndrome and SCAR Homologue (WASH) complex and the CCC complex and associated cargo adaptors as well as the degradative machinery centred on endosomal sorting complexes required for transport (ESCRT) complexes, suggest large functional importance of the retrieval versus degradative fate decisions. Indeed, genetic analysis is starting to reveal the role of the retrieval and recycling pathways in the development and physiology of many organisms. In *Drosophila melanogaster* the retromer component of the retrieval subdomain regulates: Wnt morphogenic gradient formation (through the sorting of the Wnt chaperone Wntless; this phenotype is also observed in *Caenorhabditis elegans* and *Xenopus laevis*)[174-180]; establishment of epithelial cell polarity (sorting of the apical determinant Crumbs and engagement with the Scribble polarity module)[181,182]; epithelial tube formation in trachea development (trafficking of serpentine and Crumbs, and signaling through EGF receptor)[183]; photoreceptor stimulation (regulation of light-induced rhodopsin 1 recycling)[184]; neuromuscular junction signaling (regulation of TGFβ/BMP signaling)[185]; innate immunity (regulation of the Toll receptor ligand Spätzle)[186]; and cell fate, proliferation, and differentiation (recycling of the Notch receptor and downstream signaling)[187]. In *Caenorhabditis elegans* the retromer retrieval sub-domain regulates: apoptotic cell clearance (recycling of CED-1 cell surface receptor)[56]; synaptic plasticity (recycling of the GLR-1 subunit of the AMPA-type glutamate receptors)[188]; signaling through the TGFβ/BMP superfamily (recycling of the BMP type I receptor)[189]; the development of the amphid sensory organ **[G]** (regulation of the Patched-related protein DAF-6)[190]; and the function of chemosensory neurons (recycling of the receptor-type guanylate cyclase GCY-9 to sensory cilia)[191]. A *Drosophila melanogaster* WASH null mutant displays a defect in neutralization of the acidified lysosomal possibly through perturbed actin-mediated recycling of the v-ATPase, and defects in haemocyte **[G]** cell spreading and cell migration through altered integrin recycling[192].

In vertebrates, snx3 knockdown in zebrafish leads to an anaemic phenotype as a result of perturbed recycling of the TfnR and the uptake of circulating Fe^{3+} [ref. 45]. In mice the deletion of VPS35 [ref. 193] or VPS26A (but not VPS26B [ref. 194]) leads to embryonic lethality[195], as does the deletion of WASH complex components WASH or strumpellin[196,197]. Analysis of heterozygous or conditional knockouts of these retromer components, has revealed the essential role of the retrieval subdomain in the central

nervous system. More specifically, unperturbed cargo recycling has been shown to prevent the formation of neurotoxic amyloids **[G]** in the hippocampus by controlling recycling of β-amyloid precursor protein **[G]** (APP) and some of its processing enzymes[193]. Cargo recycling pathways also control macroautophagy **[G]** and chaperone-mediated autophagy **[G]** through recycling of ATG9A and LAMP2A respectively[198], and support the maintenance of the degradative capacity of the lysosome by recycling receptors that deliver newly synthesized hydrolase enzymes (e.g. cathepsin D), thereby ensuring robust autophagic clearance (for example of. damaged mitochondria). In addition, the retrieval subdomain controls synaptic activity by recycling numerous synaptic receptors including AMPA, NMDA and dopamine receptors. Consistent with SNX27 functioning as the cargo adaptor for synaptic receptors, deletion of SNX27 leads to postnatal developmental defects highlighted by abnormal brain morphology and progressive hydrocephaly coupled with cognitive impairment, which have been linked to defects in the retrieval and recycling of synaptic receptors[199-201]. SNX27-deficient mice also display defects in bone formation and remodelling through perturbed recycling and signaling of parathyroid hormone 1 receptor[106].

BOX 3: Human diseases associated with defects in cargo retrieval and recycling

Genetic analysis has associated familial and sporadic mutations in retrieval and recycling machinery with human diseases most notably neurological disorders that include Alzhiemer disease, Parkinson disease, and hereditary spastic paraplegia **[G]** (HSP)[202,203]. In these diseases three very general features of cargo retrieval and recycling are currently considered to be important. First, by maintaining and actively remodelling the cell surface proteome endosomal retrieval and recycling regulates processes essential for neuronal health such as synaptic transmission, nutrient supply, and interactions with surrounding neurons and supporting cells and matrix. Second, through sorting receptors that are essential for delivering lyososomal hydrolases, endosomal retrieval and recycling maintains the capacity of the lysosome to degrade protein aggregates and dysfunctional organelles that accumulate in age-related pathophysiology. Thirdly, the efficiency of retrieval and recycling defines the endosomal residency time of cargo, which when increased, can result in abnormal processing of some proteins or aberrant signalling by receptors.

The retromer-based retrieval subdomain has a clear neuroprotective role in Parkinson disease[202]. Low frequency mutations in VPS35, VPS26A and VPS29 have all been

noted in patients with Parkinson disease with the clearest example of VPS35(p.D620N) mutation which is associated with late on-set disease[204,205]. This specific mutation subtly perturbs the association of retromer with the WASH complex (and its accessory proteins)[206,207] and leads to various defects[203]: perturbed sorting of the cation-independent mannose-6-phosphate receptor (CI-MPR) and the delivery of cathepsin D to the lysosome; impaired trafficking of the autophagy-associated protein ATG9A; reduced AMPA receptor recycling and synaptic activity; and impaired recycling of the D1 dopamine receptor. Further association of cargo recycling with Parkinson disease include a mutation in the WASH accessory protein DNAJC13(p.N855S)[208,209], a functional relationship between RAB7L1 and LRRK2 [ref. 210], the latter being a multi-domain GTPase and serine/threonine kinase that is frequently mutated in late onset PD[211], and through an interaction with the E3 ubiquitin ligase Parkin[212] mutations of which are the main cause of autosomal recessive early-onset Parkinson disease.

Mutations in the WASH complex component strumpellin are linked to HSP, as have mutations in spastin[162]. These mutations lead to lysosomal abnormalities through defects in CI-MPR recycling that may arise from impaired ER–endosome contacts[162]. Other genes that regulate CI-MPR recycling have also been linked to HSP[213].

A reduced expression of retromer was found in post-mortem brains of patients with Alzheimer disease[214]. A variety of animal models have now firmly established that reduced retromer expression predisposes to Alzheimer disease pathology and patient-based genetic analysis has identified additional risk factors related to retromer, including SNX1, SNX3, RAB7A, and the cargo proteins SORL1 and TREM2 [ref. 215]. SORL1 interacts with β-amyloid precursor protein (APP) and its aberrant recycling influences recycling of APP, whereas TREM2 is localised to the cell surface of microglia **[G]** and binds extracellular β-amyloid, thereby promoting its clearance and preventing spreading[216].

Retrieval and recycling are also implicated in other neurological conditions. In Down syndrome, SNX27 expression is decreased by an additional copy of miRNA from trisomic chromosome 21[ref. 200] resulting in perturbed retrieval and recycling of AMPA and NMDA receptors[217]. More extensive loss of SNX27 is associated with infantile myoclonic epilepsy[218]. Perturbed regulation of the WASH complex was linked to intellectual disability and autism spectrum disorder[121]. Mutations in CCDC22 were associated with X-linked recessive intellectual disability and hypercholesterolaemic, the latter resulting from defective retrieval and recycling of the low-density lipoprotein (LDL) receptor[94].

Consistent with coupling between the CCC and WASH complexes, a mutation in strumpellin was also linked to elevated LDL-cholesterol[94].

Given their ancient origin and conservation it is perhaps unsurprising that the core cargo retrieval and recycling machinery is targeted by a variety of viral[53,219-224] and bacterial pathogens[225-230], a number of which express proteins that mimic sorting motifs of the host. These pathogens subvert the function of cargo retrieval machineries to promote their intracellular survival and replication.

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FIGURE LEGENDS

FIGURE 1: The endo-lysosomal network. The integration between the endo-lysosomal network and the biosynthetic pathway helps to establish, maintain and re-model the cell surface proteome. Following their endocytosis, internalised integral membrane proteins enter the early endosome from where most sorting is initiated. Selected cargo can be recycled back to the cell surface by means of tubulo-vesicular transport carriers either directly, termed ‘fast recycling’ or by transit through the

endocytic recycling compartment, termed ‘slow recycling’. Recycling back to the cell surface can occur by passage through the *trans*-Golgi network and entry into the secretory pathway (termed ‘retrograde transport’). Other cargoes are targeted for degradation within the lysosome. This is principally achieved through cargo sorting into intraluminal vesicles (ILVs). Iterative rounds of cargo sorting and ILV biogenesis coupled with maturation of the early endosome, result in the formation of the late endosome (also known as the multivesicular endosome or multivesicular body). The late endosome is able to fuse with the lysosome to form an endo-lysosome within which ILVs and their accompanying cargo are degraded.

Relatively little is known of the mechanistic integration between the secretory pathway and the endo-lysosomal network. Recently, the endosome associated transcriptional regulator RNF11 has been observed to translocate from endosomes to the nucleus in response to EGF receptor signalling and receptor degradation[231]. In the nucleus RNF11 up-regulates the endoplasmic reticulum export machinery so that newly synthesized EGF receptor can be transported through the Golgi apparatus to the cell surface. The integration between degradation and synthesis maintains the physiological levels of EGF receptor at the plasma membrane.

FIGURE 2: ESCRT-mediated degradative cargo sorting. *a)* Internalised ubiquitylated (Ub) cargoes are first recognised by ESCRT-0 components HRS and STAM that are associated with the cytosolic face of the early endosome through the Fab1, YOTB, Vac1, EEA1 (FYVE) domain of HRS binding to PtdIns(3)P. ESCRT-0 provides five ubiquitin binding motifs - VHS domains, a conventional ubiquitin-interaction motif (UIM) and a double-sided ubiquitin-interaction motif (DUIM). The ESCRT-I complex components TSG101 and UBAP1 also associate with ubiquitylated cargoes via an ubiquitin E2 variant (UEV) and solenoid of overlapping ubiquitin-associated (SOUBA) domains respectively. For the ESCRT-II complex, VPS36 contains a GRAM-like ubiquitin binding in EAP45 (GLUE) domain that binds to ubiquitylated cargoes. While the precise sequence of events within these ‘early ESCRT’ complexes is presently unclear, they serve to capture and enrich ubiquitylated cargoes into a degradative sub-domain that may be further stabilised through association of ESCRT-0 with clathrin. The late ESCRT complex, ESCRT-III links the upstream acting ESCRT complexes with the downstream process of intraluminal vesicle (ILV) biogenesis by forming a filament that mediates membrane remodelling. ESCRT-III also recruits the endosomal

deubiquitylating enzymes (DUBs), USP8 and AMSH in order to recycle ubiquitin). See REF. 10 for more in depth discussion. Figure modified from[232]. *b)* ESCRT-III spiral filament surrounds the cargo and its assembly brings about dissociation of the early ESCRT complexes. Growing of the ESCRT-III filament buckles from a flat structure into a three-dimensional spring thereby deforming the membrane in to the budding ILV. Recruitment of VPS4 ATPase is responsible for the disassembly of the ESCRT-III filaments and ultimately scission, both poorly characterised events, to form the isolated cargo-enriched ILV. See REF. 26 and 32 for more detailed discussion.

FIGURE 3: Retrieval mechanisms. *a)* Retromer associates with the actin polymerising Wiskott-Aldrich syndrome and SCAR Homologue (WASH) complex through multiple L-F-acidic repeats (L-F-[D/E]$_{(3-10)}$-L-F)[123], interactions that are subtly perturbed in the Parkinson disease-linked VPS35(p.D620N) mutation[206,207]. Retromer binds to cargo proteins through the interaction of VPS26 subunit with sorting nexin-27 (SNX27)[75,235] or through an interface formed after the association with SNX3[63]. *b)* Current view of the retriever-based retrieval complex[53]. The conserved carboxy-terminus of SNX17 is necessary and sufficient for association to retriever possibly via interaction with Down syndrome critical region protein 3 (DSCR3)[53]. Retriever binds to the CCC complex[86], possibly through C16orf62[86], forming a putative COMMander complex[87,88]. Coiled-coil domain-containing protein 93 (CCDC93) binding to the 356-600 region of the FAM21 tail couples the WASH and CCC complexes[86]. *c)* A speculative model of the initial fate decision between degradation and retrieval. Both degradative (endosomal sorting complexes required for transport (ESCRT) complexes) and recycling (retromer and retriever) machineries are recruited to endosomal membranes by recognition of PtdIns(3)P and interaction with their cargo (through ubiquitin for ESCRTs and through sorting motifs for retromer/retriever). ESCRT-0, ESCRT-I and ESCRT-II recruit ESCRT-III leading to the formation of cargo-enriched ILVs (see Fig. 2). Retromer recruits the WASH complex, with the resulting polymerisation of filamentous, branched actin. Recruitment of the COMMander complex as well as independent endosomal association of the WASH complex (possibly via lipid binding) further support sequence-dependent cargo recognition into the retrieval subdomain. The model suggests that the retrieval subdomain enriches and restricts the mobility of captured cargo thereby preventing their inclusion into intraluminal vesicles (ILVs). *d)* We speculate that as the retrieval subdomain matures, the clustering of cargo and the WASH-mediated polymerisation of actin may induce initial positive membrane curvature, thereby providing a cue, together

with the presence of PtdIns(3)P, for the recruitment of membrane tubulating complexes, such as SNX-BAR (Bin/Amphiphysin/Rvs) proteins, which induce the biogenesis of tubules into which the recycling cargo is packaged for export (see also Fig. 4). Globally, cargo recycling is likely best described through a combination of multiple sequence-dependent and sequence-independent pathways (constitutive bulk membrane flow).

FIGURE 4: BAR domain-containing proteins in endosome tubule formation. *a)* A general scheme to illustrate the molecular details of BAR (Bin/Amphiphysin/Rvs) domain-containing proteins (using sorting nexin (SNX)-BARs as a model). BAR domain-containing proteins associate with membranes through recognition of electrostatic charge, the sensing of membrane curvature, insertion of amphipathic helices and the binding to specific phospholipid species. Assembly of BAR domain dimers into helical assemblies leads to the induction and/or stabilisation of membrane tubules. *b)* After induction of tubulation, tubules elongate and eventually undergo scission. The overall mechanistic details of this complex process remain to be defined but appear to require the coordination of several molecular components. Actin polymerisation mediated by Wiskott-Aldrich syndrome and SCAR Homologue (WASH) complex and neural Wiskott-Aldrich syndrome protein (N-WASP) provides pushing forces whereas coupling to microtubule motors (here dynein–dynactin complex) generates pulling forces. Eps15 homology domain (EHD) proteins in complex with F-BAR proteins such as – protein kinase C and casein kinase substrate in neurons protein (PACSIN) are recruited to the neck of the extending cargo-enriched tubule owing to the increased curvature of the membranes, driving friction-mediated or mechano-chemical driven membrane scission. The process of scission may be further assisted through formation of an ER–endosome contact at the site of final scission (see text for more detail). See also Supplementary Table S1 for a list of membrane-remodelling proteins and complexes currently implicated in endosomal recycling.

TABLE 1: Linear peptide sorting motifs required for sequence-dependent recycling of endocytosed cargo proteins.

Cargo adaptor (recognition domain)	Sorting signal[a]	Example cargos (and their sorting motifs – bold residues define the sorting signal) [b]	References
SNX17	ΦxNPx[F/Y]	P-selectin (**F**T**N**AA**F**)	80, 82, 83,

	ΦxNxx[F/Y]	LRP(**I**G**NP**T**Y**) β1-Integrin (**VV****NP**K**Y**)	233
SNX27 (PDZ domain)	[E/D]$^{-5}$x^{-4}[E/D]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [ED/pSpT]$^{-6}$[E/D]$^{-5}$x^{-4}[E/D]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [ED/pSpT]$^{-5}$x^{-4}[E/D]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [ED/pSpT]$^{-6}$[ED/pSpT]$^{-5}$x^{-4}[pS/pT]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [ED/pSpT]$^{-6}$x^{-5}x^{-4}[pS/pT]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [E/D]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0} [pS/pT]$^{-3}$[S/T]$^{-2}$x^{-1}Φ^{0}	GLUT1 (L^{-6}GA**DS**Q**V**^{0}) β2-adrenergic receptor (**S**$^{-6}$TN**DS**LL^{0}) 5-HT$_{4(a)}$ receptor (**E**$^{-6}$**S**L**ES**C**F**^{0}) GIRK (**E**$^{-5}$**SES**K**V**^{0}) Kir3.3 (**E**$^{-3}$**S**K**V**^{0})	48, 57, 70, 71, 73, 74, 234
SNX27 (FERM-like domain)	ΦxNPxpY ΦxNxxpY	VLGR1 (LK**NP**F**pY**) RET (IE**N**KL**pY**)	
SNX3–VPS26–VPS35–VPS29	Aromatic, hydrophobic motif - Φx[L/M/V]	DMT1-II (EL**Y**L**L**NTM) Sortilin (GR**F**L**V**HRY) CI-MPR (TE**W**L**M**EEI)	63, 68, 69, 235
VPS26–VPS35–VPS29	FANSHY (binding to VPS26)	SorLA (**FANSHY**)	
COMMD1	NPxY	LDLR (FD**NP**V**Y**)	94
PACS-1 **PACS-2**	Acidic cluster motif	Furin (**EE**CPpSDpS**EED**) CI-MPR (H**DD**pS**DED**LLHI) Polycystin2 (**DD**pS**EEDDDED**S)	236, 237, 238
AP-1	YxxΦ [D/E]xxxL[L/I]	Coxsackie virus and adenovirus receptor (**Y**NQ**V**) MHC-1-associated invariant chain p33 (**D**QRD**LI**)	
AP-3	[D/E]xxxL[L/I]	OCA2(**E**NTP**LL**)	239
EpsinR	Hydrophobic + electrostatic	VTI1b (H$_{abc}$ domain)	240
GGA1	DIpSLL	BACE1 - (DIpSLL)	241
GGA3	DxxL[L/V]	TrkA receptor (**D**KM**LV**) TrkB receptor (**D**KI**LV**) TrkC receptor (**D**KM**LV**)	242
ACAP1	Positively charged residues together with associated hydrophobic motif	β1-integrin (REFAKF) GLUT4 (KR, assisted by PLSLL motif) TfR (RF and LF)	44, 155, 170
SNX5, SNX6, SNX32	Hydrophobic motif	CI-MPR (TE**WLM**EEI)	60, 61

a) General sorting motifs, where Φ = hydrophobic residue; pS, pT, and pY = phosphoserine, phosphothreonine and phosphotyrosine respectively; x = any amino acid. For sorting signals recognised by the SNX27 cargo adaptor, '^{0}' represents the

carboxy-terminal hydrophobic residues and '$^{-1}$', '$^{-2}$', etc denote the subsequent residues from the carboxy-terminus. b) representative cargoes

5-HT$_{4(a)}$ receptor, 5-hydroxytryptamine receptor 4; ACAP1, Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1; BACE1, β-secretase 1; CI-MPR, Cation-independent mannose-6-phosphate receptor; DMT1-II, divalent metal transporter 1; GIRK, G-protein coupled inwardly rectifying potassium channel; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; Kir3.3, G-protein coupled inwardly rectifying potassium channel 3; LDLR, low density lipoprotein receptor; LRP1, LDL receptor related protein 1; OCA2, melanosomal transmembrane protein; RET, ret proto-oncogene tyrosine-protein kinase receptor; SorLA, sortilin related receptor 1; TfnR, transferrin receptor; TrkA, neurotrophic tyrosine kinase receptor type 1; TrkB, neurotrophic tyrosine kinase receptor type 2; TrkC, neurotrophic tyrosine kinase receptor type 3; VLGR1, adhesion G protein-coupled receptor V1; VTI1B, vesicle transport through interaction with t-SNARE 1B.

Glossary:

Intraluminal Vesicles (ILV) – small, cargo enriched vesicles within the lumen of a maturing late endosome

Endosomal Sorting Complexes Required for Transport (ESCRT) - protein complexes that mediate the sorting of ubiquitylated cargo into intraluminal vesicles for degradation in lysosomes

Sorting motifs – usually unstructured linear peptide sequences present in the cytoplasmic tail of cargo proteins that by engaging coat complexes control the sorting of said cargo through intracellular membrane trafficking

Sorting Nexin (SNX) family – large and diverse family of endosome-localized, peripheral membrane proteins defined by the presence of a PX domain

Wiskott-Aldrich syndrome and SCAR Homologue (WASH) complex – pentameric multi-protein complex that generates branched actin networks on the endosomal membrane.

BAR (Bin/Amphiphysin/Rvs) domain – a frequently occurring protein domain with α-helical coiled-coils. The domains can dimerise to form a banana-shaped structure. Oligomerization of BAR domains can deform cellular membranes

Eps15 homology domain (EHD) family – A family of four proteins (EHD1-4) that possess structural similarities to dynamin and function in intracellular trafficking

F-BAR domain (FCH-Homology Bar domain) – a BAR domain found in proteins that couple membrane remodelling with actin dynamics

Amphid sensory organ – the principal olfactory organ of nematodes

Haemocyte – a cell of the haemolymph in invertebrates.

Amyloids – Protein aggregates that can form firbrils, often associated with neurodegenerative diseases such as Alzheimer disease

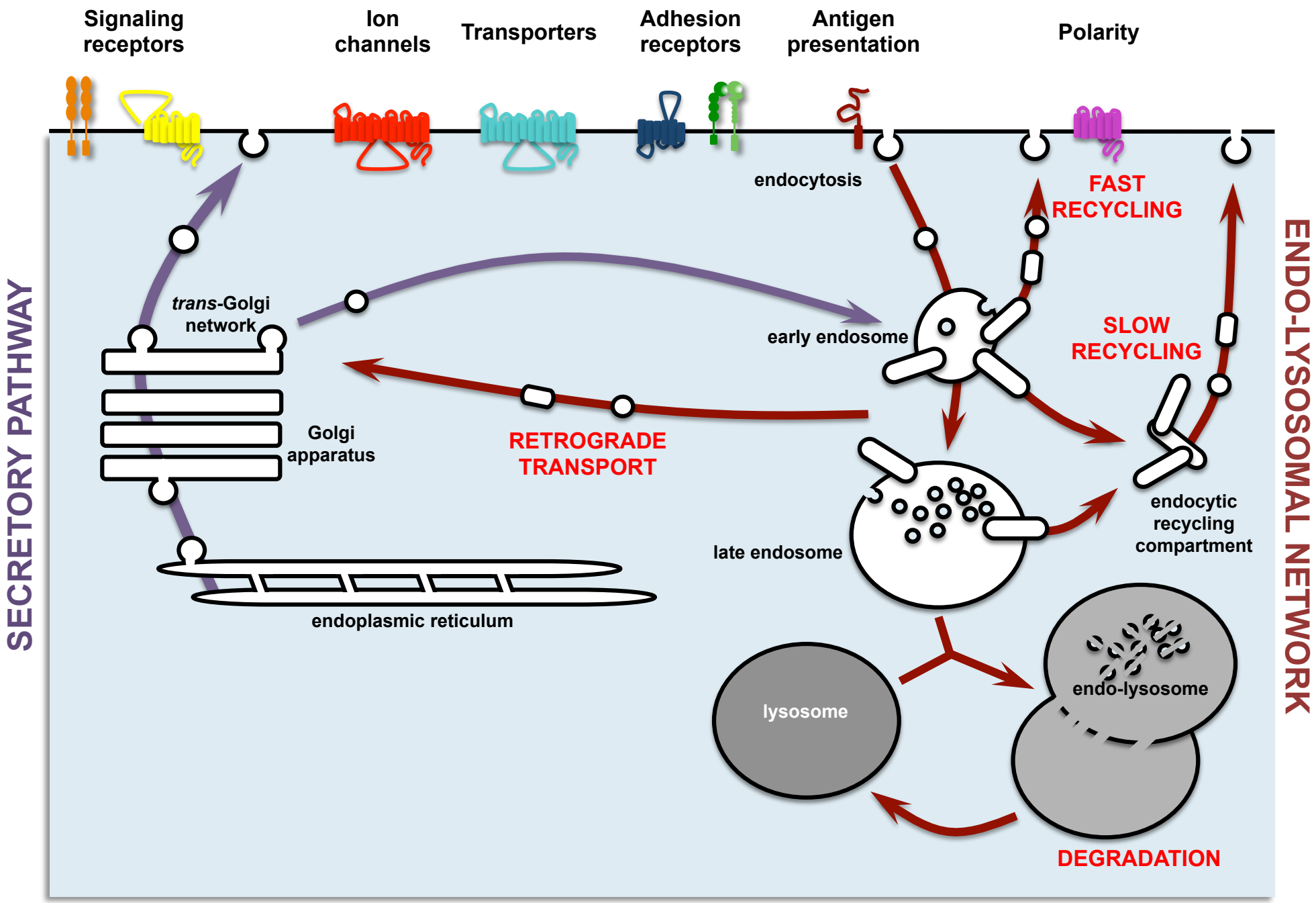
β-amyloid precursor protein (APP) – An integral membrane protein highly expressed in neuronal synapses. Proteolytic cleavage of APP generates the toxic β-amyloid polypeptide that contributes to Alzheimer disease

Macroautophagy – A degradative pathway in which a nutrient starved cell sequesters cytoplasmic content into double membraned vesicles for lysosomal degradation.

Chaperone-mediated autophagy – Specialized form of autophagy in which chaperone proteins directly shuttle cytosolic proteins into the lysosomal lumen through LAMP2a mediated channels

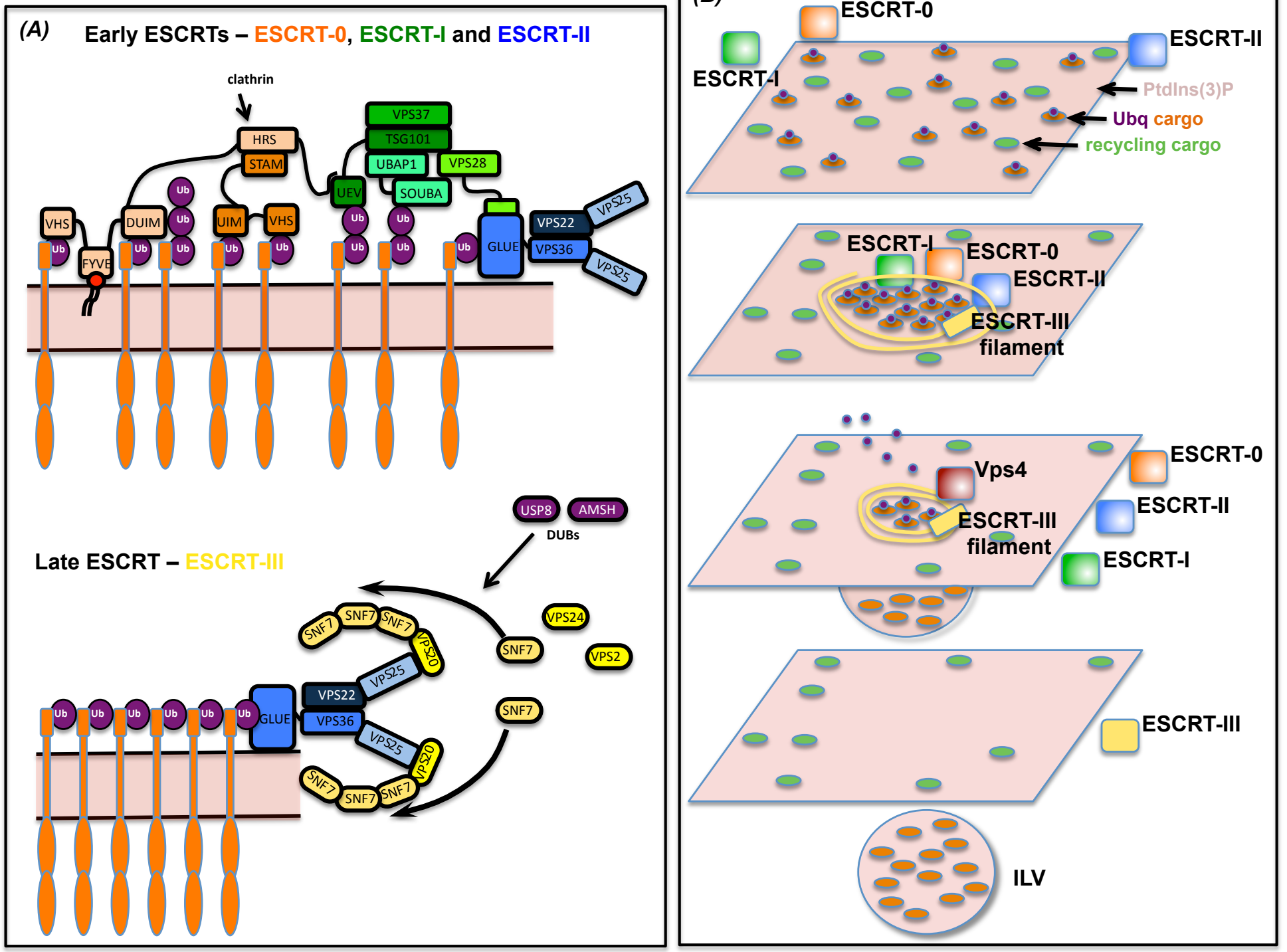
Spastic hereditary paraplegia – A group of inheritable diseases characterized by progressive gait disorders due to dysfunction of motor neurons in the spinal cord

Microglia - macrophage-related immune cells of the central nervous system

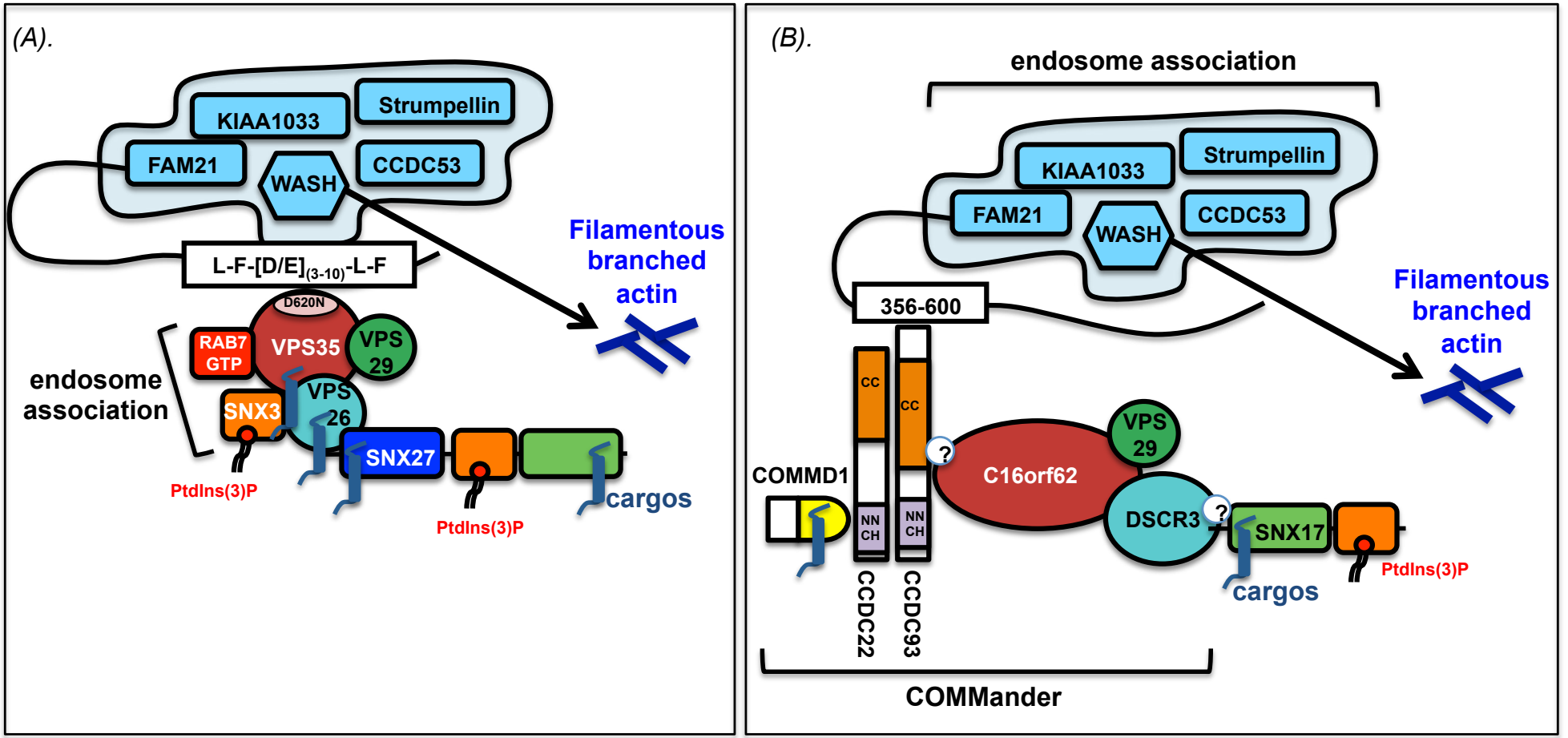


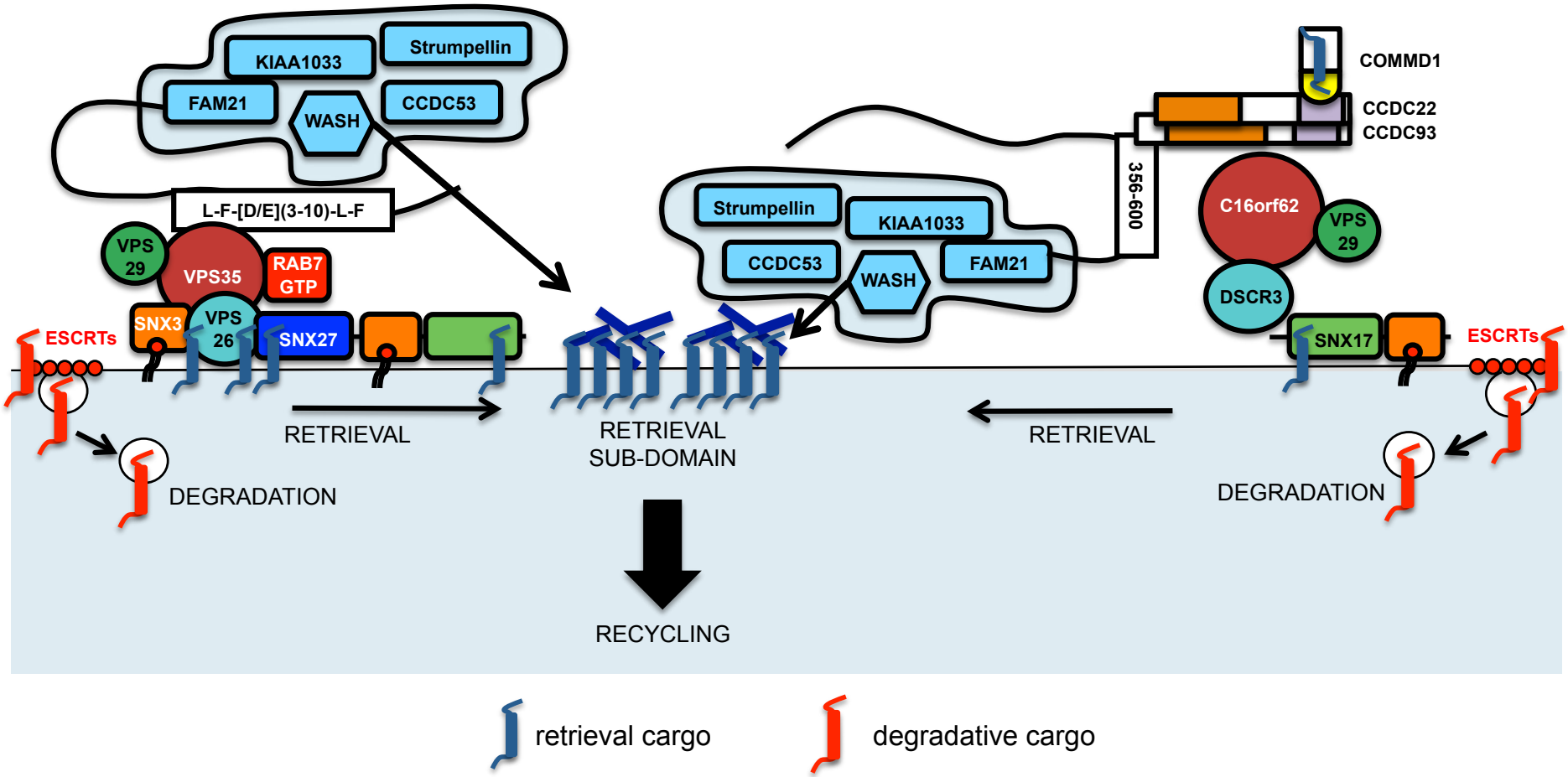
Cullen & Steinberg, Figure 1

Cullen & Steinberg, Figure 2



Cullen & Steinberg, Figure 3

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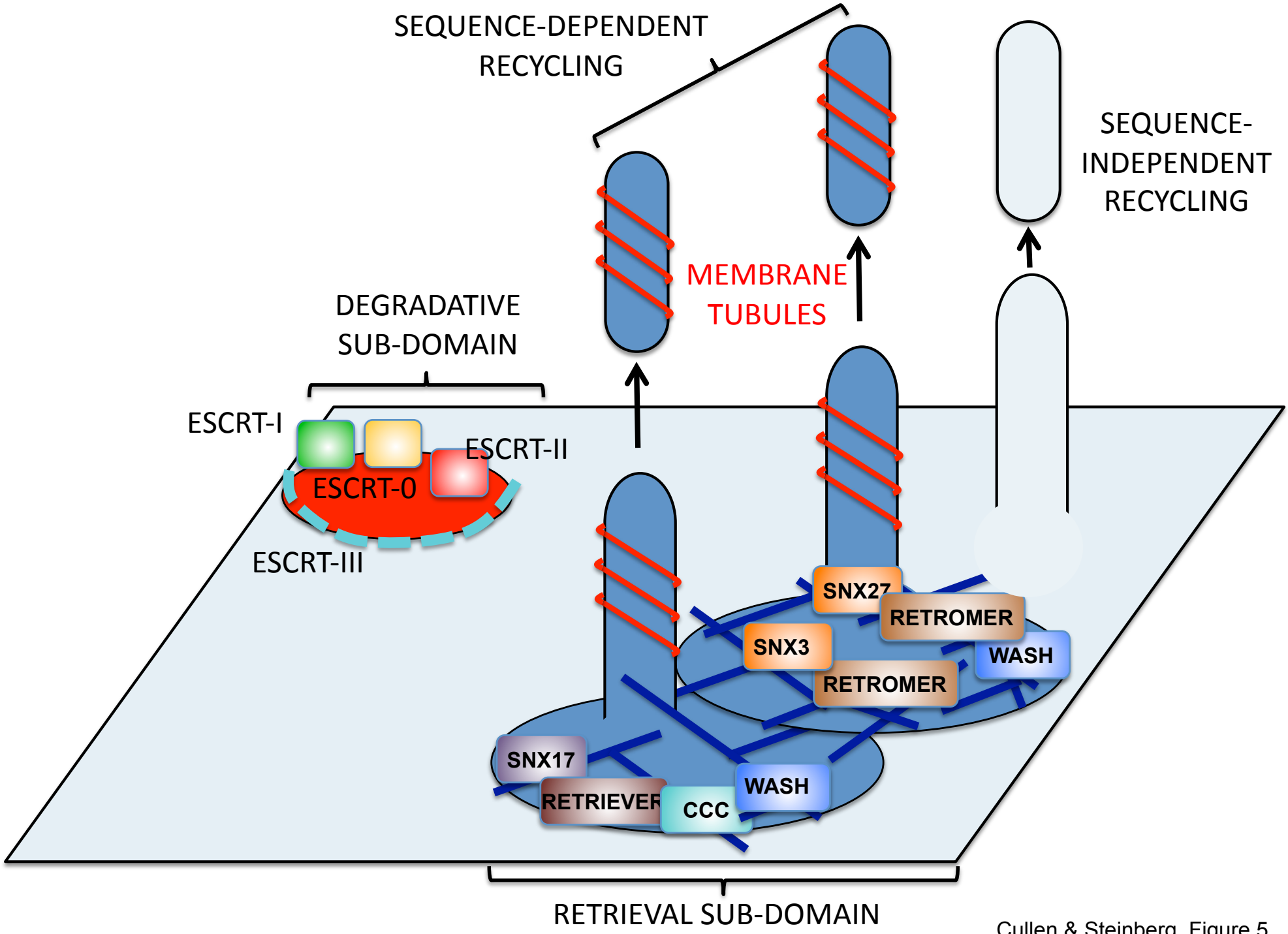
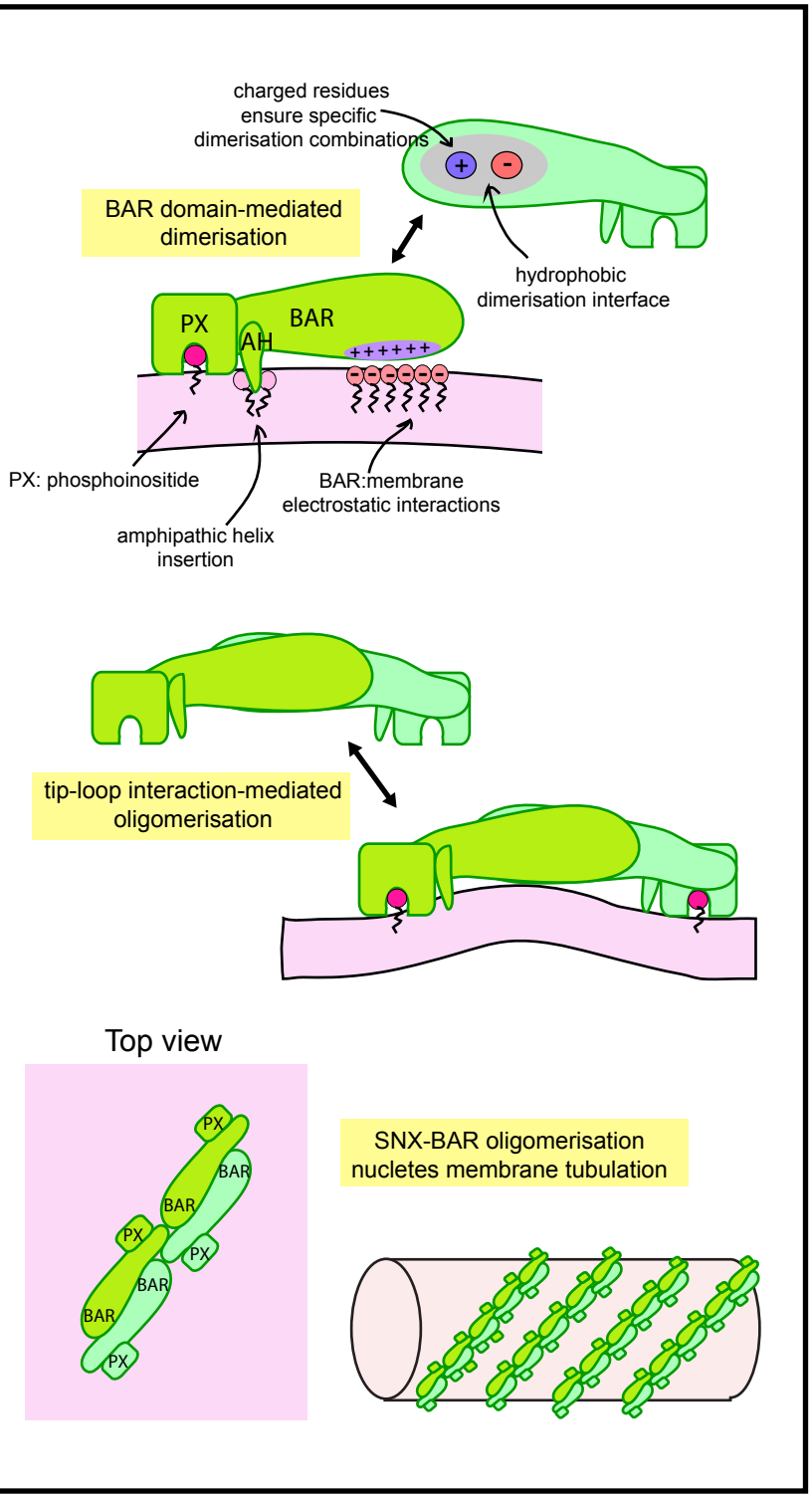
Strumpellin
FAM21
WASH
CCDC53
L-F-[D/E](3-10)-L-F
VPS 29
VPS35
RAB7 GTP
SNX3
VPS 26
SNX27
ESCRTs
RETRIEVAL
DEGRADATION
RETRIEVAL SUB-DOMAIN
RECYCLING
Strumpellin
KIAA1033
CCDC53
WASH
FAM21
356-600
COMMD1
CCDC22
CCDC93
C16orf62
VPS 29
DSCR3
SNX17
ESCRTs
RETRIEVAL
DEGRADATION
retrieval cargo
degradative cargo



Cullen & Steinberg, Figure 5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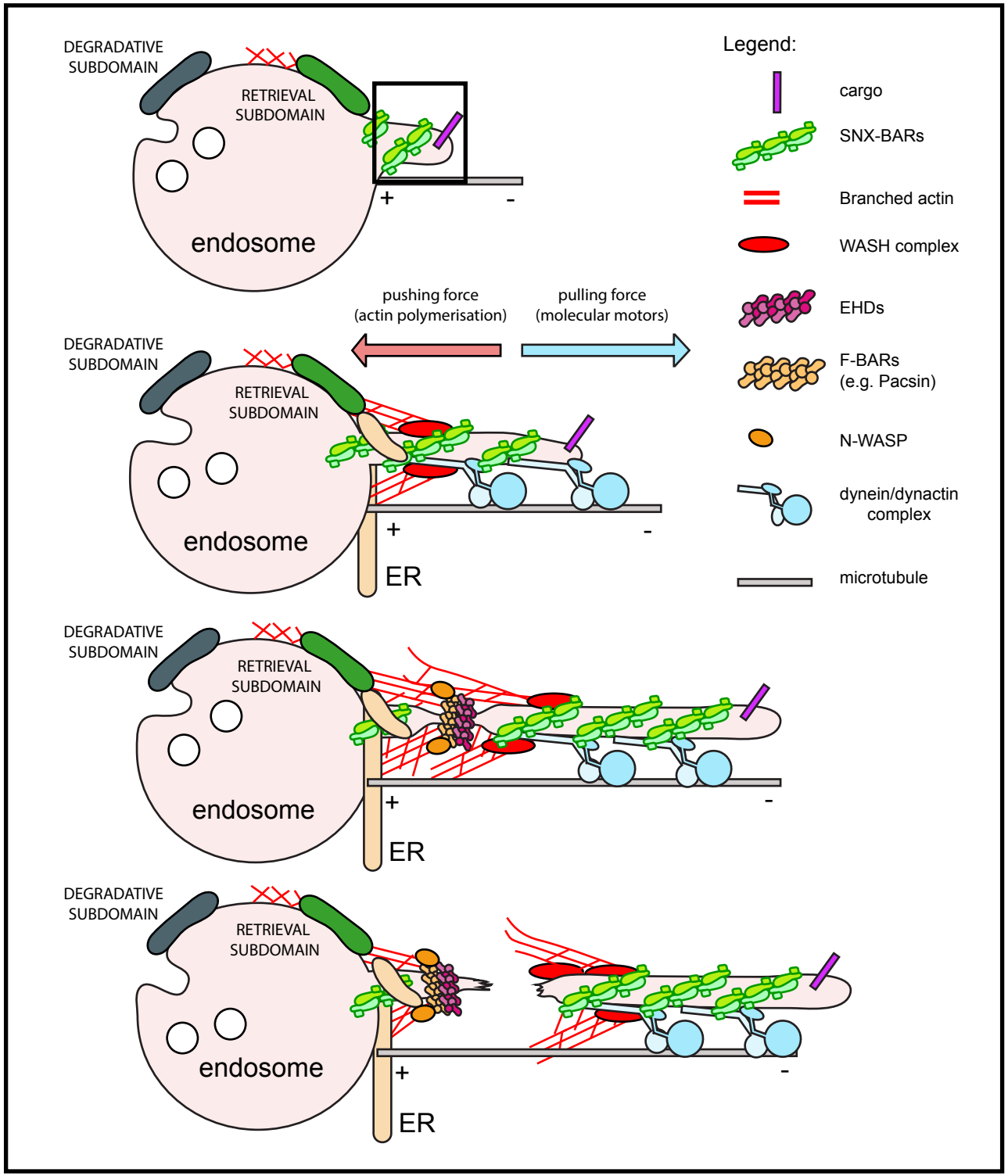
DEGRADATIVE
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pushing force
(actin polymerisation)
pulling force
(molecular motors)
ER
Legend:
cargo
SNX-BARs
Branched actin
WASH complex
EHDs
F-BARs
(e.g. Pacsin)
N-WASP
dynein/dynactin
complex
microtubule

TABLE 1: Sorting motifs required for recycling of endocytosed cargo proteins

Cargo adaptor (recognition domain)	Sorting signal	Example cargos	Reference
SNX17	ΦxNPx[F/Y] ΦxNxx[F/Y]	P-selectin - **F**T**N**AA**F** LRP – **I**G**NP**T**Y** β1-Integrin - **V**V**NP**K**Y**	Knauth et al., 2005 van Kerkhof et al., 2005 Steinberg et al., 2012 Bottcher et al., 2012
SNX27 (PDZ domain)	$[E/D]^{-5}x^{-4}[E/D]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[ED/pSpT]^{-6}[E/D]^{-5}x^{-4}[E/D]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[ED/pSpT]^{-5}x^{-4}[E/D]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[ED/pSpT]^{-6}[ED/pSpT]^{-5}x^{-4}[pS/pT]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[ED/pSpT]^{-6}x^{-5}x^{-4}[pS/pT]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[E/D]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$ $[pS/pT]^{-3}[S/T]^{-2}x^{-1}\Phi^{0}$	GLUT1 – L^{-6}GA**DS**Q**V**^{0} β2-adrenergic receptor – **S**$^{-6}$TN**DS**L**L**^{0} 5-$HT_{4(a)}$ receptor – **E**$^{-6}$**S**L**ES**C**F**^{0} GIRK – **E**$^{-5}$**SES**K**V**^{0} Kir3.3 - **E**$^{-3}$**S**K**V**^{0}	Clairfeuille et al., 2016 Steinberg et al., 2013 Lauffer et al., 2010 Joubert et al., 2004 Balana et al., 2011 Lunn et al., 2007
SNX27 (FERM-like domain)	ΦxNPxpY ΦxNxxpY	VLGR1 - LK**NP**F**pY** RET - IE**N**KL**pY**	Ghia et al., 2013
SNX3-VPS26:VPS35:VPS29	Aromatic, hydrophobic motif - Φx[L/M/V]	DMT1-II – EL**Y**L**L**NTM Sortilin – GR**F**L**V**HRY CI-MPR - TE**W**L**M**EEI	Tabuchi et al., 2010 Lucas et al., 2016 Seaman, 2007
VPS26:VPS35:VPS29	FANSHY (binding to VPS26)	SorLA - **FANSHY**	Fjorback et al., 2012
COMMD1	NPxY (direct?)	LDLR - FD**NP**V**Y**	Bartuzi et al., 2016
PACS-1 **PACS-2**	Acidic cluster motif	Furin – **EE**CPpSDpS**EED** CI-MPR – H**DD**pS**DED**LLHI Polycystin2 - **DD**pS**EEDDDED**S	Jones et al., 1995 Wan et al., 1998 Kottgen et al., 2005
AP-1	YxxΦ [D/E]xxxL[L/I]	Coxsackie virus and adenovirus receptor – **Y**NQ**V** MHC-1-associated invariant chain p33 – **D**QRD**LI**	
AP-3	[D/E]xxxL[L/I]	OCA2- **E**NTP**LL**	Sitaram et al., 2012
EpsinR	Hydrophobic + electrostatic	Vti1b – H_{abc} domain	Miller et al., 2007
GGA1	DIpSLL	BACE1 - DIpSLL	Toh et al., 2018
GGA3	DxxL[L/V]	TrkA receptor – **D**KM**LV** TrkB receptor – **D**KI**LV** TrkC receptor - **D**KM**LV**	Li et al., 2015
ACAP1	Positively charged residues + associated hydrophobic motif	β1-Integrin – REFAKF GLUT4 – KR (assisted by PLSLL motif) TfR – RF and LF	Bai et al., 2012 Li et al., 2007 Dai et al., 2004
SNX5, SNX6, SNX32	Hydrophobic motif	CI-MPR - TE**WLM**EEI	Simonetti et al., 2017 Kvainickas et al., 2017

TABLE 2: Membrane remodeling complexes implicated in endosomal cargo recycling

Membrane remodeling protein / complex	Tubulovesicular transport carrier biogenesis	Accessory proteins / lipids	Reference
SNX-BARs	N-BAR domain		
SNX1:SNX1 *SNX1/SNX2:SNX5/SNX6/SNX32*		Dynein / Dynactin – microtubule motor DNAJC13 – co-ordination with WASH complex	Carlton et al., 2004 Traer et al., 2007 Haberg et al., 2008
SNX4:SNX4 *SNX4:SNX7* *SNX4:SNX30*		Dynein / Dynactin – microtubule motor	Wassmer et al., 2009 van Weering et al., 2012 Soreng et al., 2018
SNX8:SNX8			
SNX18:SNX18		Dynamin-2 - transport carrier fission N-WASP – actin nucleation and polymerisation	Haberg et al., 2008 Soreng et al., 2018
EHDs *EHD1, EHD4 (vesiculation)* *EHD3 (stabilisation),*	EH domain	PACSIN1 / 2 – F-BAR domain mediated tubulation Amphiphysin 2 – BAR domain mediated tubulation Rabenosyn-5 – early endosome Rab5 effector Rabankyrin-5 - early endosome Rab5 effector Rab11-FIP2 - recycling endosome Rab11 effector	Pant et al., 2009
PACSINs PACSIN1, PACSIN2	F-BAR domain	EHD proteins – stabilization / vesiculation of tubules MICAL-L1 – actin organisation Phosphatidic acid – membrane recruitment Rab35 / Rab8a/ Arf6 GTPases – membrane recruitment N-WASP – actin nucleation and polymerisation Dynamin – transport carrier fission	Sharma et al., 2009 Rahajeng et al., 2012 Bahl et al., 2016
ACAP1	BAR–PH domain + association with clathrin and clathrin cage assembly	Rab10:GTP – recruits ACAP1 to endosome Arf6:GTP – activates PI(4)P5-kinase I PI(4)P5-kinase I – drives formation of $PI(4,5)P_2$	Pang et al., 2014 Li et a., 2007 Shinozaki-Narikawa et al., 2006
AP-1/clathrin	Clathrin cage assembly		Robinson & Bonifacino, 2001 Kural et al., 2012
AP-3/clathrin	Clathrin cage assembly		Robinson & Bonifacino, 2001 Kural et al., 2012
GGA3/clathrin	Clathrin cage assembly	Arf6:GTP	Puertollano & Bonifacino, 2004 Zhao & Keen, 2008

			Parachoniak et al., 2011
BLOC-1	Curvilinear chain	KIF13A – microtubule motor AnxA2:Arp2/3 – branched actin polymerisation	Delevoye et al., 2016 Delevoye et al., 2014
EHBP-1		Rab10 / actin /PtdIns(4,5)P_2	Wang et al., 2016