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PERSPECTIVES

OPINION

Getting active: protein sorting in endocytic recycling

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Abstract | Endocytic recycling returns proteins to the plasma membrane in many physiological contexts. Studies of these events have helped to elucidate fundamental mechanisms that underlie recycling. Recycling was for some time considered to be the exception to a general mechanism of active cargo sorting in multiple intracellular pathways. In recent years, studies have begun to reconcile this seeming disparity and also suggest explanations for why early recycling studies did not detect active sorting. Further articulation of this emerging trend has far-reaching implications for a deeper understanding of many physiological and pathological events that require recycling.

Endocytic recycling is crucial for many basic processes, including nutrient uptake, intracellular signalling, polarity, cytokinesis, cell adhesion and migration. Following endocytosis from the plasma membrane, internalized cargoes first reach the early endosome. This organelle is composed of two subcompartments, termed the sorting endosome and the recycling endosome. The sorting endosome acts as the main portal through which endocytic cargoes are further transported to other internal membranes. Cargoes can be targeted for degradation by transport from the sorting endosome to the late endosome (which is also known as the multivesicular body) and then to the lysosome. Alternatively, cargoes can undergo retrograde transport to the Golgi complex, and this allows access to the secretory pathway. The third major fate involves recycling to the plasma membrane, which can occur by a direct route from the sorting endosome or indirectly via the recycling endosome.

The two general routes of endocytic recycling have been elucidated through studies on multiple recycled proteins. These studies have, in turn, shed light on the many physiological events that require recycling. Some better known examples include studies on: proteins involved in nutrient uptake, such as transferrin receptor (TfR), low-density lipoprotein receptor (LDLR) and glucose transporter type 4 (GLUT4; also known as SLC2A4); proteins involved in cell adhesion and migration, such as integrins and cadherins; and proteins involved in signalling, such as G protein-coupled receptors (GPCRs) and receptor Tyr kinases (RTKs)[1–5]. Endocytic recycling also has crucial roles in other major cellular processes, such as polarity, cytokinesis and phagocytosis, which probably involve multiple cargoes being transported through the recycling pathways[6–9].

Here we discuss the mechanisms by which endocytic recycling is controlled. We outline the early observations that led to the idea that cargo sorting in this pathway might occur by default and discuss how this model has now been revisited, with increasing evidence suggesting that cargo sorting occurs by an active process instead. This emerging appreciation allows a reconciliation with a general tenet in the transport field positing that fundamental mechanisms should be conserved across the different intracellular pathways and also has practical ramifications in facilitating further dissection of the mechanisms that underlie endocytic recycling.

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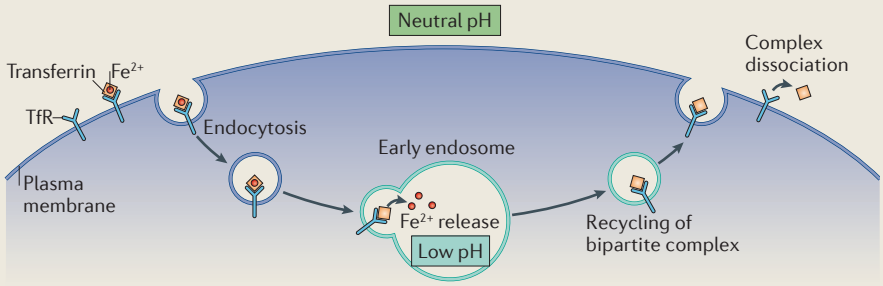
Sorting by default

One of the most intensely studied recycled proteins is TfR (BOX 1), and therefore studies on TfR recycling have led the way in advancing our fundamental understanding of endocytic recycling. Notably, however, early studies of TfR recycling led to a startling conclusion: a predicted fundamental mechanism of vesicular transport, in which coat complexes recognize sorting signals on cargoes (BOX 2), did not seem to be conserved in the recycling pathways.

A conclusive demonstration of active sorting requires the identification of both a coat complex and a sorting signal on the cargo that is recognized by the coat. Initially, a sorting signal could not be identified for TfR recycling, and this was seemingly further confirmed by the observation that truncating virtually the entire cytoplasmic domain of TfR had no appreciable effect on its recycling[10]. There was also difficulty in identifying a coat complex for this process. Early ultrastructural studies had hinted at the possibility that clathrin acts in TfR recycling[11]. However, subsequent functional studies could not provide compelling support for this role, as perturbing clathrin by several approaches, including the use of dominant-negative constructs[12], genetic elimination[13], chemical inhibition[14] and small interfering RNA (siRNA) targeting[15], led to either a modest or no effect on TfR recycling. By contrast, because these perturbations dramatically affected TfR endocytosis[12–15], it was proposed at the time that clathrin was more likely to have an indirect role in recycling[2]. It is also notable that early studies on the endocytic behaviour of lipids (which is used to track membrane transport in the endocytic pathways) had found that lipids were internalized and recycled with similar kinetics to those of TfR[16]. These observations together led to a mechanistic view that would dominate the recycling field for many years to come — that the sorting of cargoes for endocytic recycling occurs by default.

Box 1 | **TfR as the model cargo for recycling studies**

Transferrin receptor (TfR) undergoes recycling constitutively, with or without binding to its ligand, transferrin. Therefore, early recycling studies focused on TfR as a way of tracking the bulk membrane traversing the recycling pathways[16,19,71]. Another key reason for the popularity of TfR comes from a practical consideration. Receptors, in general, facilitate studies on endocytic transport, as ligand binding allows their endocytic pool to be distinguished from their biosynthetic pool. However, many ligands dissociate from their receptor when they reach the endosomal compartments (where the luminal pH is acidic), which prevents further tracking of the receptor via its ligand[72]. By contrast, transferrin remains bound to TfR throughout the recycling process, which is important for the regulation of cellular iron uptake[73]. At the cell surface, the neutral pH favours transferrin binding to both iron and TfR. When this tripartite complex is internalized, the low pH in the early endosomal compartments induces the release of iron from transferrin, leaving a bipartite complex of transferrin and TfR (see the figure). Subsequently, the restoration of neutral pH following recycling to the cell surface induces the dissociation of the remaining bipartite complex. Thus, a single round of TfR recycling can be tracked simply by adding iron-loaded transferrin to cells and then examining the eventual disappearance of transferrin from these cells.



Challenging the default model. An early challenge to this view was to explain why cargoes that undergo fluid-phase endocytosis are generally transported towards the lysosome, whereas membrane-associated endocytic cargoes, such as TfR, are generally recycled to the plasma membrane. Endocytic compartments exhibit tubular extensions[17–19], and tubules have a high surface-to-volume ratio (when compared to the central portion of endosomal compartments, which is more spherical). These considerations led to the proposal that recycling through tubular carriers allows membrane-bound cargoes to become enriched by partitioning with the tubular membrane[2]. This type of default sorting, when coupled with compartmental retention mechanisms, could in principle provide a way of generating further selective sorting. An example of a retention mechanism was suggested by studies on TUG (tether containing UBX domain for GLUT4), which was proposed to sequester GLUT4 in endosomal compartments until intracellular signalling instigated by insulin promotes GLUT4 release[20].

Further examples began to emerge that challenged the generality of default sorting. A variant of the adaptor protein 1 (AP1) complex, known as AP1B, was found to be expressed selectively in polarized cells, where it was found to target cargoes, such as TfR and LDLR, to the basolateral surface.

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As prototypical AP1 was known to act at the *trans*-Golgi network (TGN) in non-polarized cells, AP1B was initially proposed to also act at the TGN during basolateral targeting[21]. Notably, subsequent studies showed that AP1B acts at the recycling endosome in mediating the basolateral recycling of TfR and LDLR[22,23]. However, the second criterion for proof of active sorting — the identification of a sorting signal on the cargo that is recognized by the coat (BOX 2) — was still lacking. In this regard, a stretch of the cytoplasmic domain in TfR was found to be important for its basolateral recycling, but a more precise sequence could not be pinpointed[24]. Moreover, whether AP1B could bind directly to the particular region in TfR that mediates its basolateral recycling was unclear. Thus, in the absence of a conclusive demonstration of active sorting by AP1B at the time of its initial discovery, some suggested that AP1B acts indirectly during sorting of basolateral recycling cargoes, for example by promoting the formation of recycling carriers[2].

Another seeming inconsistency with the idea of default sorting came from studies on GPCRs. Following ligand binding at the plasma membrane, members of this family undergo endocytosis and are then either transported towards the lysosome for degradation or recycled to the plasma membrane[4]. In early studies, specific sequences in the cytoplasmic domain of prototypical GPCRs were shown to promote recycling of these proteins[25–27]. However, the complementary goal of identifying a coat complex, which would provide conclusive support for active sorting, was not achieved for many years. Moreover, as one could argue that GPCRs represent a special class of recycling cargoes, these early studies on GPCRs were limited in their ability to overcome the dominant view at that time that cargo sorting in the recycling pathways is achieved, in general, by a default mechanism.

Strengthening evidence for active sorting

Just as the dominance of the default model originated from studies on TfR recycling in non-polarized cells, a key turning point also came from a revisit of this recycling event. Remarkably, this re-examination led to the identification of recycling sorting signals in TfR as well as a coat component that recognized these signals[28]. This unexpected discovery emerged from insights gained into some of the better characterized transport pathways. In the secretory pathway, small GTPases of the ADP-ribosylation factor (ARF) family had been shown to recruit coat complexes to their target membranes, with ARF1 acting on COPI[29,30] and Sar1 acting on COPII[31]. As small GTPases, ARF members are, in turn, regulated by two general classes of catalytic regulators: ARF guanine nucleotide exchange factors, which catalyse ARF activation, and ARF GTPase-activating proteins (GAPs), which catalyse ARF deactivation[32]. Aside from this conventional role as negative regulators of ARF small GTPases, ARF GAPs were later discovered to possess an additional unexpected function: they act as ARF effectors within coat complexes[31,33]. In the subsequent attempt to determine whether this novel role of ARF GAPs has widespread significance, ACAP1 (ARF GAP with coiled-coil ankyrin repeat and PH domain-containing protein 1), known at the time as an ARF6 GAP[34], was found to recognize specific sequences in TfR that function as recycling sorting signals[28]. Further expanding the significance of this finding, subsequent studies found that ACAP1 also participates in the recycling of two other model cargoes, namely integrin and GLUT4 (REFS 35,36).

ACAP1 was also found to couple with clathrin to form a new type of coat

complex[36]. In the case of TfR recycling, it was initially challenging to show that clathrin has a direct role, as endocytosis of TfR also depends on clathrin[12–15]. By contrast, GLUT4 endocytosis does not require clathrin[37], and integrin endocytosis can occur through either a clathrin-dependent[38–40] or a clathrin-independent[36,41] pathway. Thus, the ability to examine integrin and GLUT4 that have been internalized through clathrin-independent mechanisms allowed the importance of clathrin to be more clearly documented[36].

Table 1 | **Coat complexes and sorting signals identified for endocytic recycling**

Pathway	Cargo	Sorting signal	Coat complex	Refs
Recycling endosome to plasma membrane	TfR	LF, RF	Clathrin–ACAP1	28
	GLUT4	KR, PLSLL	Clathrin–ACAP1	36
Sorting endosome to plasma membrane	B2AR	DSLL	Retromer–SNX27	26,43
	LRPs	NPxY	SNX17	42
	MET	Pro-rich motif	Clathrin–GGA3	48
Basolateral recycling	CAR	YNQV	Clathrin–AP1B	50,52

ACAP1, ADP-ribosylation factor GTPase-activating protein with coiled-coil ankyrin repeat and PH domain-containing protein 1; AP1B, adaptor protein 1B complex; B2AR, β2-adrenergic receptor; CAR, coxsackievirus and adenovirus receptor; GGA3, Golgi-localized γ-ear-containing ADP-ribosylation factor-binding protein 3; GLUT4, glucose transporter type 4; LRPs, low-density lipoprotein receptor-related proteins; SNX, sorting nexin; TfR, transferrin receptor.

Active sorting as a general principle. Ultimately, to validate that endocytic recycling occurs by active sorting, it is necessary to show that this principle holds true for numerous recycled cargoes, and support for this is now emerging. Whereas the ACAP1-containing clathrin complex[28,36] acts in one recycling route (from recycling endosomes to the plasma membrane), coat complexes and sorting signals have also now been identified for the other recycling route (from sorting endosomes to the plasma membrane). In one study, sorting nexin 17 (SNX17) was shown to bind directly to a sequence in the cytoplasmic domain of LDLR-related proteins (LRPs), and this was also shown to promote LRP recycling[42]. Another study demonstrated that SNX27 acts as an adaptor that links the coat protein retromer to a prototypical GPCR cargo, β2 adrenergic receptor (B2AR)[43]. A surprising aspect of this finding is that retromer acts in retrograde transport from endosomes to the Golgi[44–46]. Therefore, one possibility is that retromer acts indirectly in B2AR recycling by promoting retrograde transport to the Golgi, and this then allows B2AR to access the secretory pathway and thereby be transported to the plasma membrane. However, B2AR has more recently been shown to reside in carriers moving from the sorting endosomes to the plasma membrane[47], thus confirming a direct role for retromer in recycling. Moreover, the complexity of recycling from sorting endosomes has been further revealed by the finding that the RTK MET (also known as HGFR) recycles through this pathway, requiring clathrin in conjunction with GGA3 (Golgi-localized γ-ear-containing ARF-binding protein 3)[48]. Thus, it is becoming increasingly clear that active sorting is a widespread mechanism that operates in endocytic recycling.

This emerging theme is further illustrated by the recent advances concerning the role of AP1B in polarized cells. Aside from TfR and LDLR, other basolateral proteins are being identified that use AP1B for their recycling[49,50]. Moreover, clathrin has been shown to couple with AP1B in forming a coat complex for TfR and LDLR recycling[51]. It is also notable that a formal demonstration of active sorting by AP1B has now been achieved, as a sequence in a recycling cargo, known as coxsackievirus and adenovirus receptor (CAR), has been shown to be recognized by AP1B and to act as a recycling sorting signal[50,52].

Box 2 | **Active sorting requires coat complexes that recognize cargo-sorting signals**

Vesicular transport occurs through a series of conserved mechanistic steps. This requires coat complexes that promote vesicle formation, motor complexes that regulate translocation, tether complexes that mediate docking and SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes that drive fusion[62,74]. Aside from their effects on vesicle formation, coat complexes promote cargo sorting, driving the selective incorporation of cargoes into transport vesicles. By coupling these two roles, coat complexes act as the core machinery by which cargoes are properly sorted into the different intracellular pathways[62,63,74]. Distilled to its mechanistic core, cargo sorting involves coat complexes binding to specific sequences, known as sorting signals, in the cytoplasmic domain of protein cargoes. These signals are defined functionally as discrete sequences in the cytoplasmic domain of cargoes that direct these cargoes into specific transport pathways.

One of the earliest sorting signals to be characterized was a Tyr-based motif that promotes the endocytosis of many surface proteins. This motif was identified in transferrin receptor (TfR) through systematic mutation of its cytoplasmic domain, and this revealed the key residues required for efficient endocytosis[10,75]. In complementary studies, the sorting signal in TfR was shown to be recognized by the adaptor protein 2 (AP2) complex[76,77], which acts in conjunction with clathrin during endocytosis at the plasma membrane. Similar demonstrations of such sorting signals have been achieved for other pathways. Some of the better characterized examples, which are found in several cargoes, include a di-Lys-based motif that is recognized by the coat protein I (COPI) complex for retrograde transport in the early secretory pathway[78,79], as well as diacidic- and dihydrophobic-based motifs that are recognized by the COPII complex for anterograde transport from the endoplasmic reticulum[56,80,81].

Reconciling with earlier findings

The cumulative results of recent years have begun to reverse the long-held view of how proteins are sorted during endocytic recycling. Instead of sorting by default, there is now support for a central role of active sorting. This emerging trend has resulted from analysis of some of the most intensively investigated recycling proteins, including TfR[28], GLUT4 (REF. 36) and prototypical members of the GPCR[43] and RTK[48] families of signalling receptors. There are now many examples for which both criteria of active sorting have been met; that is, the dual demonstration of sorting signals and coat complexes that recognize such signals (TABLE 1). Notably, this advance has not only revealed distinct types of coat complexes acting in recycling (FIG. 1) but also illuminated the different ways in which these coats can achieve active sorting (FIG. 2). Importantly, these findings also provided an important confirmation of the conserved nature of the basic mechanisms that operate during intracellular trafficking.

Explaining earlier studies of TfR. With any major change in mechanistic thinking, new questions invariably follow. One obvious question has been why early studies failed to identify recycling sorting signals in TfR. One explanation is that ACAP1

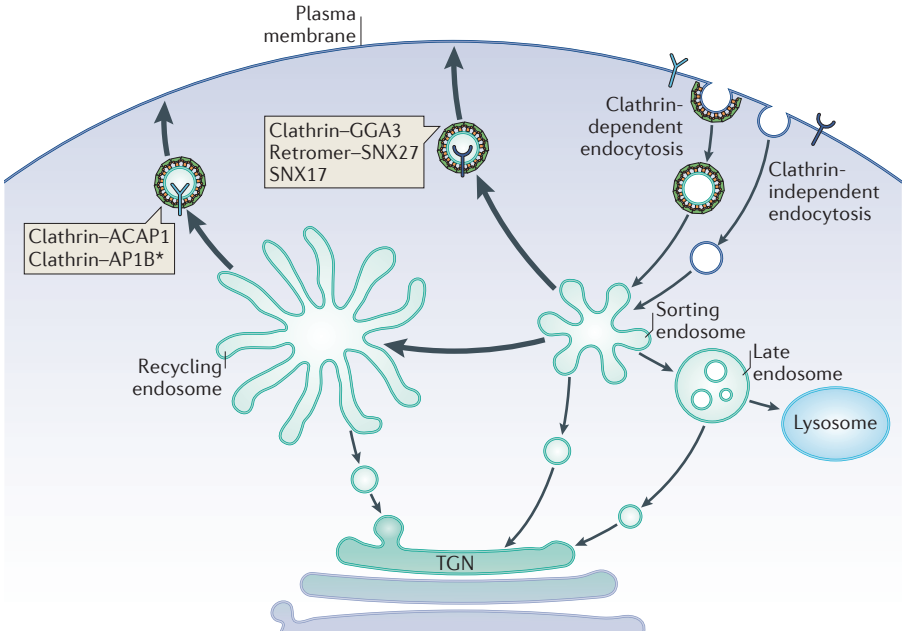

Figure 1 | **Cargo can be actively recycled during endocytosis.** Cargo can be endocytosed by both clathrin-dependent and clathrin-independent mechanisms. The three general fates of endocytic cargoes are recycling to the plasma membrane, transport towards the lysosome and retrograde transport towards the *trans*-Golgi network (TGN). The major transport pathways that underlie recycling are highlighted by bold arrows and include recycling directly from sorting endosomes or after initial sorting into recycling endosomes. The coat complexes and adaptors that have been shown to mediate active sorting in these pathways are indicated. ACAP1, ADP-ribosylation factor GTPase-activating protein with coiled-coil ankyrin repeat and PH domain-containing protein 1; AP1B, adaptor protein 1B complex; GGA3, Golgi-localized γ-ear-containing ADP-ribosylation factor-binding protein 3; SNX, sorting nexin. *Acts specifically in polarized cells.

recognizes two regions in TfR that overlap with endocytic sorting signals[28]. Thus, early recycling studies that focused on TfR probably missed these sequences owing to their dual function. However, ACAP1 localizes only to the recycling endosome[28], whereas the endocytic adaptor AP2 is localized to the plasma membrane[53,54]. This provides a possible explanation for how similar sequences in TfR mediate both its endocytosis and its recycling through distinct interactions with regulators at different subcellular locations.

Another seeming puzzle is why a mutant TfR with virtually its entire cytoplasmic domain deleted still recycles efficiently[10]. As the emerging trend suggests that active sorting is a general principle of the recycling pathways, one possibility is that the deletion of the entire cytoplasmic domain of TfR removes two counteracting signals: a sorting signal that promotes transport and a retention signal that prevents transport. Another possibility is suggested by studies in polarized cells, in which the transmembrane domain of certain cargoes has been found to possess targeting information for active sorting to the apical surface[55]. As TfR recycling has long been viewed as a key model system for studying 'generic' mechanisms of recycling, elucidating how mutant TfR recycles will be instructive.

Perspectives and future directions

The recycling pathways are actually composed of three transport segments: a direct route of recycling from sorting endosomes to the plasma membrane, and an indirect route consisting of transport from sorting endosomes to the recycling endosomes and then transport from recycling endosomes to the plasma membrane (FIG. 1). Recent results suggest key new questions to explore in each of these segments. In transport from the sorting endosome to the plasma membrane, multiple coat complexes have now been identified[42,43,48]. This is surprising because in other contexts a single type of coat complex forms transport carriers that define a pathway. One reconciling possibility could be that sorting endosomes in fact represent multiple subcompartments from which distinct transport carriers are formed. Consistent with this explanation, TfR and B2AR are concentrated in distinct tubular extensions of the early endosomal compartment[47]. Thus, an important future goal will be to determine whether other cargoes that are recycled from sorting endosomes to the plasma membrane, such as LRPs and MET, are concentrated in tubular extensions that are distinct from those containing TfR and B2AR.

With respect to transport from recycling endosomes to the plasma membrane, one peculiarity is that a single coat complex of ACAP1 and clathrin can recognize diverse sorting signals, with one class being defined by a basic residue and another class being defined by dihydrophobic residues (TABLE 1). How can this coat complex bind to such diverse signals? A potential hint comes from studies of cargo binding by the COPII complex, in which the Sec24 subunit has been shown to have distinct binding sites[56]. Therefore, it is possible that ACAP1 also has distinct binding sites that explain how it binds diverse classes of sorting signals.

For transport from sorting endosomes to recycling endosomes, coat complexes and sorting signals that underlie active sorting have yet to be identified. SNX4 can promote the transport of TfR through this pathway[57]. However, because SNX4 does not interact with TfR but instead couples with a dynein-dependent mechanism[57], SNX4 probably affects the translocation of carriers rather than promoting cargo sorting. An intriguing alternative candidate could be retromer, which acts in transport from endosomes to the Golgi[44–46], as this transport could involve not only a direct route from the sorting endosomes to the Golgi but also an indirect route via recycling endosomes. Thus, working out whether retromer acts in transport from sorting endosomes to recycling endosomes would not only confirm that active sorting occurs in this pathway but also represent a key clarification regarding how retromer acts.

Another important consideration is that clathrin couples with distinct adaptors to act in different transport pathways and so may also act in transport from sorting endosomes to recycling endosomes. For endocytic recycling, clathrin has already been shown to act in conjunction with either ACAP1 (REF. 36) or GGA3 (REF. 48). In retrograde transport towards the Golgi complex, clathrin couples with either epsin-related protein (EpsinR; also known as CLINT1)[58] or AP1 (REF. 59). Moreover, clathrin has also been implicated in endocytic transport towards the lysosome[60,61]. So, it would not be surprising if clathrin could also act in transport from sorting endosomes to recycling endosomes.

Coat complexes act not only in cargo sorting but also in carrier formation[62,63].

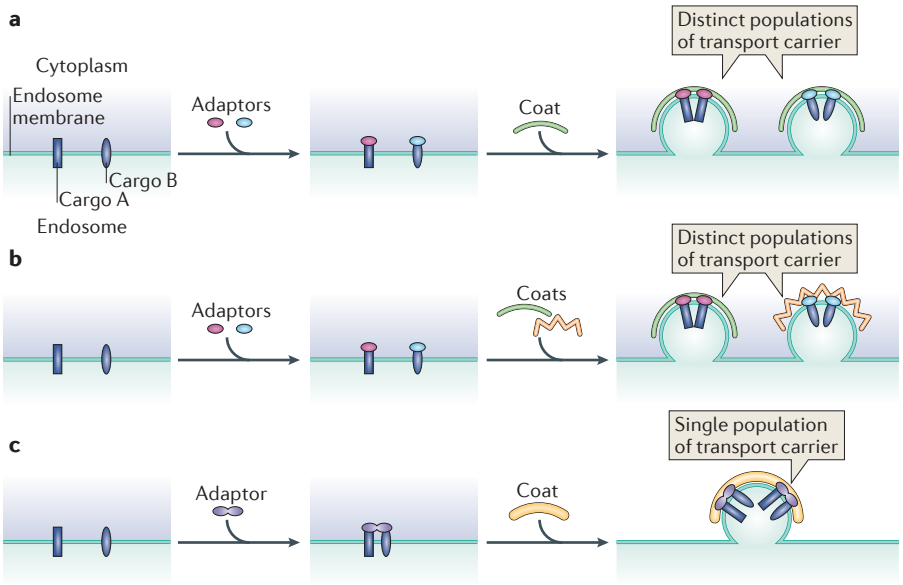

Figure 2 | **Potential mechanisms of active sorting. a** | Cargoes with different sorting signals could be recognized by distinct adaptors that couple with a common outer coat for sorting into distinct populations of carriers. **b** | Alternatively, cargoes with different sorting signals that are recognized by distinct adaptors could couple with distinct outer coats for sorting into distinct populations of carriers. **c** | A third mechanism might involve cargoes with different sorting signals that are recognized by a common adaptor containing distinct binding sites for different sorting signals, which couples with a common outer coat for sorting into a common population of carriers.

Although insights have been gained into how coats recognize sorting signals on cargoes, it has been mechanistically less clear how different coat complexes regulate carrier formation. A possible clue is that some coat components are predicted to have a BAR (Bin–amphiphysin–Rvs) domain, which can generate the membrane curvature needed for carrier formation in other pathways[64,65]. ARF-like small GTPases can deform membranes[66–69] in addition to their established role in recruiting coat complexes to target membranes[32]. Exploring these possibilities should provide a more complete mechanistic understanding of the recycling pathways.

Finally, although the notion that tubular carriers are involved in recycling has been dominant for many years, a definitive demonstration of this has been challenging. Carrier formation has been directly visualized during B2AR recycling using light-based microscopy but unfortunately could not resolve the morphology of these carriers completely[47]. By contrast, vesicle carriers have been definitively detected in the context of GLUT4 recycling using the high-resolution technique of electron microscopy[70]. Therefore, the prospect exists that the tubular extensions of endosomal compartments are capable of generating both general types of transport carriers. This could occur by a particular extension generating both vesicles and tubular carriers or by one extension generating exclusively one type of carrier and another extension generating exclusively the other type. Addressing these possibilities could provide fundamental insights into intracellular transport, as vesicles and tubules are now appreciated to represent the two general classes of intracellular membrane carriers, but their roles in different transport pathways need to be more clearly defined.

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Competing interests statement

The authors declare no competing financial interests.

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