Review assay

Sorting of cargo in the tubular endosomal network

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Abbreviations:
FERARI: Factors for endosome recycling and Rab interactions
TEN: tubular endosomal networks
TGN: trans-Golgi network
SE: sorting endosome

Abstract

Intercellular communication is an essential process in all multicellular organisms. During this process, molecules secreted by one cell will bind to a receptor on the cognate cell leading to the subsequent uptake of the receptor-ligand complex. Once inside, the cell then determines the fate of the receptor-ligand complex and any other proteins that were endocytosed together. Approximately 80% of endocytosed material is recycled back to the plasma membrane either directly or indirectly via the Golgi apparatus and the remaining 20% is delivered to the lysosome for degradation. Although most pathways have been identified, we still lack understanding on how specificity in sorting of recycling cargos into different pathways is achieved, and how the cell reaches high accuracy of these processes in the absence of clear sorting signals in the bulk of the client proteins. In this review, we will summarize our current understanding of the mechanism behind recycling cargo sorting and propose a model of differential affinities between cargo and cargo receptors/adaptors with regards to iterative sorting in endosomes.

Introduction

The biosynthetic and the endocytic routes of the intracellular transport pathway converge on endosomes where cargos destined for the plasma membrane are delivered either by a direct route (biosynthetic route) or via a recycling pathway (after endocytosis). During this process both the newly synthesized and recycled cargo proteins frequently share the same transport container on their way to the plasma membrane. However, the plasma membranes of most cells are compartmentalized into functionally different domains often accompanied by characteristic structural features. This compartmentalization is easily appreciated in polarized epithelial cells in which the apical, lateral and basal plasma membrane compartments are clearly distinguishable. Furthermore, plasma membrane compartmentalization also exists at a much smaller scale including sites of nutrient uptake, cell-to-cell communication, as well as the plasma membrane contact sites with the endoplasmic reticulum and other organelles. Each of these compartments must be populated with a distinct profile of membrane proteins at the proper concentration and the maintenance of these compartments is dependent on the correct balance of exo- and endocytosis. Transport carriers destined for the plasma membrane may contain cargo for either specific domains or for multiple domains, which could then be sorted later into different compartments. Thus, different cargo exit sites should exist at the endosomes and the trans Golgi Network (TGN) comparable to taxis and public transport systems. In support of this concept, cells appear to employ both types of transport systems as there are specialized transport vesicles (Spang, 2015) as well as more generic carriers (i.e. clathrin-coated vesicles).

Although we have a good understanding of the different routes of endocytosis such as the clathrin-dependent endocytosis pathway (Mayor and Pagano, 2007; Mettlen et al., 2018; Weinberg and Drubin, 2012), the downstream events in endocytosis and regulation thereof, which includes sorting and recycling, are much less clear. One contributing factor is that the presence or absence of a molecule from the plasma membrane is more easily assessed by light microscopy and biochemical methods compared to a protein that moves within the cell. Another reason is the diverse morphological features of the endosomal system (Fig. 1), which consists of tubular endosomal networks (TEN) also referred to as tubular-vesicular clusters, round empty or filled looking structures, multivesicular bodies, different tubular or vesicular transport carriers, which cannot be identified by light microscopy but only by electron microscopy. The advent of Correlative Light and Electron Microscopy (CLEM) has been a welcome development for the field (van der Beek et al., 2022) but the technology lacks the dynamic aspect making it incapable of tracking protein movement across various compartments. Moreover, these compartments are themselves often highly dynamic with a limited lifetime. The unambiguous identification of endolysosomal compartments by light microscopy is hampered by the fact that early endosomes mature into late endosomes, during which numerous changes are expected to occur. Most studies have only focused on Rab5 (early) and Rab7 (late) endosomal markers, both of which have clear limitations in tracking endosomal maturation. For example, Rab5 marks endocytic vesicles but also a variety of early endosomes. In addition, Rab5 plays a role in ER dynamics and nuclear envelope disassembly (Audhya et al., 2007) and is recruited to kinetochores (Serio et al., 2011). In the case of Rab7, its localization is not solely restricted to late endosomes; it can also be found on endolysosomes, lysosomes and autophagosomes (Borchers et al., 2021; Guerra and Bucci, 2016). Furthermore, EEA1, which is a commonly used marker for early endosomes, marks only a subset of early endosomes (Wilson et al., 2000). Finally, the sorting nexins such as SNX1 bind to the highly curved tubular membranes found on early and late endosomes but also on TENs (Carlton et al., 2004; Shortill et al., 2022). A related issue requiring close attention is the strict nomenclature of early and late endosomes as identified by Rab5 and Rab7. Rab5-to-Rab7 conversion takes about two minutes (Podinovskaia et al., 2021; Poteryaev et al., 2010) yet sorting, recycling and acidification often takes much longer to occur. The coordination of these events with Rab conversion is still far from being understood. Nevertheless, it appears clear that these processes are neither fully completed before Rab5-to-Rab7 conversion nor only start after the conversion event.

Another level of complication emerges when studying the recycling of proteins to the plasma membrane or the Golgi apparatus. Firstly, there are a multitude of different pathways as illustrated by the large number of different Rab GTPases, cargo adaptors and recycling complexes that act in these pathways. Do these different transport containers emerge from the same endosome as it matures from early-to-late? Are there different recycling compartments? And if so, how does the cargo enter these different compartments? Moreover, as different cell types vary in their

exo- and endocytic capacity, which is also dependent on the metabolic state and the stress level perceived by the cells, it is unsurprisingly difficult to come to a unified one-size-fits-all picture of endosomal recycling. In agreement with many researchers in the endosomal recycling field, the cargos do not adhere to a single pathway; if their preferred pathway is blocked the cargos may opt to follow a different route, albeit with lower efficiency. Even though the TEN can be detected by electron and light microscopy (Franke et al., 2019; Murk et al., 2003; Rahajeng et al., 2010; van der Beek et al., 2022), most illustrations of the endosomal recycling pathways places little to no emphasis on the TEN. This is in part due to our rather limited knowledge beyond the morphological description of TENs.
In this review, we address questions about the role of TEN in recycling and how kiss-and-run of endosomal vesicles with TEN improve the accuracy, fidelity, and efficiency in cargo sorting and recycling (Solinger et al., 2020; Solinger et al., 2022).

The role of TEN in sorting and recycling

Tubular Endosomal Networks (TEN) have been observed adjacent to early/sorting endosomes. They are described as complex and dynamic structures (Franke et al., 2019; Klumperman and Raposo, 2014; Murk et al., 2003; van der Beek et al., 2022)(see Fig.2, movie 1). The TEN and related organelles can be compared to the Golgi with regards to the complexity of their sorting processes and seem to be a long-lived compartment (Maxfield and McGraw, 2004). Surprisingly, cargos from the trans-Golgi network (TGN) are frequently sorted through early endosomal networks (Mihov et al., 2015). While TENs can often be observed and visualized, the reason for cells to produce such a complex structure is not completely understood. A longstanding hypothesis concerns geometry-based sorting where the mere increase in membrane surface would be sufficient to segregate recycling cargos away from the globular sorting endosome (SE) without requiring any sorting signals (Maxfield and McGraw, 2004). In addition, tubular membranes have been shown to selectively attract specific lipids (Roux et al., 2005) or proteins (Aimon et al., 2014). This would lead to a “bulk flow” of cargo into the direction of recycling transporter containers. On the other hand, more and more sorting motifs for specific protein sorting via cargo adaptors have been identified (Chi et al., 2015; Cullen and Steinberg, 2018; McNally and Cullen, 2018). A potential sorting mechanism involving self-assembly through prion-like domains has also been proposed (Ritz et al., 2014). The main protein sorting step has been described as happening right at the SE: short tubules emanating from the globular SE for a limited time from which vesicles (or tubules) could pinch off, and the remaining tubule would retract (Cullen and Steinberg, 2018; McNally and Cullen, 2018). Indeed, it has been proposed that the elongated tubules of the TEN serve solely as transport carriers and that no cargo exchange will happen after they detach from the sorting endosome (Xie et al., 2016).
The TEN itself seems to be enriched in sorting nexins that bind to and stabilize tubular membranes. Some of these sorting nexins (SNX5 and SNX6) are involved directly in cargo binding and function as cargo adaptors (Simonetti et al., 2019). Other sorting nexins (e.g., SNX1, SNX9) could potentially provide a structural

component of TEN by binding and tubulating membranes (Carlton et al., 2004; Pylypenko et al., 2007; van Weering et al., 2012). TEN membranes were also shown to contain clathrin and AP1 domains that could organize cargo into domains and vesicles (Klumperman and Raposo, 2014; Stoorvogel et al., 1996). Since TEN are thought to contain more than 90 % of the cargo for recycling (Griffiths et al., 1989; Marsh et al., 1986) and show fission/fusion dynamics similar to mitochondria, it seems likely that a large part of the sorting happens in these compartments. We envision the formation of cargo enriched domains through a process of membrane dynamic and diffusion that would enable cargo adaptors like AP1 and SNX5/SNX6 to connect to cargos and also potentially through the self-assembly of cargos (Fig. 2, movie 2). The latter pathway could be similar to the kin recognition model proposed for the retention of Golgi enzymes (Nilsson et al., 1993). Potential “exit sites” would form, where either new transport carriers could form and pinch off or pre-existing vesicles could dock via a kiss-and-run mechanism to pick up cargo (Solinger et al., 2020; Solinger et al., 2022). The well-described mechanisms of cargo sorting through tubule formation and pinching-off probably also operates on TEN and is indispensable for the biogenesis of new transport carriers (Chi et al., 2015; Cullen and Steinberg, 2018; McNally and Cullen, 2018). The main reason that these processes are not easily observed in HeLa cells is the relatively low frequency of endocytosis in these cells where most of the cargo in SE derives from the TGN (Podinovskaia et al., 2021). Also, cancer cells -like HeLa cells- have adapted their endocytic and recycling pathways to allow enhanced growth and cell migration (Mellman and Yarden, 2013). In polarized, intestinal cells with high endocytic throughput, extended TEN can be more readily visualized (Solinger et al., 2020). In this case, the sorting tubules contain the bulk of the membranes from which sorting would occur (as shown in Fig. 2).

Even though markers for TEN such as Appl1 (van der Beek et al., 2022), MICAL-L1 and syndapin-2 (Farmer et al., 2021; Giridharan et al., 2013; Gleason et al., 2016) have been described, the exact sorting mechanisms through these compartments are not understood.

The conundrum of affinity versus specificity in cargo sorting

Cargo adaptors need to have low affinity for their clients because they need to dissociate from the cargo when the complex reaches its destination without high energy demand. Changes in pH or ionic strength should be sufficient to drive the dissociation. Cargo adaptors come in two flavors: the first type has high specificity towards cargos, such as subunits of adaptor complexes at the trans-Golgi network and COPI and COPII coats. In these cases, specific sequence motifs are recognized (Gomez-Navarro and Miller, 2016; Guo et al., 2014; Michelsen et al., 2007; Sandmann et al., 2003). For KKXX motifs in the ER-Golgi shuttle, relatively strong binding has been measured conferring high specificity (Table 1)(Ma and Goldberg, 2013). KDEL motifs bind strongly in the Golgi and are thought to be released in a pH-dependent manner in the ER (Table 1)(Wilson et al., 1993; Wu et al., 2020). The other class of cargo adaptors bind with low specificity. These cargo adaptor-cargo

interactions are particularly prominent in the endosomal system, in which the ubiquitination stage and other parameters are read out in the decision to degrade or recycle. Thus, the cargo adaptor-cargo interactions in the SE are characterized by low affinity and low specificity. The relationship between affinity and specificity has been noted previously (Eaton et al., 1995). Essentially, high specificity often correlates with high affinity binding, as seen for antibody-epitope interactions (Landry et al., 2015), or between stable tubulin dimers (Montecinos-Franjola et al., 2016) (Table 1). Cargo adaptors have been measured by different methods to have much lower binding affinities to their respective cargo motifs (Table 1 and references therein). These basic physical properties of cargo adaptors and their cargo led to the conundrum of how to sort proteins faithfully without too many costly errors. Wrongly sorted cargo would have to be retrieved then re-sorted, which could lead to additional energetic costs, especially for cells with high cargo throughput.
To solve this conundrum, we propose that cargo can be sorted in several steps. The first step would occur as described before, and lead to biogenesis of transport carriers (Chi et al., 2015). In addition to this process, there would also be sorting in several steps with increasing cargo binding affinities of adaptors (Table 1). Initial, pre-sorting would happen by bulk flow of cargo into the extended membranes of the TEN. This would be followed by low-affinity sorting inside the TEN, leading to pre-sorted “exit sites” or domains with similar cargo in the TEN (Fig. 2, movie 2). The final steps of sorting would happen with kiss-and-run events using transport carriers, docking to these pre-sorted domains and enriching for specific cargo each time (Solinger et al., 2020; Solinger et al., 2022).

How kiss-and-run helps the sorting process

We recently discovered an unconventional tethering factor, FERARI, in the endosomal system, that combines tethering and fusion activities with membrane pinching (Table 2)(Solinger et al., 2020). In an effort to understand the implications of FERARI on cargo sorting through SE, we developed a theoretical framework in which kiss-and-run events could solve the cargo sorting problems mentioned above. We propose that TEN functions as a meeting place for endosomal cargos of different origins (plasma membrane, endosomes, TGN) (Solinger et al., 2022). In the TEN, cargos could be pre-sorted by a diffusive mixing and de-mixing mechanism according to low affinity interactions with cargo adaptors or other cargos with self-assembly domains (Fig. 2, movie 2). FERARI could already participate in this process through interactions with cargo adaptors to create specific “exit sites” in which different cargos would congregate and be transported to the same destination. FERARI also tethers different types of transport carriers (depending on their associated Rab GTPase). The following kiss-and-run event allows loading (or unloading) of cargo to enrich for specific cargos inside the vesicle (Solinger et al., 2022). This serial process of cargo enrichment solves the second conundrum of how to reach selectivity with the low affinity and low specificity of cargo adaptors. Several subsequent steps of cargo enrichment -similar to a distillation process- would allow for better sorting results.

The length of the docking (or “kiss”) of a transport carrier with the TEN would be determined by two factors: first, a catastrophe/rescue cycle of RME-1/EHD1 spiral formation on the stalk region between the transport carrier and the TEN (Fig. 3), and second, the availability of cargo in the stalk region, which could oppose or counteract pinching forces (by RME-1 itself, actin, and microtubule motors) (Fig. 4). Measurements of the size of RME-1 or EHD1 spirals around tubular membranes have shown that the space available inside the tubules is limited (Table 3) (Daumke et al., 2007; Deo et al., 2018; Pant et al., 2009). Cargos will have to move through the neck region of the docked vesicle in small packages or even in a single file. On the other hand, it is conceivable that the formation of an RME-1 spiral could be disturbed by the presence of cargo in the stalk, which would lead to difficulties in the pinching-off of the vesicle. Since RME-1 spiral formation and stability depends on ATP binding, the hydrolysis of ATP to ADP would lead to destabilization of the spiral and a “catastrophic” spiral depolymerization analogous to what has been proposed for microtubules (Fig. 3). With FERARI localized at the base of the stalk, a starting RME-1 subunit could serve as a seed and always reinitiate the spiral formation. A cycle of polymerization and catastrophic depolymerization would repeat until all cargo is successfully sorted into a particular transport container. We expect this polymerization/depolymerization process to take only a few seconds and be relatively regular, as the polymerization process should always happen with similar kinetics and the length of the spiral would be constrained by the geometry of the vesicle and its connection to the TEN (Fig. 3). In the analogous process of endocytic vesicle formation, the RME-1 related dynamin forms similar spirals (including pinching-off activity) in about 10 seconds (Shnyrova et al., 2013; Taylor et al., 2011). We believe that this regular mechanism provides an explanation for the regular residence times found in FERARI kiss-and-run events (Solinger et al., 2020; Solinger et al., 2022). Residence times are quantal with recurrent intervals of cargo loading, presumably caused by the underlying “catastrophe and rescue” mechanism. A similar effect could also be caused by the loading of defined cargo packages that would take a defined amount of time to move through the limited opening of the vesicle. It seems unlikely that the amount of cargo would always be very similar. Moreover, cargo size and topology would be rather variable and hence the restriction through the stalk geometry should affect cargo movement through the neck to varying degree. Thus, loading times would be very different, depending on the type and concentration of cargo, inconsistent with the regular intervals that were observed. We therefore prefer the “catastrophe and rescue” model.

The final decision of how many cycles of cargo loading would occur before the transport carrier is released would be at least partially dependent on the local cargo availability. We postulate several possible mechanisms of determining cargo availability: first, the cargo could clog the stalk and thereby directly undermine the stability of the RME-1 spiral. Second, cargo adaptors on the TEN, like SNX5 and SNX6, could directly or indirectly influence FERARI and pinching-off. Third, cargo adaptors on the vesicle could directly tether to FERARI and only let go when sufficient cargo is present (Fig. 4). The pinching-off would be favored by three

factors: First, the polymerization of the RME-1 spiral itself. Second, actin polymerization could provide a pushing force (similar to the endocytic process), and third, microtubule motors will try to pull the vesicle away (Fig. 4). The balance would be tipped by the presence of suitable cargo to the “stay” side. While the successful sorting of all the local cargo would lead to the “go” signal for the transport carrier. This model predicts direct interactions of cargo adaptors (e.g. AP1) with FERARI. We consider these interactions highly likely since FERARI contains several scaffold proteins with protein-protein interaction domains (e.g., ankyrin, Rabenosyn-5 and SPE-39).

The polymerization of branched actin starting from FERARI could be envisioned by the function of ankyrin (one of the subunits is UNC-44/Ank1). Roles of ankyrin-like proteins in connecting endosomes to the actin cytoskeleton have been observed previously. While Ank2 is able to bind through its ZU-5 domain to PI(3)P membranes (Qu et al., 2016) other ankyrin-like proteins can also directly interact with Arp2/3, a regulator of actin branching (shown for VARP/ANKRD27)(Koseoglu et al., 2015), or bind to Retromer and the WASH complex (which also regulates Arp2/3) (as seen for ANKRD50)(Kvainickas et al., 2017). While these functions have not yet been demonstrated for Ank1 and FERARI, it seems that ankyrins have a function in mediating contacts between endosomal membranes and actin.

Percolation model of cargo sorting at endosomes

The repeated kiss-and-run of transport carriers on TEN would lead to maturation and progressive enrichment of specific cargos in vesicles but also in the TEN itself (Fig. 5, movie 3). A similar process has been proposed for the maturation of Golgi stacks through iterative fractionation (Dunn et al., 1989). The use of successive cargo enrichment by serial contacts with low affinity binding adaptors is essentially the same in the movement of cargo through successive Golgi stacks to the TGN (Rothman, 1981). Contacts between Golgi cisternae with tubules to exchange cargo more efficiently have also been reported (Glick and Nakano, 2009). While the geometry of the TEN and Golgi look somehow different, it seems likely that similar sorting processes govern their function since the cell has to deal with similar problems in both systems.

Directionality of cargo flow during kiss-and-run

How could an intrinsically non-directional mechanism like kiss-and-run lead to a net flow of cargo into recycling vesicles and transport to their destination? The measurements of binding constants of cargo motifs to adaptors show a possible solution to this question (Table 1). In our hypothesis, recycling cargo flows from RAB-5 vesicles, where it is not bound to any particular adaptor, into the TEN, where it is bound with low affinity to SNX5 or SNX6 adaptors, and finally into RAB-11 vesicles, carrying adaptors with higher binding affinity (AP1) (Solinger et al., 2022) (Fig. 6, movie 4, Table 1). Binding of degradative cargo to ESCRT-0 on RAB-5 vesicles would ensure that these cargos remain in the degradative pathway (Fig. 6). This model is based on different RAB-5 endosomal compartments: vesicles derived

directly from endocytic events and that could undergo homotypic fusion promoted by CORVET (Peplowska et al., 2007; Solinger and Spang, 2013). These early RAB-5 vesicles already carry ESCRT-0 to sequester the degradative cargo. They would be active for kiss-and-run using the FERARI machinery and able to unload recycling cargo into the TEN. The fusion with a larger RAB-5-positive SE would lead to the deposition of all the remaining cargo into the SE. These later RAB-5 compartments would then be proficient in sorting tubule formation and biogenesis of transport carriers (Fig. 6, movie 4). After the sorting of all the cargo is achieved, RAB-5 compartments would be ready for RAB-conversion to RAB-7 (Kinchen and Ravichandran, 2010; Podinovskaia et al., 2021; Poteryaev et al., 2010).

In principle, the cargo could flow directly from the SE into the TEN through connecting tubules driven by membrane curvature and the vastly larger membrane surface of the tubular network compared to the globular SE (Maxfield and McGraw, 2004). At the same time, a central sorting step in this model is the described tubulation and pinching-off of vesicles from the SE (Chi et al., 2015; Cullen and Steinberg, 2018; McNally and Cullen, 2018). This process provides a first sorting step and produces transport carriers that would undergo kiss-and-run on TEN to further enrich for the cargo. This step will also be the determining factor for the final destination of the vesicle as well as the category of cargo that needs to be loaded. The vesicle generated at the SE would be equipped with specific SNAREs, RABs and cargo adaptors. The machineries for this vesicle biogenesis have been identified (Chi et al., 2015).

As discussed above, we doubt that a one-step sorting will be sufficient given the complexity of cargos to be sorted. For this reason, we postulate further sorting steps after the initial recycling vesicle has been formed (Fig. 6, movie 4). The higher-affinity binding cargo adaptors on the newly formed recycling vesicles (AP1, SNX17, SNX27, see Table 1) will be able to capture cargo during the kiss-and-run interactions. Wrongly packaged cargo could flow back into the TEN (it would be untethered), thereby providing a possibility to correct earlier sorting mistakes. This mis-sorted cargo could even be picked up by a RAB-5 vesicle and brought all the way back to the SE. It would primarily be attracted by the cogent cargo adaptor in the TEN (or by cargo self-assembly) to an "exit site" and await the next kiss-and-run by an appropriate vesicle.

Conclusion

Our model is in agreement with the observed data that after the SE very little to no sorting happens in transport carriers (Xie et al., 2016). Most of the sorting would happen inside the TEN and not in the recycling vesicles themselves. The transport carriers would not interact in a stable manner with the TEN but only through short-term kiss-and-run. The adjustments and "proof-reading" of vesicle cargo would maybe go unnoticed in a global analysis but would still be crucial in the avoidance of sorting mistakes. TEN as a whole would still contain all cargo types that need to be sorted but local differences in cargo content between pre-sorted sub-domains may be rather large. More high-resolution imaging (also resolving events in time, not only

space) would be needed to fully understand the processes involved. We observed changes over the time scale of a few seconds (4 sec in HeLa, 7 sec in intestinal *C. elegans* cells for one kiss-and-run event) (Solinger et al., 2022). The type of imaging required to obtain the necessary resolution for the highly dynamic events is much faster than the imaging routinely used for trafficking events. Therefore, kiss-and-run has so far been completely overlooked and not taken into consideration.

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Figure legends

Figure 1: The many shapes of endosomes. Schematic representation of the endosomal system, showing the degradative pathway on the right and the recycling/secretory pathway on the left. Incoming material from endocytic vesicles and from the Golgi are transported to early/sorting endosomes and associated TEN where sorting occurs. From this hub, the sorted substances can flow to the dedicated plasma membrane domains (e.g. apical, basal-lateral, adherens junctions), the TGN or the degradative pathway (late endosomes, lysosomes). While degradation is achieved through the formation of intralumenal vesicles inside the endosomes, sorting and recycling happens mainly through tubular-vesicular structures. Since many of these processes occur in parallel, markers for specific machineries will overlap on some structures and be different on others. Early/sorting endosomes may contain Rab5, SNX1, Rab11 and other markers, while later, maturing endosomes will still have Rab5 and SNX1 but also Rab7 (and no Rab11). Cargo will flow through these structures and be handed from one machinery to the next. Thus, each marker by itself can appear to have very different morphological shapes depending on the combination with other markers and its position in the pathway, e.g. Rab5 might appear as small vesicles directly after endocytosis, as larger sorting endosomes associated with tubules, or as even bigger multivesicular bodies, before switching to Rab7.

Figure 2: Cargo sorting inside the TEN. The TEN is a highly dynamic structure constantly exchanging membranes through fission and fusion. Left: movie stills from movie 1, showing the dynamic nature of TEN labelled with mCherry-SNX-1 in *C. elegans* intestinal cells. Right: schematic representation of the process of cargo sorting through tubule movement and diffusion to form cargo “exit sites” with similar cargo with the same destination. Shown are self-assembling cargos with prion-like domains, cargos bound to higher affinity binding adaptors (adaptor 1) that will be transported directly to their respective membranes, and cargos bound to low affinity

adaptors (adaptor 2). The low affinity adaptor (e.g., SNX5, SNX6) will stay in the TEN and function as preliminary sorting stations where FERARI can also bind and form “exit sites” for kiss-and-run interactions. On these “exit sites” transport carriers with higher affinity adaptors can dock and pick up cargo released form the low affinity adaptors. In this step, a gradual enrichment of specific cargos could be achieved and account for the precise sorting without having to use high affinity binding interactions. For the animated model, see movie 2.

Figure 3: 7 seconds away, just as long as I stay, I’ll be waiting. Schematic representation of the “catastrophe and rescue” hypothesis for regulation of residence times of vesicles docked through FERARI to TEN. Top: established mechanism of catastrophe and rescue in microtubules as a comparison. GTP-bound tubulin dimers will assemble into microtubules, which will undergo a depolymerization event upon GTP hydrolysis but can be rescued by repolymerization of fresh GTP-bound tubulin. Bottom: The microtubule mechanism applied to RME-1 spiral formation around a stalk connecting a vesicle to the TEN. FERARI will always supply at least one RME-1 subunit as a crystallization point for filament formation. The growth of the RME-1 spiral will be constrained by the length of the stalk region. The stalk region will also be constrained by the size of the vesicle and the physical properties of the TEN tubules. We expect the stalk to have a rather consistent length, allowing for a consistent amount of time for each cycle of polymerization/depolymerization.

Figure 4: Should I stay or should I go? Schematic representation showing positive and negative mechanisms determining residence time of transport carriers on TEN. In addition to the process shown in Fig.2, there is an additional level of control to determine the residence times of vesicles during cargo loading/unloading. Since high amounts of cargo cause more cycles of “catastrophe and rescue”, it seems reasonable to assume that cargo availability will play a role in making transport carriers wait until the cargo is properly loaded. We envision 3 possible mechanisms: first, a direct interference of cargo in the stalk with the stability of the RME-1 spiral. Second, a possible regulation through SNX5/SNX6 and FERARI to stop pinching-off while cargo is still bound. Third, a direct binding/interaction of vesicle cargo adaptors with FERARI that would only release once binding sites are saturated. We also hypothesize 3 mechanisms that would promote pinching-off: first, the polymerization of RME-1 into a spiral. Second, a pushing force generated by branched actin near the stalk, possibly starting with the ankyrin subunit of FERARI. Third, microtubule motors pulling forces since it has been shown that these motors are transporting vesicles and the speed of vesicles suggests active transport along microtubule tracks. The net outcome of staying or going would be determined by these opposing forces.

Figure 5: Percolation mechanism of cargo sorting at TEN. Schematic representation of vesicle movement through the TEN. See movie 3 for an animated version to appreciate the dynamics of the mechanism. RAB-5 vesicles would move inward from

the plasma membrane and fuse with the progressively growing transport carriers by CORVET. These could also unload recycling cargo by performing kiss-and-run on TEN structures. The fusion with a larger SE would then be final and any remaining cargo including all the ESCRT-0 bound cargo for degradation would be unloaded. A fast-recycling option that bypasses the SE would be available for the recycling cargo unloaded at an earlier stage. Cargo that was falsely sorted could be recovered and brought back to the SE for re-sorting. Formation of new transport carriers at the SE would then provide the next step of sorting and generate vesicles carrying cargo bound to adaptors. These vesicles would become progressively enriched in cargo with each successive kiss-and-run event. Again, this would provide an opportunity to unload wrongly-sorted cargo. The net outcome of this mechanism would be analogous to Golgi cargo sorting with the TEN being contacted by transport vesicles moving in opposite directions and enriching cargo by selective binding to cargo adaptors.

Figure 6: Cargo flow through TEN using kiss-and-run by FERARI. Schematic representation of cargo movement and directionality during RAB-5 and RAB-11 kiss-and-run events. See also movie 4 for a dynamic representation of the mechanism. A first step of RAB-5 vesicle docking to TEN would allow the unloading for fast-recycling cargo (presumably unbound inside RAB-5 vesicles). The presence of ESCRT-0 adaptors on these transport carriers would preclude the loss of cargo for degradation. The large membrane surfaces inside the TEN would ensure cargo diffusion into the network. Cargo adaptors with low binding affinity would then be retained in the TEN and distributed to appropriate “exit sites” with similar cargo and possibly FERARI to be recovered later. The RAB-5 vesicle would then travel to the SE and fuse with the help of CORVET to deliver all remaining cargo. From the SE, the well-described mechanism of tubulation, cargo sorting by adaptors, followed by pinching-off would generate RAB-11 recycling vesicles. RAB-11 vesicles kiss-and-run would provide opportunities to load additional cargo but also to proof-read and deliver cargo to appropriate “exit sites” thus providing more precise sorting.

Data availability statement

Data sharing is not applicable to this article as no new data were created.

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Table 1: Binding affinities of cargo adaptors in the endosomal recycling pathway

Cargo	Adaptor	K_D	Method	Publication
CI-MPR motif	SNX5	18 µM	1	[51]
CI-MPR motif	SNX6	36 µM		
CI-MPR motif	SNX32	18 µM		
IncE	SNX5	0.5-1 µM		
APP peptide	SNX17	33 µM	1	[52]
APP peptide	SNX17-FERM	23 µM		
P-selectin	SNX17	2.7 µM	1	[53]
APP peptide	SNX17	22 µM		
APP peptide	SNX27	28 µM		[52]
LRFN2 motif	SNX27-PDZ	1.6 µM	1	[54]
LRFN2 motif	SNX27-PDZ*	< 1.0 µM		
Kir3.3 motif	SNX27-PDZ	15 µM	1	[55]
Kir3.3 motif	SNX27-PDZ*	0.84 µM		
MPR46 tail**	AP1 complex	11-15 µM	2	[56]
Furin cytosolic tail	AP1 µ1	100-200 µM	1	[57]
Furin cytosolic tail (phos.)***	AP1 µ1	22 µM		
Furin cytosolic tail (phos.)***	AP1 µ1	35 µM	2	
TGN38 cytosolic domain	AP2 µ2	58 µM	3	[58]
CWRPKETLYRRF selected peptide	AP4 µ4	7 µM	2	[59]
APP sorting signal	AP4 µ4	28 µM	1	[60]
hWbp1-KEKSD	α-COP	2.9 µM	1	[45]
	β'-COP	3.4 µM		
Emp47p-KTKLL	α-COP	16.1 µM		
	β'-COP	22.5 µM		
YTSEKDEL	Golgi-Erd2	0.078-0.2 µM	4	[47]
ubiquitin	Hrs	127 µM	1	[61]
ubiquitin	STAM	485 µM		
α-Tubulin	β-Tubulin	0.8-0.08 µM	5	[50]
Epitope	Antibody	10-200 pM	6	[49]

1: isothermal titration calorimetry with peptides
2: surface plasmon resonance
3: fluorimetry
4: Scatchard analysis
5: global analysis combining sedimentation velocity and fluorescence anisotropy
6: microarray-based label-free assay

* with recombinant VPS26
** contains 3 binding sites
*** phosphorylated

Table 2: Previously described roles of FERARI members

FERARI member	Interactor	Process	Publication
SPE-39/VIPAS39/VPS16B	VPS-33.2/VPS33B	ARC syndrome, CHEVI, platelet biogenesis	[62-65]
VPS-45/VPS45	Rabenosyn-5	early endosome fusion	[66-69]
RABS-5/Rabenosyn-5	VPS18	function with CORVET/HOPS	[70,71]
RME-1/EHD1	MICAL-L1, Syndapin2, cPLA2α	membrane tubule fission	[72-77]
UNC-44/ANK1	Spectrin	plasma membrane organization	[78-82]
RFIP-2/Rab11FIP5/RIP11	Rab11	endocytic protein recycling	[83-86]

Table 3: Size constraints inside dynamin, RME-1/EHD helices.

Helix protein	**inner diameter**	**outer diameter**	**Method**	**Publication**
dynamin open	20 nm	50 nm	1	[90]
dynamin close	7 nm	40 nm		
dynamin super-constricted	3.7 nm	37 nm		
EHD1	20-60 nm	340 nm	3	[89]
EHD2	25-75 nm	50-100 nm	4	[87]
RME-1	95 nm	120 nm	4	[88]
Cargo	**size**			
hTfR	11 x 11 nm		2	[91]
Ptch1	6 x 11 nm		1	[92]

1: cryo-EM
2: crystal structure
3: fluorescence microscopy
4: electron microscopy

Figure 1

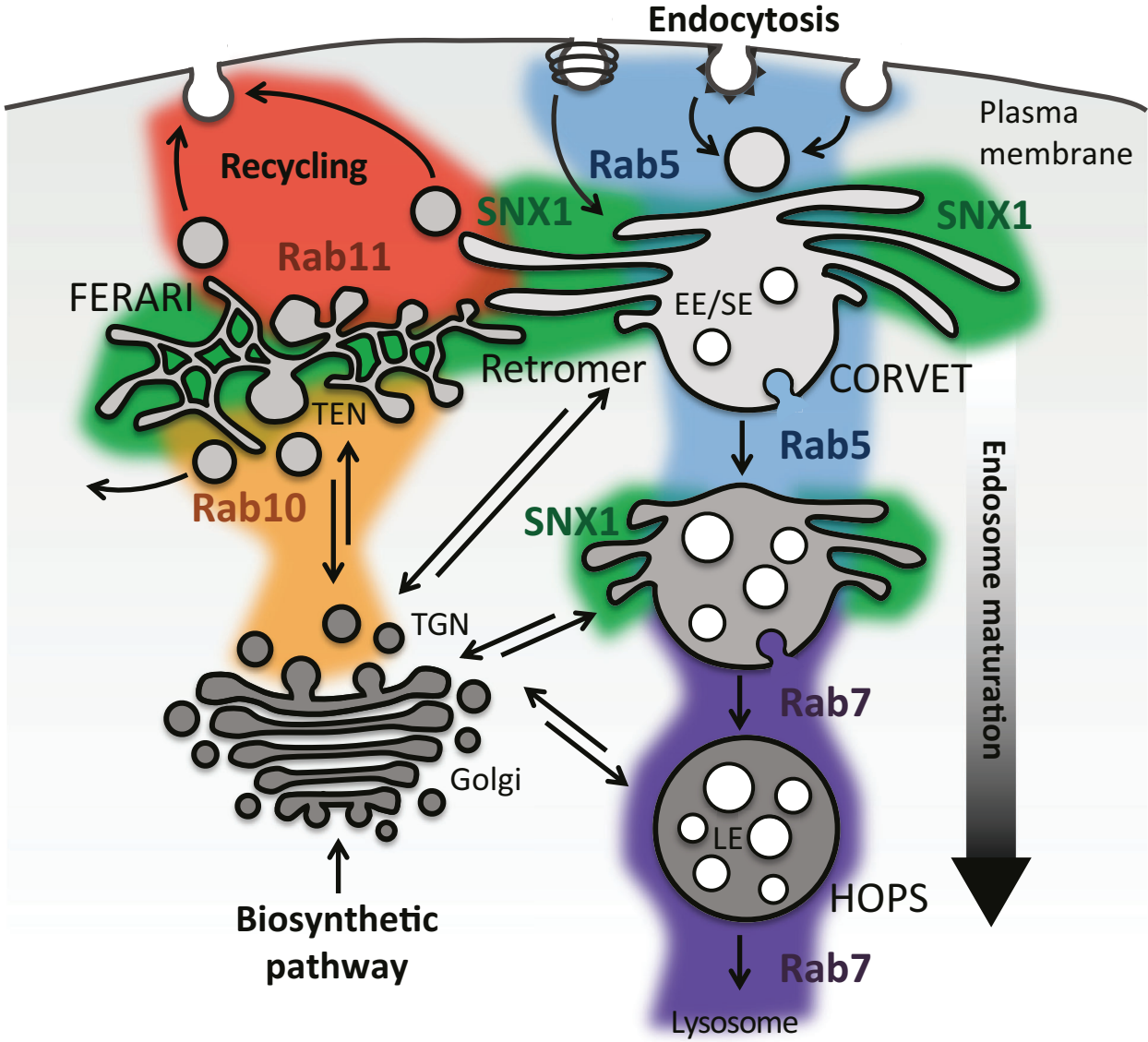
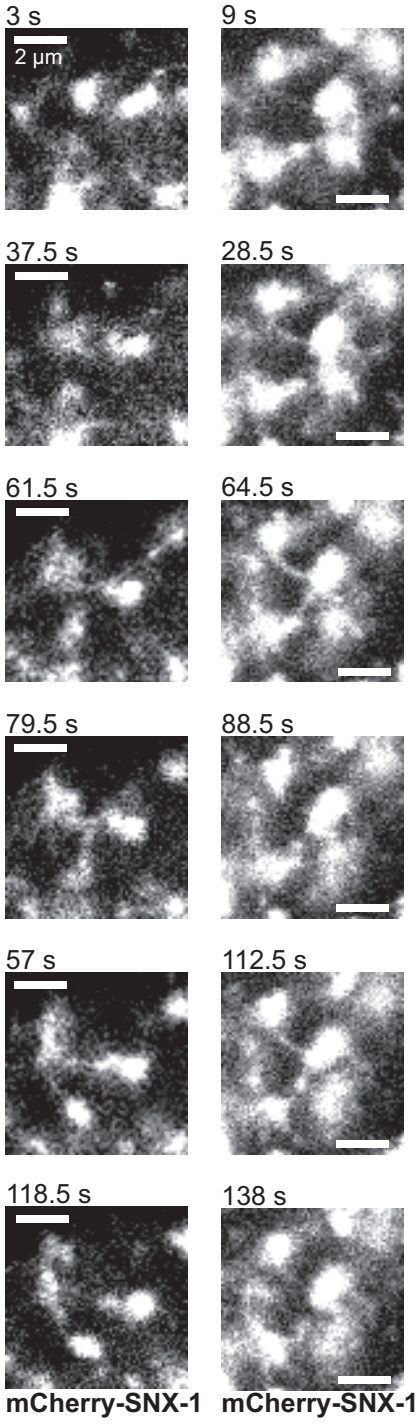

Endocytosis
Plasma membrane
Recycling
Rab5
SNX1
SNX1
Rab11
FERARI
EE/SE
Retromer
CORVET
TEN
Rab5
Rab10
SNX1
TGN
Rab7
Golgi
LE
HOPS
Biosynthetic pathway
Rab7
Lysosome
Endosome maturation

Figure 2


3 s
2 µm
9 s
37.5 s
28.5 s
61.5 s
64.5 s
79.5 s
88.5 s
57 s
112.5 s
118.5 s
138 s
mCherry-SNX-1
mCherry-SNX-1

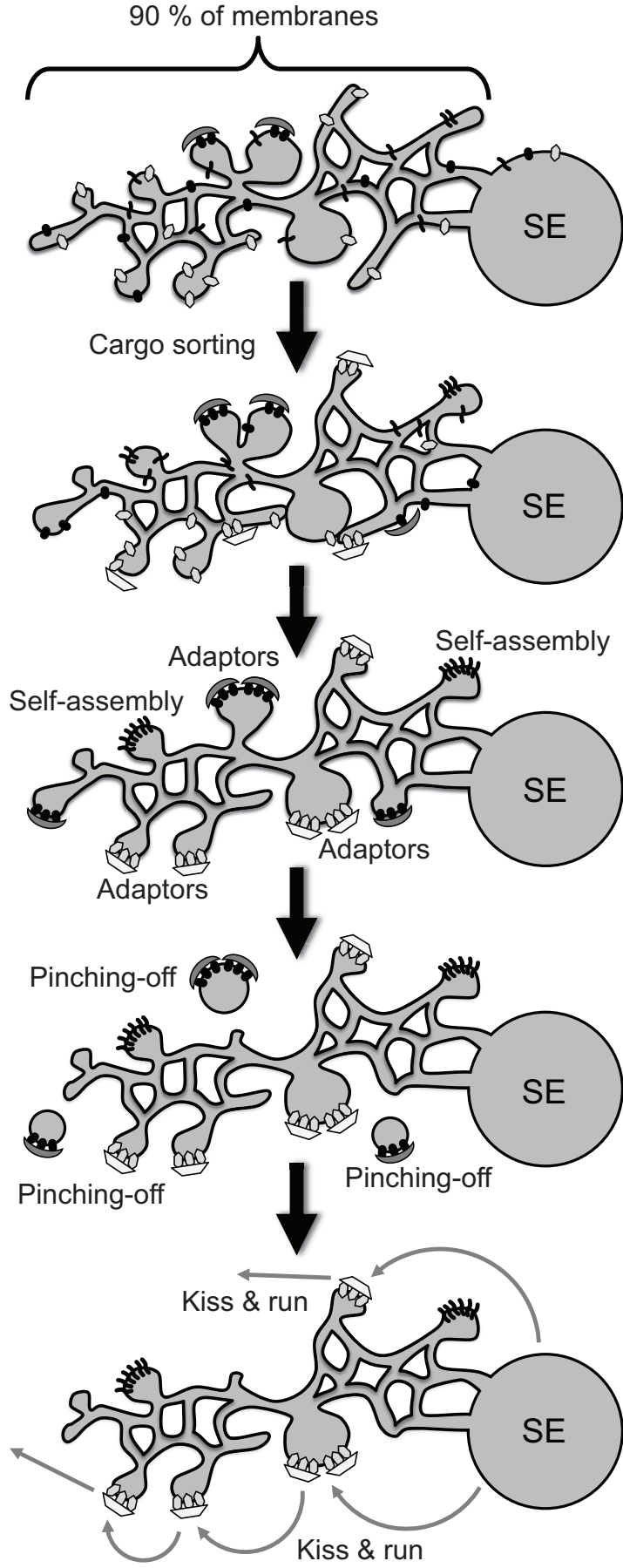

90 % of membranes
SE
Cargo sorting
SE
Adaptors
Self-assembly
Self-assembly
SE
Adaptors
Adaptors
Pinching-off
SE
Pinching-off
Pinching-off
Kiss & run
SE
Kiss & run
Self-assembling cargo
Cargo binding adaptor 1
Cargo adaptor 1
Cargo binding adaptor 2
Cargo adaptor 2

Figure 3

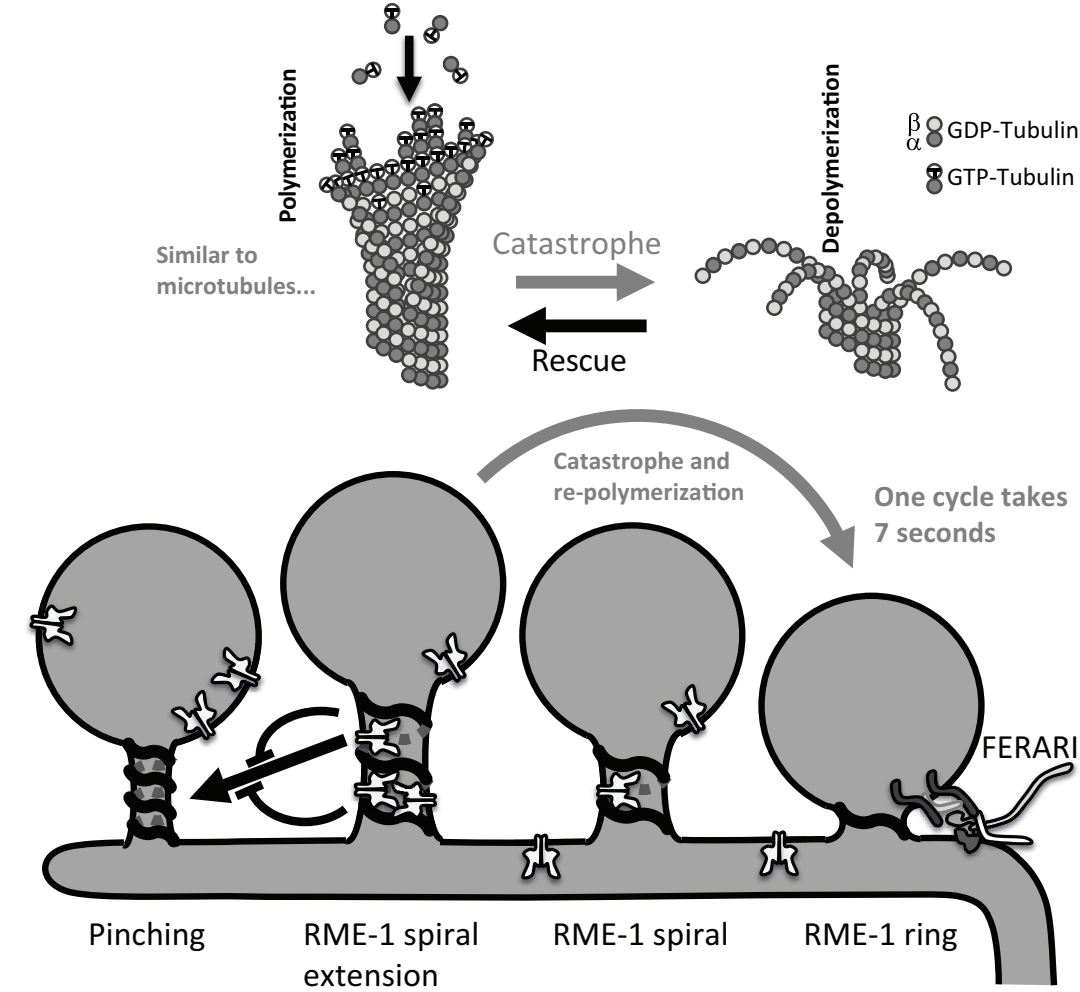
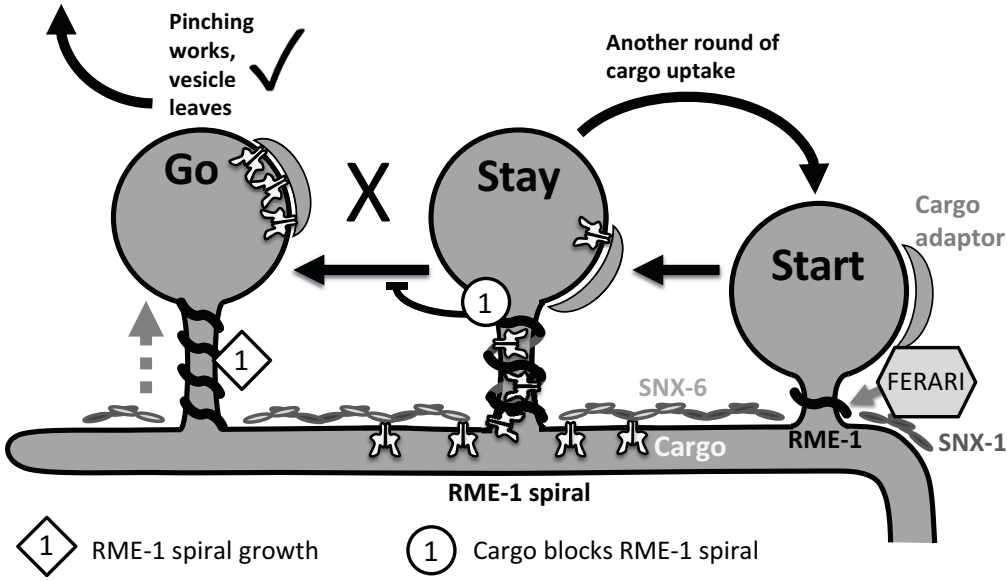
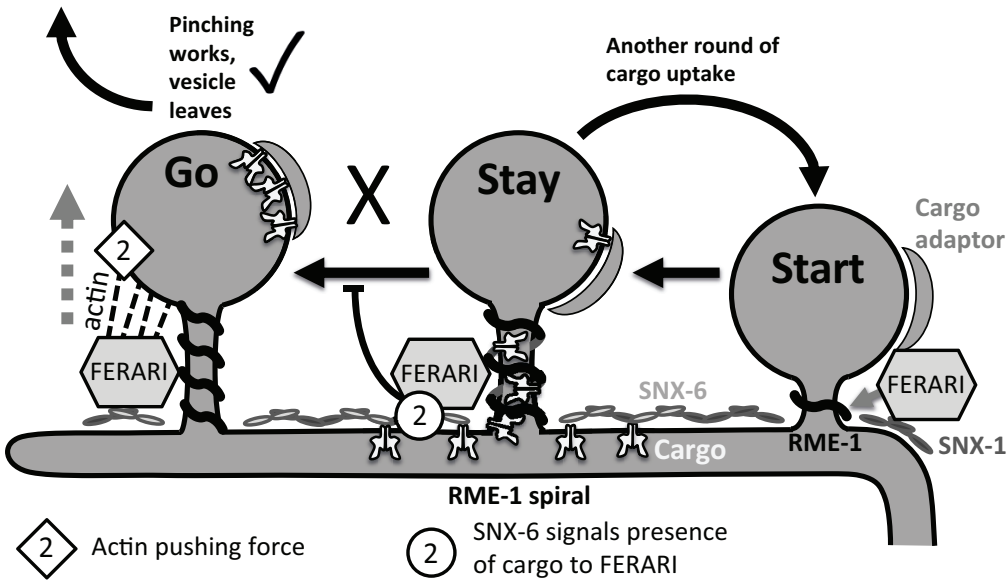

Polymerization
Similar to microtubules...
Catastrophe
Rescue
Depolymerization
β
α
GDP-Tubulin
GTP-Tubulin
Catastrophe and re-polymerization
One cycle takes 7 seconds
FERARI
Pinching
RME-1 spiral extension
RME-1 spiral
RME-1 ring

Figure 4


Pinching works, vesicle leaves
Another round of cargo uptake
Go
X
Stay
Start
Cargo adaptor
1
1
SNX-6
FERARI
Cargo
RME-1
SNX-1
RME-1 spiral
1 RME-1 spiral growth
1 Cargo blocks RME-1 spiral


Pinching works, vesicle leaves
Another round of cargo uptake
Go
X
Stay
Start
Cargo adaptor
2
actin
FERARI
FERARI
2
SNX-6
FERARI
Cargo
RME-1
SNX-1
RME-1 spiral
2 Actin pushing force
2 SNX-6 signals presence of cargo to FERARI

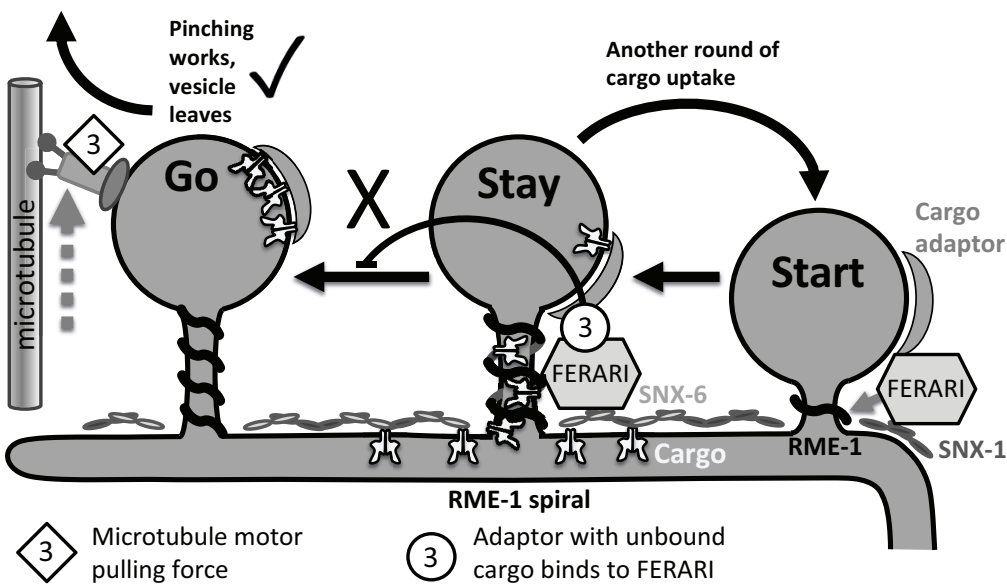

Pinching works, vesicle leaves
Another round of cargo uptake
3
microtubule
Go
X
Stay
Start
Cargo adaptor
3
FERARI
SNX-6
FERARI
Cargo
RME-1
SNX-1
RME-1 spiral
3 Microtubule motor pulling force
3 Adaptor with unbound cargo binds to FERARI

Figure 5

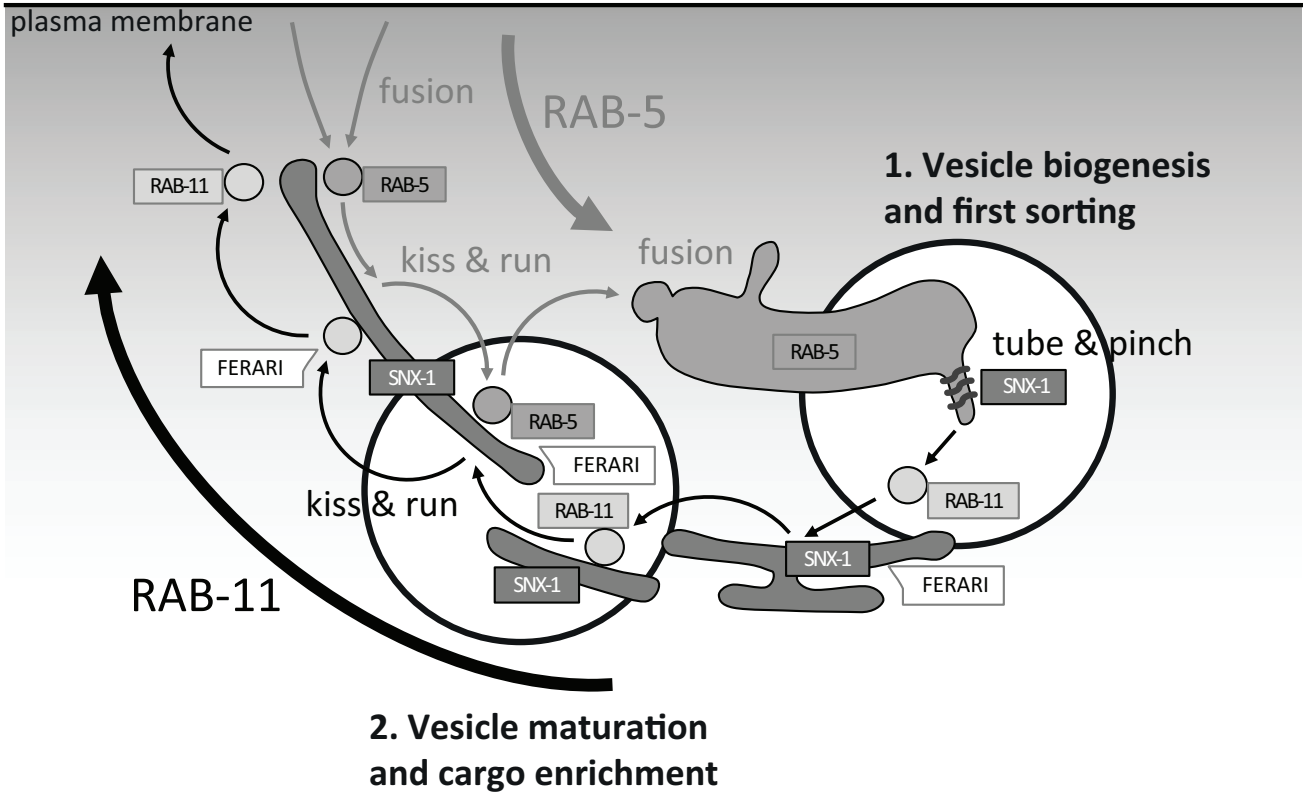
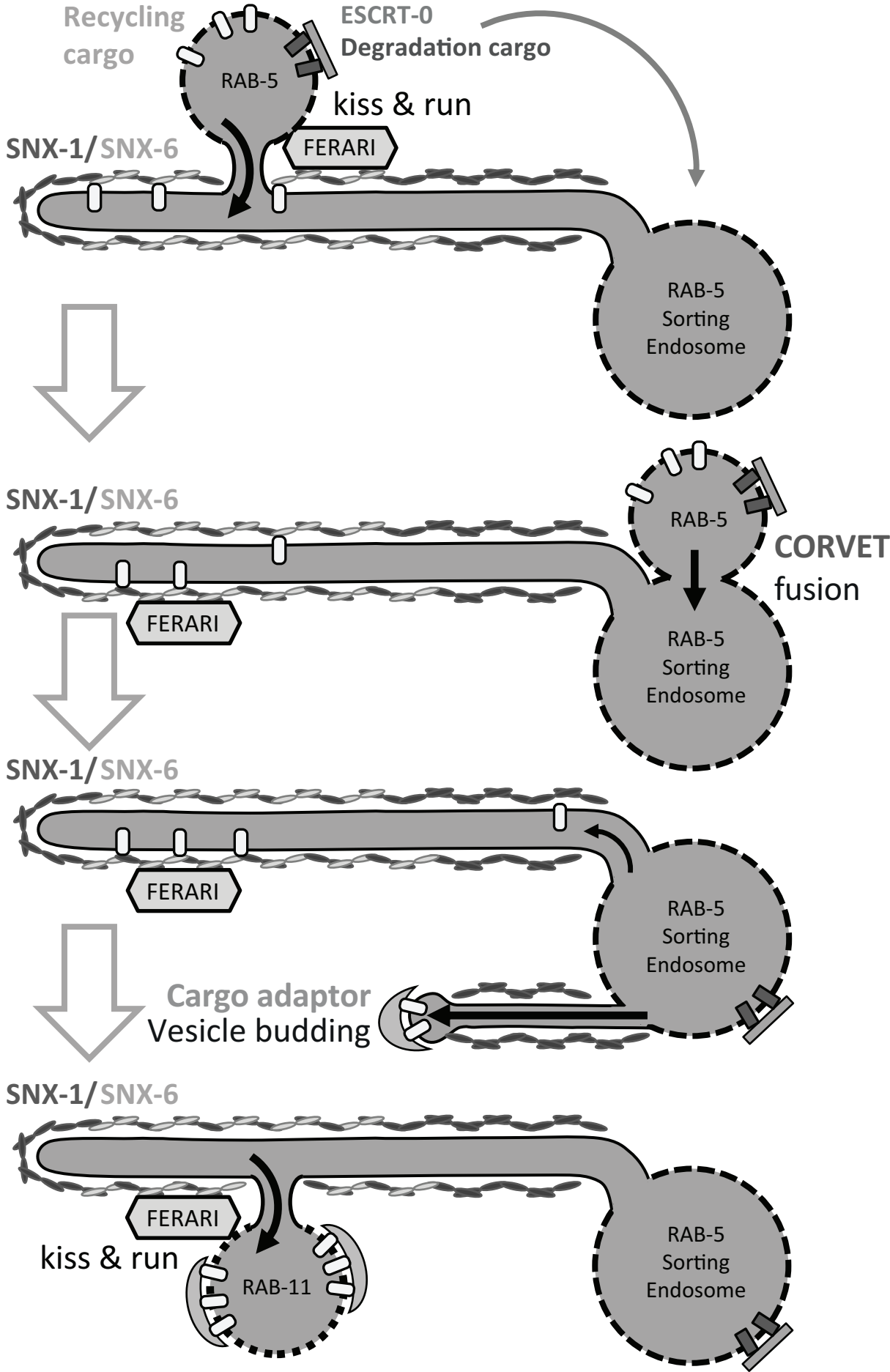

plasma membrane
fusion
RAB-5
RAB-11
RAB-5
1. Vesicle biogenesis
and first sorting
kiss & run
fusion
tube & pinch
FERARI
SNX-1
RAB-5
RAB-5
SNX-1
FERARI
kiss & run
RAB-11
RAB-11
SNX-1
SNX-1
FERARI
RAB-11
2. Vesicle maturation
and cargo enrichment

Figure 6


Recycling cargo
ESCRT-0
Degradation cargo
RAB-5
kiss & run
SNX-1/SNX-6
FERARI
RAB-5 Sorting Endosome
SNX-1/SNX-6
RAB-5
CORVET fusion
FERARI
RAB-5 Sorting Endosome
SNX-1/SNX-6
FERARI
RAB-5 Sorting Endosome
Cargo adaptor
Vesicle budding
SNX-1/SNX-6
FERARI
kiss & run
RAB-11
RAB-5 Sorting Endosome