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Retromer guides STxB and CD8-M6PR from early to recycling endosomes, EHD1 guides STxB from recycling endosome to Golgi

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Abstract

Retrograde trafficking transports proteins, lipids and toxins from the plasma membrane to the Golgi and ER. To reach the Golgi, these cargos must transit the endosomal system, consisting of early endosomes, recycling endosomes, late endosomes and lysosomes. All cargos pass through early endosomes, but may take different routes to the Golgi. Retromer dependent cargos bypass the late endosomes to reach the Golgi. We compared how two very different retromer dependent cargos negotiate the endosomal sorting system. Shiga toxin B, bound to the external layer of the plasma membrane, and chimeric CD8-Mannose-6-Phosphate Receptor, which is anchored via a transmembrane domain. Both appear to pass through the recycling endosome. Ablation of the recycling endosome diverted both of these cargos to an aberrant compartment and prevented them from reaching the Golgi. Once in the recycling endosome, Shiga toxin required EHD1 to traffic to the TGN, while the CD8-Mannose-6-Phosphate Receptor was not significantly dependent on EHD1. Knockdown of retromer components left cargo in the early endosomes, suggesting that it is required for retrograde exit from this compartment. This work establishes the recycling endosome as a required step in retrograde traffic of at least these two retromer dependent cargos. Along this pathway, retromer is associated with EE to recycling endosome traffic, while EHD1 is associated with recycling endosome to TGN traffic of STxB.

Keywords

Cation-independent Mannose 6-phosphate receptor; Membrane Traffic; EHD1; SNX1; BSC-1; Endosomes; Endocytosis; Retrograde Traffic; Retromer; Shiga Toxin; Shiga B; Recycling Endosome; Early Endosome; VPS26

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Introduction

Retrograde cargo broadly describes material that is transported from the plasma membrane to the Golgi and the ER; that is, opposite in direction to cargo in the secretory pathway. Retrograde cargos include various bacterial toxins, viruses and plasma membrane proteins (1) (2). Additionally, trans-Golgi network (TGN) resident proteins, such as TGN38 and Furin routinely escape the TGN and are retrieved from the plasma membrane using this route(3). To reach the TGN, internalized retrograde traffic must pass through some combination of early endosomes, late endosomes and/or recycling endosomes(4–6). A recent review of these pathways presents the possibility of traffic either directly from EE to the TGN or via the recycling endosome (7). However, the requirement or degree of passage through the recycling endosome remained unresolved. What seems clear is that the route taken through the endosomal system varies depending upon the protein(8). For example, Furin, a resident TGN protease, passes through early endosomes to late endosomes to the TGN. In contrast, chimeric tac-TGN38, which also cycles between TGN and plasma membrane, passes through early endosomes (EE) to the recycling endosome to the TGN(8). Alternatively, at least one report suggests that wild type TGN38 may traffic from early endosomes directly to the TGN in HeLa cells(9). A more complex route may be followed by the Cation Independent Mannose 6-Phosphate Receptor (CI-M6PR) which, at steady state, cycles between the TGN, plasma membrane and late endosomes (10). Kinetic evidence suggests that CI-M6PR at the plasma membrane, traffics to the TGN passing through early endosomes to the recycling endosome to the TGN(11). Retrieval of CI-M6PR from late endosomes appears to progress through a TIP47/Rab9 dependent pathway from late endosomes to TGN(12). This complex sorting system requires precise routing through the endocytic system to assure correct delivery.

In non-polarized cells, this system includes early endosomes, recycling endosomes, late endosomes and lysosomes. At least one level of specificity in this system is provided at the point of fusion by tethering complexes and Golgins associated with the TGN (13). For example GCC185 is required for delivery of STxB into the TGN, whereas GCC88 is required for delivery of CI-M6PR and TGN38. Although these tethering proteins provide specificity upon fusion, they do not define the source organelle for the vesicular traffic that they capture.

Shiga toxin (STx) and cholera toxin exploit the retrograde pathway to reach the Golgi and subsequently the ER. Both are A–B₅ toxins whose catalytic A subunit is delivered to the cytosol via retrotranslocation out of the ER(14). The pentameric B subunit binds to lipid receptors (Gb3 for Shiga and GM1 for Cholera) and directs traffic along the retrograde pathway. We have recently demonstrated that passage through the Golgi is required for delivery of STx to the ER (15). It is also well accepted that Shiga toxin passes through EE after internalization (4). It has been suggested that Shiga toxin traffics directly from the EE to the TGN, however, we and others have observed STx passing through a juxtanuclear endosomal structure (most likely the recycling endosome) before entering the TGN(9, 15, 16). The physiologic importance of each of these pathways has never been tested, leaving an open question as to which sorting mechanisms may be involved at which organelle, and whether sorting at the recycling endosome is required for STx trafficking to the TGN.

One protein complex that is particularly important for retrograde traffic is the retromer (for reviews see (17, 18)). Retromer allows cargo to be sorted away from the EE to lysosomal pathway. Mammalian retromer is a 5 component complex consisting of the mammalian homologues of yeast VPS26/VPS29/VPS35 in one subcomplex, and sorting nexins (SNX) in the other subcomplex. In the case of STx, the important sorting nexins are SNX1 and SNX2. The VPS26/VPS29/VPS35 core is thought to mediate cargo selection, while SNX1/2 are

thought to be scaffolding proteins involved in generating vesicular traffic(19). STx delivery to the TGN is retromer dependent as knockdown of either VPS26 or a combination of SNX1 and SNX2 results in a decrease in delivery of STx to the TGN. It remains unclear where in the endocytic sorting system retromer is acting (20, 21). Similarly, CI-M6PR delivery to the TGN is dependent upon retromer function, as is delivery of TGN38(9, 22). In contrast, Furin traffic via the late endosomes is unaffected by knockdown of retromer(23).

Retromer has been observed on tubules of EEA-1 positive EE where it mediates sorting of retrograde traffic such as STxB into SNX1 positive tubules (22, 24, 25). Since these tubules are depleted of transferrin, it has been suggested that they carry traffic directly from the EE to the TGN (25). However, retromer dependent cargos such as CI-M6PR and STxB have also been observed in juxtanuclear accumulations near the TGN or even explicitly in the recycling endosome(11). Retromer component VPS26 interacts directly with the recycling endosome associated protein EHD-1(26). EHD-1 itself appears to be required for at least some retrograde traffic of CI-M6PR to the TGN under low temperature conditions. These studies would suggest a role for the recycling endosome in retrograde traffic of retromer dependent cargos. The functional importance of the recycling endosome in this traffic has not been evaluated. If it is an important way-station in retrograde traffic, then sorting at the early endosome cannot be equated with direct delivery to the TGN, and juxtanuclear accumulations involve a second sorting step in the pathway. However, if retromer dependent cargos at the early endosome do traffic directly to the TGN, then juxtanuclear accumulations of retrograde traffic may merely be transport intermediates accumulating near the TGN. It may also be the case that retromer mediates traffic along both routes in parallel.

To test the importance of the recycling endosome in retromer dependent retrograde traffic in living cells, we examined the trafficking of two very different cargoes from the early endosomes to the TGN. The first is a CD8-CI-M6PR chimera, developed by Seaman, that features the ectodomain of CD8 and the cytoplasmic tail of the CI-M6PR(23). While trafficking of this construct is not identical to that of the full length CI-M6PR, it has nonetheless been used to follow trafficking from the plasma membrane to the Golgi(27). This trafficking is retromer dependent. It is therefore a representative transmembrane protein engaged in retromer dependent traffic. The second is the B subunit pentamer of Shiga Toxin (STxB) (28). This protein has been observed to traffic identically to the full A-B₅ toxin, but lack toxic properties. Traffic of STxB to the TGN is also retromer dependent. We find that trafficking through the recycling endosome is the normally predominant pathway for retrograde transport of both of these proteins through the endosomal system.

Results

To examine retrograde sorting through the endocytic system, we used BSC-1 cells. These cells express Gb3, the STxB receptor, are readily transfected, and have a flat morphology that allows ready discrimination of juxtanuclear recycling endosomes from both peripheral early endosomes and the Golgi (Supplementary Figure 1 B, C arrows) (15). The recycling endosome is located at the microtubule organizing center, and is visually distinguishable from the surrounding TGN (Supplementary Figure 1 A, C). Quantitation of possible overlap between recycling endosome and Golgi was performed using multiple images (n=11). The global Pearson's Correlation Coefficient (PCC) for this comparison was only 0.11 +/- 0.083 for endosomes (labeled with Tfn) vs. Golgi (labeled with GM130). The PCC may be erroneously low if the pattern of distribution of two markers differs within a compartment. We therefore followed the protocol described by Dunn et Al. and also determined the Mander's Correlation Coefficients (MCC) for overlap between the compartments (29). Comparing recycling endosome and Golgi, the $MCC_{\text{Endosomes}} = 0.161 \pm 0.081$ and $MCC_{\text{Golgi}} = 0.119 \pm 0.095$ (n=11 for both). We interpret this to mean that only 16% of the

labeled recycling endosome overlapped Golgi, and 12% of Golgi overlapped the recycling endosome. See methods for details on how images were processed and quantified. Similarly the PCC for Tfn labeled recycling endosome vs. TGN46 labeled TGN was 0.147 ± 0.019 ($n=4$) and the $MCC_{\text{Endosomes}} = 0.198 \pm 0.022$, $MCC_{\text{TGN46}} = 0.136 \pm 0.011$ ($n=4$). Together these suggest that the recycling endosome and Golgi compartments can be readily differentiated in these cells using fluorescence microscopy. EE and recycling endosomes can be similarly distinguished using Alexa 488-Tfn internalized for 2.5 min for early endosomes, and Alexa 546-Tfn internalized for 25 min for recycling endosomes in BSC-1 cells expressing the human transferrin receptor (Supplementary Figure 1 B). We have previously used this system to physically exclude recycling endosomes from cytoplasts as well as to follow STxB trafficking through the TGN and cis/medial Golgi(15, 30).

Both STxB and CI-M6PR colocalize with recycling endosomes during retrograde transport

We applied both Alexa 546-STxB and Alexa 488-Tfn to BSC-1 cells on ice, and then internalized the ligands at 37°C for various times to follow sorting through the endocytic system. (Figure 1A). The cells were then immunolabeled for TGN46 (to identify the TGN) and Rab11a (to identify the recycling endosome) (31, 32). At 1 min, STxB (red) colocalized with Tfn (green, left) in peripheral puncta corresponding to EE (Figure 1A arrows, 1 min and Table 1). The STxB did not colocalize with TGN46 (blue) or with the recycling endosome marker Rab11a (labeled with Alexa 350 but shown here also in green, panels on right). After 7 min, STxB colocalized to a lesser degree with Tfn (but not significantly less), now located in both EE and the recycling endosome. The PCC for Tfn vs STxB shifted from 0.649 ± 0.079 ($n=6$) at 1 min to 0.464 ± 0.146 ($n=14$) at 7 min. The MCC_{STxB} for this pair yields essentially the same result (Table 1). Although the majority of Tfn is present in recycling endosomes, some remains in early endosomes at this point, so colocalization with Tfn does not necessarily mean that STxB is entering the recycling endosome. Rab11a is another marker for the recycling endosome. We therefore compared the colocalization of STxB with Rab11a in the same cells (Figure 1A right panels). STxB appears to be in the recycling endosome at 7 min with this marker as well. The PCC for STxB vs Rab11a peaked at 7 min (see table 1), significantly more than at 2.5 min ($p < 0.001$) and dropping significantly between 7 and 19 min ($p < 0.001$) (Table 1). These results suggested STxB passage through the recycling endosome at 7 min. In contrast comparison of STxB and TGN46 showed relatively low colocalization at 1 and 7 min (see both table 1, TGN vs STxB and Figure 1A), increasing significantly ($p < 0.001$) at 19 min ($PCC = 0.502 \pm 0.186$, $n=17$). These results suggest a progression of STxB from the recycling endosome to TGN.

We next followed the retrograde trafficking of a mannose 6-phosphate receptor chimera through the endosomal system. Like STxB, cation independent mannose 6-phosphate receptor (CI-M6PR) requires SNX1 to traffic through the endosomal system to the TGN (22). In this case, the cytoplasmic tail of CI-M6PR is thought to also interact with the VPS26/VPS29/VPS35 subunit of retromer, through direct binding to VPS35 (22, 23, 27). We used a chimeric construct containing the ectodomain of CD8 fused to the transmembrane and cytoplasmic domains of CI-M6PR (termed CD8-M6PR, a kind gift from Matthew Seaman). This construct provides a tight antibody binding site for live cell labeling while preserving the trafficking characteristics of the CI-M6PR. While the trafficking characteristics are slightly different than that of the wild type CI-M6PR, it does remain retromer dependent and kinetic evidence suggests that both it, and the wild type, may pass through a juxtanuclear endosomal compartment(23, 33).

BSC-1 cells were stably transfected with the CD8-M6PR chimera. Alexa 546-anti-CD8 Ab and Alexa 488-Tfn were applied to the cells on ice and internalized at 37°C as for STxB (Figure 1B). For convenience we will refer to the labeled anti-CD8 Ab bound to CD8-M6PR simply as CD8-M6PR. Unlabeled construct is not visible in our assay, and the antibody has

been shown to remain quantitatively associated with the construct inside of the cell (23). Internalization of CD8-M6PR was not as efficient as that of STxB resulting in persistent labeling of the plasma membrane. For this reason, it was necessary to acid wash (pH 3.0) the surface of the cells to remove non-internalized antibody after 3 min internalization. Internalized CD8-M6PR colocalized with Tfn in the EE after 5 min ($PCC = 0.488 \pm 0.237$, $n=7$, see Table 2) (Figure 1B). After 7 min STxB colocalized with Tfn, which had shifted to the recycling endosome ($PCC = 0.621 \pm 0.171$, $n=13$, Figure 1B, 7 min panels, arrow). Colocalization of CD8-M6PR with Tfn (see table 2) decreased at 20 min ($PCC = 0.250 \pm 0.128$, $n=10$) while colocalization of CD8-M6PR with the TGN increased at 20 min ($PCC = 0.777 \pm 0.063$, $n=10$), and remained in the TGN at 30 min internalization (Figure 1B, 30 min panels). This would suggest a progression similar to that of STxB, with at least some of the internalized CD8-M6PR passing through the recycling endosome (Figure 1B, 7 min arrow), but it was unclear whether this was a significant portion, or if the bulk of CD8-M6PR passed directly from early endosomes to the TGN.

Our results led us to question what fraction of retromer dependent retrograde traffic passes through the recycling endosome. EM studies have found retromer components associated with EEA-1 positive endosomes, which describes EE, but the recycling endosome is EEA-1 negative (25, 30). Further, knockdown of retromer has little, if any, effect on Tfn recycling through the endosomal system, a pathway which includes traffic from the EE through the recycling endosome as well as directly from the EE to the plasma membrane (25, 34). Thus it remained possible that the juxtanuclear STxB and CD8-M6PR represented accumulations of transport intermediates in the area of the recycling endosome and not visibly distinguishable from the recycling endosome.

Passage through the recycling endosome is a major pathway for retrograde traffic

To test the importance of the recycling endosome for retromer dependent retrograde traffic, we performed a functional knockout of the organelle. Tfn trafficking in BSC-1, CHO, MDCK, and HeLa cells is well characterized (34–37). All have very similar kinetics of Tfn passage through the recycling pathways allowing for specific targeting of Tfn and Tfn conjugates to either EE, or recycling endosomes. As seen in Supplementary Figure 1, binding of Tfn on ice followed by a 25 minute chase provides specific labeling of the recycling endosomes. We, and others, have made use of this to specifically target horse radish peroxidase labeled Tfn (HRP-Tfn) to the recycling endosome (38, 39). The recycling endosome was ablated using a well established H_2O_2 /DAB protocol on ice for 1 hour (39, 40). This ablation functionally removes the recycling endosome while leaving the recycling and sorting functions of early and late endosomes intact (supplementary Figure 2 A). To assure specificity, we tested the ability of EE to recycle Alexa 488-Tfn. Ablated cells were labeled with a continuous pulse of Alexa 488-Tfn for 30 min at 37° (no chase). This resulted in labeling of the entire recycling pathway (See diagram Supplemental Figure 2 A) (34). Peripheral EE were visible in both control and ablated cells (Supplemental Figure 2 B), but juxtanuclear recycling endosomes were visible only in the control cells (arrow in same figure). After a 30 min chase with unlabeled Tfn, the ablated cells retained no Tfn and the control cells retained only trace Tfn in the recycling endosomes (lower panels, Supplementary Figure 2 B, arrow). This demonstrated that the rapid recycling pathway out of the early endosomes remained functional in ablated cells. To test if sorting from early endosomes to late endosomes/lysosomes was affected, ablated cells were loaded with Alexa 488-Dextran, a fluid phase endocytic marker (41). The dextran was delivered to late endosomes and lysosomes labeled with lysotracker as in untreated cells (Supplementary Figure 2 C). To test whether the ablation protocol had inactivated trafficking into the TGN, we followed trafficking of a Flag-Furin construct. Furin is a TGN resident protein that is recovered from the plasma membrane via retrograde traffic. Tac-furin and Flag-furin are

known to traffic to the TGN via late endosomes (see diagram Supplementary Figure 2 A), rather than via recycling endosomes (8, 42). Flag-furin was expressed in BSC-1 cells. Anti-Flag Ab was bound to the cells and internalized for 60 min to follow internalization of the furin construct (Supplementary Figure 2 D). In control cells, Flag-furin was delivered to the TGN where it colocalized with TGN46 ($PCC = 0.424 \pm 0.151$, $MCC_{\text{furin}} = 0.246 \pm 0.110$, $MCC_{\text{TGN}} = .698 \pm 0.125$). It is notable that the TGN was largely occupied by the furin construct, whereas the furin construct was visualized at the TGN and in a pattern reminiscent of plasma membrane (the MCC_{TGN} was higher than the MCC_{furin}). In the ablated cells, Flag-furin was also delivered to the TGN ($PCC = 0.743 \pm 0.169$, $MCC_{\text{TGN}} = 0.824 \pm 0.145$, $MCC_{\text{furin}} = 0.564 \pm 0.226$). It is unclear why Flag-furin internalization/sorting appear to be more efficient in ablated cells, but these results do demonstrate that the retromer independent pathway (via late endosomes) remains functional after ablation of the recycling endosome.

Taken together these results suggest that ligand sorting in the EE, maturation into late endosomes, and delivery into the TGN via late endosomes were not affected by ablation of the recycling endosome. Others have previously demonstrated that this ablation protocol does not affect delivery of non- μ_1 B binding basolateral cargo, or apical cargo from the TGN, again suggesting that the ablation, as performed here, is specific for the recycling endosome (39, 43).

We next examined the effects of recycling endosome ablation on retrograde traffic. Alexa 546-STxB is normally delivered to the Golgi (here labeled with the cis/medial marker GM130) within 60 min (at 60 min: $PCC = 0.689 \pm 0.103$, $MCC_{\text{STxB}} = 0.556 \pm 0.158$, $n=12$) and to the ER within 180 min (Figure 2A, control cells, arrows). However, in ablated cells, internalized Alexa 546-STxB took on a dramatically different morphology. At 60 min it did not appear in the Golgi showing significantly ($p < 0.0001$) lower colocalization with GM130 ($PCC = 0.038 \pm 0.080$, $MCC_{\text{STxB}} = 0.029 \pm 0.048$, $n=12$) STxB remained in peripheral puncta for at least 3 hours after internalization (Figure 2A, Ablated Cells, arrows), nor did it colocalize with ER resident PDI during this time (Figure 2B, Ablated, arrow) (44). This suggested that STxB in the EE could not bypass the recycling endosome implying that passage through the recycling endosome was a significant pathway for retrograde STxB traffic.

We performed a similar experiment with CD8-M6PR to test if the trafficking of CD8-M6PR was inhibited in cells where the recycling endosome was ablated (Figure 3). CD8-M6PR normally cycles between the plasma membrane and the TGN, but not the cis/medial Golgi. We therefore stained the cells for TGN46 rather than GM130. We also took advantage of the staining conditions to internalize Alexa 488-Tfn along with the CD8 antibody. This allowed for identification of the recycling endosome (after 30 minutes) in control cells (Figure 3A). In ablated cells, all Tfn was recycled out of the cells by 60 min confirming that ablation did not block recycling from the EE compartment (Figure 3B, green channel).

In control cells, there was colocalization of CD8-M6PR with the TGN after 30 min internalization ($PCC = 0.835 \pm 0.087$, $MCC_{\text{CD8-M6PR}} = 0.793 \pm 0.0138$, $n=4$; Figure 3A, arrows). CD8-M6PR continued to colocalize with the TGN after 60 min (Figure 3A). In the ablated cells, CD8-M6PR partially colocalized with peripheral Tfn in early endosomes after 10 or even 30 min internalization (Figure 3B). Some CD8-M6PR remained in peripheral puncta that did not visibly colocalize with Tfn (Figure 3B, red puncta in bottom panels). This material may represent CD8-M6PR retained in early endosomes after Tfn has exited, or ligand that is mis-sorted in the absence of a target recycling endosome. At longer times, the Tfn recycled out of the cell, preventing colocalization. More importantly, internalized CD8-M6PR did not colocalize with the TGN marker at 30 min ($PCC = 0.002 \pm$

– 0.019 $MCC_{CD8-M6PR}$, $n=6$). The same was true, even after 60 min ($PCC = 0.039 \pm 0.031$, $MCC_{CD8-M6PR} = 0.045 \pm 0.034$, $n=6$; Figure 3B). In both cases the lack of colocalization with TGN was significantly ($p < 0.0001$) different than in control cells. Rather, the CD8-M6PR remained in peripheral puncta, (Figure 3B, Ablated cells, arrows). This suggested that as with STxB, ablation of the recycling endosome blocked CD8-M6PR from trafficking to the TGN. The simplest interpretation of these results is that passage through the recycling endosomes is a required step in trafficking of both of these retromer cargos from the early endosomes to the Golgi. However, it remained possible that the ablation treatment may have inactivated cytosolic retromer or adversely affected traffic through off-target organelles.

The fate of mis-sorted cargo

Under ablation conditions, both CD8-MPR and STxB were observed in peripheral puncta. We first tested if they were directed to the same structures. Cells expressing CD8-M6PR were labeled with both CD8 antibody and STxB for 60 min. The cells were also stained for TGN46. Both CD8-M6PR and STxB colocalized in the same peripheral structures ($PCC = 0.421 \pm 0.125$, $MCC_{CD8-M6PR} = 0.409 \pm 0.140$, $MCC_{STxB} = 0.361 \pm 0.159$, $n=9$; Figure 4A, yellow arrows), and neither was delivered to the TGN (Figure 4A blue arrows). This allowed us to use Alexa 546-STxB for further characterizations of the peripheral structures. We preferred STxB for these studies for technical ease.

To better define where STxB was being delivered in ablated cells, we internalized Alexa 546-STxB for 60 min. Our other results suggested that this was ample time for delivery to the Golgi. Lysosomes are an obvious destination for mis-sorted membrane traffic. We therefore looked for colocalization of internalized STxB with lysosomes using lysotracker. In control cells, as expected, STxB did not colocalize with lysotracker (Figure 4B, upper panels). Somewhat surprisingly, STxB was not mis-directed to lysosomes in the ablated cells. (Figure 4B lower panels) The vesicular structures containing STxB were usually adjacent to lysosomal structures, but were never observed to colocalize with them (for quantification see Table 2). We then sought to determine if STxB was delivered to late endosomes or poorly acidified lysosomes in ablated cells. Such structures label with LAMP1 and LAMP2 but may not be visualized by lysotracker (Figure 4C). Comparison with LAMP1/2 (combined antibodies to LAMP1 and LAMP2) demonstrated that STxB was not delivered to these late endosomal compartments in ablated cells (see Table 2), which was not significantly different than control cells. It was possible that in the absence of functional recycling endosomes, STxB might either remain in EE or be delivered to a recycling endosome-like compartment. We compared STxB with EEA-1 positive EE (Figure 4C, white arrow.) Ablation did not cause significant overlap of these compartments (Table 2). Rab11a is found primarily on the recycling endosome, but is also present to a much lesser extent on early endosomes. STxB did not colocalize with Rab11a in ablated cells (Figure 4C bottom panels) suggesting that it was not sequestered in a fragmented recycling endosome. Taken together, these results suggest that when the recycling endosome is ablated, retromer associated cargo still exits the EE, but enters an aberrant compartment.

The ablation protocol creates a DAB precipitate in the recycling endosome. However, the precipitate, or perhaps the reagents used may inactivate retromer components, thus making it unavailable for trafficking. In this case, we would expect that one or both of the subcomplexes would no longer be associated with the STxB cargo when it is mis-sorted. We therefore examined the localization of both SNX1 (SNX1/SNX2 sub-complex) and VPS35 (VPS26/VPS298/VPS35 subcomplex) compared to STxB internalized in ablated cells (Figure 4D). STxB and SNX1 had different but largely overlapping distributions in both control cells and ablated cells ($PCC = 0.327$ and $PCC = 0.505$ respectively, see Table 2). Additionally, VPS35 was also localized to the aberrant compartment in ablated cells ($PCC =$

0.370 $\pm$ 0.118, $MCC_{VP835} = .290 \pm 0.086$ n=9). This suggested that both retromer and STxB were in this compartment. Thus STxB is not trapped in the compartment by a lack of access to retromer. It is still formally possible that this retromer is in some way inactivated, but it was clearly not sequestered at the ablated recycling endosome, nor segregated from STxB in the EE.

EHD1 dependence separates STxB and CD8-M6PR at the recycling endosome

EHD1 is a scaffolding protein located at the recycling endosome that is required for exit of Tfn during recycling. (45) We tested whether retrograde traffic may also depend on EHD1. It has been suggested that EHD1 interacts with retromer at the EE, however, this may present a conceptual difficulty as EHD1 is located in the recycling endosome rather than at the EE (46, 47).

Expression of wild type GFP-EHD1 at low levels had no effect on the delivery of STxB to the TGN. Alexa 546-STxB was applied to cells expressing the wild-type GFP-EHD1 and internalized as before. It passed through early endosomes at 2.5 min and colocalized with EHD1 in the recycling endosome at 10 min (Figure 5A, yellow arrows). After 20 min (not shown) and continuing through 60 min (Figure 5A, purple arrows), STxB colocalized with TGN46. (at 60 min $PCC = 0.672 \pm 0.134$, $MCC_{STxB} = 0.735 \pm 0.158$ n=8;). as was observed in untreated cells (Figure 1). Overexpression of GFP-EHD1 can lead to excessive tubularization of the recycling endosome, so only cells expressing lower levels (determined visually) of the construct were selected.

To perturb traffic of STxB from the recycling endosome, we expressed a dominant negative (DN) GFP-EHD1 (G429R, a kind gift from Barth Grant). This construct is known to block recycling of Tfn from the recycling endosome, however, it differs from both the P-loop mutation (T72N) and the EH domain deletion used in previous retromer association studies(26, 45). The G429R mutant remains associated with the recycling endosome facilitating identification of the organelle. DN GFP-EHD1 had a profound effect upon STxB trafficking out of the recycling endosome. STxB was sequestered in the recycling endosomes at all times after 10 min of internalization (Figure 5B, yellow arrows). Even after 60 min STxB did not colocalize with TGN46 ($PCC = 0.065 \pm 0.105$, $MCC_{STxB} = 0.095 \pm 0.182$ n=6), but remained in the recycling endosome. This colocalization was significantly ($p < 0.0001$) different than in control cells. Exit from the EE appeared unaffected (Figure 5B). In a convenient extra control, some cells remained untransfected (one is indicated by the asterisk in Figure 5B) and in these cells STxB completely colocalized with the TGN.

To further quantify of the effect of DN-EHD1, we used a biochemical assay to follow trafficking of STxB. Modified STxB carrying two sulfation sequences (STxB-SS, kindly provided by Ludger Johannes) was applied to cells in the presence of $^{35}SO_4$ (16). The sulfation sequences are sulfated by sulfonyle transferase in the TGN. Sulfation was measured by homogenizing the cells and running equal amounts of protein on SDS PAGE gels. A unique 7.5 KD radiolabeled band was obtained only in the presence of STxB-SS and sulfate (a typical result is shown in Figure 5C). The band was measured using a phosphorimager. This assay has been used extensively to measure STxB delivery to the Golgi (16, 48). We found that delivery to the Golgi was inhibited by 69% ($\pm 3\%$) by the dominant negative construct (Figure 5C lower panel). The residual 31% may have resulted from non-transfected cells, or residual traffic in the presence of the dominant negative construct. Taken together with the morphological results, this suggests that much of the retrograde traffic of STxB to the TGN is dependent upon EHD1. Since the traffic appeared to be sequestered in the recycling endosome, it is likely that EHD1 is required for retrograde trafficking out of the recycling endosome.

We next examined the effect of DN GFP-EHD1 on CD8-M6PR trafficking. Previous studies of EHD1 requirements for CD8-M6PR delivery to the TGN demonstrated differential effects of the different DN mutants. The T72N P-loop mutant had the greatest effect and also had an effect on retromer distribution (26). These measurements reflected the delivery at steady state rather than the pulse-chase format used here. We therefore examined the effect of the G429R DN- EHD1 mutant on CD8-M6PR passage through endosomal compartments in our assay system.

In our assay, DN- EHD1 expression did not result any sequestration CD8-M6PR in the recycling endosome, or the EE (Figure 5D). By 20 min, CD8-M6PR colocalized with the TGN ($PCC = 0.721 \pm 0.134$, $MCC_{STxB} = 0.743 \pm 0.200$ $n=4$; Figure 5D, arrow). This was not significantly different than in control cells. We did note that cells expressing the DN GFP-EHD1 did appear to take up less total anti-CD8 Ab as reported but this did not appear to significantly affect the sorting of the label to the TGN(26). Similar, essentially negative, results were obtained with the wt EHD1 construct (not shown).

Dominant negative constructs often act through sequestration of other required proteins. DN GFP-EHD1 could exert its effect on retrograde trafficking through sequestration of VPS26 or another required protein at the recycling endosome. As such, the perturbation we observed in trafficking could be an indirect result. Indeed, SNX1 is normally associated with early and recycling endosomes, but over-expression of wild type GFP-EHD1 (sufficient to tubularize the organelle) resulted in recruitment of SNX1 to the periphery of the recycling endosome (Supplementary Figure 3, upper panels, white arrows) (19, 24). Expression of the DN GFP-EHD1 resulted in further colocalization of SNX1 with the compacted recycling endosome (Supplementary Figure 3, lower panels, yellow arrows). It was thus possible that the effects of DN-GFP-EHD1 on STxB trafficking were mediated by sequestration of retromer.

To test for direct dependence on EHD1, we performed a knockdown of EHD1 using siRNA in BSC-1 cells. A scrambled oligo matching no known sequence was used as a negative control. Because EHD1 and VPS26 interact, knockdown of one may affect the stability or expression of the other. Therefore, we also performed a knockdown of VPS26 and monitored the levels of both EHD1 and VPS26 in both knockdowns (Figure 6A). The degree of knockdown was determined by western blot with beta-tubulin as a loading control. EHD1 was depleted by greater than 85% with no effect on retromer component VPS26. Similarly, knockdown of VPS26 (greater than 80%, Figure 6A) did not change expression of EHD1. Retrograde STxB traffic was measured using sulfation of STxB-SS as before. EHD1 knockdown resulted in a $77\% \pm 4$ ($n=3$) inhibition of sulfation compared to a scrambled oligo control (Figure 6B). This result agreed with the DN-EHD1 result. Surprisingly, knockdown of EHD1 provided a greater inhibition of sulfation than did knockdown of VPS26 (Figure 6B). In our hands, VPS26 knockdown resulted in a $39\% \pm 20$ ($n=3$) inhibition, a value comparable to that found by other investigators (25). This suggested that for trafficking of STxB, EHD1 is at least as important as the retromer component VPS26.

As suggested by the dominant negative EHD1 data, knockdown of EHD1 had no discernable effect on CD8-M6PR trafficking (not shown).

Retromer activity in the endosomal sorting system

Because we had seen DN-EHD1 primarily affected the recycling endosome, we compared this to the effects of retromer depletion on traffic through endosomal compartments.

VPS26 knockdown is known to delay retrograde cargo delivery(23). Knockdown of VPS26 disrupts the retromer complex, and as this component has no redundancy, unlike SNX1/

SNX5 and SNX2/SNX6 pairs, it cannot be bypassed (21, 49). Tfn was continuously internalized for 30 min to label both early and recycling endosomes. A pulse of STxB, was chased into the cells for 30 min. Colocalization of STxB with the TGN in knockdown cells ($PCC = 0.268 \pm 0.125$, $MCC_{STxB} = 0.206 \pm 0.101$) was significantly ($p = 0.0001$) less than in control cells ($PCC = 0.570 \pm 0.058$, $MCC_{STxB} = 0.744 \pm 0.077$) as expected from the sulfation assay results. Depletion of VPS26 also resulted in a redistribution of STxB to EE (Supplementary Figure 4 A arrows). To provide some quantification of this effect, we relied on the morphology of BSC-1 cells. The TGN typically surrounds the recycling endosome, so we quantified STxB inside a circle covering the TGN (and recycling endosome) and compared to STxB in the rest of the cell. STxB outside of the circle colocalized with Tfn (Supplementary Figure 4 A, B) and so was presumed to be in EE. $60 \pm 16\%$ ($n=4$) of the STxB was in EE in knockdown cells compared to $19 \pm 12\%$ ($n=10$) in control oligo treated cells at 30 min. This is also significantly ($p < 0.001$) more STxB in the EE than in untreated cells ($8.8 \pm 9.3\%$ ($n=4$)) (compare Figure 6C to Figure 5B) This result suggested a significant sequestering of cargo in the EE in the absence of functional retromer. Recycling of Tfn appeared unaffected by the VPS26 knockdown (compare Tfn in upper and lower panels Supplementary Figure 4 A) as described elsewhere(25).

We next examined the effect of VPS26 KD on trafficking of CD8-M6PR. VPS26 is known to be required for CD8-M6PR delivery to the TGN (23), but our focus was on whether the cargo would be delayed in EE or in recycling endosomes. A pulse of anti-CD8-M6PR was internalized for 30 min as was done for STxB (Supplementary Figure 4 B). Using the central/peripheral measurement described for STxB, $90 \pm 10\%$ ($n=6$) of the anti-CD8-M6PR was significantly ($p < 0.0001$) delayed in EE, compared to $20 \pm 10\%$ ($n=6$) in control cells. The CD8-M6PR in EE appeared to colocalize with internalized Tfn in the periphery, and to a lesser extent in the center of the cell (Supplementary Figure 4 B). This suggests that at a minimum, retromer is required for exit from the EE compartment. This result is similar to that obtained by others following CI-M6PR with a knockdown of either SNX1 or p150^{glued} (an SNX1 binding partner) (49). Taken together, these results suggest that VPS26 (and by implication retromer) is required for both STxB and CD8-M6PR cargos and acts along an early endosome to recycling endosome pathway.

Retromer is recruited to cargo in early endosomes

Retromer components are recruited to membranes containing retrograde cargo through the actions of Rab5 and Rab7 at the EE(50, 51). We reasoned that a pulse of retromer dependent cargo might induce a shift in the distribution of the recruited retromer along steps of the pathway mediated by retromer. Indeed, movement of SNX1 (along with STxB) from peripheral endosomes to a juxtanuclear area has been visualized by Bujny et al. although association with the recycling endosome was not determined (20). Here, we focused on redistribution of retromer as traffic passed through the recycling endosome. STxB was used because exogenous STxB applied can be applied as a pulse to the cell surface, while the expressed CD8-M6PR construct is present throughout the pathway at steady state. We labeled BSC-1 for both SNX1 and VPS35 as STxB moved from early endosomes (2.5 min) to recycling endosomes (7 min) to TGN (30 min) (Figure 7). In the absence of STxB, the retromer components are distributed in both peripheral puncta and in the juxtanuclear area (Figure 7, No STxB). Both VPS35 and SNX1 (SNX1 quantified in Table 3) wererecruited to EE and colocalized with labeled with STxB after 2.5 min (Figure 7, small arrows, quantified in Table 3). After 7 min, SNX1 colocalized significantly more ($p < 0.001$) with STxB as it passed through the recycling endosome. (Figure 7 large arrows). By 30 min, the STxB was in the Golgi, and the SNX1 and VPS35 no longer colocalized with STxB (Table 3, Figure 7, 30 min). Retromer components were not observed at the TGN. Together this suggested that retromer trafficked with STxB from early endosomes to recycling endosomes.

Discussion

Not all retrograde cargos follow the same pathway from the plasma membrane to the TGN. Retromer dependent cargos such as CD8-M6PR, TGN38 and STxB have been reported to pass sequentially from plasma membrane to early/recycling endosomes to the TGN, although the degree to which they pass through recycling endosomes has been controversial (8, 9, 40, 52). M6PR also delivers lysosomal hydrolases to the late endosomes and is retrieved along the Rab9/Tip47 pathway to the TGN (12, 53). Retrograde traffic of TGN38 requires the TGN Golgin GCC88, while STxB delivery requires the TGN Golgin GCC185 suggesting that these too may traffic along different routes (9).

Despite extensive investigation of molecules required for the delivery of each cargo, it remains unclear which endosomal sorting compartments are of physiologic significance for each cargo (4, 24, 54–56). Indeed, Mukhopadhyay and Linstedt report that inactivation of GPP130 by addition of Mn^{++} to the culture medium results in retention of STxB in endosomal structures distributed throughout the cytoplasm of HeLa cells (57). In this case, the internalized STxB is redirected into the late endosomal (Rab7) pathway, suggesting that GPP130 acts at the level of sorting within the early endosome. As retromer is also located on early endosomes, some groups have suggested that retromer dependent traffic, such as STxB and CD8-M6PR flows directly from early endosomes to the TGN, avoiding both late and recycling endosomes (21, 23, 25, 51). On the other hand STxB has been found to overlap recycling endosomes during retrograde traffic in HeLa cells (9). However it remains unclear from this work what percentage of the STxB may bypass the recycling endosomes. Indeed Lieu et Al. find no passage of WT TGN38 through the recycling endosomes, while Ghosh et Al. observe that tac-TGN38 chimera passes through the recycling endosomes (9, 52). Some of the differences may be ascribed to the poor differentiation of EE from recycling endosomes in HeLa cells. Our findings, in BSC-1 cells with well defined EE and recycling endosomes suggest that retromer dependent traffic through the endosomal system requires the recycling endosome as well as the EE. By following two distinct retromer cargos through the endosomal system, we dissected this pathway, and the compartments involved.

For purposes of this study, we defined early endosomes as EEA-1 positive, EHD1 negative, Rab11a negative peripheral puncta accessed by Tfn internalized for 2.5 min (34, 35, 58). The recycling endosome was defined as a juxtanuclear structure that is EEA-1 negative, EHD1 positive and Rab11a positive that is accessed by Tfn after 7 min, and specifically labeled by Tfn after a 25 min internalization (32, 34, 35). Both are readily distinguished from TGN46 positive TGN and GM130 positive cis/medial Golgi in BSC-1 cells used here (15).

Retromer dependent traffic passes through the recycling endosome

Our fundamental observation is that both STxB and CD8-M6PR pass through the recycling endosome during retrograde traffic. Because our methodology only labels retrograde traffic originating at the plasma membrane, traffic of CD8-M6PR (or endogenous M6PR) from the late endosomes is not addressed. Retromer (at least the VPS26/VPS29/VPS35 with SNX1/2) is clearly required for delivery of these cargos to the TGN (19, 22, 23). However, sorting of retromer dependent traffic at the EE does not necessarily imply direct delivery to the TGN. Using pulse chase assays and fluorescently tagged ligands, we directly observed STxB and CD8-M6PR (and by analogy CI-M6PR) passing sequentially from EE, to the recycling endosome to the TGN. We were able to follow that cargo into the recycling endosome by colocalization with Tfn internalized for 25 min and by colocalization with the recycling endosomal markers Rab11a, and GFP-EHD1. Colocalization in the recycling endosome also appeared to precede localization of the cargo to the TGN. These observations suggest that a significant fraction of the retromer dependent retrograde traffic passes through the recycling endosome.

Still unclear was whether passage through the recycling endosome was physiologically important. We directly addressed this issue by ablating the recycling endosome. The HRP-DAB ablation protocol has been used to selectively ablate the recycling endosome in a number of different cell lines, without effects on early endosomal tfn traffic or effects on retrograde traffic such as Flag-furin which does not pass through the recycling endosome (Supplementary Figure 2). Secretory traffic that does not pass through the recycling endosome is also not affected (i.e. apical and non- μ -1B associated basolateral traffic) (38, 39). The protocol differs from that used by Stoorvogel to label the endocytic pathway in that a timed pulse is used to specifically target the recycling endosomes (59). We ensured specificity by demonstrating that recycling traffic through the EE, sorting to the late endosomal/lysosomal pathways and sorting to the TGN via late endosomes remained functional. Under ablation conditions, both STxB and CD8-M6PR in the early endosomes were no longer able to reach the TGN, Golgi or ER. Instead the cargo was diverted to an aberrant compartment. Curiously, ablation of the recycling endosome did not result in diversion of STxB or CD8-M6PR into late endosomes, supporting the idea that sorting out of the EE and into the retrograde pathway had already occurred when these cargos were diverted by the ablation protocol.

The simplest explanation of our results is that a large fraction, if not all, of retromer dependent retrograde traffic passes through the recycling endosome and cannot bypass it. An alternative explanation is that retromer function was specifically inhibited by the ablation protocol. We find such an explanation unlikely because inactivation of retromer would likely have resulted in an inability to sort the cargo out of the EE. In fact, this was the phenotype that we observed when we knocked down the retromer component VPS26. Further, we found that retromer was co-sorted with STxB to the aberrant compartment suggesting that cargo delivery was not prevented by a lack of available retromer but rather by an inability of cargo and retromer to target the correct compartment.

EHD1 and retrograde traffic

EHD1 is physically associated with the recycling endosome. It is required for recycling of Tfn from this organelle (46, 60). It directly interacts with VPS26 in retromer and expression of dominant negative EHD1 disrupts the distribution of both retromer and TGN38 (26) (47). This interaction is puzzling because EM studies have found retromer located predominantly at the EE. If retromer directs traffic from the early endosomes directly to the TGN, then it is difficult to understand how interaction with EHD1 at the recycling endosome might occur. We observed both retromer dependent cargos passing through the recycling endosome, and expression of DN EHD1 caused a redistribution of retromer to the recycling endosome. Together these suggest that retromer and EHD1 interact as traffic passes through the recycling endosome.

Expression of, DN-EHD1, or knockdown of EHD1 caused sequestration of STxB at the recycling endosome. Since little or no STxB reached the TGN under these conditions, this would suggest that the pathway through the recycling endosome is the major trafficking pathway for STxB. The lack of effect of DN-EHD1 and EHD1 knockdown on CD8-M6PR trafficking is surprising. Gokool et Al. observed a slowing of CD8-M6PR trafficking to the TGN using a P-loop mutant of EHD1 (26). We are not certain as to why this difference arises. It is possibly due to a difference in experimental conditions, Gokeel et Al. observed the block only at 32° C but not at 37° C. We worked exclusively at 37° C. Neither we, nor Gokool et Al. observed any change in trafficking at 37° C. We did note that knockdown of EHD1, or expression of the dominant negative construct resulted in decreased total labeling with the anti-CD8 antibody. This may reflect changes in delivery of CD8-M6PR to the cell surface. Our assay was insensitive to delivery of M6PR cargo to the late endosomes and lysosomes, however that traffic is not thought to be EHD1 dependent. (61). (12). Our results

do suggest that for this retrograde pathway, CD8-M6PR is far less reliant upon EHD1 function than is STxB.

Retromer along the retrograde trafficking pathway

Retromer has been associated with multiple functions (62). It is associated with EE to Golgi traffic, retrieval of M6PR from late endosomes to the Golgi, and transcytosis of pIgAR (22, 25) (63). Our results suggest that one role for retromer may be in the guiding of retrograde traffic from the EE to the recycling endosome. This view is supported by the recruitment of retromer to early endosomes by Rab7/9 (50). Knockdown of VPS26 slowed trafficking through the endosomes and notably sequestered retrograde cargo in the EE. This pattern is similar to the pattern observed with knockdown of EHD3, a protein required for EE to recycling endosome transport (64). Retromer is recruited to EE, but SNX1/2 also binds through p150^{glued} to dynein (49). Because p150^{glued} also interacts with Rab6IP1 (found at the TGN) it has been suggested that retromer dependent cargo from the EE is transported directly to the TGN. However, Rab6IP1 is also found at the recycling endosome, where it interacts with Rab11a and is required for normal Tfn recycling. Furthermore, the recycling endosome is located at the MTOC (at the minus end of the microtubules) and is thus also a possible target for dynein directed traffic. In light of our findings, it seems more likely that retromer derived traffic from the EE is being delivered to the recycling endosome.

Ablation of the recycling endosome was not phenotypically equivalent to knockdown of VPS26. Exit from the EE appears to require retromer, whereas ablation of the recycling endosome misdirected traffic that had already left the EE. We interpret this to mean that retromer dependent sorting had already occurred in the latter case. We also observed that, in ablated cells retromer remained associated with the cargo in an aberrant compartment, suggesting that it had not reached a point where it could release the cargo. We interpret these results to mean that retromer is facilitating sorting of retrograde cargo away from other EE cargo, and may remain associated with the cargo at least to the recycling endosome. It is difficult to say from these results, whether retromer is also involved in delivery of cargo from the recycling endosome to the TGN.

Is there a direct EE to TGN pathway?

Our work does not exclude the existence of a direct EE to TGN pathway. Such a pathway may be present in other cell lines (perhaps HeLa) or involve other cargoes in BSC-1 cells. Here we have found that in our hands, in BSC-1 cells, at least a majority of each of our retromer dependent retrograde cargoes must pass through the recycling endosome.

Our conclusion disagrees with conclusions drawn by others that cargo may traffic directly from EE to the TGN. In light of our findings, it may be prudent to reexamine those conclusions. Wassamer et. Al couple EE exit of retromer dependent cargo to the TGN protein Rab6IP1, but this protein is also found at the recycling endosome, and thus does not exclude passage through the recycling endosome (49). Rme8, Retromer and SNX8 are required for traffic of multiple cargoes between EE and TGN, but while the endpoints are clear, it remains unclear whether this traffic proceeds via the recycling endosome (65) (66) (67). The retromer complex is recruited to EE, and is clearly required for delivery of cargoes such as ours for delivery to the TGN (21, 23, 25, 51). Involvement of retromer at the EE and selective Golgins at the TGN suggest a direct pathway in a variety of systems (68). However just as we cannot exclude such a pathway in these systems, neither has bypass of the recycling endosome been demonstrated in these other organisms and cell lines..

A model for endocytic transport of retromer dependent retrograde traffic

Our data allow us to integrate the role of the recycling endosome into established pathways (for a review see (4)). We propose a model in which transport of STxB and CD8-M6PR progresses through three distinct stages (Figure 8). In the first stage, retrograde (ex. CI-M6PR, STxB, etc), recycling (Tfn) and degradative (LDL) cargoes are all delivered to the early endosome (Figure 8, 1). This is supported by a wide body of literature (69) (6). Delivery to the EE in this case reflects traffic arriving from the plasma membrane. The second stage involves early endosomal sorting and delivery (Figure 8, 2). At a minimum, we would suggest at least four exit pathways from the EE. First released ligands and cargo such as furin are sorted into vesicular domains which mature into late endosomes (Figure 8, furin pathway) (70–72). Second is a direct recycling pathway returning to the plasma membrane. Third recycling traffic directed to the recycling endosome, exemplified by the Tfn receptor (Figure 8, dashed lines) (34). This traffic is not dependent upon retromer (as seen in our results and in (25)). Fourth, retromer dependent sorting of STxB, and CD8-M6PR, providing a second separate pathway to the recycling endosome (Figure 8, 2, red pathway) (73). This is evidenced by the early endosomal distribution of STxB and CD8-M6PR when VPS26 is knocked down as well as our observation that STxB and CD8-M6PR are separated from EE when the recycling endosome is ablated. We do not include a direct EE to TGN pathway in this model as our evidence does not directly support it. However, such a pathway may also be present

Finally, retrograde traffic must be directed to the TGN from the recycling endosome (Figure 8, 3). We at least two routes, only one of which (STxB pathway) is EHD1 dependent. By including the recycling endosome along this pathway, we believe that several difficult aspects of retrograde traffic can be explained. If both cargoes are included in the same retromer mediated sorting event at the EE, then it is difficult to explain the difference in EHD1 dependence. However if delivery by retromer is to the recycling endosome, then separate pathways may exist for these two cargoes from the recycling endosomes to the closely apposed TGN. Resorting at the recycling endosome may also explain how the recycling endosome associated protein EHD1 can interact with EE recruited retromer in the transport of STxB. Passage through the recycling endosome does not rule out participation of retromer in the recycling endosome to TGN stage, and inclusion of retromer at this point has been suggested (49). Divergent pathways at the recycling endosome would also explain differential dependence of TGN38 and STxB upon GCC88 and GCC185 respectively for delivery to the TGN (9).

It has been suggested that different retromer forms, incorporating different sorting nexins in different complexes may incorporate different cargoes from the EE directly to the TGN. (17, 19, 21). This may equally be the case in sorting from the EE to the recycling endosome. Given the increasing complexity and selectivity of sorting and trafficking pathways through the endosomal system, it would not be surprising to find that many alternative retrograde trafficking pathways exist for different cargoes.

Materials and Methods

Cell culture and transfections

BSC-1 cells were a generous gift from the Mellman laboratory (Genentech, South San Francisco, CA) and were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovineserum, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA) in 5% CO₂, 95% air. ATCC HeLa cells were cultured as described (74). Where transferrin was used, BSC-1 cells were infected with an adenoviral expression construct containing the human TfnR as previously described (15,

30). EHD1 and GFP-EHD-1 and DN GFP-EHD1 recombinant DNA constructs were transiently transfected into BSC-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Silencing RNA

BSC-1 cells were transfected with 2 μ L per 22 mm well with RNAi-max according to manufacturers directions, 72 hours before use. Cells were transfected with a scrambled sequence control (either 20 or 8 nM concentration to match treatment), 20 nM concentration EHD1 siRNA (AAGGAGAGAUCUACCAGAAGA) duplexes validated by Gokool et Al. (26), or a combination of 2 siRNA oligos to target VPS26. These were 40 nM (GGAAAGAGCUAGCGCUGAA), and 40 nM (AGAACCACGUGAUAAGUA) (both from siGENOME SMART Oligos from Invitrogen) Essentially identical results were obtained with each oligo alone. All transfections were in a total of 600 μ L Optimem (Gibco) in the 22 mm well for a minimum of 2 hours at 37°. Efficiency of knockdown was assessed by western blot of EHD1 and VPS26. Additionally, in IF experiments involving VPS26 KD, cells were stained for VPS26 and only cells lacking the normal punctate pattern were selected for IF imaging of KD effects. EHD1 Abs were not suitable for IF, however testing of EHD1 KD on cells transfected with GFP-EHD1 demonstrated nearly 100% of the cells had decreased expression of the construct, in agreement with western blot testing. All sequences were found not to correspond to any other known genomic sequences using BLAST.

Antibodies and reagents

The primary antibodies used were as follows: mouse anti-LAMP1 and mouse anti-LAMP2 (J.T. August and J.E.K. Hildreth, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-EEA-1 and mouse anti-GM130 (BD Biosciences, San Jose, CA), mouse anti-PDI (Assay Designs, Ann Arbor, MI), polyclonal rabbit anti-Rab11 (Invitrogen, Carlsbad, CA, USA), polyclonal sheep anti-TGN46 (Serotec, Raleigh, NC), and mouse anti-SNX1 (Transduction Labs Franklin Lakes, NJ). Secondary antibodies used were Alexa 488 goat anti-mouse IgG and Alexa 633 donkey anti-sheep IgG (Invitrogen Carlsbad, CA, USA). Purified STxB was labeled using the Alexa Fluor 546 labeling kit (Invitrogen, Carlsbad, CA) according to manufacture's instructions. Alexa 488/Alexa 546 human holotransferrin and LysoTracker® Red were purchased from Invitrogen (Carlsbad, CA). HRP-conjugated human transferrin (Pierce, Rockford, IL) or unconjugated human transferrin (Sigma-Aldrich, St. Louis, MO) was used in the RE ablation experiments. 70 kDa FITC-labeled Dextran (Sigma-Aldrich, St. Louis, MO) was used to follow fluid phase endocytosis.

STxB, Tfn, and dextran internalization—BSC-1 cells were grown on glass coverslips. For Tfn uptake experiments, cells were infected with a human TfnR expressing adenovirus 24–36 hours prior to use. On the day of use, cells were preincubated in serum-free media for 30 min at 37°C to clear Tfn from the cell. For all experiments, cells were chilled on ice and labeled with 1:200 dilution of 0.22 mg/ml Alexa-546 STxB, and/or 1:100 dilution of 5 mg/ml Alexa 488-Tfn in PBS. Dextran was used at 10 mg/ml in MEM media at 37°C for 30 min before chasing with unlabeled media. Internalization was performed by placing the cells in 37°C MEM for indicated times.

Recycling endosome ablation

Recycling endosomes were specifically ablated using HRP-Tfn (Pierce Chemical) essentially as described (38, 39). Briefly, BSC-1 cells expressing the hTfnR were allowed to bind Tfn conjugated to horseradish peroxidase (HRP-Tfn), 10 μ g/ml, in MEM on ice for 45 min. The

ligands were then internalized in MEM supplemented with 10% FCS (after the first min) for 30 min at 37°C. Although this time is longer than the 25 min used for maximal labeling, it ensured that all HRP-Tfn had cleared the EE. Residual surface Tfn was removed with a pH 5.0 wash for 5 min followed by a wash at pH 7.4 for 5 min on ice. Mock treated cells were subjected to the same conditions, except that transferrin without HRP was used. Cells were placed on ice and washed with PBS⁺⁺ (phosphate-buffered saline with Ca⁺⁺ and Mg⁺⁺) three times. The cells were placed in PBS with 0.1 mg/ml 3,3'-diaminobenzidine(Sigma) and 0.025% H₂O₂ for 1 hour on ice in the dark. The reaction was stopped with PBS/bovine serum albumin (BSA; 1%, wt/vol). Following the ablation, STxB was bound to the cells on ice, and then warmed to start internalization. For Tfn, cells were allowed to continuously internalize Alexa 488-Tfn for 30 min, washed (0 min) or washed and chased for 30 min in complete media. For dextran uptake, cells were incubated at 37°C with FITC-dextran, washed, then chased in complete media for 60 min. LysoTrackerTM Red was used at 1:1000 dilution for 10 min to label lysosomes. Cells were then fixed at the noted times in 3% PFA, immunolabeled if noted, then imaged.

Furin internalization assay

Full-length furin tagged with a Flag (Sigma) epitope tag at the C-terminal end was a kind gift from Gary Thomas. Flag-furin was subcloned into mammalian expression vector pCDNA3 (kindly provided by Paul Gleeson). The construct was transfected into BSC-1 cells using Lipofectamine LTX at 10 µL per µg DNA. At the same time adenovirus containing hTfn receptor was added to the cell cultures (MOI = approx. 50). After 24 hours, internalization protocols were performed essentially as described in Chia et. al. (42). Recycling endosomes were ablated in some cells, as detailed above, before internalizing ligands. Rabbit anti-Flag Ab (Sigma, St. Louis MO) was applied at 10 µg/ml to the cells on ice for 45 min. After washing in PBS, the Ab was internalized for 60 min at 37°. During the last 5 min of internalization, Alexa-488 Tfn was included in the media to confirm that individual cells had ablated recycling endosomes. Cells were fixed and stained for TGN46, and probed with anti-rabbit and anti-sheep secondary Abs.

STxB sulfation—The sulfation analysis of STxB-SS was performed essentially as described (16, 64, 75). Briefly, BSC-1 cells were transfected with EHD1 or EHD1 DN constructs 72 hours prior to treatment. BSC-1 cells were transfected once each day for 3 days with the Lonza NucleofectionTM device and Kit V according to manufacture's instructions (Lonza Group, Basel, Switzerland). Cells were starved for 2 hours in sulfate-free media. 0.01 mg of purified STxB-SS and 0.15 mCi carrier-free ³⁵S-Sulfate (PerkinElmer, Waltham, MA) was added to each 10 cm culture dish of cells for 60 min. This resulted in a single wave of internalization (76). The cells were lysed, run on an SDS-PAGE 4–20% acrylamide gradient gel, which was stained with Coomassie Blue R and protein quantified by fluorescence using an Odyssey Fluorescence reader. The gel was dried and ³⁵S-imaged using FLA-7000 Bioimaging System and Image Reader software (Fujifilm, Tokyo, Japan). Sulfation was calculated as normalized sulfation = ((density of the image band in an ROI) – (background intensity in the lane in an equal area ROI))/(Protein in the lane in Fluorescence units).

Recombinant DNA constructs

The EHD1 (Rme-1) constructs, GFP-mRme-1 and GFP-mRme-1 (G429R), were kindly provided by Barth Grant at Rutgers University (47). Constructs are referred to as EHD1 and DN EHD1 respectively to simplify the naming conventions. The Shiga toxin B subunit (STxB) and STxB-SS plasmids were a kind gift from Ludger Johannes (Institut Curie, Paris, France) and were prepared and purified as previously described (16, 75, 77). CD8-M6PR was kindly provided by Matthew Seaman at Cambridge Institute for Medical Research.

Image analysis

All images were acquired on a Zeiss 200M (Thornwood NY) inverted Microscope equipped with a 63X water immersion lens and a Hamamatsu ER camera operated by Openlab from Improvision (Coventry, UK) on a Macintosh G4 computer from Apple (Cupertino CA). Low exposure and high exposure images of cells were obtained. Contrast in images was optimized using Photoshop from Adobe (San Jose, CA) on an Apple G5 computer. Specifically, each image was composed of separate micrographs that were captured in different channels (wavelengths of fluorophores) within the same microscope field. The contrast of each channel image (grayscale) was optimized using linear adjustments in Adobe Photoshop (Adobe, San Jose, CA). Colocalization in images was quantified essentially as described in Dunn, Kamocka and McDonald (29). To minimize background from out of focus light and diffuse staining, 1344 by 1024 pixel grayscale images for each color channel were subjected to a median filter set to a radius of 14 pixels for small endosomes, or 36 pixels when larger endosomal or Golgi structures were included. The filtered image, which consists largely of a blurred background was then digitally subtracted from the original image so produce an image in which endosomal and Golgi structures appear on a black background (gray value of 0 on a scale of 0–65536). This minimizes false colocalization based on low levels of background in pixels throughout the image. Red/green, red/blue, and blue/green image pairs were composed in Volocity (Perkin Elmer). Individual cells in each pair were measured using the modified global Pearson's Correlation Coefficient (PCC), and the Mander's Correlation Coefficient (MCC). Modified PCC compares all pixels within the ROI, and ignores pixels with a zero value in either channel, to avoid false colocalization based on both channels being zero. PCC remains sensitive to variations of each channel within a structure. PCC values from the aggregate images fell naturally into 3 categories that matched observation by eye: 0-0.25, no colocalization, 0.26-0.4 partial colocalization, 0.41-1.0, colocalized. To calculate the MCC, the maximum value in each image was measured in each channel, and a threshold set at 10% of the maximum value in the image. The MCC is the sum of pixels over the threshold in both channels (colocalized by definition) as a fraction of pixels over the threshold in the target channel. The MCC_{STxB} is thus the fraction of signal in pixels positive for STxB that colocalizes with another marker. MCC values from the aggregate images fell naturally into 3 categories that matched observation by eye: 0-0.2, no colocalization, 0.21-0.5 partial colocalization, 0.41-1.0, colocalized. MCC values typically followed PCC values in these images. MCC values for cargo are given in the text as the localization of the cargo, rather than of the organelle is of concern. Significance of measurement differences was calculated from PCC values using an unpaired two-tailed t-test. Calculations based on MCC values followed those calculated from PCC, but are not shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CI-M6PR

Cation Independent Mannose 6-Phosphate Receptor

CD8-M6PR	Chimeric CD8 luminal domain fused to the cytoplasmic carboxy terminal tail of CI-M6PR
EE	Early Endosome
EHD1	Eps15 Homology Domain-containing protein 1
ER	Endoplasmic Reticulum
RE	Recycling Endosome
SNX1	Sorting Nexin 1
STxB	Shiga Toxin B
Tfn	Transferrin
TfnR	Transferrin Receptor
TGN	Trans-Golgi Network

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Figure 1.

Timecourse of STxB and CD8-M6PR internalization. A) Alexa 546 STxB (red) and Alexa 488-Tfn (green-left panels) were applied to BSC-1 cells on ice and then internalized at 37°C for the times shown. The cells were fixed and stained for TGN46 (blue). A merge of these channels is shown. The same cells were also stained for the recycling endosome marker Rab11a (green-right panels). A merge of the STxB and Rab11a channels is shown. Arrows indicate the position of STxB at each time. Note STxB in recycling endosomes at 7 min and in TGN at 19 min. B) Alexa-546 anti-CD8(red) and Alexa 488-Tfn (green) applied to BSC-1 cells on ice and internalized for various times as shown. Arrows indicate positions of internalized CD8-M6PR at each time. Note colocalization with Tfn in recycling endosome at

7 min and with TGN46 at later times. Typical images shown. Insets are magnified views of areas indicated. Quantification of colocalizations from multiple images are presented in Table 1. Bars = 10 μ m.

Figure 2. Ablation of recycling endosomes blocks STxB access to Golgi. A) STxB and Golgi. Alexa 546 STxB (red) applied to BSC-1 cells and internalized for times shown. Cells were stained for the Golgi marker GM130 (green). Arrows in upper panels indicate colocalization with Golgi. Arrows in lower panels indicate STxB not colocalized with Golgi. B) STxB and ER. STxB internalized for 180 min as in A, cells were stained for the ER marker PDI. Arrows indicate colocalization with ER in control cells, and not colocalized with ER in ablated cells. ER stain is often brighter in the center of the cell as an artifact of TX-100 permeabilization used for ER staining. Quantification of multiple images is provided in the text. Bars = 10 μ m

Figure 3.

Ablation of recycling endosomes blocks CD8-M6PR access to Golgi. A) Alexa-546 anti CD8 (red) applied to BSC-1 cells and internalized along with Alexa 488-Tfn for times shown. Cells were stained for TGN46 (blue). Tfn (green) is entirely recycled out of the cells by 60 min. Arrows indicate internalized CD8-M6PR in Golgi after 30 min. B) Recycling endosomes were ablated using a pulse of TFN-HRP and DAB/H₂O₂ (ablated cells, lower panels). Tfn without HRP was in control cells (upper panels). Anti CD8 and Tfn internalized into BSC-1 cells as in A after ablation of the recycling endosomes. Cells were stained for TGN46 (blue). Arrows indicate CD8-M6PR not colocalized with Golgi. (See Supplementary Figure 2 for additional controls). Typical cells shown. Quantification of colocalization in multiple cells is provided in the text. Bars = 10 μm.

Figure 4.

Destination of retrograde cargo when the recycling endosome is ablated. A) CD8-M6PR(green) and STxB (red) were applied and internalized for 60 min in BSC-1 cells with ablated recycling endosomes. Cells were stained for TGN46(blue) STxB and CD8-M6PR localize to the same peripheral structures (yellow arrows) but not to TGN (blue arrows). B) STxB internalized for 60 min (red) in control and ablated BSC-1 cells labeled with lysotracker (green). White arrows indicate STxB not colocalized with lysotracker, green arrows indicate lysotracker. C) STxB (red) internalized for 60 min, in ablated BSC-1 cells stained for late endosome/lysosome markers LAMP1 and Lamp2 (mixed mouse Ab's for greater sensitivity), EEA-1, or recycling endosome marker Rab11a as indicated. White arrows indicate location of STxB, Green arrows indicate each marker where it does not colocalize with STxB, yellow arrows indicate partial colocalization of EEA-1 with STxB. D) STxB (red) internalized for 60 min in ablated BSC-1 cells stained for retromer components SNX1 or VPS35 as indicated. Yellow arrows indicate colocalization of retromer and STxB. Typical cells are shown. Quantification of colocalization in multiple images is presented in Table 2 and in the text. Bars = 10 μ m.

Figure 5.

Dominant negative EHD1 inhibits STxB but not CD8-M6PR trafficking. A) Alexa 546-STxB (red) applied to BSC-1 cells expressing GFP-EHD1 wild type (green) and internalized for various times. The cells were stained for TGN46 (blue). Yellow arrows indicate colocalization of STxB and EHD1 in the recycling endosome. Purple arrows indicate colocalization of STxB with TGN46 in the Golgi. B) STxB internalized as in A, in cells expressing dominant negative GFP-EHD1 (green). Yellow arrows indicate colocalization of STxB and GFP-DN EHD1 in the recycling endosome. Asterisk in lower panel indicates a cell not expressing GFP-DN EHD1 as an internal control. C) Sulfatable STxB-SS applied to BSC-1 cells and internalized for 60 min in the presence of $^{35}\text{S}\text{-SO}_4$. STxB is sulfated upon reaching the Golgi. It is visualized as a unique, radiolabeled 7.5 KD band by SDS-PAGE. Sulfation was quantified by phosphorimager in indicated areas. A typical gel is shown. Lower panel is quantified results, $n=3$ error bars are 1 SEM. D) Alexa 546 anti-CD8 applied to BSC-1 cells expressing CD8-M6PR (red) and GFP-DN EHD1 (green). CD8-M6PR was internalized for times shown. Internalized CD8-M6PR reached the TGN (blue) by 20 min (white arrow). See Supplementary Figure 3 for effects of DN EHD1 on SNX1 distribution. Quantification of multiple images is presented in the text. Bars = 10 μm .

Figure 6.

Knockdown of EHD1 and VPS36. A) BSC-1 cells in which either EHD1 or VPS26 was knocked down using siRNA (see methods). A) Western blot of equal amounts of protein probed for EHD1, and VPS26 with β-tubulin as a loading control. Blot was visualized using fluorescent secondary antibodies. EHD1 KD was > 85%, VPS26 KD was >80% compared to control. B) Effects of knockdowns on STxB-SS sulfation. Sulfatable STxB-SS was applied to scrambled oligo control and knockdown cells on ice and internalized for various times. Sulfation was determined as in Fig. 5. EHD1 KD (red) and VPS26 KD (blue) resulted in 39% and 77% inhibition of sulfation at 60 min. n=3, error bars are std. dev.

Figure 7.

STxB and retromer move together from early endosomes to recycling endosomes. Alexa 546-STxB (red) was applied to BSC-1 cells and internalized for times shown. The cells were fixed and stained for retromer components SNX1(green) and VPS35(blue). Initially, SNX1 and VPS35 are recruited to peripheral early endosomes as STxB is internalized (2.5 min, small arrows). After 7 min, SNX1 and VPS35 colocalize with STxB in the recycling endosome (large arrows). By 30 min STxB in the Golgi and no longer colocalizes with SNX1 or VPS35. Quantification of multiple STxB vs SNX1 images is presented in Table 3. Bars = 10 μ m.

Figure 8.

Schematic representation of retromer directed retrograde traffic and recycling traffic in BSC-1 cells. 1) STxB, CD8-M6PR and Tfn are bound to the surface, internalized and delivered to the early endosome (EE). This may include clathrin dependent and possibly clathrin independent pathways. 2) Tfn is recycled along the dashed lines. Tfn delivery to the recycling endosome (RE) is not retromer dependent. STxB and CD8-M6PR delivery to the recycling endosome (red arrow) is retromer dependent. 3) At the recycling endosome, EHD1 and retromer interact. EHD1 is required for delivery of STxB to the TGN (red arrow), but not for delivery of CD8-M6PR (and by extension CI-M6PR, blue arrow). Retromer is thus essential for EE to RE transport of retrograde cargo. It may also be involved in RE to TGN transport. Dotted lines indicate retrograde pathways for Furin (EE to late endosome-LE) and for cycling of CI-M6PR between TGN and late endosomes, both non-retromer dependent pathways.

Table 1

Quantification of STxB and CD8-M6PR colocalization with compartment markers during retrograde trafficking. Upper panels: STxB bound to the surface of cells was internalized along with Tfn. Lower panels: anti-CD8 Ab was bound to CD8-M6PR and internalized along with Tfn in cells expressing both receptors. Cells were stained for TGN46 and/or Rab11a to mark the TGN and recycling endosome respectively. Each table is a pair-wise comparison of STxB fluorescence with another fluorescent marker. Internalization times are given in the column headers along with the number of images evaluated in parenthesis. Background subtraction, image processing, calculation of Pearson's Correlation Coefficient (PCC) and Manders Correlation Coefficient MCC. Are provided in Methods. PCC is 0 for no correlation between fluors. 1.0 for absolute correlation. PCC is sensitive to differential distribution within a compartment. Based on aggregate data and comparison to visual images for two fluors. $PCC < 0.25$ is not colocalized, $0.26 < PCC < 0.4$ is partly colocalized and $0.41 < PCC$ is colocalized. Error margin as standard deviation about the mean. MCC represents the percentage of each fluor, that is in the same compartment as the other fluor. It is less sensitive to relative distribution in the compartment. $MCC < 0.2$ is not colocalized, $0.21 < MCC < 0.5$ is partly colocalized. $0.51 < MCC$ indicates the fluor, for which the MCC is calculated is colocalized with another fluor. Note that MCC_{cargo} may colocalize with a compartment while $MCC_{compartment}$ may not colocalize indicating that not all of the compartment is filled with the cargo (or *vice versa*).

STxB Internalization:			
Tfn vs STxB	1 min (6) ^{*f}	7 min (14) ^{*f}	19 min (11) ^{*f}
PCC	0.649 +/- 0.079	0.464 +/- 0.146	0.245 +/- 0.093
MCC _{Tfn}	0.547 +/- 0.149	0.376 +/- 0.197	0.238 +/- 0.134
MCC _{STxB}	0.624 +/- 0.096	0.370 +/- 0.211	0.141 +/- 0.129

TGN vs STxB	1 min (8) [*]	7 min (8) ^{*f}	19 min (17) ^{*f}
PCC	0.229 +/- 0.176	0.165 +/- 0.051	0.502 +/- 0.186
MCC _{TGN}	0.314 +/- 0.168	0.274 +/- 0.091	0.620 +/- 0.142
MCC _{STxB}	0.163 +/- 0.150	0.131 +/- 0.041	0.495 +/- 0.221

Rab11 vs STxB	1 min (5) [*]	7 min (11) ^{*f}	19 min (4) ^{*f}
PCC	0.077 +/- 0.038	0.427 +/- 0.128	0.085 +/- 0.050
MCC _{Rab11}	0.056 +/- 0.019	0.620 +/- 0.100	0.257 +/- 0.068
MCC _{STxB}	0.089 +/- 0.053	0.424 +/- 0.186	0.054 +/- 0.037

CD8-M6PR Internalization:			
Tfn vs CD8-M6PR	5 min (7)	7 min (13)	20 min (10)
PCC	0.488 +/- 0.237	0.621 +/- 0.171	0.250 +/- 0.128
MCC _{Tfn}	0.476 +/- 0.300	0.630 +/- 0.241	0.223 +/- 0.139
MCC _{CD8-M6PR}	0.545 +/- 0.284	0.651 +/- 0.257	0.198 +/- 0.159

TGN vs CD8-M6PR	5 min (6) ^{*f}	7 min (8) [*]	20 min (10) ^{*f}
PCC	0.012 +/- 0.022	0.102 +/- 0.057	0.777 +/- 0.063
MCC _{TGN}	0.120 +/- 0.175	0.343 +/- 0.232	0.672 +/- 0.203
MCC _{CD8-M6PR}	0.033 +/- 0.064	0.178 +/- 0.081	0.793 +/- 0.155

^{*}, [†], or [‡] in a table indicates a pair of columns that are significantly different with $p < 0.001$.

Table 2

Colocalization of STxB with compartment markers in cells with ablated recycling endosomes. STxB was bound and internalized for 60 min in either control cells or cells with ablated recycling endosomes and then. There is no significant colocalization with EEA1 (EE) or LAMP ½ (late endosomes/lysosomes). There is significant colocalization with SNX 1 in ablated cells. Number of images evaluated is in parenthesis. Error is standard deviation about the mean. No pair showed significant difference. Calculations are as for Table 1.

In ablated cells:		
EEA1 vs STxB	60 min Control (9)	60 min Ablated (13)
PCC	0.009 +/- 0.014	0.006 +/- 0.049
MCC _{EEA1}	0.010 +/- 0.010	0.035 +/- 0.082
MCC _{STxB}	0.013 +/- 0.014	0.017 +/- 0.031

LAMP vs STxB	60 min Control (6)	60 min Ablated (7)
PCC	0.048 +/- 0.070	0.092 +/- 0.132
MCC _{LAMP}	0.035 +/- 0.034	0.078 +/- 0.101
MCC _{STxB}	0.137 +/- 0.136	0.109 +/- 0.117

SNX1 vs STxB	60 min Control (5)	60 min Ablated (7)
PCC	0.327 +/- 0.268	0.505 +/- 0.107
MCC _{SNX1}	0.269 +/- 0.234	0.470 +/- 0.210
MCC _{STxB}	0.557 +/- 0.184	0.666 +/- 0.230

Table 3

Sorting Nexin 1 and STxB stay together during passage through the EE and recycling endosomes. Colocalization of SNX1 and STxB during various times of internalization. Number of images evaluated is in parenthesis. Errors are standard deviation about the mean.

SNX1 vs STxB	2.5 min (16) ^{‡$\hat{I}$}	7 min (11) ^{*$\hat{I}$}	30 min (18) ^{*$\hat{I}$}
PCC	0.578 $\pm$ 0.110	0.746 $\pm$ 0.045	0.186 $\pm$ 0.064
MCC _{SNX1}	0.668 $\pm$ 0.126	0.758 $\pm$ 0.071	0.169 $\pm$ 0.066
MCC _{STxB}	0.394 $\pm$ 0.122	0.623 $\pm$ 0.076	0.171 $\pm$ 0.087

^{*}, [‡] or ^{$\hat{I}$} in a table indicates a pair of columns that are significantly different with $p < 0.001$. Calculations are as for Table 1.