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Sorting Nexin-1 Mediates Tubular Endosome-to-TGN Transport through Coincidence Sensing of High-Curvature Membranes and 3-Phosphoinositides

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Summary

Background: Sorting nexins (SNXs) are phox homology (PX) domain-containing proteins thought to regulate endosomal sorting of internalized receptors. The prototypical SNX is sorting nexin-1 (SNX1), a protein that through its PX domain binds phosphatidylinositol 3-monophosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2]. SNX1 is associated with early endosomes, from where it has been proposed to regulate the degradation of internalized epidermal growth factor (EGF) receptors through modulating endosomal-to-lysosomal sorting.

Results: We show here that SNX1 contains a BAR (Bin/Amphiphysin/Rvs) domain, a membrane binding domain that endows SNX1 with the ability to form dimers and to sense membrane curvature. We present evidence that through coincidence detection, the BAR and PX domains efficiently target SNX1 to a microdomain of the early endosome defined by high curvature and the presence of 3-phosphoinositides. In addition, we show that the BAR domain endows SNX1 with an ability to tubulate membranes in-vitro and drive the tubulation of the endosomal compartment in-vivo. Using RNA interference (RNAi), we establish that SNX1 does not play a role in EGF or transferrin receptor sorting; rather it specifically perturbs endosome-to-*trans* Golgi network (TGN) transport of the cation-independent mannose-6-phosphate receptor (CI-MPR). Our data support an evolutionarily conserved function for SNX1 from yeast to mammals and provide functional insight into the molecular mechanisms underlying lipid-mediated protein targeting and tubular-based protein sorting.

Conclusions: We conclude that through coincidence detection SNX1 associates with a microdomain of the early endosome—characterized by high membrane curvature and the presence of 3-phosphoinositides—from where it regulates tubular-based endosome-to-TGN retrieval of the CI-MPR.

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Introduction

The endosomal system functions to sort and deliver internalized proteins to appropriate subcellular destinations. Molecules internalized at the cell surface are delivered to the early endosome, a highly pleiomorphic organelle composed of cisternal, tubular, and vesicular regions [1–3]. Thus, internalized receptors for transferrin and epidermal growth factor (EGF), as well as the cation-independent mannose-6-phosphate receptor (CI-MPR), all enter early endosomes and are sorted to various destinations [4]. While transferrin receptors are enriched in tubules and are recycled back to the plasma membrane through the endocytic recycling compartment (ERC), EGF receptors remain within the endosome, which maturates into late endosomes/multivesicular bodies (MVBs) that are competent to fuse with the degradative lysosome. In contrast, the CI-MPR is removed from the degradative pathway via retrieval through the ERC to the *trans*-Golgi network (TGN) by the retromer complex [5, 6, 7]. From here, it regulates delivery of newly synthesized acid hydrolases to the endosomal system [5, 8, 9]. In mammalian cells, the machinery required for endosomal sorting remains largely undefined. However, it is clear that the endosomal lipid phosphatidylinositol 3-monophosphate [PtdIns(3)P] acts to localize regulators of endosomal function that contain PtdIns(3)P binding FYVE or phox homology (PX) domains and thus helps to define endosomal identity [10–14].

A major family of PX domain-containing proteins are the sorting nexins (SNXs) [15–17]. In those examined, the SNX-PX domain functions as a phosphoinositide binding domain that aids in localizing these proteins to cellular membranes [16]. Indeed, sorting nexin-1 (SNX1), originally identified as an interacting partner for the EGF receptor [18], associates in a 3-phosphoinositide-dependent manner with the early endosome [19, 20]. Under conditions of overexpression, SNX1 has been proposed to regulate EGF receptor degradation by modulating endosome-to-lysosomal sorting [17–20]. This contrasts with the role of its yeast ortholog, Vps5p [21, 22], which acts to retrieve proteins away from vacuole-mediated degradation (reviewed in [23]). Vps5p is a component of the yeast retromer complex, a membrane coat complex also containing Vps17p, Vps26p, Vps29p, and Vps35p. This complex functions to retrieve the lyso-

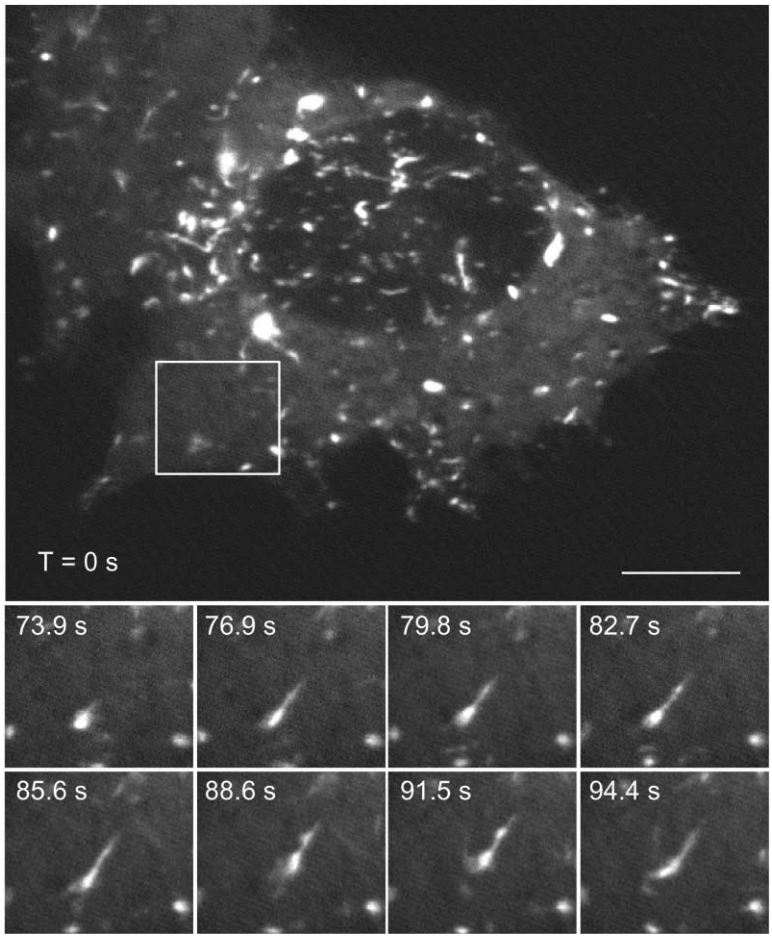

Figure 1. GFP-SNX1 Localizes to a Tubulo-Vesicular Early Endosomal Compartment

HeLa cells were transfected with pEGFPC1-SNX1 and imaged live after 22 hr. Frames were captured every 2.9 s for 5 min. Frames from the highlighted region depicting the formation of GFP-SNX1-decorated tubules from a GFP-SNX1-positive vesicle are provided. The scale bar represents 10 μm.

somal hydrolase receptor Vps10p from the prevacuolar compartment to the late Golgi [23–25]. With the exception of Vps17p, mammalian orthologs of the yeast retromer have been identified [26]. The retromer has been proposed to assemble from a cargo selective Vps26p/Vps29p/Vps35p subcomplex and a membrane associated Vps5p/Vps17p subcomplex [25]. Suppression of the cargo selective complex in mammalian cells has recently been shown to prevent retrieval of the CI-MPR to the TGN [6, 7], indicating a conserved role for the retromer from yeast to mammalian cells. The role of SNX1 within this complex has not been examined.

Here, we show that in addition to a phosphoinositide binding PX domain, SNX1 contains a membrane binding and curvature-sensing BAR (Bin/Amphiphysin/Rvs) domain [27, 28]. We present evidence that the BAR and PX domains, through coincidence detection, target SNX1 to a microdomain of the early endosome defined by high membrane curvature and the presence of 3-phosphoinositides. We show that the BAR domain endows SNX1 with an ability to tubulate membranes in-vitro, and drive the formation of tubular carriers from the endosomal compartment in-vivo. Finally, by using RNA interference (RNAi), we establish that SNX1 does not function in EGF or transferrin receptor sorting; rather it specifically perturbs endosome-to-TGN transport of the CI-MPR. Coupled with evidence that other components of the mammalian retromer regulate endosome-to-TGN transport [6, 7], our data support an evolutionarily conserved function for SNX1 from yeast to mammals and provide functional insight into the molecular mechanisms underlying lipid-mediated protein targeting and tubular-based protein sorting.

Results

SNX1 Is Enriched on Tubular Elements of the Early Endosome

Previous studies have established that both endogenous and GFP-tagged SNX1 are associated with the tubular and vesicular elements of the early endosome [15, 18–20, 29–31]. We examined the dynamics of this compartment by imaging GFP-SNX1-expressing HeLa cells live over a 5 min period (Figure 1). In all cells examined, we observed GFP-SNX1-decorated tubules exiting GFP-SNX1-labeled compartments in budding processes sustained over periods of seconds. (Figure 1). Although we regularly observed fusion of SNX1-positive vesicles, we failed to detect the fusion of SNX1-decorated tubules with SNX1-decorated vesicles, indicating that tubulation of SNX1 occurs vectorially, the direction being out of the endosome.

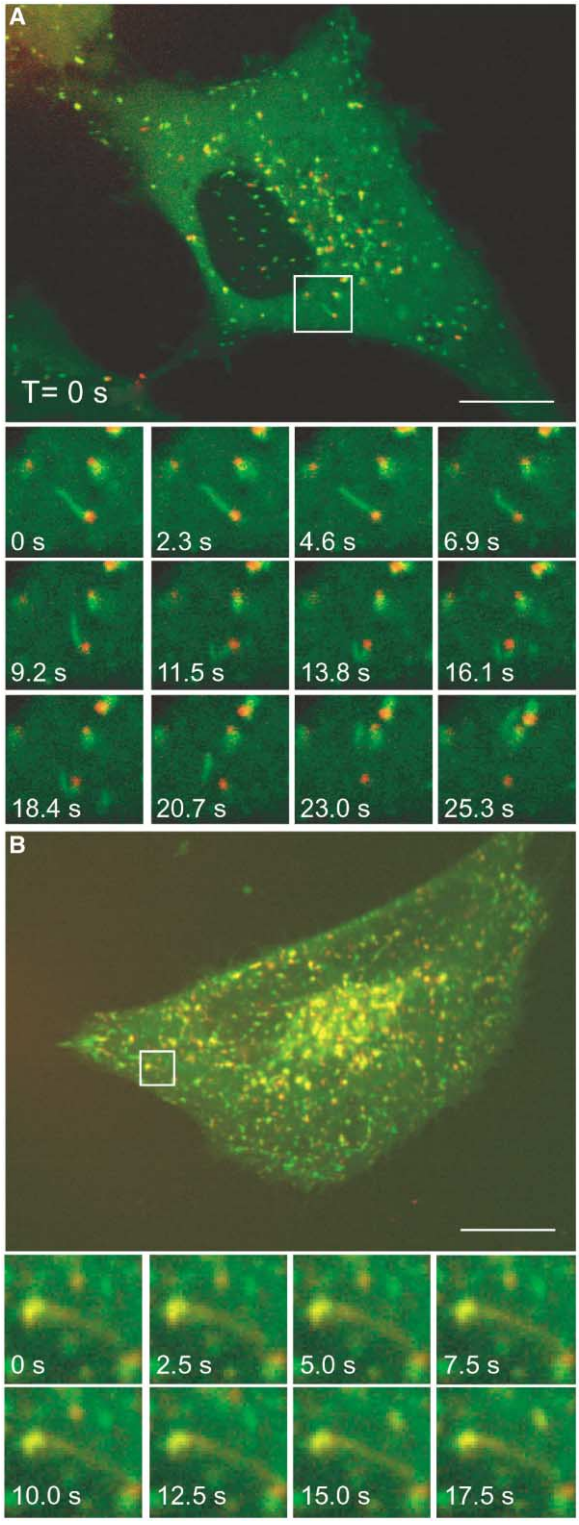

Figure 2. GFP-SNX1 Decorated Tubules Can Transport Internalized Alexa^{568}-Transferrin but Not Texas Red-EGF

HeLa cells were transfected with pEGFPC1-SNX1 for 22 hr and serum starved for 3 hr prior to live imaging live. Cells were incubated with either 200 ng/ml TxR-EGF (A) or 20 μg/ml Alexa^{568}-transferrin (B) for 20 min. TxR-EGF reaches the GFP-SNX1-positive compartment after 20 min and is not transported along GFP-SNX1-positive tubules but remains in the endosomal body (A). A series of frames depicting a GFP-SNX1 positive tubule exiting an endosome positive for GFP-SNX1 and TxR-EGF is shown and is available as Movie 1. Alexa^{568}-transferrin also enters the GFP-SNX1-positive compartment after 20 min, but is able to traffic along GFP-SNX1-positive tubules (B). A series of frames depicting a GFP-SNX1-decorated tubule transporting Alexa^{568}-transferrin is shown and is available as Movie 2. Similar data were observed in greater than 10 imaged cells for each case. The scale bar represents 10 μm.

To examine the sorting of endogenous receptors within the SNX1-labeled endosome, we examined fluorescent ligand trafficking by using dual-wavelength, live-cell confocal microscopy. To follow lysosomal trafficking of the EGF receptor, we stimulated serum-starved HeLa cells expressing low levels of GFP-SNX1 with Texas Red-labeled EGF (TxR-EGF). After 20 min of stimulation, TxR-EGF reached vesicular elements of the GFP-SNX1-labeled early endosome (Figure 2A). In all cells imaged, we did not observe detectable trafficking of TxR-EGF along GFP-SNX1-decorated tubules. Interestingly, we did observe the retention of TxR-EGF within the body of the endosome while GFP-SNX1 labeled membrane tubules exited this organelle (Figure 2A and Movie 1 in the Supplemental Data available with this article online).

Given the tubular nature of the SNX1-positive compartment, we examined the sorting of recycling cargo. We incubated serum-starved HeLa cells expressing low levels of GFP-SNX1 with Alexa^{568}-labeled transferrin (Alexa^{568}-Tf) for 20 min. After 20 min, we observed the delivery of Alexa^{568}-Tf to the vesicular element of the SNX1-labeled endosome and in a limited number of instances, trafficking of Alexa^{568}-Tf along GFP-SNX1-decorated tubules (Figure 2B and Movie 2).

To perform a higher resolution analysis of cargo sorting, we performed immunogold labeling of ultrathin cryosections. Endogenous SNX1 was enriched on tubular membranes in close vicinity to endosomal vacuoles (Figure 3). SNX1 labeling was also observed on the limiting membrane of vacuoles. These endosomal vacuoles could be labeled with internalized transferrin, indicating that they are early endosomes (data not shown). Double-immunogold labeling showed a high level of colocalization of SNX1 and the CI-MPR in the endosome-associated tubules and vesicles (Figure 3). Consistent with previous studies [7], these data establish that SNX1 is enriched on the tubular elements of early endosomes.

SNX1 Contains a Carboxy Terminal BAR Domain that Conveys an Intrinsic Ability to Sense Membrane Curvature and Drive Membrane Tubulation

During these studies, we observed that chronic overexpression of SNX1 resulted in the formation of extensive tubular networks (Figure 4A). This expression level-dependent tubulation-required membrane association as prolonged expression of SNX1(K214A), a PX domain mutant rendered cytosolic as a result of being unable to bind 3-phosphoinositides [19], failed to generate membrane tubules (Figure 4B). Interestingly, when ex-

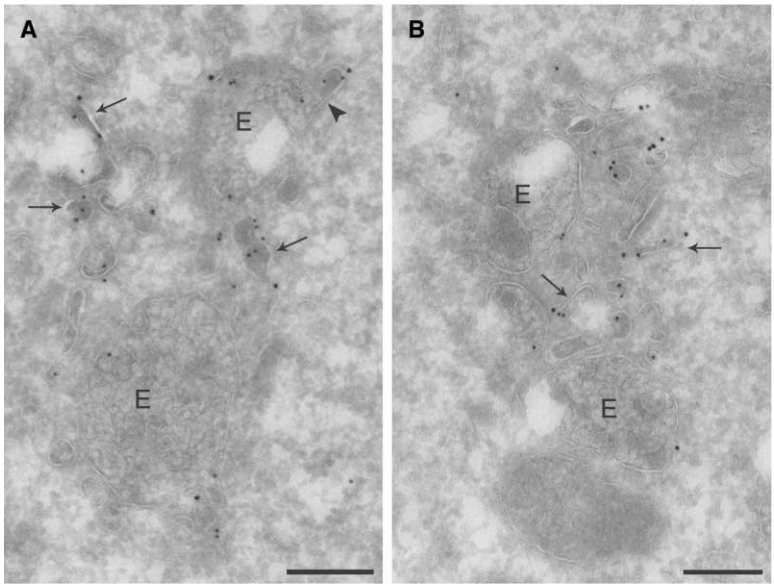

Figure 3. Endogenous SNX1 Is Enriched on the Tubular Elements of the Early Endosome

Ultrathin cryosections of HepG2 cells showing the colocalization (arrows) of SNX1 (15 nm gold particles) and CI-MPR (10 nm gold particles) on endosomal tubules. The arrowhead in (A) points to a tubule that is clearly connected to the endosomal vacuole (E) within the plane of the section. The scale bar represents 200 nm.

pressed at high levels, membrane association of wild-type SNX1 became insensitive to the addition of the PI 3-kinase inhibitor wortmannin (data not shown). As SNX1 has been reported to self-associate into an oligomeric complex [17, 20, 30], these data suggest that under conditions of overexpression, SNX1 may assemble into a stable complex that can drive and/or stabilize membrane tubulation from the early endosome.

SNX1 has been predicted to possess a BAR domain [32], a dimerization, membrane binding and membrane curvature-sensing module that allows proteins to bind and tubulate membranes [27, 28]. Because SNX1 is associated with high-curvature membrane tubules and is able to generate membrane tubules in vivo, we analyzed whether the predicted BAR domain functioned in these processes.

Consistent with previous data [20, 30], SNX1 existed as a dimer in solution (analytical ultracentrifugation, data not shown). In assays using liposomes of differential curvature, membrane association of SNX1 was enhanced as curvature increased (Figure 4C). At high concentrations, recombinant SNX1 was capable of inducing curvature, as assessed by the tubulation of liposomes visualized by electron microscopy (Figure 4D). This effect was concentration dependent; at low concentrations, a few "pan-handle" buds on liposomes were observed, whereas at higher concentrations, these buds and longer tubules were more common.

Point mutations of charged residues in either the PX domain (K214A) or the BAR domain (K429E, K430E, R431E) [SNX1(KKR-EEE)] blocked the ability of SNX1 to tubulate liposomes. Furthermore, like SNX1 (K214A), SNX1 (KKR-EEE) was cytosolic when expressed in HeLa cells (Figure 4E). Therefore, both a functional BAR domain (to bind curved membranes) and a functional PX domain (to bind 3-phosphoinositides) were required for efficient targeting of SNX1 to tubular elements of the early endosome. SNX1 therefore constitutes a coincidence detector, which associates preferentially with highly curved, 3-phosphoinositide-containing membranes.

Suppression of Endogenous SNX1 Has no Effect on EGF Receptor Degradation

In a number of independent studies, overexpression of SNX1 has been shown to enhance degradation of the EGF receptor [18, 19]. As overexpression of SNX1 leads to extensive endosomal tubulation (Figure 4A), we became concerned by the functional interpretation of these data. To dissect the function of endogenous SNX1, we employed RNAi to suppress SNX1 expression in HeLa cells. After 72 hr treatment with SNX1-specific siRNA duplexes, the level of endogenous SNX1 was suppressed by greater than 90% as detected by Western blotting or by immunofluorescence, compared to cells treated with control duplexes (Figures 5A and 5B). In cells exhibiting RNAi against SNX1, the EEA1-positive compartment appeared morphologically normal, as did the internalization of EGF and transferrin receptors (Figure 5B, data not shown). Initial steps of endocytosis and the gross morphology of the early endosomal compartment are thus unaffected by SNX1 suppression.

We examined ^{125}I-EGF trafficking in HeLa cells suppressed for SNX1 expression. The rate of ^{125}I-EGF internalization, recycling, and degradation was unaffected by SNX1 suppression when compared to control cells (Figure 5C). Such data demonstrate that endogenous SNX1 plays no role in the sorting of the EGF receptor—a conclusion that supports recent data from Gullapalli et al., 2004 [31]. Given that a population of GFP-SNX1-decorated tubules was observed to transport Alexa^{568}-Tf, we examined the trafficking of ^{125}I-labeled transferrin in SNX1-suppressed HeLa cells. We did not observe a significant perturbation of transferrin receptor internalization, degradation, or recycling (Figure 5D); therefore, endogenous SNX1 does not appear to regulate the sorting and trafficking of either EGF or transferrin receptors.

SNX1 Is Necessary for Proper Endosome-to-TGN Trafficking of the Cation-Independent Mannose 6-Phosphate Receptor

Given the genetic precedent for a role for yeast SNX1 in endosome-to-TGN retrieval [21, 22] and the functional

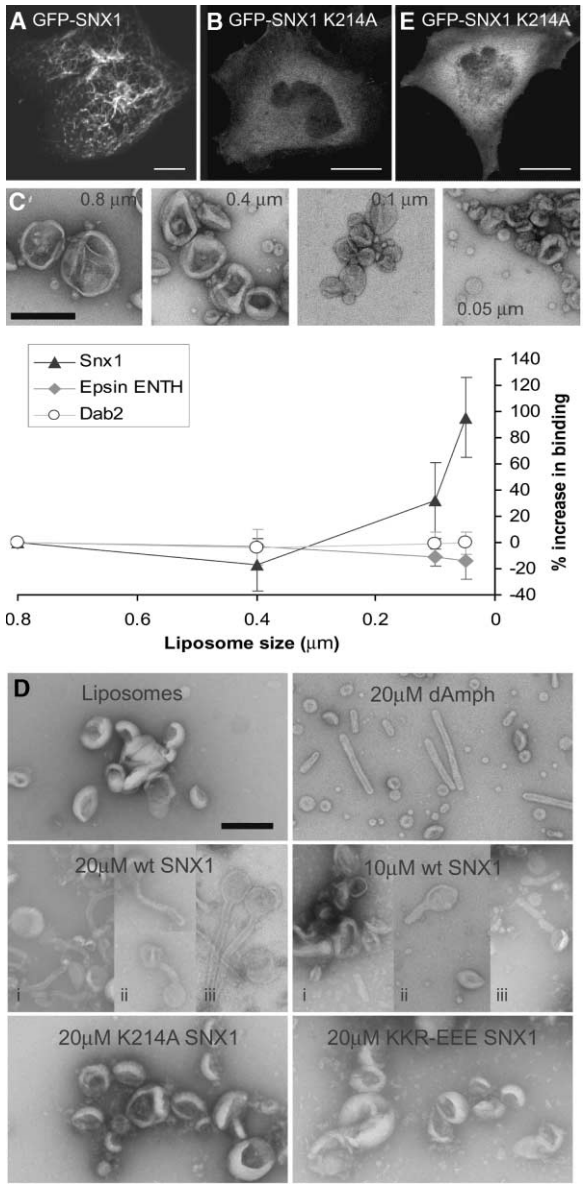

Figure 4. SNX1 Is Able to Tubulate Membranes Both In Vitro and In Vivo

(A) HeLa cells were transfected with pEGFPC1-SNX1 for 48 hr, fixed, and imaged. Chronically overexpressed GFP-SNX1 decorates extensive tubular networks. (B) SNX1 must associate with membranes to induce tubulation; cells expressing pEGFPC1-SNX1 K214A exhibited no tubules. (C) Variously curved liposomes were formed by extrusion, and binding of proteins was assayed by sedimentation. SNX1 bound preferentially to highly curved membranes, the Epsin1 ENTH domain (which strongly tubulates membranes), and Dab2 (which does not change liposome shape) were insensitive to curvature. Amount of bound protein was normalized to the value for 0.8 μm liposomes. Average values for three experiments ± SD are shown. (D) SNX1 can tubulate membranes in-vitro. At 10 μM SNX1, liposomes with budding tubules were rare, whereas at 20 μM these structures were more common, and occasionally tubular networks were seen (i–iii in each case represents three independent experiments). Liposome tubulation by SNX1 was rarer than tubulation by *Drosophila* amphiphysin (top left). At the 20 μM concentrations shown, *Drosophila* amphiphysin tubulated approximately 45% of the liposomes in a field while SNX1 tubules accounted for approximately 2% of the liposomes (data not shown). (E) Point mutations of charged residues in the BAR domain (KKR-EEE) blocked the tubulation ability of SNX1.

In all cases, the scale bar represents 300 nm.

evidence for a mammalian retromer [6, 7, 26], we examined the relationship between SNX1 and the CI-MPR. We followed the trafficking of internalized CI-MPR en route to its steady-state distribution by using HeLaM cells stably expressing a CD8-CI-MPR chimera [7]. HeLaM cells expressing low levels of GFP-SNX1 were labeled at 4°C with an anti-CD8 antibody and then incubated at 37°C for various times before fixation. By staining against CD8, we determined the distribution of the internalized CD8-CI-MPR chimera. After 10 min of internalization, the CD8-CI-MPR entered the SNX1-labeled early endosome, from where the receptor was sorted and transported to the TGN via SNX1-labeled membrane tubules (Figure 6A).

We next determined the steady-state distribution of endogenous CI-MPR. In control cells, endogenous CI-MPR localized predominantly to a perinuclear structure (Figure 6B) previously identified as the TGN [6]. In contrast, in SNX1-suppressed cells the CI-MPR failed to localize to the TGN and was found in dispersed peripheral structures (Figure 6C), although the TGN (Figure 6D) and Golgi complex (data not shown) appeared normal. The peripheral CI-MPR-labeled structures exhibited significant colocalization with endogenous SNX2 (Figure 6C). As endogenous SNX2 normally colocalizes with SNX1 (Figure 6E), this defines the CI-MPR-positive peripheral structures as early endosomes. Thus, when SNX1 is suppressed, the CI-MPR is poorly retrieved from endosomes to the TGN. Consistent with this failure in retrieval, the rate of degradation of the CI-MPR was significantly enhanced in SNX1-suppressed cells, compared with control (half-life of 10.1 ± 1.8 and 34.5 ± 0.4 hr respectively [n = 4]) (Figure 7). Thus, in SNX1-suppressed cells, the CI-MPR is retained within the early endosome, from where it is delivered to and degraded within the lysosome. A similar phenotype has been previously demonstrated for cells in which the cargo selective subcomplex of the retromer has been suppressed [6]. These data establish a role for endogenous SNX1 in the retrieval of the CI-MPR from early endosomes to the TGN. Taken with the data on other components of the mammalian retromer [6, 7, 26], we suggest that SNX1 plays an evolutionarily conserved role in endosome-to-TGN retrieval.

Discussion

In this study, we have examined the role of endogenous SNX1. We have established that in addition to a 3-phosphoinositide binding PX domain [19, 20], SNX1 contains a carboxyl terminal BAR domain. This domain aids in dimerization and acts as a membrane binding domain that can sense membrane curvature and drive membrane tubulation.

Previous work has demonstrated that the binding of the SNX1-PX domain to 3-phosphoinositides is crucial

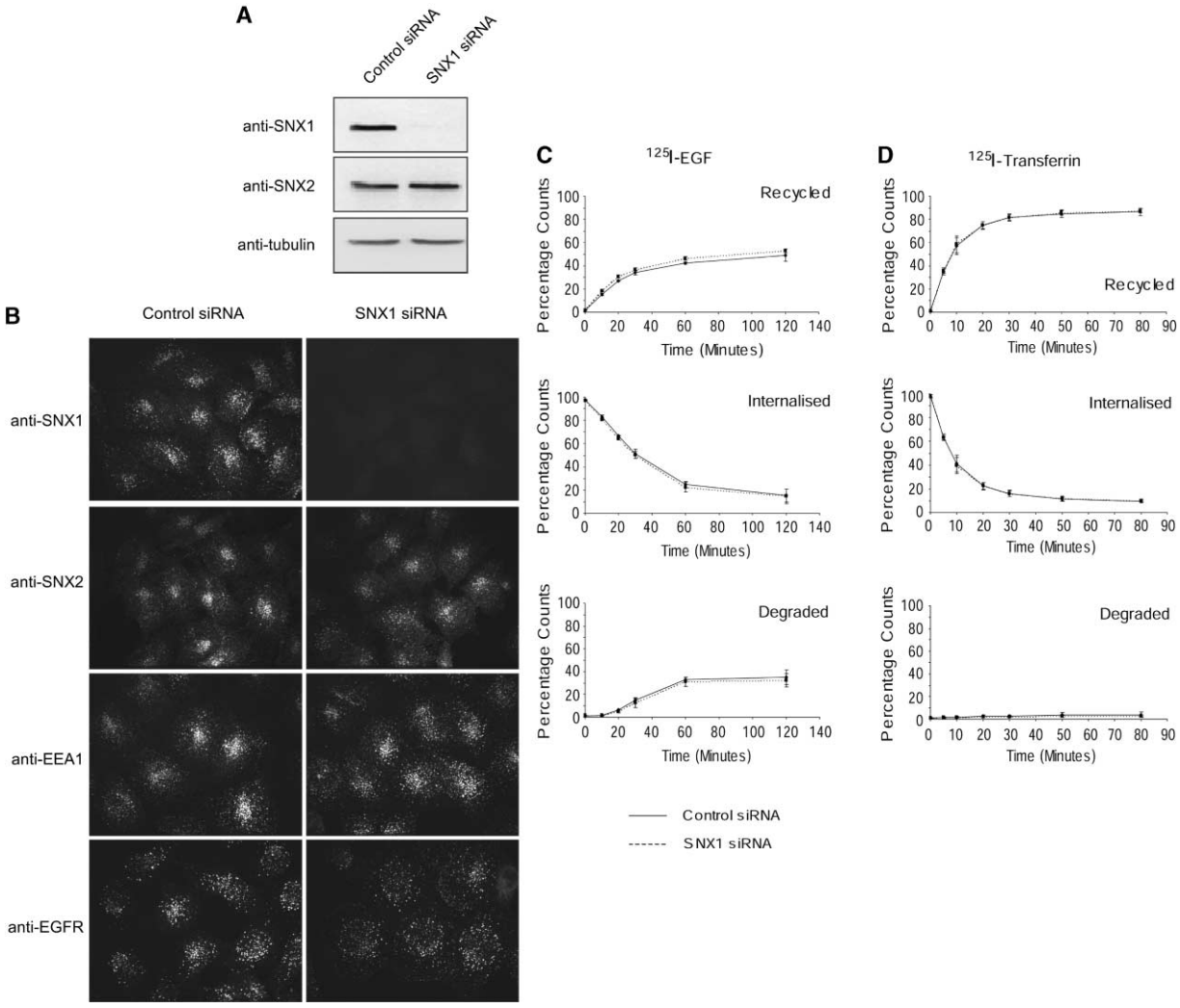

Figure 5. siRNA Induced Suppression of SNX1 Has No Gross Affect on Endosomal Trafficking

(A) HeLa cells were treated for 72 hr with scrambled or SNX1-specific siRNA duplexes. SNX1, SNX2, and tubulin levels were examined by Western blotting. Specific suppression of SNX1 can be achieved with no compensatory up-regulation of SNX2 compared to cells treated with scrambled siRNA duplexes. (B) HeLa cells were treated with either scrambled or SNX1-specific siRNA duplexes for 72 hr, stimulated with 100 ng/ml EGF for 10 min as indicated, and then fixed. Cells were stained for SNX1, SNX2, EEA1, or EGFR as indicated. (C) HeLa cells were treated with either scrambled or SNX1-specific siRNA duplexes for 72 hr. Cells were serum starved for 3 hr and labeled with 1 kBq per well ^{125}I-EGF for 1 hr at 4°C, allowed to internalize surface bound ^{125}I-EGF for 5 min at 37°C, and then returned to 4°C. Cells were chased into 100 ng/ml cold EGF-containing media for various times at 37°C. Recycled, degraded, and internalized fractions were subjected to γ counting. (D) HeLa cells were treated with either scrambled or SNX1-specific siRNA duplexes for 72 hr. Cells were serum starved for 3 hr and labeled with 1 kBq per well ^{125}I-transferrin for 60 min at 37°C. Cells were chased into 50 μg/ml cold transferrin-containing media for various times at 37°C. Recycled, degraded, and internalized fractions were subjected to γ counting.

for association with the early endosome [19, 20]. The data presented here establish that for efficient membrane association, SNX1 also requires a functional BAR domain. We propose that the targeting of SNX1 to endosomal tubules is mediated by coincidence detection. Although a number of proteins associate with endosomes through binding 3-phosphoinositides, SNX1 is preferentially enriched within an endosomal microdomain defined by two parameters: the presence of 3-phosphoinositides and high membrane curvature.

To define the functional consequence of SNX1's association with such a microdomain, we employed RNAi to suppress SNX1 expression. In contrast to other studies reliant upon ectopic expression, we find that endogenous SNX1 plays no detectable role in the internalization, recycling, or degradation of receptors for transferrin or EGF; in the case of the latter receptor, our detailed kinetic analysis supports and extends the conclusions drawn by Gullapalli et al., 2004 [31].

The proposed roles for SNX1 generated through either overexpression or suppression are clearly contradictory. We feel that given the extensive tubulation of the endosomal network, observed upon overexpression of SNX1, a likely explanation for this discrepancy is that overexpression of SNX1 perturbs global transport and sorting of endosomal cargo and, therefore, has the effect

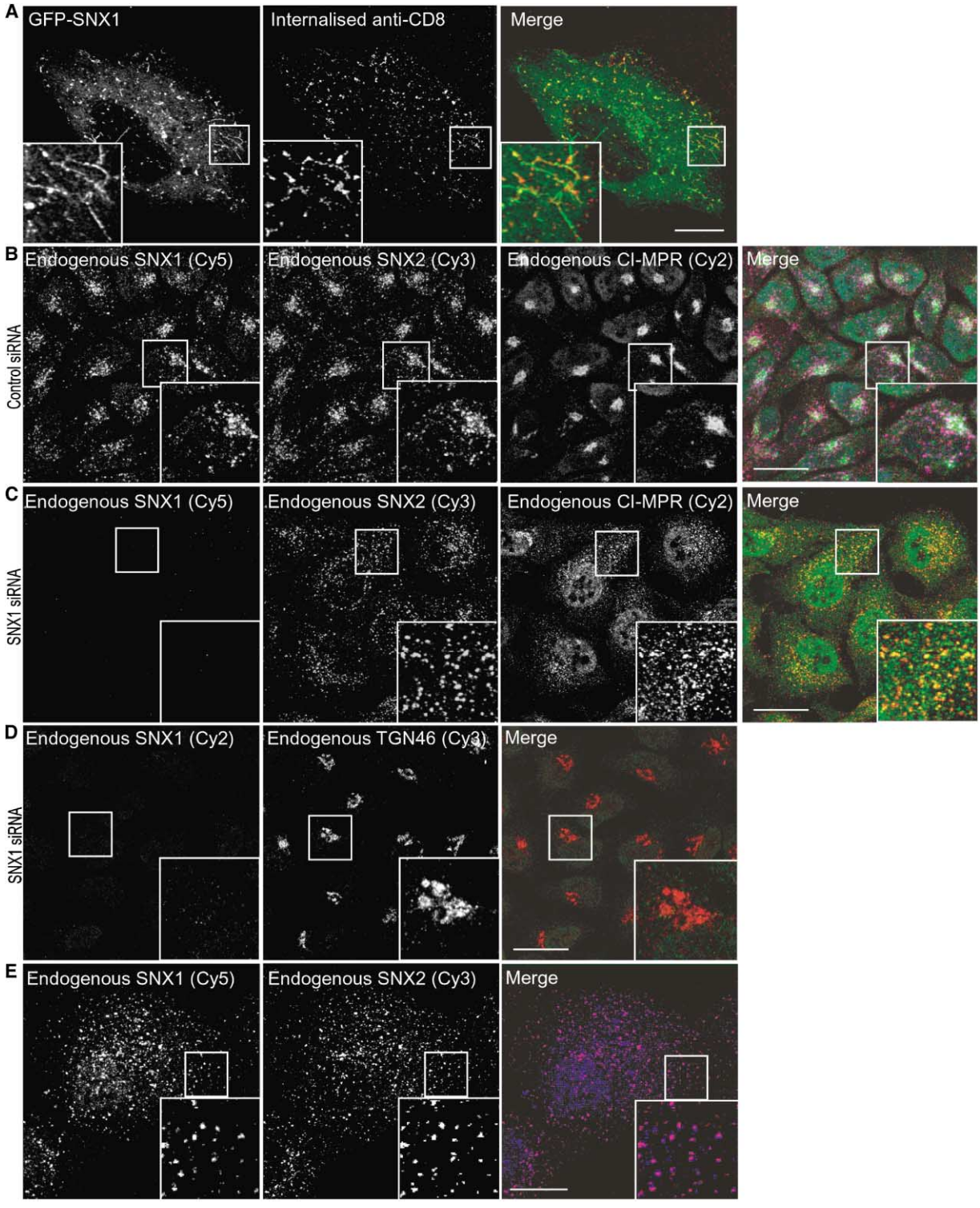

Figure 6. siRNA Induced Suppression of SNX1 Alters the Steady-State Distribution of the CI-MPR

(A) HeLaM cells stably expressing the CI-MPR C terminus fused to CD8 were transfected with pEGFPC1-SNX1 for 22 hr. Cells were labeled with anti-CD8 for 30 min at 4°C and then warmed to 37°C for 10 min, fixed, and stained against CD8. GFP-SNX1-decorated tubules contain anti-CD8, indicating that they are intermediates that transport CI-MPR from early endosomes. The scale bar represents 10 μm. (B and C) HeLa cells were treated for 72 hr with scrambled (B) or SNX1-specific (C) siRNA duplexes. Cells were fixed and stained against SNX1 (Cy5, pseudocolor blue), SNX2 (Cy3, pseudocolor red), and CI-MPR (Cy2, pseudocolor green) and imaged. Suppression of SNX1 results in a redistribution of the CI-MPR from the TGN to peripheral SNX2-positive endosomes. The scale bar represents 20 μm. (D) HeLa cells were treated for 72 hr with scrambled or SNX1-specific siRNA duplexes. Cells were fixed and stained against SNX1 and TGN46. TGN morphology is not grossly disrupted in cells that have reduced SNX1 expression. The scale bar represents 20 μm. (E) HeLa cells were fixed and stained against SNX1 and SNX2. Near-complete overlap of SNX1 and SNX2 is observed in the merged image. Images are representative of greater than ten imaged cells in each case. The scale bar represents 10 μm.

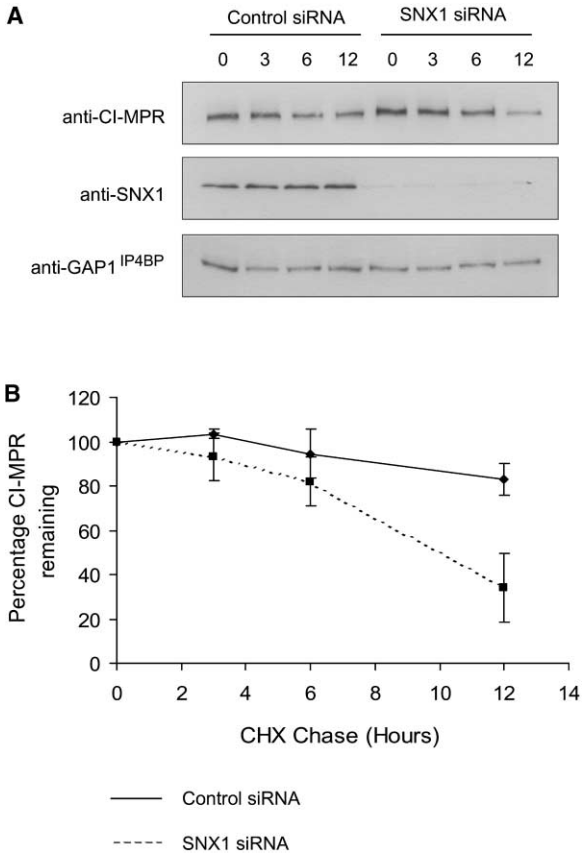

Figure 7. The CI-MPR Is Destabilized in SNX1 Suppressed Cells

(A) HeLa cells were treated twice with either control or SNX1-specific siRNA duplexes, each time for 72 hr (total suppression time of 144 hr). Cells were chased into DMEM containing 40 μg/ml cycloheximide for the indicated time period, lysed, and blotted against the CI-MPR. Representative results are shown. (B) Results from four independent experiments were quantified. The half-life of the CI-MPR is reduced to 10.1 ± 1.8 hr in cells treated with SNX1-specific siRNA duplexes.

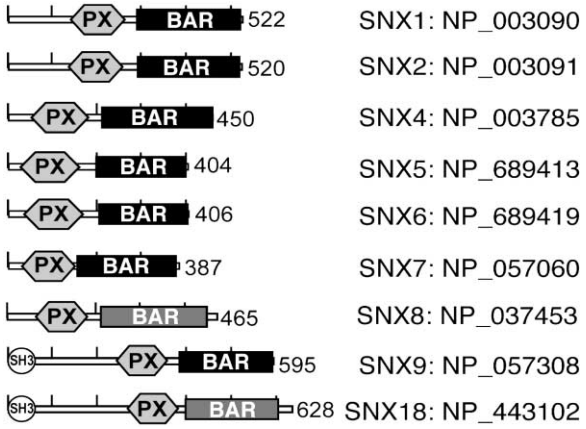

Figure 8. BAR Domains in Mammalian Sorting Nexins

Domain structures and accession numbers of the nine sorting nexins containing predicted BAR domains. BAR domain alignments can be found ([32] and on http://www2.mrc-lmb.cam.ac.uk/groups/hmm/BARdomains/BARS.html), and PX domain alignments can be found in [14]. The gray color of the BAR domains in SNX8 and SNX18 indicates weaker homology. BAR alignments were found using repeated iterations of Psi-BLAST against arfaptin, amphiphysin, SNX1, and SNX9, selecting only known BAR domains in each iteration. To confirm the alignments, positive sequences were back-BLASTed and then aligned to the BAR domains of arfaptin2 and *Drosophila* amphiphysin by using ClustalW; sequences were checked for helical secondary structure using Coils; and the Hydrophobic Cluster Analysis profiles were compared to known BAR domains. The coils, clustalW, and HCA programs can be found at www.expasy.org.

of masking the true physiological function of SNX1. In this context, our data suggest that endogenous SNX1 controls endosome-to-TGN transport of the CI-MPR; a conclusion supported by the recent demonstration that other components of the mammalian retromer also regulate this pathway [6, 7]. This evidence, when incorporated with the data presented here, clearly establish that, together with other components of the mammalian retromer complex, endogenous SNX1 regulates endosome-to-TGN retrieval.

Our data add significantly to the mechanistic understanding of retromer function. The mammalian retromer complex associates and is enriched on tubular profiles connected to the vacuolar portion of endosomes [6, 7]. How this specific membrane targeting is achieved is unclear. By proposing coincidence detection, our data taken with the evidence that SNX1 directly associates with the cargo selective retromer subcomplex [26, 31], suggests that retromer targeting arises indirectly from the PX and BAR domain-mediated association of SNX1 to high-curvature, 3-phosphoinositide-containing membrane tubules. We propose, as the BAR domain also conveys an ability for SNX1 to form oligomers, that the fundamental role of SNX1 is to sense the defined endosomal microdomain, from where it acts as a coat complex that aids the formation of membrane tubules. Within these tubules, SNX1 does not select cargo, rather this is achieved by SNX1 acting as a scaffold for the association of the cargo selective mVps35/mVps29/mVps26 subcomplex [6, 7, 31]. This complex could then partition the CI-MPR into the SNX1-defined tubular microdomain. Fission of the tubule and docking with the TGN would allow for retrieval of the CI-MPR. Through a process of iterative, tubular-based fractionation, the retromer complex is thus able to retrieve the CI-MPR from the endosome [33, 34]. Interestingly, narrow-diameter tubules have a high surface area to volume ratio and will thus efficiently partition transmembrane cargo over soluble content. Retrieval of the transmembrane CI-MPR will occur most efficiently through a tubular rather than a vesicular carrier. Thus, by geometric sorting [34, 35], SNX1-decorated membrane tubules constitute an efficient means for retrieving the CI-MPR over soluble lumenal content.

Finally, by establishing that SNX1 possesses a functional BAR domain, our data have raised the issue of whether other sorting nexins may also possess this domain. As currently defined, sorting nexins are a family of proteins that are characterized by the presence of a SNX-PX domain [15, 16]. Although this implies that sorting nexins form a family of phosphoinositide binding proteins, it does not necessarily follow that all of these proteins will modulate membrane traffic. Of the mammalian sorting nexins, there is only strong evidence of a BAR domain in nine family members (Figure 8 and [32]).

We propose that these SNX-PX/BAR domain-containing proteins may have a general role in regulating membrane traffic by acting as coincidence detectors that sense membrane curvature and induce or stabilize membrane tubulation from compartments enriched in phosphoinositides. Because mammalian PX domains are more promiscuous in their phosphoinositide specificity than their yeast counterparts, it is tempting to speculate that SNX-PX/BAR-containing sorting nexins may control transport from distinct intracellular compartments enriched in phosphoinositides other than PtdIns(3)P [36, 37].

Conclusions

We conclude that through coincidence detection, SNX1 associates with a microdomain of the early endosome, characterized by high curvature and the presence of 3-phosphoinositide, from where it regulates tubular-based endosome-to-TGN retrieval of the CI-MPR.

Experimental Procedures

Antibodies and Ligands

Anti-SNX1, anti-SNX2, and anti-EEA1 monoclonal antibodies were from Becton Dickinson. Anti-SNX1 goat polyclonal was from Santa Cruz. Anti-tubulin monoclonal was from Sigma. Anti-CD8 monoclonal was from Alexis. Anti-TGN46 sheep polyclonal was from Serotec. Anti-EGFR monoclonal was a kind gift from Professor Peter Parker (Cancer Research UK, London, UK). Anti-CI-MPR rabbit polyclonal was a kind gift from Professor Paul Luzio (Cambridge Institute for Medical Research, Cambridge, UK). Secondary antibodies conjugated to fluorophores were from Jackson Immunoreagents. Fluorescent ligands were from Molecular Probes.

siRNA Transfection

siRNA duplexes designed against SNX1 (target: AAGAACAAGAC CAAGAGCCAC) or control duplex (target: AAGACAAGAACCAGAA CGCCA) were from Dharmacon. Cells were seeded in 6-well plates at 1.15×10^5 cells per well prior to transfection with siRNA duplexes by oligofectamine (Invitrogen) and 0.2 nM duplex for 72 hr.

cDNA Constructs, Transfection, and Fixed and Live Cell Imaging

Plasmids encoding SNX1 were as previously described [19]. HeLa cells were transfected with cDNA using Genejuice (Novagen). Cells were fixed in paraformaldehyde (4% w/v) for 15 min at room temperature or used live for biochemical or live-cell imaging studies. Indirect immunofluorescence was performed on cells permeabilized with 0.1% Triton X-100 for 5 min at room temperature and treated with 0.1% sodium borohydride for 10 min. Fixed and live cell imaging was performed on a Leica AOBS confocal microscope and a Perkin Elmer Ultra*VIEW* LCI respectively.

Electron Microscopy, Liposome Tubulation and Sedimentation, and Radioligand Trafficking Assays

See Supplemental Data.

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures and two movies are available at http://www.current-biology.com/cgi/content/full/14/20/1791/DC1/.

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