Regulation of endosomal clathrin and retromer-mediated endosome to Golgi retrograde transport by the J-domain protein RME-8

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After endocytosis, most cargo enters the pleiomorphic early endosomes in which sorting occurs. As endosomes mature, transmembrane cargo can be sequestered into inwardly budding vesicles for degradation, or can exit the endosome in membrane tubules for recycling to the plasma membrane, the recycling endosome, or the Golgi apparatus. Endosome to Golgi transport requires the retromer complex. Without retromer, recycling cargo such as the MIG-14/Wntless protein aberrantly enters the degradative pathway and is depleted from the Golgi. Endosome-associated clathrin also affects the recycling of retrograde cargo and has been shown to function in the formation of endosomal subdomains. Here, we find that the *Caenorhabditis elegans* endosomal J-domain protein RME-8 associates with the retromer component SNX-1. Loss of SNX-1, RME-8, or the clathrin chaperone Hsc70/HSP-1 leads to over-accumulation of endosomal clathrin, reduced clathrin dynamics, and missorting of MIG-14 to the lysosome. Our results indicate a mechanism, whereby retromer can regulate endosomal clathrin dynamics through RME-8 and Hsc70, promoting the sorting of recycling cargo into the retrograde pathway.

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Introduction

RME-8 was originally identified in *Caenorhabditis elegans* in our earlier genetic screens, isolated as a temperature sensitive lethal mutant defective in endocytosis (Grant and Hirsh, 1999; Zhang *et al.*, 2001). Further analysis showed that RME-8 is normally abundant on endosomes, but not the plasma membrane, suggesting a function for RME-8 in endosome function rather than plasma membrane uptake processes (Zhang *et al.*, 2001). Subsequently, *rme-8* mutants were

also identified in genetic screens in *Drosophila*, showing endocytic defects in several tissues (Chang *et al.*, 2004). Further analysis also showed that as in *C. elegans*, *Drosophila* RME-8 protein was associated with endosomes and not the plasma membrane (Chang *et al.*, 2004). Likewise, the RME-8 orthologue in mammals is tightly associated with the membranes of endosomes, but is not enriched on the plasma membrane (Girard *et al.*, 2005; Fujibayashi *et al.*, 2008). In plants, RME-8 is also endosome associated and is required for gravitropism, probably because of abnormalities in the vacuole of *rme-8* mutant cells that alters amyloplast sedimentation (Silady *et al.*, 2008). Despite all of these observations, an understanding of the precise membrane trafficking defect in *rme-8* mutants has remained elusive.

RME-8 in all these species is a large protein (>200 kDa) with highly conserved features including a central DNA J-domain and four repeated motifs (IWN repeats) of unknown function (Zhang *et al.*, 2001; Chang *et al.*, 2004; Silady *et al.*, 2004; Girard *et al.*, 2005). DNA J-domains are known to bind to the ubiquitous Hsc70 chaperone, and J-domains in general are a characteristic feature of Hsc70 co-chaperones, proteins that recruit Hsc70 to specific cellular compartments and stimulate Hsc70 ATPase activity (Walsh *et al.*, 2004). For example, the auxillin DNA J-domain protein recruits and activates Hsc70 on clathrin-coated vesicles to facilitate uncoating, a prerequisite for downstream fusion steps (Ungewickell *et al.*, 1995). Although auxillin and its paralogue GAK mediate this function for CCVs derived from the plasma membrane and Golgi (Greener *et al.*, 2001; Eisenberg and Greene, 2007), RME-8 is the only DNA J-domain protein reported to localize to endosomes. *Drosophila* and human RME-8 J-domains have been shown to physically interact with Hsc70 (Chang *et al.*, 2004; Girard *et al.*, 2005). *Drosophila* Hsc70 mutants interact with *rme-8* mutants genetically, and dominant negative Hsc70 expressed in mammalian cells impairs endosome function (Newmyer and Schmid, 2001; Chang *et al.*, 2002). These results suggested that RME-8 could mediate its effects on endosomes through a role as an endosomal Hsc70 co-chaperone.

The early endosome is known to be a hub for the sorting of membrane proteins after endocytosis. In the endosome, cargo proteins can either be delivered to lysosomes through multivesicular bodies (MVBs) for degradation, recycled to the plasma membrane directly or indirectly through the recycling endosome, or can be recycled through retrograde transport from endosomes to the trans-Golgi network (TGN). The retrograde pathway and retromer, the major regulatory complex associated with this pathway, are vital for the retrieval of the Golgi sorting receptors from endosomes to the TGN in yeast and mammalian cells (Bonifacino and Hurley, 2008). This includes receptors that transport degradative enzymes to the vacuole/lysosome such as Vps10 and CI-MPR (cation-independent mannose 6-phosphate receptor) (Marcusson

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et al, 1994; Arighi *et al*, 2004; Carlton *et al*, 2004; Seaman, 2004). Recently, retromer-dependent retrieval of the Wnt-ligand chaperone MIG-14/Wntless has also been shown (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008).

Retromer consists of a core complex Vps5-Vps17 (SNX1/2-SNX5/6 in mammals) and a cargo recognition complex Vps26-Vps29-Vps35 (Bonifacino and Hurley, 2008; Collins, 2008). In addition to retromer, clathrin and clathrin-related proteins (such as epsinR and AP-1) have also been reported to function as components of the retrograde transport machinery. Clathrin and clathrin adaptor epsinR have been shown to be required for retrograde transport of the Shiga toxin B subunit (Lauvrak *et al*, 2004; Saint-Pol *et al*, 2004). Clathrin adaptor AP-1 is also required for retrograde transport of mannose 6-phosphate receptor MPR46 (Meyer *et al*, 2000). Vps27/Hrs together with the ESCRT-I,II,III (endosomal sorting complex required for transport) complexes also functions on early endosomes and MVBs, but rather than promoting retrograde recycling, these proteins are best known for promoting degradation of integral membrane proteins (Katoh *et al*, 2003; Odorizzi *et al*, 2003). Importantly, Hrs has been linked to the accumulation of endosomal clathrin, promoting the formation of degradative ESCRT-enriched endosomal subdomains (Raiborg *et al*, 2001a, 2002).

In this study, we analyse retrograde transport in *C. elegans* and identify an earlier unsuspected mechanism for the regulation of endosomal clathrin that is required for retrograde transport from endosomes to the Golgi. We show that the J-domain protein RME-8 binds to SNX-1, and that loss of function in *rme-8* or *snx-1*, or depletion of *C. elegans* Hsc70 (HSP-1) by RNAi, disrupts endosome to Golgi transport of the retromer-dependent cargo protein MIG-14/Wntless. In the absence of any of RME-8, SNX-1, or HSP-1/Hsc70, MIG-14 is missorted to the late endosome and lysosome and is depleted from the Golgi. Furthermore, we show that loss of any of these three proteins leads to accumulation of clathrin on endosomes and loss of endosomal clathrin dynamics. Our work shows that retromer, through RME-8 and Hsc70, acts to limit clathrin accumulation, a prerequisite for the recycling of retrograde cargo. In the absence of this regulation, the retrograde transport route is lost and cargo that should be retrieved from the endosome is instead degraded.

Results

RME-8 physically interacts with SNX-1

To better understand the function of RME-8 as an endosomal regulator, we screened for interacting proteins using the yeast two-hybrid system. The bait construct included part of the DNA J-domain and the entire C-terminal half of the RME-8 protein (amino-acids 1337–2279; Figure 1C) including IWN repeats 3 and 4 and the intervening Armadillo (ARM)-like region. One clone encoding a portion of the retromer component SNX-1 was recovered from this interaction screen (see Materials and methods). Further analysis showed that full-length SNX-1 also interacts with RME-8 in this assay (data not shown). Successively, smaller regions of RME-8 were used as bait, narrowing the interacting region to amino-acids 1388–1950 (Figure 1B and C). Deletion of amino-acids 1388–1619 or 1732–1950 of RME-8 abrogated the interaction, indicating a requirement for RME-8 sequences corresponding

to C-terminal region beyond the DNA J-domain, including the adjacent linker between the J-domain and IWN3, IWN3 itself, and the ARM-like domain (Figure 1B and C). Sequences C-terminal to the ARM-like domain, including IWN4, were dispensable for the interaction.

Residues 423–466 of SNX-1, corresponding to a portion of helix 3 of the SNX-1 VPS5/BAR domain, were necessary and sufficient for the interaction in the yeast two-hybrid system (Figure 1A and C). This region of the SNX-1 BAR domain is phylogenetically highly conserved, and molecular modelling of the SNX-1 BAR domain suggests that it would be exposed to the cytoplasm, away from the membrane-binding surface, in which it could interact with other proteins (data not shown).

We also confirmed the binding and the specificity of the RME-8/SNX-1 interaction using an independent glutathione S-transferase (GST)-pull down assay. Recombinant GST-SNX-1 (aa 271–472), GST-Y59A8B.22/Snx5, GST-LST-4/Snx9, or GST only was immobilized on glutathione sepharose beads and incubated with *in vitro* transcribed and translated HA-tagged RME-8 (aa 1337–2279). Only GST-SNX-1, but not other SNX proteins, pulled down RME-8 (Figure 1D; Supplementary Figure 1).

RME-8 colocalizes with SNX-1

SNX-1 is the only *C. elegans* homologue of mammalian Sorting Nexin 1 and Sorting Nexin 2, PX and BAR domain proteins that function in endosome to Golgi retrograde transport with VPS-26, VPS-29, and VPS-35 as part of the retromer complex (Bonifacino and Hurley, 2008). The physical association of RME-8 with SNX-1 suggested that RME-8 might function with the retromer complex on endosomes to mediate retrograde transport. If the RME-8/SNX-1 physical interaction is functionally relevant *in vivo*, then the two proteins would be expected to colocalize on endosomes. Indeed, we found that mCherry-RME-8 colocalized extensively with GFP-SNX-1 in several different tissues, including the intestine, the hypodermis, and coelomocytes (Figure 2A–A''; Supplementary Figure 2A–B'').

RME-8 and SNX-1 localize to early endosomes but not Golgi

In other organisms, the retromer complex, including Snx1 and Snx2, is found primarily on early endosomes, and to a lesser extent on or near the Golgi apparatus (Bonifacino and Hurley, 2008). As expected, we found that in *C. elegans*, mCherry-SNX-1 colocalized well with early endosomal markers GFP-EEA-1 and GFP-RAB-5 in several tissues of living animals (Figure 2B–B''; Supplementary Figure 3). GFP-RME-8 also colocalized well with early endosome markers (Figure 2D–D''; Supplementary Figure 4). Similar to all invertebrate Golgi, *C. elegans* Golgi appears as dispersed ministacks throughout the cell rather than in one large juxtanuclear stack (Grant and Sato, 2006). In the intestine, the Golgi ministacks are similar in size to early endosomes (Chen *et al*, 2006). We found very little direct overlap of mCherry-SNX-1 or mCherry-RME-8 with Golgi marker Mannosidase (MANS)-GFP (Figure 2C–C'' and E–E''). Rather, we noted that RME-8 and SNX-1-labelled endosomes were very often directly juxtaposed to MANS-labelled Golgi, a localization that could potentially facilitate retrograde transport (Figure 2C'' and E'', note insets). GFP-RAB-5 also shows a similar high incidence of juxtaposition to MANS-labelled Golgi, further

Figure 1 RME-8 physically interacts with SNX-1. (A) The interaction between RME-8 and SNX-1 requires C-terminal SNX-1 residues span 423–466. RME-8 (residues 1337–2279) was expressed in a yeast reporter strain as a fusion with the DNA-binding domain of LexA (bait). SNX-1 and its truncated forms were expressed in the same yeast cells as fusions with the B42 transcriptional activation domain (prey). Interaction between bait and prey was assayed by complementation of leucine auxotrophy (LEU2 growth assay). Colonies were diluted in liquid and spotted on solid growth medium directly or after further 10X dilution. (B) The interaction of RME-8 with SNX-1 requires RME-8 sequences C-terminal to the DNA J-domain. SNX-1 (residues 221–472) was expressed in a yeast reporter strain, a fusion with the B42 transcriptional activation domain (prey). Mutant forms of RME-8 were expressed in the same yeast cells as a fusion with the DNA-binding domain of LexA (bait). Interaction between bait and prey was assayed by complementation of leucine auxotrophy (LEU2 growth assay) as above. (C) Schematic representations of SNX-1 and RME-8 and the regions of each used in the Y2H analysis. Protein domains are displayed as boxes (white for the ARM-like domain, dark for others) above protein sequences used in the study (shown as dark lines). Amino-acid numbers are indicated. (D) Glutathione beads loaded with recombinant GST or GST-SNX-1(271–472) were incubated with *in vitro* expressed HA-RME-8(1337–2279), and then washed to remove unbound proteins. Bound proteins were eluted and analysed by western blot using anti-HA (top) or anti-GST (bottom) antibodies. Input lane contains *in vitro* expressed HA-RME-8(1337–2279) used in the binding assays (10%).

suggesting a close association of a population of endosomes with Golgi ministacks (data not shown).

MIG-14/Wntless is missorted to the late endosome and lysosome in *rme-8* and *snx-1* mutants

Earlier analysis of Golgi-resident retromer-dependent cargo proteins, including the mammalian CI-MPR (cation-independent mannose-6-phosphate receptor) and the *C. elegans*, *Drosophila*, and human MIG-14/Wntless proteins, showed that in the absence of retromer function, such cargos were depleted from the Golgi and missorted to the late endosome and lysosome (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). The resulting increase in CI-MPR and MIG-14/Wntless lysosomal degradation leads to reduced steady-state levels of these cargo proteins in the cell (Rojas *et al*, 2007). Thus, we reasoned that if RME-8 functions with SNX-1 in retrograde transport, rather than in endocytic uptake at the plasma

membrane, then *rme-8* mutants should missort cargo in the same manner as *snx-1* mutants. Conversely, defective endocytosis of such cargo would be expected to lead to cargo accumulation at the plasma membrane, as was earlier observed for MIG-14-GFP in *dpy-23/mu2-adaptin* mutants (Pan *et al*, 2008). To date, the only retromer-dependent cargo protein known in *C. elegans* is MIG-14 (Pan *et al*, 2008; Yang *et al*, 2008). Thus, to determine whether RME-8 is required for retrograde transport, we assayed for changes in MIG-14-GFP endocytic sorting, comparing wild-type animals with *rme-8* mutants. We also assayed for changes in MIG-14-GFP localization in *snx-1* deletion mutants.

As expected in wild-type animals, MIG-14-GFP colocalized well with the early endosome marker mCherry-RAB-5 and the Golgi marker MANS-mCherry, but not with mCherry-RAB-7, a marker for late endosomes and lysosomes (Supplementary Figure 5A–A'', E–E''; Figure 3O–O''). Consistent with the proposition that RME-8 functions with SNX-1 in retrograde

Figure 2 RME-8 colocalizes with SNX-1 and RAB-5 on early endosomes. Representative images from deconvolved 3D image stacks are shown. All images were acquired in intact living animals expressing GFP and mCherry-tagged proteins specifically in intestinal epithelial cells. (A–A'') mCherry-RME-8 colocalizes with GFP-SNX-1. Arrowheads indicate endosomes labelled by both GFP-SNX-1 and mCherry-RME-8. (B–B'') mCherry-SNX-1 colocalizes with early endosome marker GFP-RAB-5. Arrowheads indicate endosomes labelled by both GFP-RAB-5 and mCherry-SNX-1. (C–C'') mCherry-SNX-1 does not colocalize well with Golgi marker MANS-GFP. Arrowheads and the inset indicate mCherry-SNX-1 positive endosomes juxtaposed to MANS-GFP-labelled Golgi ministacks. (D–D'') mCherry-RME-8 colocalizes with early endosome marker GFP-RAB-5. Arrowheads indicate endosomes labelled by both GFP-RAB-5 and mCherry-RME-8. (E–E'') mCherry-RME-8 does not colocalize well with Golgi marker MANS-GFP. Arrowheads and the inset indicate mCherry-RME-8 positive endosomes juxtaposed to MANS-GFP-labelled Golgi ministacks. Enlarged images ($\times 4$) of boxed regions are shown in the insets. In each image, autofluorescent lysosome-like organelles can be seen in all three channels with the strongest signal in blue, whereas GFP appears only in the green channel and mCherry only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or mCherry signals, respectively. Scale bar represents 10 μ m.

transport, both *rme-8* and *snx-1* mutants displayed a greater than 10-fold reduction in average intestinally expressed MIG-14-GFP fluorescence intensity compared with wild-type con-

trols (Figure 3A–C'', quantified in Figure 3H). Furthermore, overexpression of RFP fused to SNX-1(423–466), the 43 amino-acid fragment of SNX-1 identified above that interacts

with RME-8, resulted in a three-fold reduction in MIG-14-GFP fluorescence, presumably by interfering with the association of the endogenous SNX-1 and RME-8 proteins (Supplementary Figure 11A–C). The reduction in MIG-14-GFP signal in all these backgrounds is equivalent to the phenotype earlier reported for MIG-14-GFP in *vps-35* mutants (Pan *et al*, 2008; Yang *et al*, 2008). In the case of the *rme-8* mutant, which is temperature sensitive, the dramatic redistribution of MIG-14-GFP only occurred at the restrictive temperature (data not shown). We observed similar reductions in MIG-14-GFP levels in *rme-8* mutant early embryos, indicating that the requirement for RME-8 in sorting of retrograde cargo is not cell-type specific (Supplementary Figure 5I and J).

As controls, we assayed the distribution of earlier characterized model transmembrane cargos that recycle through the recycling endosome and not the Golgi: the human transferrin receptor (hTfR-GFP) and the IL-2 receptor alpha chain (hTac-GFP) (Chen *et al*, 2006; Shi *et al*, 2007). The localization and steady-state levels of these receptors in the intestine were unaffected by mutation of *rme-8* or *snx-1* (Figure 3I–N). Consistent with our results, transferrin endocytosis in mammalian cells is normal after depletion of RME-8 by RNAi (Girard *et al*, 2005).

In subsequent experiments, we boosted the remaining signal for intestinal MIG-14-GFP in mutant animals to determine the fate of MIG-14 under these conditions (Figure 3P–R'; Supplementary Figure 5B–D' and F–H'). Most of the remaining MIG-14-GFP protein in *rme-8* and *snx-1* mutants was found in ring-like late endosomes and lysosomes positive for mCherry-RAB-7, a localization not found in wild-type animals (Figure 3P–Q'). In *rme-8* and *snx-1* mutant cells, MIG-14-GFP colocalized with mCherry-RAB-7 on the apparent limiting membrane of these large endosomes and on mCherry-RAB-7 negative puncta apparently contained within the endosome, possibly intraluminal vesicles (Figure 3P–Q'). MIG-14-GFP could also still be observed in enlarged mCherry-RAB-5 labelled early endosomes in *rme-8* and *snx-1* mutants (Supplementary Figure 5B–C'). However, even with the boosted MIG-14-GFP signal, most MANS-labelled Golgi displayed only weak or adjacent MIG-14-GFP labelling in *rme-8* and *snx-1* mutants (Supplementary Figure 5F–G').

MIG-14/Wntless trafficking defects occur after endocytosis in *rme-8* and *snx-1* mutants

To further establish the altered trafficking itinerary of MIG-14-GFP in *rme-8* and *snx-1* mutants, we blocked endocytic uptake of MIG-14-GFP in the *rme-8* and *snx-1* mutant backgrounds by RNAi-mediated depletion of the mu2 subunit of the clathrin adaptor complex AP-2 (DPY-23) (Pan *et al*, 2008). This blockade at the cell surface restored MIG-14-GFP fluorescence to levels equal to or above wild type and enhanced cell surface localization of MIG-14-GFP in both *rme-8* and *snx-1* mutants (Figure 3D–E' and H), indicating that the trafficking defects associated with MIG-14 in *rme-8* and *snx-1* mutants occur after endocytosis, consistent with a function for both proteins in endosomal sorting events. We also inhibited lysosome-mediated degradation by depletion of CUP-5/mucopolipin1, a transmembrane protein required for normal lysosome biogenesis and normal levels of hydrolytic activity (Treusch *et al*, 2004). Such depletion of CUP-5 by RNAi blocked much of the abnormal degradation of MIG-14-GFP in *rme-8* and *snx-1* mutants (Figure 3F–G' and H),

indicating that loss of *rme-8* or *snx-1* leads to degradation of MIG-14 through missorting into the lysosomal pathway. Similarly, the loss of MIG-14-GFP in *rme-8* and *snx-1* mutants was also ameliorated by RNAi-mediated depletion of VPS-37, a component of the ESCRT-I complex (data not shown).

The increased degradation of MIG-14-GFP suggested that MVB-mediated transport of membrane proteins to the lysosome does not require RME-8 or SNX-1. To examine this point further, we also assayed degradation of CAV-1-GFP in early embryos (Sato *et al*, 2006; Shi *et al*, 2007). CAV-1 is a known transmembrane cargo protein that is degraded in the one cell embryo after the metaphase to anaphase transition (Sato *et al*, 2006, 2008a; Bembenek *et al*, 2007). Degradation of CAV-1-GFP requires endocytosis and ESCRT-mediated endosomal sorting (Sato *et al*, 2006; Audhya *et al*, 2007). CAV-1-GFP degradation was unaffected in *rme-8(b1023)* and *snx-1(tm847)* mutants, further suggesting that RME-8 and SNX-1 are not required for ESCRT-mediated degradation of integral membrane proteins (Supplementary Figure 8A–C').

Neuronal cell polarity is impaired in *rme-8* and *snx-1* mutants

The observed abnormal degradation of MIG-14 in *rme-8* and *snx-1* mutants would be expected to impair Wnt signalling, as shown earlier for *vps-35* mutants. To test for an effect of loss of RME-8 and SNX-1 on Wnt signalling, we examined the control of mechanosensory neuron polarity, a process that requires MIG-14 and retromer regulated Wnt signalling (Prasad and Clark, 2006; Pan *et al*, 2008). As *rme-8(b1023)* is lethal at 20°C or higher, we examined the *rme-8* effect on this phenotype at the permissive temperature (15°C), in which *rme-8* mutants display partially penetrant phenotypes. We found that *rme-8(b1023)* and *snx-1(tm847)* display defective ALM posterior processes at a penetrance similar to that shown earlier for *vps-35* mutants (Supplementary Figure 6A–C and G) (Pan *et al*, 2008). Defective PLM posterior processes were also observed in both mutants, although in this case, the *snx-1* null mutant showed a higher penetrance than the partial loss of RME-8 (Supplementary Figure 6D–F and H). These results are consistent with the requirements we identified for RME-8 and SNX-1 in the control of MIG-14 trafficking.

Depletion of *C. elegans* Hsc70 (HSP-1) causes MIG-14/Wntless missorting to the late endosome and lysosome

We next sought to better understand the mechanism by which RME-8 influences endosome function and regulates retrograde transport. Recent work has shown that retrograde transport of the glycosphingolipid-binding bacterial Shiga toxin (STxB), and another retrograde cargo protein TGN46, from the early endosome to the Golgi requires endosomal clathrin and the clathrin adaptor epsinR (Saint-Pol *et al*, 2004; Popoff *et al*, 2007). Depletion of RME-8 from the cells of several organisms has been shown to produce abnormal clathrin distribution *in vivo* (Chang *et al*, 2004; Girard *et al*, 2005), and similar to human and *Drosophila* RME-8, we found that the *C. elegans* RME-8 J-domain binds to HSP-1/Hsc70 *in vitro* (Supplementary Figure 11D). Thus, to better understand how RME-8 functions, we examined the relationship between endosomal clathrin, RME-8, and SNX-1. One simple model that could explain these observations would be that SNX-1 cooperates with RME-8 to activate endosomal Hsc70, and in turn Hsc70 chaperone activity on the endosome

Figure 4 Clathrin accumulates on endosomes in animals lacking RME-8, SNX-1, or HSP-1/Hsc70. Panels show confocal micrographs of intestinally expressed GFP-tagged clathrin heavy chain (GFP-CHC-1) in intact living animals (A–D). Note the increased GFP-CHC-1 intensity in *rme-8(b1023)*, *snx-1(tm847)*, and *hsp-1(RNAi)* animals. (E) Bar graph indicates average GFP-CHC-1 fluorescence intensity in the indicated genetic backgrounds. Asterisks indicate a significant difference in the one-tailed Student's *t*-test ($P < 0.01$). P -values for GFP-CHC-1 were 4.27×10^{-17} for wild type (WT) versus *rme-8(b1023)*, 5.92×10^{-26} for WT versus *snx-1(tm847)* and 3.05×10^{-19} for WT versus *hsp-1(RNAi)*. For panels F–M', images from deconvolved 3D image stacks are shown. All images were acquired in intact living animals expressing GFP and mCherry-tagged proteins specifically in intestinal epithelial cells. Autofluorescent lysosome-like organelles are shown in blue. GFP-CHC-1 could be visualized on WT and mutant mCherry-RAB-5-labelled endosomes in WT and mutants. Arrowheads indicated colocalization of the GFP-CHC-1 and mCherry-RAB-5 signals (F–I). GFP-CHC-1 could be visualized on WT and *snx-1* mutant mCherry-RME-8-labelled endosomes. Arrowheads indicated colocalization of the GFP-CHC-1 and mCherry-RME-8 signals (J–K'). GFP-CHC-1 could be visualized on WT and *rme-8* mutant mCherry-SNX-1-labelled endosomes. Arrowheads indicated colocalization of the GFP-CHC-1 and mCherry-SNX-1 signals (L–M'). Scale bars represent 10 μ m.

controls endosomal clathrin dynamics to promote retrograde sorting.

Consistent with this model, we found that depletion of *C. elegans* Hsc70 (HSP-1) by RNAi resulted in aberrant sorting of MIG-14-GFP, very similar to the phenotype observed for *rme-8* and *snx-1* mutants (Figure 3R–R'; Supplementary Figure 5D–D'', H–H''). After HSP-1 RNAi, MIG-14-GFP levels were reduced and most of the remaining MIG-14-GFP signal was found within large mCherry-RAB-7-labelled endosomes/lysosomes (Figure 3R–R'). Similar to the effects observed in *rme-8* and *snx-1* mutants, *hsp-1(RNAi)* led to MIG-14-GFP labelling of the mCherry-RAB-7-labelled limiting membrane and smaller mCherry-RAB-7 negative puncta that appeared to be within the RAB-7-labelled ring (Figure 3R–R'). GFP-SNX-1 and GFP-VPS-35 protein levels were not reduced in *rme-8(b1023)* or *hsp-1(RNAi)* animals, suggesting that the defects in retrograde transport were not through effects on retromer protein stability (Supplementary Figure 7A and B). The congruence of *snx-1*, *rme-8*, and *hsp-1* phenotypes indicates that all three proteins function in a common process.

Loss of SNX-1, RME-8, or HSP-1 leads to clathrin accumulation on endosomes

Next, we examined the localization of a fully functional GFP-tagged clathrin heavy chain (GFP-CHC-1) (Greener *et al*, 2001; Sato, 2009). In *rme-8* and *snx-1* mutants, GFP-CHC-1

accumulated in abnormally bright puncta throughout the cell, suggesting an accumulation of membrane-bound clathrin (Figure 4A–C, quantified in Figure 4E; Supplementary Figure 9E and F). A similar effect was observed in GFP-CHC-1 expressing animals after RNAi-mediated depletion of HSP-1/Hsc70 (Figure 4D and E). Further analysis showed that many of the intracellular GFP-CHC-1 puncta were positive for mCherry-RAB-5, indicating that much of the accumulated clathrin was on early endosomes (Figure 4F–I; Supplementary Figure 9A–D''). The observed enlargement of early endosomes did not require overexpression of clathrin, as enlargement was also found in GFP-RAB-5 and GFP-RAB-10 positive early endosomes in *rme-8* mutants not expressing GFP-CHC-1 (Supplementary Figure 9G–J). Further analysis showed that in *rme-8* mutants, mCherry-SNX-1 and GFP-CHC-1 accumulated together in these structures (Figure 4L–M''). Single-labelled GFP-SNX-1 or GFP-CHC-1 puncta also appeared morphologically abnormal in other tissues of *rme-8* mutants (Supplementary Figure 9E, F, and K–N). Likewise in *snx-1* mutants, mCherry-RME-8 and GFP-CHC-1 co-accumulated on the same structures (Figure 4J–K'). These results indicate that RME-8, SNX-1, and HSP-1/Hsc70 are all important for the regulation of endosomal clathrin, and that the presence of only RME-8 or SNX-1 on the endosome is not sufficient for proper clathrin regulation.

Loss of SNX-1, RME-8, or HSP-1/Hsc70 impairs clathrin dynamics

Expression of ATPase-defective forms of Hsc70 in mammalian cells is known to result in the loss of the unassembled cytosolic pool of clathrin (Newmyer and Schmid, 2001). Likewise, in *Drosophila* Hsc70 partial loss-of-function mutants, GFP-tagged clathrin was shown to aggregate within the cell, although the labelled compartment was not identified (Chang *et al*, 2002). These results are consistent with the phenotype we show above for *hsp-1/Hsc70* RNAi in *C. elegans*. *In vivo* membrane-bound clathrin is highly dynamic as evidenced by its rapid exchange with free cytosolic clathrin (Newmyer and Schmid, 2001; Lee *et al*, 2005). This dynamic exchange of cytosolic and membrane-bound clathrin is generally measured using fluorescence recovery after photobleaching (FRAP) in cells expressing GFP-tagged clathrin (Greener *et al*, 2001; Wu *et al*, 2001). In the absence of Hsc70 co-chaperone activity, or in the absence of Hsc70 itself, the levels of membrane-associated GFP-clathrin would be expected to increase, and exchange of such GFP clathrin with cytosolic pools would be expected to decrease, interfering with fluorescence recovery. We compared the recovery after photobleaching of GFP-CHC-1 puncta in wild-type animals with those lacking RME-8, SNX-1, or HSP-1/Hsc70 because of mutation or RNAi. Consistent with a function for these three proteins functioning together to regulate clathrin dynamics/disassembly on endosomes, GFP-CHC-1 fluorescence recovery in cytoplasmic puncta was greatly impaired in animals deficient in RME-8, SNX-1, or HSP-1/Hsc70 (Figure 5A–E). HSP-1 RNAi produced a weaker effect than mutation of RME-8 or SNX-1, likely because of residual HSP-1 after RNAi treatment, and/or the expression of additional Hsc70 homologues in the animal.

Intramolecular interactions within RME-8

The above data suggests that SNX-1 is required for RME-8 function, but not for RME-8 stability or localization. Many proteins are not constitutively active, but are rather autoinhibited and can only achieve full activity when autoinhibitory interactions are disrupted through intermolecular binding to a partner protein. As first step towards understanding the mechanism of RME-8 regulation, we sought to determine whether the RME-8 DNA J-domain has the potential to form intramolecular interactions with other domains within RME-8. To test this idea, we separated the J-domain (aa 1322–1388) from regions of RME-8 C-terminal to the J-domain (aa 1388–2279) and assayed for interaction between the two pieces of RME-8. Yeast two-hybrid and GST-pull down assays both indicated that the J-domain of RME-8 can bind to the C-terminal half of RME-8 (Supplementary Figure 10A and B), indicating that *in vivo* the J-domain of RME-8 may be occupied in an intramolecular interaction.

Discussion

In this work, we have shown that RME-8 and SNX-1 are required to rescue MIG-14 from degradation after its endocytosis. Our results showing the aberrant sorting and degradation of MIG-14 in *snx-1* and *rme-8* mutants are quite similar to those earlier shown for CI-M6PR after co-depletion of Snx1 and Snx2, or single depletion of other retromer components, in mammalian cells (Rojas *et al*, 2007). Our results are also

reminiscent of the aberrant sorting and degradation of the EGF-R after siRNA-mediated depletion of RME-8 in mammalian cells (Girard and McPherson, 2008). In fact, this similarity in phenotype may indicate that a fraction of EGF-Rs recycle through the retrograde endosome to Golgi pathway.

However, CI-M6PR steady-state levels were not reported to be reduced after siRNA-mediated depletion of RME-8 (Girard *et al*, 2005). Rather, CI-M6PR was reported to aberrantly accumulate in or near the Golgi (Girard *et al*, 2005). We do not yet understand this apparent difference in RME-8 and Snx1/2 phenotype with respect to M6PR, but suspect that they may reflect differences in the requirements for RME-8 and retromer in sorting of specific cargo, and/or their requirements in alternative retrograde pathways. For instance, in addition to the early endosome to Golgi route, the CI-M6PR is known to recycle to the Golgi through the late endosome, using a Rab9-dependent pathway (Lombardi *et al*, 1993). In addition, after endocytosis, CI-M6PR retrieval to the Golgi has been shown to be mediated by the recycling endosome (Lin *et al*, 2001, 2004). RME-8 may be specific to the early endosome, whereas retromer may be more generally required. In the worm, MIG-14-GFP trafficking was identically affected by loss of RME-8 or SNX-1. It is not known whether worm and mammalian MIG-14 have access to alternative retrograde routes. It is also worth noting that in the case of the worm, the Rab9 pathway may not even exist, as the *C. elegans* genome lacks a Rab9 homologue.

With regard to the early endosome, our data indicates that RME-8 and SNX-1 are important clathrin regulators, and strongly suggests that SNX-1 and RME-8 regulate endosomal clathrin disassembly through HSP-1/Hsc70. Earlier analysis has indicated that clathrin functions on endosomes, at least in part, to create endosomal subdomains (Raiborg *et al*, 2001b, 2002, 2006). Such subdomains have been implicated in the sorting of cargo into the degradative compartment through their association with the ubiquitin and clathrin scaffolding protein Hrs. Hrs, along with its partner STAM, is often referred to as ESCRT-0 to denote its physical connection to the TSG101 component of the ESCRT-I complex and its function in cargo recognition. The ESCRT machinery functions to sort mono-ubiquitinated integral membrane cargo into intraluminal vesicles of the endosome, leading to their degradation in the lysosome. Hrs binds to ubiquitinated cargo and to endosomal clathrin and when Hrs is depleted, endosomal clathrin is lost (Raiborg *et al*, 2001b, 2002, 2006). In the absence of Hrs or endosomal clathrin, the normal subdomain organization of the early endosome is disrupted (Raiborg *et al*, 2001b, 2002, 2006).

Within these same endosomes, clathrin has been recently shown to regulate endosomal sorting into the retrograde recycling pathway, somehow cooperating with the retromer complex to recycle cargo from endosomes to the Golgi (Saint-Pol *et al*, 2004). Related work suggests that clathrin functions early in the sorting process (Popoff *et al*, 2007). In the absence of clathrin, retrograde sorting is altered (Saint-Pol *et al*, 2004; Popoff *et al*, 2007; Skanland *et al*, 2009). In the case of ricin toxin, clathrin depletion increases transport from endosomes to the Golgi (Skanland *et al*, 2009). Our work indicates that the SNX-1 component of retromer, earlier known to provide membrane-binding and curvature-inducing abilities to the complex, also physically interacts with RME-8 to negatively regulate endosomal clathrin accumulation and

Figure 5 Clathrin dynamics are impaired in *rme-8(b1023)*, *snx-1(tm847)*, and *hsp-1(RNAi)* knock-down animals. (A–D'') Representative images of GFP-CHC-1 in wild-type control, *rme-8(b1023)*, *snx-1(tm847)*, and *hsp-1(RNAi)* animals before bleaching (prebleach), immediately after bleaching (bleach) and 100 s after bleaching (100 s). Bleached areas are shown by white-bordered circles. Fluorescence intensities in bleached areas were recorded every 10 s. (E) Mean time courses of FRAP ($\pm$ s.d.) from eight animals of each genotype as indicated with WT (wild-type control), *rme-8(b1023)*, *snx-1(tm847)*, and *hsp-1(RNAi)*. Error bars represent standard deviations from the mean ($n=8$ each time point, eight animals of each genotype were sampled in the intestine). Scale bar represents 10 μ m.

promote clathrin dynamics, such that sorting into the retrograde pathway can proceed. In the absence of the SNX-1/RME-8/Hsc70-mediated regulation, degradative sorting appears to continue, but is deregulated, depleting the cell of cargo that would normally be recycling to the Golgi. Thus, with respect to clathrin, SNX-1, RME-8, and HSP-1/Hsc70 appear to act oppositely to Hrs in the endosome (Supplementary Figure 10C). The opposing activities of Hrs and RME-8 on clathrin accumulation could maintain an equilibrium between endosomal subdomains, such that degradative and recycling functions can coexist in the same endosome.

Unlike the well-studied clathrin co-chaperones auxilin and GAK, RME-8 does not appear to bind directly to clathrin (Girard *et al*, 2005). Interestingly, recent studies showed that the N-terminal PX domains of several sorting nexins, including human SNX-1 orthologues Snx1 and Snx2, bind to clathrin through an inverted clathrin box motif (Skanland *et al*, 2009). This inverted clathrin-binding motif is identical in *C. elegans* and human SNX-1/Snx1, suggesting that SNX-1 family proteins could act to bridge RME-8 and clathrin, thus completing the Hsc70 to clathrin connection.

An additional, and not mutually exclusive, model for the function of RME-8/SNX-1 binding is that the RME-8 J-domain might normally be autoinhibited by interaction with other domains within RME-8. If so, then SNX-1 binding to RME-8 might relieve this autoinhibition, allowing the J-domain to bind and stimulate Hsc70. This idea is supported by our observation that the isolated RME-8 J-domain can bind to the C-terminal half of RME-8 *in vitro*. Significant future work will be required to better understand the relationship between RME-8 and SNX-1, testing such models to obtain greater insight into the formation and maintenance of endosomal subdomains and retrograde transport.

Materials and methods

General methods and strains

All *C. elegans* strains were derived originally from the wild-type Bristol strain N2. Worm cultures, genetic crosses, and other *C. elegans* husbandry were performed according to standard protocols (Brenner, 1974). Strains expressing transgenes were grown at 20°C. A complete list of strains used in this study can be found in Supplementary Table 1.

RNAi was performed using the feeding method (Timmons and Fire, 1998). Feeding constructs were either from the Ahringer library (Kamath and Ahringer, 2003) or prepared by PCR from EST clones provided by Dr Yuji Kohara (National Institute of Genetic, Japan) followed by subcloning into the RNAi vector L4440 (Timmons and Fire, 1998). For most experiments, synchronized L1 or L3 stage animals were treated for 48–72 h and were scored as adults.

Antibodies

The following antibodies were used in this study: mouse anti-HA monoclonal antibody (16B12) Covance Research Products (Berkeley, CA) and rabbit anti-GST polyclonal antibody (Z-5) Santa Cruz Biotechnologies (Santa Cruz, CA).

Yeast two-hybrid analyses

The yeast two-hybrid screen was performed using the Gal4-based Proquest System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The pDBLeu-RME-8 bait plasmid, containing the C-terminal 951 amino acids of RME-8 (aa 1337–2279), was transformed into yeast strain MaV203. MaV203 bearing the bait plasmid was amplified and transformed with the Vidal *C. elegans* library in prey vector PC86, essentially as described (Walhout *et al*,

2000). Approximately four million clones were screened on histidine dropout plates. A single positive clone was recovered and re-tested as positive in the His and β -Gal assays after isolation and re-transformation with fresh bait plasmid. This positive clone contained a portion of a *snx-1* cDNA encoding the C-terminal 251 amino acids (aa 221–472). The LexA-based DupLEX-A yeast two-hybrid system (OriGene Technologies Inc., Rockville, MD) was used for all subsequent analysis, according to the manufacturer's instructions. pSH18-34 was used as reporter in all the yeast two-hybrid experiments. Constructs were introduced into the yeast strain EGY48 included in the system. To assess the expression of the *LEU2* reporter, transformants were selected on plates lacking leucine, histidine, tryptophan, and uracil, containing 2% galactose/1% raffinose at 30°C for 3 days. Blue/white β -galactosidase assays, performed according to manufacturer's instructions, confirmed results shown for growth assays.

Protein expression and coprecipitation assays

N-terminally HA-tagged proteins: RME-8 (aa 1337–2279), RME-8 (aa 1388–2279), and HSP-1 were synthesized *in vitro* using the TNT coupled transcription-translation system (Promega) using DNA templates pcDNA3.1-2xHA-RME-8(1337–2279), pcDNA3.1-2xHA-RME-8(1388–2279), and pcDNA3.1-2xHA-HSP-1, respectively (1.6 μ g/each 50 μ l reaction). The reaction cocktail was incubated at 30°C for 90 min. Control GST, GST-SNX-1(aa 271–472), GST-Y59A8B.22, GST-LST-4, GST-RME-8(aa 1322–1388) fusion proteins were expressed in the ArcticExpress strain of *Escherichia coli* (Stratagene). Bacterial pellets were lysed in 10 ml B-PER Bacterial Protein Extraction Reagent (Pierce) with Complete Protease Inhibitor Cocktail Tablets (Roche). Extracts were cleared by centrifugation and supernatants were incubated with glutathione sepharose 4B beads (Amersham Pharmacia) at 4°C overnight. Beads were then washed six times with cold STET buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20). *In vitro* synthesized HA-tagged protein (10 μ l TNT mix diluted in 500 μ l STET) was added to the beads and allowed to bind at 4°C for 2 h. After six additional washes in STET, the proteins were eluted by boiling in 30 μ l 2 $\times$ SDS-PAGE sample buffer. Eluted proteins were separated on SDS-PAGE (10% polyacrylamide), blotted to nitrocellulose, and probed with anti-HA (16B12). Subsequently, the blots were stripped and re-probed with anti-GST (Z-5) antibodies.

Plasmids and transgenic strains

The Proquest system two-hybrid bait plasmid pDBLeu-RME-8(1337–2279) was generated as a PCR product from template cDNA clone yk398h12, cloned into the unique SalI-NotI sites of the vector. All Origene system two-hybrid plasmids were generated as PCR products with Gateway attB.1 and attB.2 sequence extensions, and were introduced into the Gateway entry vector pDONR221 by BP reaction. They were then transferred into the final destination vector using Gateway recombination cloning (Invitrogen) LR reaction. The bait vector pEG202-Gtwy and target vector pJG4-5-Gtwy have been described earlier (Sato *et al*, 2008b). Prey plasmids encoded full-length SNX-1 (aa 1–472) and SNX-1 truncations (aa 221–472, aa 271–472, aa 221–422, aa 271–466, and aa 423–466) were amplified from *snx-1* cDNA clone yk290f7. All amplified regions were confirmed by DNA sequencing. RME-8(1337–2279), RME-8(1388–2279), and HSP-1 (gift of Jon Audhya) cDNA clones were transferred into an in-house modified vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) with 2xHA epitope tag and Gateway cassette (Invitrogen, Carlsbad, CA) for *in vitro* transcription/translation experiments. For GST-pull down experiments, an equivalent *snx-1*(271–472) PCR product, Y59A8B.22/SNX5 PCR product (from cDNA clone yk1476h03), *lst-4/Snx4p* PCR product (from cDNA clone yk1061f06), and *rme-8*(1322–1388) PCR product were introduced in frame into vector pGEX-2T (GE Healthcare Life Sciences) modified with a Gateway cassette.

To create the GFP-SNX-1 plasmid driven by the *snx-1* promoter, 738 bp of *snx-1* promoter sequence was PCR amplified from *C. elegans* genomic DNA with primers containing Sal I and Kpn I restriction sites and cloned into the same sites in the *C. elegans* GFP vector pPD117.01 (gift of Andrew Fire). The entire *snx-1* gene body and 3' UTR (3848 bp) was then PCR amplified with primers including Ngo MI and Apa I restriction sites and cloned into the same sites downstream of GFP. The equivalent RFP-SNX-1 plasmid was created by cloning mRFP1 (gift of Roger Tsien) into the unique Kpn I and EcoR I sites, replacing GFP. The GFP-RME-8 and

mRFP1-RME-8 fusion plasmids and strains driven by the *rme-8* promoter was described earlier (Zhang *et al*, 2001; Treusch *et al*, 2004). To construct GFP or mCherry/RFP fusion transgenes for expression specifically in the worm intestine, two earlier described Gateway destination vectors (Chen *et al*, 2006) were used that contain the promoter region of the intestine-specific gene *vha-6* cloned into the *C. elegans* pPD117.01 vector, followed by GFP or mCherry/RFP coding sequences, a Gateway cassette (Invitrogen, Carlsbad, CA), and *let-858* 3' UTR sequences, followed by the *unc-119* gene of *C. briggsae*. The sequences of *C. elegans rme-8* (*minigene*), *snx-1*(cDNA), *snx-1*(423–466), and *vps-35*(genomic DNA) were cloned individually into entry vector pDONR221 by PCR and BP reaction, and then transferred into intestinal expression vectors by Gateway recombination cloning LR reaction to generate N-terminal fusions. The *mig-14* expression plasmid was created by PCR amplification and Gateway cloning of the *mig-14* cDNA (gift of Iva Greenwald), lacking a stop codon, into an earlier described *vha-6* promoter driven vector modified with a Gateway cassette inserted at the Asp718I site just upstream of the GFP coding region (Chen *et al*, 2006). Germline MIG-14 expression was driven by the *pie-1* promoter using Gateway vector pJK7 (gift of Jayne Squirrel). Complete plasmid sequences are available on request. Low copy integrated transgenic lines for all these plasmids were obtained by the microparticle bombardment method (Praitis *et al*, 2001).

Microscopy and image analysis

Live worms were mounted on 2% agarose pads with 10 mM levamisole as described earlier (Sato *et al*, 2005). Multi-wavelength fluorescence images were obtained using an Axiovert 200 M (Carl Zeiss MicroImaging, Oberkochen, Germany) microscope equipped with a digital CCD camera (C4742–12ER, Hamamatsu Photonics, Hamamatsu, Japan), captured using Metamorph software ver 6.3r2 (Universal Imaging, West Chester, PA), and then deconvolved using AutoDeblur Gold software ver 9.3 (AutoQuant Imaging, Watervliet, NY). Images taken in the DAPI channel were used to identify broad-spectrum intestinal autofluorescence caused by lipofuscin-positive lysosome-like organelles (Clokey and Jacobson, 1986; Hermann *et al*, 2005). To obtain images of GFP fluorescence without interference from autofluorescence, we used argon 488 nm excitation and the spectral fingerprinting function of the Zeiss LSM510 Meta confocal microscope system (Carl Zeiss MicroImaging) as described earlier (Chen *et al*, 2006). Quantification of images was performed with Metamorph software ver 6.3r2 (Universal Imaging).

Most GFP/mCherry colocalization experiments were performed on L3 and L4 larvae expressing GFP and mCherry markers as described earlier. In colocalization assays of MIG-14-GFP with mCherry-RAB-7, mCherry-RAB-5 and MANS-mCherry (Figure 3O–R"; Supplementary Figure 5A–H") in *rme-8(b1023)* and *snx-1(tm847)* mutants, or *hsp-1(RNAi)* knock-down animals, we boosted MIG-14-GFP intensity by doubling the exposure time (200 ms) compared with wild-type controls (100 ms). We further altered the scaling factor in the Metamorph software to reveal the low-intensity GFP signal in the mutant backgrounds. Without these changes to the imaging protocol, the MIG-14-GFP intensity was too weak to localize in these mutants.

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FRAP analysis

Worms were grown at 25°C overnight, then mounted live on 2% agarose pads with 10 mM levamisole and imaged at room temperature (approximately 22°C) on the stage of a Zeiss LSM 510 laser scanning confocal microscope using the argon 488 nm 25 mW laser and water-immersion objective ($\times 63$). GFP-CHC-1 fluorescence was photobleached in a small defined region with 30 iterations at 25% laser power/100% excitation. After bleaching, fluorescence intensity in the bleached area was monitored over time by scanning at 5% excitation every 10 s. Data sets in which the focal planes shifted because animal movements were discarded.

To assess differences in GFP-CHC-1 recovery in different mutants or RNAi knock-down animals, we determined recovery curves of fluorescence intensities at the same photobleaching setting and corrected for background versus time. Error bars represent standard deviations from the mean ($n = 8$ each time point, eight animals of each genotype were sampled). All data points shown were collected on the same day. Similar experiments carried out on two other occasions gave equivalent results.

Neuronal polarity assay

Transgenic line *zdl5[Pmec-4::gfp]* was used to assay neuronal polarity of mechanosensory ALM and PLM neurons as described earlier (Prasad and Clark, 2006; Pan *et al*, 2008). An ALM posterior process that was longer than five ALM cell body diameters in length was scored as defective (numbers of axons scored: wild type: 100; *rme-8(b1023)*: 120; *snx-1(tm847)*: 100). A PLM posterior process that extends to the tip of the tail was scored as defective (numbers of axons scored: wild type: 60; *rme-8(b1023)*: 90; *snx-1(tm847)*: 80).

Western analysis

Total protein extracts were made from synchronized *C. elegans* adult animals ($n = 40$ each). A measure of 40 μ l worm boil buffer (100 mM Tris pH 6.8, 8% SDS, 20 mM β -mercaptoethanol) was added to the pellet. The worm mixture was boiled for 60 min at 100°C, then checked for complete solubilization of protein under a dissecting microscope. Proteins were size separated by SDS-PAGE and transferred to nitrocellulose. Probing and visualization of GFP-SNX-1 and GFP-VPS-35 were performed using HRP-conjugated GFP Abs (1:10 000 dilution) incubated with the blot overnight at 4°C.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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