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A SNX3-dependent Retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion

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Abstract

Wnt proteins are lipid modified glycoproteins that play a central role in development, adult tissue homeostasis and disease. Secretion of Wnt proteins is mediated by the Wnt-binding protein Wntless (Wls), which transports Wnt from the Golgi network to the cell surface for release. It has recently been shown that recycling of Wls through a retromer-dependent endosome-to-Golgi trafficking pathway is required for efficient Wnt secretion, but the mechanism of this retrograde transport pathway is poorly understood. Here, we report that Wls recycling is mediated through a novel retromer pathway that is independent of the retromer sorting nexins SNX1-SNX2 and SNX5-SNX6. We found that the unrelated sorting nexin, SNX3, has an evolutionarily conserved function in Wls recycling and Wnt secretion and show that SNX3 interacts directly with the cargo-selective sub-complex of the retromer to sort Wls into a morphologically distinct retrieval pathway. These results demonstrate that SNX3 is part of an alternative retromer pathway that functionally separates the retrograde transport of Wls from other retromer cargo.

The classical retromer complex consists of a membrane-bound coat formed by the sorting nexins SNX1-SNX2 and SNX5-SNX6 (referred to as SNX-BAR sorting nexins)¹⁻⁴ and a cargo-selective sub-complex comprised of the subunits VPS26, VPS29 and VPS35^{5,6},

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which binds to a sorting motif in the cytoplasmic tail of cargo proteins⁷. The SNX-BAR sorting nexins are recruited to cargo containing endosomes through a phosphatidylinositol-3-monophosphate (PI3P) binding Phox homology (PX) domain, and utilize the carboxy-terminal Bin-amphiphysin-Rvs (BAR) domain to drive membrane deformation and to generate membrane tubules. In recruiting the cargo-selective sub-complex to the forming tubules, the SNX-BAR coat complex is thought to traffic cargo into a tubular-based endosomal sorting pathway⁸. One of the principal functions of this pathway is to mediate retrograde transport between endosomes and the *trans*-Golgi network (TGN), as has been established for cargo proteins such as Vps10p in yeast⁹ and the cation-independent mannose 6-phosphate receptor (CI-MPR) in mammals^{7, 10, 11}. It has recently been shown that Wls (also known as Evi or Sprinter)¹²⁻¹⁴ is also a retromer cargo¹⁵⁻¹⁹. Wls binds to the cargo-selective sub-complex and in mutants of the cargo-selective subunits, Wls is missorted and degraded in lysosomes, leading to a strong defect in Wnt secretion and downstream signaling¹⁵⁻¹⁹.

To further examine the function of the retromer complex in the Wnt secretion pathway, we studied the function of the SNX-BAR coat components in Wls recycling. We made the surprising discovery that the SNX-BAR sorting nexins, which are required for the retromer-dependent trafficking of all retromer cargo proteins that have been studied to date^{1, 6, 20-22}, are fully dispensable for Wls recycling and Wnt secretion. We show that the unrelated sorting nexin, SNX3, has an evolutionarily conserved function in the Wnt secretion pathway. SNX3 directly interacts with the cargo-selective subunits of the retromer in a complex that does not contain the SNX-BAR coat components. Furthermore, we show that the SNX3 retromer pathway sorts Wls into a retrieval pathway that is morphologically distinct from the SNX-BAR retromer pathway. Our results demonstrate that Wls recycling is mediated by a novel retromer pathway that separates the recycling of Wls from cargo proteins that take the classical SNX-BAR dependent retromer pathway. We propose that such uncoupling may be essential to achieve the tight regulation of Wnt secretion that is necessary for normal development and adult tissue homeostasis.

RESULTS

The SNX-BAR retromer sorting nexins are dispensable for Wls recycling and Wnt signaling in *C. elegans* and *Drosophila*

In *C. elegans*, mutation of the cargo-selective subunits of the retromer induces defects in several Wnt dependent processes^{23, 24}, including the EGL-20 (Wnt) dependent posterior migration of the left Q neuroblast descendants (QL.d). In contrast, we found that mutation of the single SNX1-SNX2 ortholog *snx-1*²³ and the single SNX5-SNX6 ortholog *snx-6*²⁵ did not induce defects in QL.d positioning (Fig. 1A). A comprehensive analysis of other Wnt dependent processes did not reveal defects either (Fig. S1D, Table S1), indicating that *snx-1* and *snx-6* are not required for Wnt signaling in *C. elegans*. Consistently, we found that *snx-1* and *snx-6* are dispensable for the retromer-dependent recycling of the *C. elegans* Wls ortholog MIG-14 (Fig. 1D). In contrast, recycling of the retromer cargo protein CED-1 was fully dependent on *snx-1* and *snx-6* (Fig. S2C)²⁵.

To extend these observations, we knocked down the single SNX5-SNX6 ortholog *Dsnx6* in the posterior compartment of the *Drosophila* wing imaginal disc by transgene mediated RNAi. The wing pouch is patterned along the dorsoventral axis by the Wnt protein Wingless (Wg)²⁶, which is expressed by cells that are located at the dorsoventral boundary of the disc (Fig. 2A). In the absence of Dvps35, Wg secretion is strongly reduced, resulting in accumulation of Wg in the producing cells and a loss of expression of the Wg target gene *senseless*^{15, 16, 18}. We found that knock down of *Dsnx6* did not induce accumulation of Wg (Fig. 2G) and that it also did not reduce the expression of *senseless* (Fig. 2D). Furthermore, knock down of *Dsnx6* did not affect the levels of endogenous Wls (Fig. 2J), whereas in the absence of Dvps35, Wls levels are strongly reduced in Wg producing cells^{15, 16, 18}. Taken together, we conclude that the *C. elegans* and *Drosophila* SNX-BAR orthologs are dispensable for Wls trafficking and Wnt signaling. To our knowledge this is the first example of the cargo-selective sub-complex of the retromer functioning independently of the SNX-BAR retromer sorting nexins.

The PX domain-only sorting nexin SNX3 is required for Wnt signaling

In a genome-wide RNAi screen in *C. elegans* (Table S3), we found that the PX domain-only sorting nexin, *snx-3*, which is closely related to yeast Grd19p, *Drosophila* DSnx3 and vertebrate SNX3 and SNX12 (Fig. S1B)⁸, is required for the EGL-20 (Wnt) dependent posterior migration of the QL.d, a result that we confirmed using the predicted *snx-3* null allele *tm1595* (Fig. 1A, S1C). EGL-20 induces posterior migration of the QL.d by activating the target gene *mab-5*^{27, 28}. In *snx-3(tm1595)* mutants, *mab-5* expression was lost in the QL lineage (Fig. 1B), consistent with the notion that *snx-3* is required for the EGL-20 dependent activation of *mab-5*. This conclusion is supported by the rescue of the QL.d migration defect of *snx-3* by EGL-20 independent activation of *mab-5*. Thus, the QL.d localize at their normal posterior positions in double mutants between *snx-3* and the *mab-5* gain of function allele *e1751*²⁸ and in double mutants with the Axin ortholog *pry-1* (Table S2)^{29, 30}. In addition to the defect in QL.d migration, *snx-3(tm1595)* showed a range of other Wnt related phenotypes. Thus, the final position of the QR.d and the HSN neurons was shifted towards the posterior and the polarity of the ALM and PLM mechanosensory neurons was lost or reversed (Table S1, Fig. S1D). Taken together, these results show that *snx-3* is required for several Wnt dependent processes and that the range and penetrance of these phenotypes is similar to mutations in components of the cargo-selective retromer sub-complex^{23, 24}.

To investigate whether SNX3 is required for Wnt signaling in *Drosophila*, we knocked down the single SNX3 ortholog *Dsnx3* in the posterior compartment of the wing imaginal disc. As shown in Fig. 2E, there was a strong reduction in expression of the high-threshold Wg target gene *senseless*. Furthermore, knock down of *Dsnx3* in the posterior compartment or in clones frequently resulted in typical *wg* loss of function defects in the adult wing, such as notches and a loss of sensory bristles at the wing periphery (Fig. 2F). These results establish that SNX3 has an evolutionarily conserved function in Wnt signaling.

SNX3 is required in Wnt producing cells for Wnt secretion

We found that *snx-3* is ubiquitously expressed in *C. elegans*, with prominent expression in coelomocytes, the pharynx and in rectal epithelial cells, which include the cells that produce

and secrete EGL-20 (Fig. S2A). We have previously shown that the cargo-selective sub-complex of the retromer is specifically required in Wnt producing cells²³. To investigate whether *snx-3* has a similar site of action, we tested whether specific expression of wild type *snx-3* in the EGL-20 producing cells of *snx-3(tm1595)* mutants restores the EGL-20 dependent posterior migration of the QL.d. We found that *egl-20* promoter directed expression of *snx-3* significantly rescued QL.d migration (Table S2). In contrast, expression of *snx-1* did not restore QL.d migration. We conclude that the presence of *snx-3* in Wnt producing cells is necessary and sufficient for its function in Wnt signaling. Furthermore, these results show that *snx-3* and the cargo-selective retromer sub-complex do not only produce similar mutant phenotypes, but also share a common site of action.

The requirement of *snx-3* in Wnt producing cells suggests a function of SNX-3 in Wnt secretion. To investigate this possibility, we tested whether the secretion of EGL-20 is affected in *snx-3* mutants. EGL-20 (visualized using the immunoglobulin binding domain of protein A as a tag) forms a punctate concentration gradient that ranges from the *egl-20* expressing cells in the tail to the mid-body region (Fig. 1C)²³. In *vps-35* mutants, this gradient is strongly reduced or absent. We found that the EGL-20 gradient was similarly reduced in *snx-3(tm1595)* (Fig. 1C), indicating that *snx-3* is necessary for EGL-20 secretion. To determine whether DSnx3 is also required for Wnt secretion and gradient formation in *Drosophila*, we depleted *Dsnx3* in the posterior compartment of the wing imaginal disc and stained for endogenous Wg. As shown in Fig. 2H and S3C, D, knock down or mutation of *Dsnx3* resulted in a strong accumulation of Wg within the stripe of *wg* expressing cells along the dorsoventral boundary, indicating that Wg secretion is strongly reduced. Indeed, staining of extracellular Wg showed that there was a strong reduction in the level of Wg outside of the *wg* expressing cells (Fig. 2I). Taken together, these results are consistent with a conserved function of SNX3 in Wnt secretion.

To address the specificity of SNX3 for Wnt secretion, we tested whether knock down of *Dsnx3* affects the secretion of two other morphogens in the wing disc: the lipid-modified Hedgehog (Hh) protein³¹ and the *Drosophila* BMP ortholog decapentaplegic (Dpp)³². Depletion of *Dsnx3* in the dorsal compartment or in large clones spanning the *hh* expressing domain of the wing imaginal disc did not interfere with Hh secretion, as determined by monitoring endogenous Hh protein (Fig. S3D). To examine effects on Dpp secretion, Dpp signaling activity was measured by staining of phosphorylated Mad protein (pMad), a downstream effector in the Dpp pathway³³, but again no effects were observed when *Dsnx3* was depleted (Fig. S3E). Although these experiments assay only a limited set of secreted proteins, these results clearly indicate that DSnx3 is not generally required for protein secretion. This conclusion is further supported by the *snx-3* mutant phenotype in *C. elegans*: apart from a defect in Wnt signaling, *snx-3* mutants have no other obvious developmental defects (data not shown).

SNX3 has a conserved function in regulating Wls stability

To examine the function of SNX-3 in the Wnt secretion pathway, we tested whether depletion of *snx-3* has an effect on the subcellular localization or stability of MIG-14 (Wls)¹²⁻¹⁴. As shown in Fig. 1D and E, there was a striking decrease in MIG-14 protein

levels in *snx-3(tm1595)*, which was similar to the reduction observed in the cargo-selective sub-complex mutant *vps-26*. In contrast, steady state levels of the retromer cargo protein CED-1 were not significantly changed in the absence of *snx-3* (Fig. S2C). Also in *Drosophila*, depletion of *Dsnx3* resulted in a loss of endogenous Wls protein (Fig. 2K, S3C, D). Interestingly, Wls levels were only reduced within the *wg* expressing cells, an effect that was also observed upon knock down of *Dvps35*¹⁸. We conclude that SNX3 has a conserved function in maintaining Wls protein levels.

To determine whether the reduction in MIG-14 protein levels is the result of lysosomal degradation, we performed co-localization studies between MIG-14 and the late endosomal and lysosomal marker LMP-1::mCherry in *C. elegans*¹⁹. In wild type animals, no significant co-localization between MIG-14::GFP and LMP-1::mCherry could be observed in *egl-20* expressing cells (Fig. 1F). However, in *snx-3(tm1595)* mutants, the remaining MIG-14::GFP was mostly localized to LMP-1::mCherry positive structures. These results indicate that MIG-14 is missorted into the lysosomal degradation pathway in *snx-3* mutants, consistent with a function of SNX-3 in the retromer dependent endosome-to-TGN transport of MIG-14.

To investigate whether MIG-14 becomes limiting for Wnt signaling in *snx-3* mutants, we tested whether *mig-14* overexpression can rescue EGL-20 signaling. Overexpression of a functional MIG-14::GFP fusion protein from a multi-copy transgene fully rescued the QL.d migration defect of *snx-3(tm1595)* (Table S2), supporting the hypothesis that the Wnt secretion defect of *snx-3* mutants is caused by a decrease in MIG-14 (Wls) protein level.

Human SNX3 co-localizes with Wls and VPS26 on endosomes and facilitates membrane association of the cargo-selective retromer sub-complex

To investigate how SNX-3 and the cargo-selective sub-complex functionally interact in Wls recycling, we examined human SNX3 in HeLa cells, which express SNX3 but lack detectable expression of the related sorting nexin SNX12 (Fig. S4A, B). Using a lentivirally expressed GFP-SNX3 fusion protein, we found that SNX3 co-localizes with the cargo-selective retromer subunit VPS26 on endosomes (Fig. 3A), as was confirmed by immunoelectron microscopy (EM) (Fig. 3B). Furthermore, we found that GFP-SNX3 co-localizes with Wls-mCherry (Fig. 4A, and Fig. S2B for co-localization in *C. elegans*). Knock down of SNX3 led to a significant reduction in endogenous human Wls protein levels (Fig. 4D), consistent with the conserved function of SNX3 in maintaining Wls protein levels. SNX3 depletion also induced a striking reduction in co-localization between Wls-mCherry and endogenous VPS26 (Fig. 4B, C). As the VPS26 staining pattern appeared more cytoplasmic than in control siRNA treated cells, we tested whether SNX3 is required for membrane association of VPS26. Similar to knock down of Rab7, which has been shown to mediate membrane recruitment of the cargo-selective sub-complex to late endosomes^{34, 35}, depletion of SNX3 induced a decrease in membrane associated VPS26 and a corresponding increase in cytoplasmically localized VPS26 (Fig. 4E). These results are consistent with a function of SNX3 in aiding the association of the cargo-selective subunits to Wls containing endosomes. Whether this function is independent of other retromer recruitment mechanisms, such as mediated by Rab7^{34, 35} and Hrs³⁶, remains to be established.

SNX3 physically interacts with the cargo-selective retromer subunits in a complex that does not contain the SNX-BAR sorting nexins

To investigate whether SNX3 and the retromer physically interact, we performed co-immunoprecipitation (CoIP) experiments with GFP-SNX3. As shown in Fig. 3C, there was significant CoIP between SNX3 and both endogenous VPS35 and VPS26, indicating that SNX3 and the cargo-selective retromer sub-complex can associate *in vivo*. This interaction is direct, as recombinant full length SNX3 co-precipitated with purified VPS26-VPS29-VPS35 trimeric complex (Fig. 3D and S4C). Next, we compared IPs of SNX3 with IPs of the SNX-BAR sorting nexins SNX1 and SNX5 (Fig. 3C). Although in both cases the cargo-selective subunits were co-precipitated (note however that the interaction with SNX1-SNX5 is weaker than with SNX3), we failed to detect endogenous SNX1 and SNX5 in the SNX3 IP and endogenous SNX3 in the SNX1 and SNX5 IP. Based on these results, we conclude that there are two distinct retromer complexes: a SNX-BAR containing retromer complex and a novel complex in which SNX3 interacts with the cargo-selective retromer sub-complex.

SNX3 localizes to early endosomes and segregates WIs into a retrieval pathway that is morphologically distinct from the SNX-BAR retromer pathway

To examine the spatial distribution of SNX3 and the SNX-BAR sorting nexins along the endosomal maturation pathway, we expressed GFP-SNX3 and GFP-SNX1 in HeLa cells. Using markers for early (EEA1 and Rab5) and late endosomes and lysosomes (LAMP1 and Rab7), we found that GFP-SNX3 primarily localizes to early endosomes (Fig. 3A), in agreement with the previously reported localization of SNX3 in A431 cells³⁷. In contrast, the classical SNX-BAR retromer sorting nexins are most abundant on endosomes that are at the early-to-late endosomal transition point²² (J.R.T. van Weering and P.J. Cullen, unpublished). Consistent with this distinct but overlapping distribution, co-localization between SNX1 and SNX3 was only partial (Fig. 3A), supporting the notion that SNX3 and SNX1 display an element of spatial segregation along the endosomal maturation pathway (see Fig. S6).

The SNX-BAR retromer sorting nexins contain a membrane curvature sensing BAR domain which drives membrane deformation to segregate cargo into a tubular-based endosomal trafficking pathway⁸. SNX3 lacks a BAR domain, indicating that it may direct WIs and the cargo-selective retromer sub-complex into a morphologically distinct sorting pathway. Using live cell confocal imaging to define the dynamic relationship between SNX3, the SNX-BAR sorting nexins and WIs, we found that WIs is not sorted into SNX-BAR labeled tubular endosomal profiles. Thus, in cells expressing GFP-SNX1 and WIs-mCherry, WIs was not enriched in SNX1 decorated tubules emerging from endosomes labeled for both proteins (Fig. 5A, B, S4D) (18/22 tubules were negative for WIs, while 4/22 were weakly positive). Instead, we observed the emergence of small GFP-SNX3 and WIs-mCherry labeled transport vesicles from endosomes co-labeled for both proteins (Fig. 5C). These results are consistent with our observation that the SNX-BAR sorting nexins are dispensable for WIs trafficking and indicate that WIs exits SNX3-labelled early endosomes through vesicular carriers rather than through SNX-BAR decorated tubular profiles. This conclusion is further supported by the observation that SNX3 interacts with clathrin³⁸, a result that we

have confirmed here through endogenous CoIP (Fig. S5A). Although SNX3 has been suggested to contain an inverted clathrin box³⁸, the interaction appears indirect, as recombinant SNX3 did not associate with recombinant clathrin (residues 1-579) either in the absence or presence of PI3P-containing liposomes (Fig. S5B, C). Immunostaining of endogenous clathrin revealed that a small population of SNX3 decorated endosomes co-localizes with clathrin (Fig. S5D). At the resolution of immuno-EM, these appear as clathrin coated, small 50-75 nm vesicles (Fig. S5E) that are morphologically distinct from the previously characterized retromer decorated endosome-to-TGN transport carriers (ETC's), which appear as clathrin-negative, non-branched tubules (average length 170-230 nm and diameter 20-50 nm)²². These results suggest that SNX3 may direct the Wls-retromer complex into a clathrin-dependent vesicular transport pathway. Future studies will examine how Wls trafficking is mediated by the SNX3 retromer complex.

DISCUSSION

The identification of the Wnt sorting receptor Wls has shown that the secretion of Wnt proteins is mediated by a specialized trafficking pathway that provides an important layer of regulation to Wnt signaling^{39, 40}. A key step in this pathway is the retromer-dependent transport of Wls from endosomes to the TGN¹⁵⁻¹⁹. Here, we report that Wls retrieval is mediated by a novel retromer pathway that functions independently of the SNX-BAR retromer coat components.

The retromer consists of a cargo-selective sub-complex that interacts with sorting nexins of the SNX-BAR family to segregate cargo into a tubular endosomal sorting pathway⁸. Our results show that the cargo-selective sub-complex also interacts with SNX3 as part of an alternative retromer pathway that mediates the recycling of Wls. Three lines of evidence suggest that these are functionally distinct retromer pathways. First, genetic analysis in *C. elegans* showed that *snx-3* and the SNX-BAR sorting nexins function in parallel pathways. Thus, retrieval of the phagocytic receptor CED-1²⁵ is dependent on the SNX-BAR sorting nexins but independent of SNX3, whereas Wls recycling requires SNX3 but not the SNX-BAR sorting nexins. Second, CoIP experiments showed that the interaction of the cargo-selective sub-complex of the retromer with the SNX-BAR sorting nexins and SNX3 is mutually exclusive. Finally, live cell imaging revealed that the SNX3 retromer pathway sorts Wls into a vesicular retrieval pathway that is morphologically distinct from the SNX-BAR dependent tubular endosomal sorting pathway⁸. Based on these results we conclude that the SNX-BAR and SNX3 pathways are independent and mechanistically distinct retromer pathways.

Studies in yeast have shown that the SNX3 ortholog Grd19p also functions in retromer-dependent endosome to Golgi retrieval^{21, 41-43}, but in contrast to SNX3, Grd19p functions together with the SNX-BAR sorting nexins Vps5p and Vps17p in the retrieval of cargo proteins such as the iron transporter Fet3p-Ftr1p. Grd19p physically interacts with a sorting sequence in the cytoplasmic tail of Ftr1p and with the SNX-BAR retromer complex, which led to the hypothesis that Grd19p acts as a cargo-specific adaptor that links Ftr1p to the SNX-BAR retromer complex⁴³. We did not observe an interaction between SNX3 and Wls in CoIP experiments (data not shown) and also did not find co-precipitation of SNX3 with

the SNX-BAR sorting nexins. Furthermore, we found that mutation of the SNX-BAR sorting nexins did not affect the SNX3 dependent retrieval of Wls, indicating that the function of SNX3 is fundamentally different from Grd19p in yeast.

How do the two distinct SNX3- and SNX-BAR-retromer complexes regulate sorting of different endosomal cargo? One simple model to answer this question relies on the spatial segregation of SNX3 and the SNX-BAR sorting nexins along the endosomal maturation pathway. While there is significant co-localization between these sorting nexins, SNX3 is predominantly localized to early endosomes by its high affinity interaction with PI3P⁴⁴, while the SNX-BAR retromer sorting nexins reside at the interface between early and late endosomes²². Endocytosed Wls therefore initially enters SNX3-labeled early endosomes where it engages the VPS26-VPS29-VPS35 trimeric complex, recruited to this compartment by the interaction with SNX3 (Fig. S6). Through a vesicular pathway, possibly dependent upon indirect binding to clathrin as well as additional membrane remodeling proteins, the SNX3 retromer complex sorts Wls for retrieval back to the TGN. In the absence of SNX3, Wls can be missorted into intraluminal vesicles and hence lysosomal degradation, or can be recycled through SNX-BAR retromer back to the TGN. The relative flux through these two distinct pathways therefore determines the steady-state level of Wls. As the level of Wls is severely reduced upon loss of SNX3, the flux into the lysosomal degradative pathway appears to be dominant. Thus, while a proportion of Wls may undergo SNX-BAR retromer-mediated recycling in the absence of SNX3, this is insufficient to maintain the required level of Wls for Wnt gradient formation during iterative rounds of Wnt secretion and Wls retrieval from the cell surface.

Interestingly, the steady-state trafficking of the classical SNX-BAR retromer cargo CI-MPR is primarily defined by intracellular cycling between the TGN and late endosomes with retrieval back to the TGN via the SNX-BAR retromer. The spatial segregation model therefore suggests that the lack of effect of SNX3 suppression on steady-state CI-MPR distribution arises from CI-MPR entering the endosomal network at a point downstream of SNX3³. That said, the complexity of CI-MPR trafficking - a proportion of this receptor traffics to the plasma membrane before undergoing endocytosis and retrograde transport to the TGN⁴⁵⁻⁴⁷ - suggests that such a simple spatial segregation model may be an oversimplification. We therefore speculate that alongside spatial segregation, cargo binding to the VPS26-VPS29-VPS35 complex may be an important element in selecting the sorting nexin coat that specifies the subsequent retrograde trafficking route. Thus, binding of VPS26-VPS29-VPS35 to Wls may favor association with SNX3, while engagement with CI-MPR favors binding to the SNX-BAR coat complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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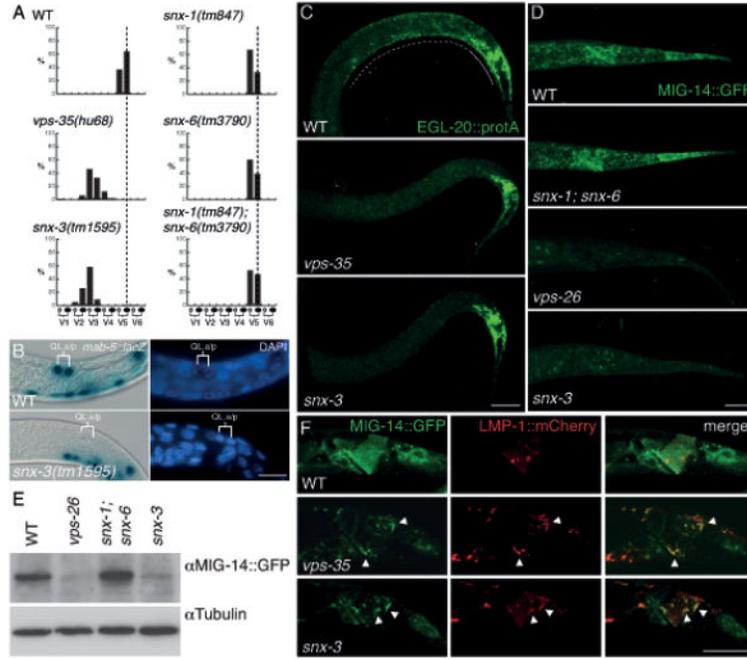


Fig. 1. SNX-3 is required for EGL-20 (Wnt) signaling and MIG-14 (Wls) recycling in *C. elegans*. (A) The final positions of the QL.paa and QL.pap cells relative to the invariant positions of the seam cells V1 to V6 ($n > 100$). Both *snx-1(tm847)* and *snx-6(tm3790)* are viable as single or double mutants and could be propagated as homozygous strains, excluding a contribution of maternally provided protein in our assays. (B) Expression of the EGL-20 target gene *mab-5* in the QL descendants QL.a and QL.p. Cell nuclei are shown by DAPI staining. Scale bar is 10 μ m. (C) Staining of EGL-20::proteinA with rabbit anti-goat-Cy5²³ in wild type, *vps-35(hu68)* and *snx-3(tm1595)*. Expression is visible within the *egl-20* expressing cells (closed line) and as a punctate posterior to anterior gradient (dotted line). In all images, anterior is to the left and dorsal is up. Scale bar is 10 μ m. (D) Confocal images of MIG-14::GFP (*huSi2*) at identical exposure settings in wild type and in *snx-1(tm847)*; *snx-6(tm3790)*, *vps-26(tm1523)* and *snx-3(tm1595)*. Scale bar is 10 μ m. (E) Western blot quantification of MIG-14::GFP (*huSi2*) protein levels. (F) Confocal images of MIG-14::GFP (*huIs71*) (green) and LMP-1::mCherry (red) in wild type, *vps-35(hu68)* and *snx-3(tm1595)*. Arrowheads indicate examples of co-localization between MIG-14::GFP and LMP-1::mCherry. Scale bar is 10 μ m.

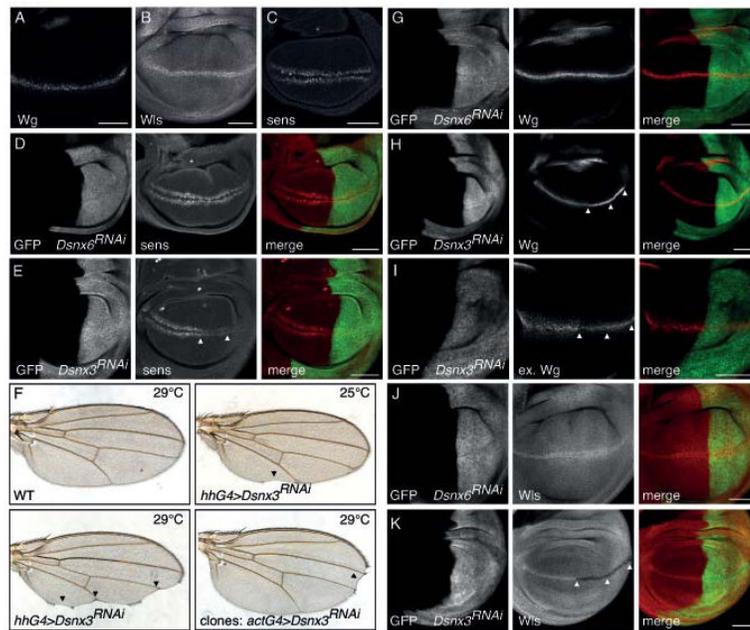


Fig. 2. DSnx3 is required for Wg secretion and Wls recycling in the *Drosophila* wing imaginal disc. (A, B, C) Immunostaining of Wg, Wls and Senseless in wild type wing disc. (D, E, G, H, I, J, K) Expression of *Dsnx6* or *Dsnx3* RNAi transgenes was induced in the posterior compartment of the wing disc (marked by mCD8-GFP in green) using a *hhGal4* driver (see Fig. S3A, B for quantification of knock down efficiency). (D, E) Immunostaining of Senseless (red). Arrowheads indicate loss of *senseless* expression in the *Dsnx3* RNAi expressing posterior compartment. (F) *Dsnx3* RNAi was induced in the posterior compartment using *hhGal4* or in clones using an *actinGal4* driver. Arrowheads indicate notches and loss of sensory bristles. (G, H) Immunostaining of total Wg (red). Arrowheads indicate Wg accumulation in the *Dsnx3* RNAi expressing posterior compartment. (I) Immunostaining of extracellular Wg (red). Arrowheads indicate loss of extracellular Wg staining. (J, K) Immunostaining of Wls (red). Arrowheads indicate loss of Wls in *wg* expressing cells in the *Dsnx3* RNAi expressing posterior compartment. Scale bars, 50 μ m.

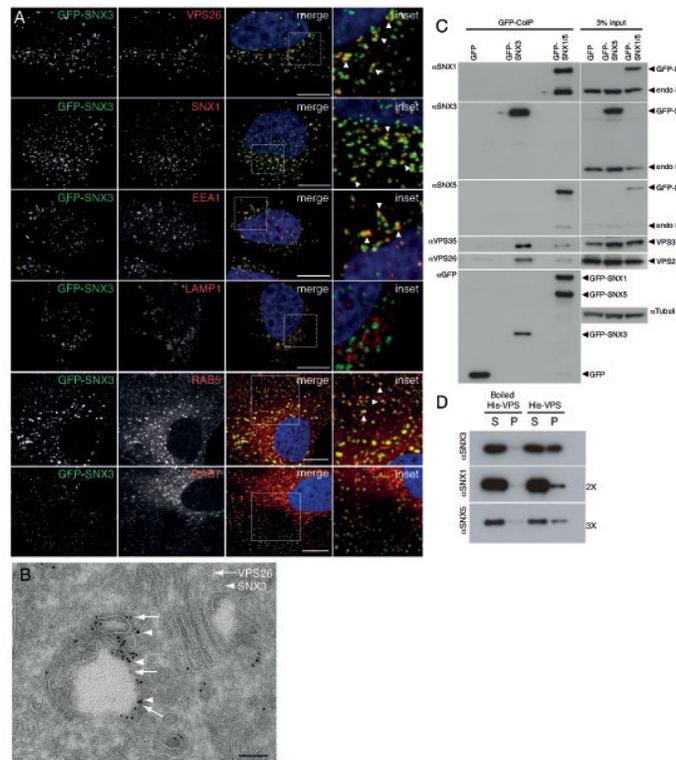


Fig. 3. Co-localization and physical interaction of SNX3 with the cargo-selective sub-complex of the retromer. **(A)** SNX3 partially co-localizes with VPS26-positive early endosomes. HeLa cells lentivirally transduced to express GFP-SNX3 (green) were fixed and stained for VPS26, SNX1, EEA1 or LAMP1 (red). Co-localization between GFP-SNX3 and VPS26, SNX1, EEA1, LAMP1, Rab5 and Rab7 was quantified as 0.43 ± 0.05 , 0.55 ± 0.04 , 0.38 ± 0.02 and 0.07 ± 0.04 , 0.61 ± 0.02 , and 0.34 ± 0.02 , respectively (Pearson's coefficient, mean \pm SD, $n=3$ with 30 cells per condition, for Rab5 and Rab7, $n=20$ cells). Scale bar, 11 μm . **(B)** At the ultrastructural level, SNX3 and VPS26 localize to common vesicular endosomal profiles. GFP-SNX3 is labeled with 10 nm gold and mCherry-VPS26 with 6 nm gold. The image is representative of that observed from the analysis of 5 other endosomal vacuoles. Scale bar, 100 nm. **(C)** SNX3 interacts with the cargo-selective sub-complex of the retromer. Cell extracts derived from HeLa cells lentivirally transduced with GFP, GFP-SNX3 or both GFP-SNX1 and GFP-SNX5 (GFP-SNX1/5), were subjected to GFP-nanotrap. The classical retromer SNX-BARs form heterodimeric complexes leading to the presence of both endogenous SNX1 and SNX5 in the GFP-SNX1/5 IPs³. **(D)** 3xFLAG-VPS26-VPS29-VPS35-His₆ complex (His-VPS) was isolated from BL21 *E. coli* onto TALON resin and incubated with 2 μM of recombinant SNX3, SNX1 or SNX5 for 2 hours at 4°C. Supernatant (S) and TALON containing resin (P) were isolated. SNX3 directly associates with His-VPS as do SNX1 and SNX5 although this is less well pronounced (longer exposures are shown). Control: boiled His-VPS resin.

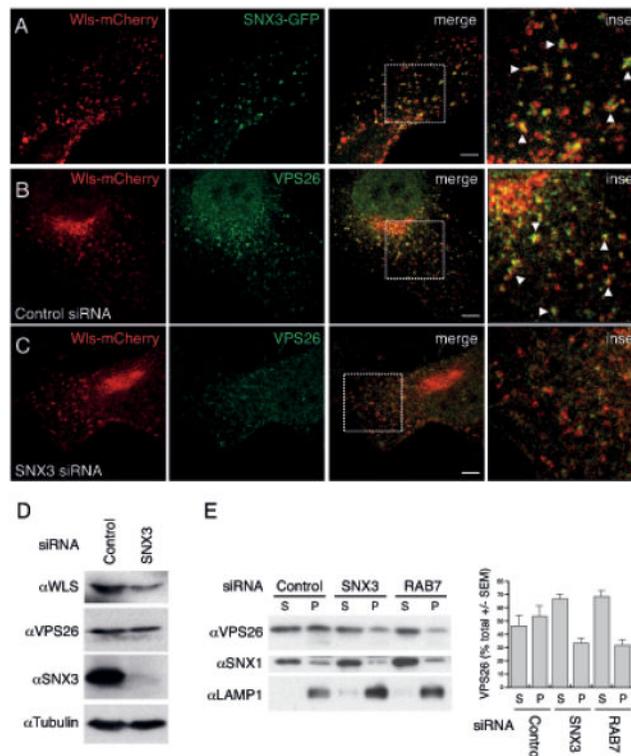
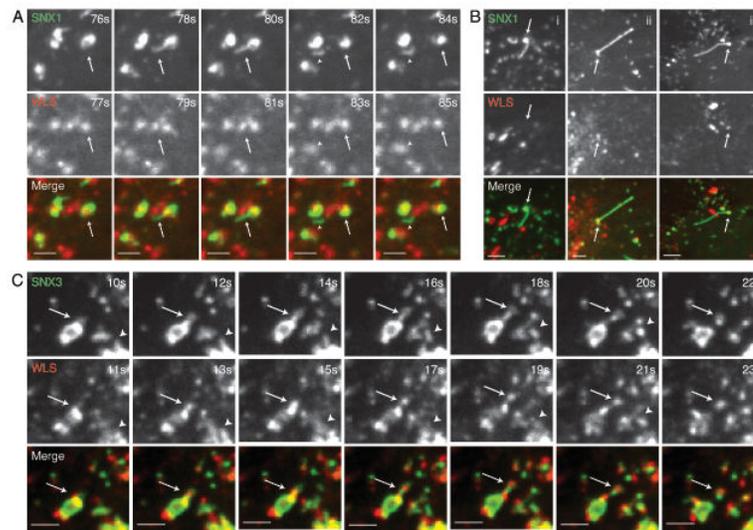


Fig. 4. SNX3 co-localizes with Wls and facilitates membrane association of the cargo-selective sub-complex of the retromer. **(A)** Co-localization between SNX3-GFP (green) and Wls-mCherry (red) in HeLa cells was quantified as $0.25 \pm 0.02\%$ (Pearson's coefficient; mean \pm SEM, $n=2$ with 23 and 11 cells). Arrowheads show examples of co-localization. Scale bar, $10 \mu\text{m}$. **(B, C)** Co-localization between Wls-mCherry and endogenous VPS26 (green) in HeLa cells treated with control or SNX3 siRNA was quantified as $0.19 \pm 0.02\%$ and $0.08 \pm 0.02\%$, respectively (Pearson's coefficient; mean \pm SEM, $n=4$ with 7-10 cells each). Arrowheads show examples of co-localization. **(D)** HeLa cells were transfected with control or SNX3 siRNA and assayed for endogenous Wls, VPS26, SNX3 and tubulin protein levels. **(E)** HeLa cells treated with control, SNX3 or RAB7 siRNA were separated into a supernatant (S) fraction containing cytosol and a pellet fraction (P) containing membranes³⁴ and were stained for endogenous VPS26, SNX1 and LAMP1. The amount of VPS26 in the supernatant and pellet fractions was quantified using densitometry and is shown as percentage of the total. Data are presented as mean \pm SEM and represent three independent experiments. There was no significant change in SNX1 membrane association upon SNX3 knock down ($17.8 \pm 3.8\%$ versus $22 \pm 8.1\%$). Knock down of RAB7 was included as a positive control³⁴.

**Fig. 5.**

Wls is contained within SNX3 positive vesicular carriers but is absent from SNX1 retromer decorated tubular carriers. **(A)** RPE-1 cells were transiently co-transfected with pEGFP-SNX1 (green) and Wls-mCherry (red) and cells were subsequently imaged live after a 16 hour incubation period. Frames depicting the formation and scission of a GFP-SNX1 tubule from a vesicle positive for both SNX1 and Wls are shown (arrows indicate the dual expressing vesicle, while arrowheads indicate the carrier post scission) (see Supplementary Information, Movie 1). Scale bars represent 6 μm . Of the 100 SNX1 decorated tubulating endosomes that were analyzed, 22 were positive for Wls; 18/22 tubules emanating from these endosomes were negative for Wls, while 4/22 were weakly positive. Quantification of Wls-mCherry and GFP-SNX1 levels in an endosome and corresponding tubule is shown in Fig. S4D. **(B)** Further examples of SNX1 retromer tubules negative for Wls. (i) An example of a SNX1 retromer positive endosome and tubule both of which are negative for Wls. (ii and iii) Further examples of SNX1-labelled endosomes positive for Wls, but with tubules negative for Wls. Scale bars represent 6 μm . **(C)** RPE-1 cells were transiently co-transfected with pEGFP-SNX3 (green) and Wls-mCherry and cells were subsequently imaged live after a 16 hour incubation period. Frames depicting the formation and scission of GFP-SNX3 labeled buds from vesicles positive for both SNX3 and Wls are shown. Note the 1 second delay between acquisitions for a given image pair. Arrows and arrowheads show two examples of buds positive for both Wls and SNX3 (see Supplementary Information, Movie 2). Scale bars represent 6 μm .