

Title

Interaction of the sorting nexin 25 homolog Snazarus with Rab11 balances endocytic and secretory transport and maintains the ultrafiltration diaphragm in nephrocytes

Running Head

Snz counteracts Rab11 in nephrocytes

Authors

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Abstract

Proper balance of exocytosis and endocytosis is important for the maintenance of plasma membrane lipid and protein homeostasis. This is especially critical in human podocytes and the podocyte-like *Drosophila* nephrocytes that both use a delicate diaphragm system with evolutionarily conserved components for ultrafiltration. Here we show that the sorting nexin 25 homolog Snazarus (Snz) binds to Rab11 and localizes to Rab11-positive recycling endosomes in *Drosophila* nephrocytes, unlike in fat cells where it is present in plasma membrane/lipid droplet/ER contact sites. Loss of Snz leads to redistribution of Rab11 vesicles from the cell periphery and increases endocytic activity in nephrocytes. These changes are accompanied by defects in diaphragm protein distribution that resemble those seen in Rab11 gain-of-function cells. Of note, co-overexpression of Snz rescues diaphragm defects in Rab11 overexpressing cells, whereas *snz* knockdown in Rab11 overexpressing nephrocytes or simultaneous knockdown of *snz* and *tbc1d8b* encoding a Rab11 GAP lead to massive expansion of the lacunar system that contains mislocalized diaphragm components: Sns and Pyd/ZO-1. We find that loss of Snz enhances while its overexpression impairs secretion, which, together with genetic epistasis analyses, suggest that Snz counteracts Rab11 to maintain the diaphragm via setting the proper balance of exocytosis and endocytosis.

Introduction

Eukaryotic cells contain a remarkably complex endomembrane system consisting of numerous membrane-bound organelles. Continuous, directed flow of material through this system is a prerequisite of normal cell function and is primarily achieved by vesicle-mediated transport. In the case of endocytosis, extracellular material is enwrapped by portions of the plasma membrane (PM). The resulting endocytic vesicles fuse to form early endosomes (EEs), which then mature into late endosomes (LEs). During endosomal maturation, portions of the endosomal membrane and its integral membrane proteins are sorted into the intraluminal vesicles of LEs/multivesicular bodies and are forwarded towards lysosomes where degradation takes place (Huotari and Helenius, 2011). Notably, a significant portion of transmembrane proteins are targeted from the endosomes back to the PM either directly (through the so-called fast recycling pathway) or indirectly through the recycling endosomal compartment (slow recycling pathway) (Naslavsky and Caplan, 2018). Proper functioning of the endocytic and endosomal recycling routes is essential for establishing a steady-state proteome of integral membrane proteins in the PM and for dynamic remodeling in response to changing physiological conditions. Generating vesicles that bud from the donor membrane with a specific content, moving them through the cytoplasm and ensuring their fusion exclusively with the appropriate target organelle all play key roles in this complex system. These processes are driven by proteins and multiprotein complexes and defect of any of these factors (for example due to mutations) leads to compromised cell function and the pathogenesis of severe diseases such as cancer and neurodegeneration.

Sorting nexin (Snx) proteins belong to the PX (phox homology)-domain containing protein family that has almost 50 members in humans (Teasdale and Collins, 2012). Besides their common phosphoinositide-binding PX-domain that enables their membrane recruitment, different subgroups of Snx proteins contain various other domains that define their further molecular interactions. Many Snx proteins have well-characterized roles in the endosomal system as parts of the retromer complex, while other members of the family have retromer-independent functions (Gallon and Cullen, 2015). Among the latter group, the SNX13/SNX14/SNX19/SNX25 subgroup of mammalian Snx proteins is characterized by a unique domain structure consisting of two predicted N-terminal transmembrane regions followed by the PXA-RGS-PX-PXC domains (for simplicity, these proteins hereafter will be referred to as Snx-TM proteins). Snx-TM proteins are also involved in different human diseases (Teasdale and Collins, 2012). Among these, the effects of SNX14 mutations are extensively investigated as they lead to a distinct type of cerebellar ataxia syndrome with features of cerebellar atrophy (due to the loss of Purkinje-cells), intellectual disability and coarse facial features (Thomas *et al.*, 2014; Akizu *et al.*, 2015). A recent study showed that SNX14 is a part of endoplasmic reticulum (ER) - lipid droplet (LD) contact sites promoting the incorporation of fatty acids into triacyl-glycerol in mammalian cells (Datta *et al.*, 2019, 2020). Other members of the Snx-TM subgroup also function at contact sites from yeast to mammals and a growing body of evidence points to their involvement in human diseases (Jacques *et al.*, 2005; Bare *et al.*, 2007; Li *et al.*, 2014; Olsson *et al.*, 2014; Henne *et al.*, 2015; Hariri *et al.*, 2019).

The fruitfly *Drosophila melanogaster* has a single Snx-TM protein (encoded by the *CG1514* gene) that is most closely related to human SNX25. The first study about *CG1514* reported the massive expression of this gene in larval fat bodies and described the lifespan extending effect of loss-of-function mutations (Suh *et al.*, 2008), that is why the authors named this gene *sorting nexin lazarus, snazarus* (*snz*). The first molecular function of Snz was recently demonstrated: Snz is involved in normal lipid droplet distribution and lipid transport in larval fat body cells, through maintaining triorganellar membrane contact sites between the ER, the PM and peripheral LDs (Ugrankar *et al.*, 2019). Recently, Snz has also been identified as a regulator of autophagic flux in the fat tissue (Lauzier *et al.*, 2022).

Here, we report a novel function of Snz in larval garland nephrocytes, the highly endocytic, podocyte-like cells of *Drosophila* (Helmstädter, Huber and Hermle, 2017). Garland nephrocytes are spherical cells that continuously take up material from the hemolymph and display a highly organized endosomal system (Denholm and Skaer, 2009). Loss of *snz* leads to prominent morphological and

functional changes in the endomembrane system of nephrocytes. These include upregulation of endocytosis, redistribution of recycling endosomes (REs) and alterations of the nephrocyte diaphragm (ND). We show that the effects of *snz* loss are linked to its role in Rab11-dependent recycling and are independent of ER-PM-LD contact sites in this cell type. We propose a model in which Snz negatively regulates exocytosis downstream of Rab11, thus contributing to the maintenance of membrane balance in the endosomal system of nephrocytes.

Results

Loss of Snz enhances endocytic activity in nephrocytes

Labeling endosomal subpopulations is a relatively simple method that gives an overview about endosomal maturation in nephrocytes (Lőrincz *et al.*, 2016). We evaluated the effect of *snz* loss-of-function in nephrocytes by colabeling Rab5 and Rab7 to identify EEs and LEs, respectively. We knocked down *snz* in nephrocytes of wandering stage larvae by using two independent UAS RNAi lines driven by prospero-Gal4 (Bechtel *et al.*, 2013). In wild type cells (**Figure 1A**), EEs and LEs form separate layers in the cytoplasm of the cells. Beneath the PM, small Rab5-positive EEs are formed, then they mature into large, Rab7-positive LEs as they move deeper into the cells. We detected a striking increase in the size of LEs as a result of *snz* knockdown in case of both RNAi lines (**Figures 1B and C**). Compared to control cells, nephrocytes expressing *snz* RNAi 1 or *snz* RNAi 2 contain significantly larger LEs, with 2,23 μm^2 mean area in the control versus 4,89 μm^2 and 3,52 μm^2 mean area in the case of RNAi 1 and RNAi 2 knockdowns, respectively (**Figure 1O**). We used *snz* RNAi 1 line in our further experiments and hereafter we will refer to it as *snz* knockdown. Ultrastructural analysis also confirmed the presence of enlarged LEs in *snz* knockdown cells (**Figure 1K and L**). Importantly, *snz* null mutant nephrocytes also contain abnormally enlarged LEs (**Figure 1D**), and this phenotype can be rescued by the expression of a full-length, C-terminally GFP-tagged Snz fusion protein that restores LE size from 4,25 μm^2 mean area of the mutant to 2,29 μm^2 in the rescue (**Figures 1E and O**). Notably, the population of EEs also shows an expansion in *snz* loss-of-function cells (**Figures 1A-E and P**). These results show that *snz* loss leads to severe alterations in the main endosomal compartments of nephrocytes.

Next, we carried out *ex vivo* tracer uptake experiments to assess the functional consequences of the morphological changes detected in the endosomal system of *snz* loss-of-function cells. Live nephrocytes were pulse-labeled with BSA-Alexa Fluor 647 conjugate (hereafter referred to as BSA⁶⁴⁷), then fixed after allowing the cells to internalize the tracer during the chase. To evaluate the kinetics of earlier steps of endocytosis, we applied 1-minute pulse followed by a 9-minute chase interval. We quantified the amount of the tracer in the subcortical endosomal population (**Figure 1J**), as during this relatively short chase it does not access the perinuclearly located lysosomes. In this experimental setup, excessive uptake manifests in an increase of the tracer-positive area compared to control cells (**Figures 1F, J, and S1A**), as in the case of Rab5 overexpression, a known enhancer of endocytic activity (Bucci *et al.*, 1992; Atienza-Manuel *et al.*, 2021) that we used as a positive control (**Figures 1I, J, and S1D**). Similarly, *snz* knockdown (**Figures 1G, J, and S1B**) or *snz* knockout (**Figures 1H, J, and S1C**) leads to a significant increase in the tracer-covered area compared to the control cells, and large endosomes are present in the subcortical region of these cells. Notably, the presence of fewer but larger tracer-positive endosomes indicates a more advanced stage of endosomal maturation. We observed similar changes upon expression of GTP-locked Rab11, a key positive regulator of recycling endosomes (**Figures 1J and S1E**). These suggest increased endocytic activity in *snz* LOF nephrocytes.

To monitor later steps of endosomal maturation, live nephrocytes expressing the GFP-Rab7 reporter were allowed to take up BSA⁶⁴⁷ from the culture medium for 5 minutes (pulse) and were subjected to live imaging after 20 minutes (chase). In control cells (**Figure 1M**), the tracer appeared in the inner regions of the cells where it filled up spherical vesicles encircled by GFP-Rab7 and additional Rab7-negative compartments with irregular shape and with signs of tubulation (**Figure 1M'**). Live imaging revealed the extensive formation of tracer-positive tubules with a highly dynamic behavior after a 30-minute chase in control cells (**Figure 1U¹-U³ and Supplementary movie V1**). Such tubules are

considered as the hallmarks of actively digesting lysosomes that constitute the so-called terminal lysosomal network in the perinuclear region of nephrocytes (Lund, Madsen and Kjaerulff, 2018). In the case of *snz* knockdown cells (**Figure 1N**), BSA⁶⁴⁷ accumulated in compartments demarcated by GFP-Rab7, showing that these enlarged LEs receive the incoming cargo. Tubulation of tracer-positive vesicles can also be frequently observed in these cells (**Figure 1N'**) suggesting that actively digesting lysosomes are present in *snz* knockdown nephrocytes (**Figure 1W¹-W³ and Supplementary movie V3**).

To test this more directly, we added BSA⁶⁴⁷ and BSA^{DQ Green} to the medium of cultured nephrocytes for a 5-minute pulse period followed by 1-hour chase, allowing the tracers to reach lysosomes. Fluorescence of BSA^{DQ Green} is quenched in its intact form, while its degradation by proteases leads to the formation of highly fluorescent products. In control nephrocytes (**Figure 1R**), fluorescence of BSA^{DQ Green} can be detected in vesicles with variable sizes as well as in the cytosol indicating that degradation products are present in, and also released from, lysosomes. In contrast, inhibiting lysosomal delivery of the tracer by knocking down the gene encoding the key HOPS subunit Vps39 strongly decreases cytoplasmic green fluorescence (**Figure 1S**). In this case, the tracers are trapped in enlarged LEs that are located at the nephrocyte periphery due to the lack of HOPS-mediated fusion of LEs and lysosomes. Here, BSA^{DQ Green} can only be subjected to partial degradation (indicated by patches of the green signal) without substantial release to the cytoplasm. This leads to a significantly higher vesicular vs. cytoplasmic green fluorescence ratio in *vps39* knockdown cells compared to control nephrocytes (**Figure 1Q**). Importantly, tracer-positive compartments with signs of tubulation are absent, indicating the lack of actively digesting lysosomes in *vps39* knockdown cells (**Figure 1V¹-V³ and Supplementary movie V2**). In the case of *snz* knockdown cells (**Figure 1T**), the ratio of vesicular vs. cytosolic fluorescence of BSA^{DQ Green} does not differ significantly from the control (**Figure 1Q**). Notably, dynamic tubulation of tracer-positive compartments is readily detected in *snz* knockdown cells, indicating efficient trafficking to digesting lysosomes (**Figure 1W¹-W³ and Supplementary movie V3**). To further assess lysosomal function, we performed LysoTracker Red and Lamp-GFP assays and quantified the area of cells covered by these markers (**Figures S1J and S1O**). Using the LysoTracker Red dye, acidic lysosomes can be readily detected in control (**Figure S1F**) and *snz* knockdown (**Figure S1G**) or null mutant cells (**Figure S1H**), unlike in the case of the *vha16-1* V-ATPase subunit knockdown where acidification of lysosomes is abrogated (Formica *et al.*, 2021) (**Figure S1I**). To further confirm that functional lysosomes are present in *snz* loss-of-function cells, we assessed lysosomal turnover of the Lamp-GFP reporter. The signal of its luminal GFP is quenched upon delivery to digesting lysosomes, while it remains fluorescent in the case of lysosomal degradation defects (Lund, Madsen and Kjaerulff, 2018; Milosavljevic *et al.*, 2022). Accordingly, the acidification-defective *vha16-1* knockdown cells show increased GFP-fluorescence and contain large GFP-positive vesicles (**Figure S1N**), most probably corresponding to dysfunctional lysosomes. These structures are absent from control cells that show low GFP fluorescence (**Figure S1K**). GFP signal in *snz* knockdown (**Figure S1L**) or null mutant (**Figure S1M**) cells is even mildly decreased compared to control cells. Based on these results, we assume that endosomal flux, that is, the uptake and processing of cargo including its transport to and degradation in lysosomes is not impaired in *snz*-depleted cells.

Taken together, these results show that lysosomal degradation proceeds normally in nephrocytes with decreased *snz* function. The increased amount of endocytic cargo taken up by enhanced endocytic activity appears to slightly exceed the degradative capacity of *snz* LOF nephrocytes, leading to a partial congestion of the endosomal system that manifests in the expansion of the EE population and the presence of enlarged LEs over time during the 5 days of larval development.

Snz colocalizes with and binds to Rab11 in nephrocytes independently of ER-PM-LD contact sites

Next, we analyzed the localization of Snz in nephrocytes to understand its role in the endosomal system. We labeled various endomembrane compartments in nephrocytes expressing the GFP-tagged, full-length Snz reporter. Snz-GFP is peripherally distributed in these cells and forms a network composed of irregularly shaped, tubulo-vesicular structures with sizes ranging from a few hundred nm-

s up to 2-3 μm -s (**Figures 2A-D, and S2**). Surprisingly, and in contrast to the pronounced effect of *snz* loss on the morphology of Rab7-positive LEs, Snz-GFP does not colocalize with Rab7 (**Figure 2E**). In fact, the two signals occupy different regions of the cells with Snz-GFP being more peripheral than the layer of LEs (**Figure S2A**). Rab5-positive EEs are close to Snz-GFP-positive structures in the cell periphery (**Figure S2B**), and mild colocalization of the two signals can be detected (**Figure 2E**). Based on anti-Hrp staining, Snz-GFP-positive structures are positioned right beneath the PM and the two signals do not overlap (**Figure 2E**). Moreover, a mutually exclusive pattern of Snz-GFP and anti-Hrp can be recognized in the cortical zone of the cells (**Figure 2A**), indicating that Snz is excluded from the PM of nephrocytes. Thus, structures that are positive for Snz-GFP seem to be clustered in the nephrocyte periphery, more precisely in the interlacunar spaces created by the invaginations of the PM.

Snz was described in *Drosophila* fat cells as a member of membrane contact sites in triorganellar junctions formed by ER cisterns, the PM and peripheral LDs (Ugrankar *et al.*, 2019). We wanted to know whether nephrocytes have similar contact sites and if such contacts exist, whether Snz is recruited to these in nephrocytes or not. To this end, we used the dMAPPER-Cherry reporter (Chang *et al.*, 2013) that marks ER-PM contacts and forms strongly fluorescent foci in the periphery of fat cells. Interestingly, dMAPPER-Cherry does not form discrete foci in nephrocytes, it rather shows a bipartite distribution in the perinuclear and peripheral regions of the cells, reminiscent of the organization of ER cisterns (**Figure 2B and S2C**). Notably, no Snz-GFP colocalization can be observed with either dMAPPER-Cherry or the ER marker Cnx99A (**Figure 2E**). Based on our observations, unlike fat cells, nephrocytes do not seem to contain ER-PM contact sites that would recruit dMAPPER-Cherry and therefore this reporter is distributed throughout the ER. We also wanted to test whether Snz is recruited to LDs in nephrocytes. LDs, while present in nephrocytes (**Figure 2C**), are mostly located in the inner regions of the cells and do not colocalize with Snz-GFP-positive structures (**Figure 2E**). These results indicate that in contrast to fat cells, nephrocytes do not contain ER-PM-LD contact sites that would recruit Snz.

In search for the identity of Snz-GFP-positive structures, we labeled further endomembrane compartments such as the cis- and trans-Golgi and the exocyst complex with anti-Gmap, anti-Golgin245 and anti-Sec5 antibodies, respectively (**Figure S2D-F**). No colocalizations with Snz-GFP can be detected in the case of the Gmap and Sec5 markers, and rare colocalization in the case of Golgin245 (**Figure 2E**). Ultimately, we found a striking colocalization of Snz-GFP with the Rab11-positive recycling compartment (**Figure 2E**) that also shows peripheral distribution in nephrocytes (**Figure 2D**). Moreover, physical interaction between full-length Snz-GFP and Rab11-3xHA proteins was evident in co-immunoprecipitation experiments using fly lysates (**Figure 2F**).

Given the colocalization and interaction of Snz and Rab11, we wanted to test the effect of *snz* loss-of-function on the localization of Rab11-positive structures in nephrocytes. Compared to control cells (**Figure 2G**), endogenous Rab11-positive structures relocalize from the cell periphery to the inner regions and are scattered throughout the cytoplasm of *snz* knockdown nephrocytes (**Figure 2H**). The distance of Rab11 dots from the PM significantly increases in *snz* knockdown cells compared to controls (**Figure 2I**), indicating that proper distribution of REs depends on *snz* in nephrocytes.

Taken together, these results point to an ER-PM-LD contact site-independent function of Snz in nephrocytes, at the Rab11-positive RE compartment.

Snz modulates Rab11 during the maintenance of diaphragm and lacunar channel integrity in nephrocytes

Next, we aimed to analyze the Rab11-related functional consequences of *snz* loss in nephrocytes. Components of the nephrocyte diaphragm (ND) are in continuous turnover that requires intact EE and RE functions, which are controlled by Rab5 and Rab11, respectively (Wen *et al.*, 2020). Thus, changes in ND pattern potentially reflect on impaired endosomal trafficking in nephrocytes that can be a consequence of altered Rab5 or Rab11 functions. To this end, we immunolabeled Sticks and stones (Sns) and Polychaetoid (Pyd) proteins to visualize ND in heat-fixed samples. These diaphragm

proteins reveal a strictly organized, fingerprint-like pattern - corresponding to the entrances of the lacunar channels - at the cortical region of control nephrocytes (**Figure 3A and A'**) (Weavers *et al.*, 2009; Zhuang *et al.*, 2009), while in medial cross-sections, the Sns and Pyd signals appear as a line of tightly arranged ND units (**Figures 3A', A'' and S3A**). In *snz* knockdown nephrocytes, such straight and parallel lines are rarely seen at the cortical region (**Figure 3B and B'**), indicating that the architecture of lacunar channels is mildly affected. We detected the accumulation of Sns and Pyd in the inner regions of *snz* knockdown cells as well (**Figures 3B', B'' and S3B**), suggesting that Snz is involved in the trafficking of ND proteins. Of note, the intracellular Sns and Pyd accumulation in *snz* knockdown cells can be rescued by abrogating exocyst function through the simultaneous knockdown of *sec5* (**Figure S3E**), raising the possibility that decreasing the rate of exocytosis compensates for the loss of *snz*. *Rab11* knockdown results in severe disorganization of the ND (Fu *et al.*, 2017; Wen *et al.*, 2020) observed in the cortical region of smaller than control nephrocytes (**Figure S3H**) without accumulation of Sns and Pyd in the inner regions (**Figure S3H' and H''**). Interestingly, in nephrocytes expressing the EYFP-tagged, GTP-locked and presumably constitutively active form of Rab11 (for simplicity, hereafter referred to as Rab11^{CA}), we observed similar ND rearrangements and intracellular Sns and Pyd accumulation as in the case of *snz* loss (**Figures 3C, C' and S3C**). In line with the binding of Snz to Rab11 and opposite effects of their modulation (Rab11 GOF looks similar to Snz LOF), the intracellular Sns and Pyd accumulation in Rab11^{CA} expressing cells can be rescued by the coexpression of Snz-GFP (**Figure S3D**).

Ectopic NDs are observed as Sns- and Pyd-positive intracellular protrusions in nephrocytes expressing Rab11^{CA} (**Figure 3C' and C''**). Formation of ectopic NDs was described in recent studies (Kampf *et al.*, 2019; Atienza-Manuel *et al.*, 2021) and in the case of Rab11 overexpression, it was interpreted as a result of increased secretion. Importantly, we found a striking enhancement of this phenotype when we combined *snz* knockdown with Rab11^{CA} expression. In this case, ectopic Sns and Pyd form ruffles at the cell surface and also demarcate a network that penetrates deeply into the inner regions of the cells (**Figures 3D', D'' and S3F**). In the cortical view of these nephrocytes, severely disorganized ND sections alternate with PM-regions without recognizable ND units (**Figure 3D and D'**). We also carried out formaldehyde fixation to enable the simultaneous visualization of the Pyd signal and Rab11^{CA}. In nephrocytes expressing only Rab11^{CA}, the protein localizes to the cortical region and it shows a pattern that most probably corresponds to the outline of the lacunar system (**Figure 4E**). It is likely that in this case, Rab11^{CA} cannot be released after Rab11-positive secretory vesicles fuse with the PM and it gets trapped in the membrane of the lacunar channels. Importantly, when Rab11^{CA} is expressed on a *snz* knockdown background, the resulting network-like Pyd signal overlaps with Rab11^{CA} (**Figure 4F and F'**), indicating that Pyd is found in ingressions formed by the PM. These results demonstrate that knockdown of *snz* strongly enhances ectopic ND formation in elongated lacunar channels in Rab11-overexpressing nephrocytes, and that Snz overexpression counteracts Rab11^{CA}.

To further corroborate the functional interaction of Snz with Rab11, we used additional tools to modulate Rab11 function in nephrocytes. First, we knocked down *tbc1d8b* (a known GAP activator of Rab11 (Kampf *et al.*, 2019)) alone or in combination with *snz* RNAi. As expected, loss of *tbc1d8b* leads to the formation of ectopic diaphragms (**Figure 3E' and E''**) and it has only a minor effect on the cortical diaphragm pattern (**Figure 3E and E'**). This effect is indistinguishable from Rab11^{CA} expression (**Figure 3C and C'**) or Rab11^{WT} overexpression (**Figure 3G and G'**) and thus represents a Rab11 gain-of-function phenotype. When combined with *snz* RNAi, *tbc1d8b* knockdown leads to much more severe disorganization of the diaphragm, which manifests in large-scale disruption in the cortical region (**Figure 4F and F'**) and formation of membrane ingressions containing ectopic diaphragm protein deposits in the medial region of nephrocytes (**Figure 3F' and F''**). This effect is highly similar to the effect of Rab11^{CA} expression (**Figure 3D**) or Rab11^{WT} overexpression combined with *snz* knockdown (**Figure 3H**). These results indicate that Rab11 gain-of-function perturbs diaphragm integrity, and support a role for Snz as a negative regulator of Rab11 in nephrocytes.

Ectopic ND deposits form in abnormally elongated lacunar channels of nephrocytes when either endocytosis is blocked or exocytosis is upregulated (Kampf *et al.*, 2019; Atienza-Manuel *et al.*, 2021).

Under these circumstances, excess membrane results in the elongation of lacunar channels towards the inner regions of the cells and PM proteins also populate these ingressions in the form of seemingly intracellular deposits. To visualize the lacunar network of nephrocytes, we carried out tannic acid impregnation followed by EM imaging. Tannic acid does not penetrate through the PM, so it only labels those membranes and intermembrane spaces that are exposed to the extracellular milieu (Kosaka and Ikeda, 1983). In control nephrocytes (**Figure 4A**), lacunar channels form in the cortical region of the cells in an approx. 2-3 μm average depth. Somewhat deeper channels can be found in *snz* knockdown (**Figure 4B**) or Rab11^{CA} overexpressing cells (**Figure 4C**). Strikingly, tannic acid-positive, membrane-bound ingressions penetrate deep into nephrocytes when Rab11^{CA} is expressed together with *snz* RNAi (**Figure 4D**). In these cells, the PM invaginates into the inner regions of nephrocytes and lacunar channels form an extensive network of membrane ingressions that corresponds to the Pyd-labeled ectopic ND network in this genotype. Notably, intracellular diaphragm protein deposits can be recognized in EM images as electron dense ectopic diaphragms in the labyrinthine channels that penetrate to the deeper regions of nephrocytes (**Figure S3G**). In a parallel set of experiments, we used the fluorescence-based channel diffusion assay (Milosavljevic *et al.*, 2022) where we applied BSA^{Alexa-647} to fill up the labyrinthine channels (**Figure 4G-J**). We quantified the deepest channel depth to cell diameter ratio (**Figure 4K**). Compared to the control (**Figure 4G**), we found only a mild increase in *snz* knockdown (**Figure 4H**) or Rab11^{CA} expressing cells (**Figure 4I**). Strikingly, when we expressed Rab11^{CA} in a *snz* knockdown background (**Figure 4J**), tracer-filled channels penetrate much deeper into the cells, in an asymmetric manner in most of the cases. Thus, large amount of excess plasma membrane is generated and forms membrane ingressions in Rab11^{CA} expressing cells with decreased *snz* function.

Taken together, these results show that *snz* is required for maintaining ND and lacunar channel integrity, most probably through restricting Rab11-mediated recycling and exocytosis towards the PM.

Snz inhibits exocytosis

As endocytic degradation is not compromised and endocytic activity seems enhanced in *snz* knockdown cells, we hypothesized that loss of *snz* contributes to the formation of membrane ingressions through the upregulation of exocytosis in nephrocytes. To investigate this, we examined the status of the secretory system by colabeling Golgin245 and Sec5 in nephrocytes as markers of the trans-Golgi network and the exocyst complex, respectively. In control cells, Golgin245-positive structures are present in the nephrocyte periphery, and they can also be found in small patches in the deeper regions of the cells (**Figure 5A**). Sec5 is distributed along the cell periphery in the form of small dots, which most probably represent exocyst complexes assembled on the surface of secretory vesicles prior to fusion and/or at docking sites where these vesicles are tethered to the PM. In *snz* knockdown nephrocytes expressing Rab11^{CA} (**Figure 5D**), the Sec5-positive area is greatly increased compared to the control cells (**Figure 5E**). This can indicate the increased number of secretory vesicles that are distributed along membrane ingressions formed by the network of elongated lacunar channels. Interestingly, the distribution of Golgin-245-positive structures is also altered in these cells. The peripheral Golgin245 signal is decreased or completely missing in most cells, while the number of patches in the deeper regions is markedly increased compared to the control. We interpret these results as the expansion and reorganization of the trans-Golgi network in *snz* knockdown nephrocytes that also express Rab11^{CA}. Importantly, knockdown of *snz* (**Figure 5B**) or expression of Rab11^{CA} (**Figure 5C**) alone does not lead to such large-scale alterations of Sec5 or Golgin-245, indicating the synergistic effect of *Snz* loss and Rab11 gain in the secretory system of nephrocytes.

Next, we combined *snz* knockdown with *sec5* knockdown to investigate the role of exocytosis in the formation of *snz* loss-of-function phenotypes. As the enlargement of LEs is a very robust response of nephrocytes to *snz* loss, we compared LE size in *snz* knockdown cells to *snz-sec5* double knockdown cells in an epistasis analysis (**Figure 5H**). In contrast to the enlarged LEs of *snz* knockdown nephrocytes (**Figure 5F**), LE size was restored to normal in *snz-sec5* double knockdown cells (**Figure 5G**). Thus, exocytic transport is required for the formation of enlarged LEs in *snz* knockdown cells.

To assess the role of *snz* in the regulation of exocytosis in another tissue, we monitored the release of secretory glue from *snz* knockdown and *snz* overexpressing salivary glands (SGs) at the onset of metamorphosis. Knockdown or overexpression of *snz* were driven by the *fkh-Gal4* driver in SGs expressing a Glue-dsRed reporter (Costantino *et al.*, 2008; Csizmadia *et al.*, 2018). Glue is synthesized in and secreted from SGs of wandering larvae and it mediates the strong attachment of the (pre)pupa to a solid surface during puparium formation. In the case of control animals (**Figure 5I**), traces of glue released from SGs can be observed beneath the prepupae facing the surface of the culture vial. Importantly, a significant amount of glue remains in SGs that can be seen inside the pupal case. In age-matched animals with *snz* knockdown in SGs (**Figure 5J**), the released glue covers a much larger surface under the prepupae, and SGs containing residual glue appear fainter compared to the control. This indicates increased glue release from SGs upon *snz* knockdown. Finally, we detected intense remaining dsRed fluorescence in SGs that overexpress *snz*, and at the same time no traces of glue are seen on the surface of prepupae, indicating glue release failure (**Figure 5K**). These observations indicate that Snz also regulates secretion in SGs, which further supports our findings in nephrocytes.

Discussion

Based on our results described here, we favor a model in which the main role of Snz is the negative regulation of Rab11-mediated recycling in *Drosophila* nephrocytes (**Figure 6**), supported not only by its colocalization with and binding to Rab11 but also by the mutant/overexpression phenotypes and extensive genetic interaction data. We propose that decreased *snz* function enhances membrane flow from REs towards the PM. This would lead to the formation of membrane ingressions; however, no prominent elongation of lacunar channels can be observed in *snz* loss-of-function nephrocytes. We assume that in this case, increased endocytosis compensates and it is sufficient to counterbalance the excess amount of membrane that arrives from REs to the PM. This increased rate of endocytosis leads to a slight overload of the endosomal system, culminating over time (that is, during the 5 days of larval development) in the striking expansion of the EE and LE endosomal populations in *snz* loss-of-function nephrocytes. This role of exocytosis in the development of enlarged LEs is strongly supported by our finding that depletion of exocyst restores LE size and eliminates intracellular Sns and Pyd accumulation in *snz* knockdown nephrocytes. Similar to the effect of *snz* knockdown, Rab11 gain-of-function (via overexpressing the wild type or GTP-locked form of Rab11 or knocking down *tbc1d8b* encoding a Rab11 GAP) does not lead to the formation of deep membrane ingressions on its own either, while Sns and Pyd again form intracellular deposits in Rab11^{CA} expressing cells, similarly to *snz* knockdown. Notably, this phenotype can be rescued by the overexpression of Snz, indicating that Snz counteracts Rab11. Ectopic diaphragms are also frequently observed in Rab11^{CA} expressing nephrocytes as the result of increased exocytosis (Kampf *et al.*, 2019). Importantly, expression of Rab11^{CA} on a *snz* loss-of-function background leads to partial breakdown of the nephrocyte surface diaphragm network and to the formation of deep membrane ingressions. We assume that in this case, excess membrane derived from REs is channeled towards the PM via massive exocytosis that far exceeds the capacity of compensatory endocytosis and leads to the uncontrolled elongation of labyrinthine channels. Similarly, rearrangements in the secretory system involving the trans-Golgi network and exocyst-positive vesicles are also apparent only in the case of simultaneous Rab11^{CA} expression and *snz* knockdown. Snazarus was identified as a potential binding partner of Rab11 in a comprehensive interactome study of Rab small GTPases (Gillingham *et al.*, 2014), which we have now confirmed in our study. Together with our genetic interaction data, the binding of Snz to Rab11 raises the possibility that Snz itself restricts Rab11 activity.

Although the exact biochemical mechanism is yet to be confirmed, we assume that decreased *snz* function leads to the disinhibition of a molecular step in the recycling route that promotes membrane flow from REs towards the PM. Snz functionally interacts with the fatty acyl desaturase Desat1 in *Drosophila* fat tissue as part of ER-PM-LD contacts (Ugrankar *et al.*, 2019) and the contact site-dependent role of human SNX14 in lipid metabolism is also known (Datta *et al.*, 2019, 2020).

Importantly, a recent study using AlphaFold2-based prediction reported that the PXA and PXC domains of Snx-TM proteins interact with each other to form a hydrophobic cavity with a presumable function of lipid binding and/or transfer (Paul *et al.*, 2022). These findings indicate the conserved role of Snx-TM proteins in lipid metabolism and transfer as parts of membrane contact sites. Thus, lipid transfer regulated by Snz might be also involved in its function. In line with this, ethanolamine addition rescues the autophagic defect caused by loss of Snz in *Drosophila* fat cells (Lauzier *et al.*, 2022). Nephrocytes must also depend on the trafficking of lipids as their endomembrane system undergoes continuous and intensive remodeling. In these cells, maintaining the balance of exocytosis and endocytosis is key to preserve the steady-state amount of not only proteins at the PM but also lipids/membrane. We think that Snz is a key player in regulating this kind of balance, and we cannot exclude the possibility that altered lipid composition also affects Rab11-dependent recycling events in nephrocytes in addition to the binding of Snz to Rab11.

The Rab11 small GTPase is a master regulator of cell surface protein composition in many cell types as it acts at the intersection of vesicular trafficking events directed towards the PM (Welz, Wellbourne-Wood and Kerkhoff, 2014). Accordingly, Rab11 is present on recycling endosomes, post-Golgi vesicles and also at the trans-Golgi network. Regulated by its activator GEF (guanine nucleotide exchange factor) and deactivator GAP (GTP-ase activating protein) partners, Rab11 interacts with a variety of effector proteins including different motor protein complexes and the exocyst complex (Welz, Wellbourne-Wood and Kerkhoff, 2014). Through these interactions, Rab11 is involved in a variety of fundamental cellular processes such as receptor and adhesion protein recycling, cytokinesis, oogenesis etc. *Drosophila* Rab11 is extensively investigated, and its function is described in glue granule maturation in the salivary gland, rhodopsin recycling in photoreceptor cells and also in diaphragm maintenance in nephrocytes (Satoh *et al.*, 2005; Wen *et al.*, 2020; Neuman *et al.*, 2021).

The involvement of Snx proteins in Rab11-mediated recycling has not been described previously, although the role of many sorting nexins in various vesicular trafficking processes is well established. The presence of the PI(3)P-binding PX domain in sorting nexins predestines these proteins to be involved primarily in endosome-associated sorting. Indeed, as parts of different sorting complexes such as the retromer, retriever and recycler, Snx proteins contribute to the recycling of a multitude of endosomal transmembrane proteins from early or late endosomes towards various locations of the endosomal system (Chen, Healy and Collins, 2019; Zhou *et al.*, 2022). Interestingly, both *Snz* and human SNX25 contain a non-canonical PX-domain, and alterations in their phospholipid-binding surfaces confers a broad specificity towards di- or triphosphorylated PI-derivatives. PI(4,5)P₂ is the most abundant PI-derivative in the PM, and in *Drosophila* fat cells, *Snz* interacts with the PM through its noncanonical PX domain. As it has been recently shown, *Snz* has a dual role in *Drosophila* fat cells as it is involved in lipid metabolism and also in autophagy, which might also involve its binding to PI(3)P that is typically found in forming and mature autophagosomes. Thus, the role of *Snz* in both vesicular and nonvesicular trafficking within the same tissue is already known in *Drosophila* fat cells. Another interesting feature of Snx-TM proteins is the presence of an RGS-domain, and it is tempting to speculate that *Snz* might directly regulate Rab11 in nephrocytes through this domain. This unique RGS (short for regulators of G protein signaling) domain is known to function as a GAP on trimeric G protein α subunits (Amatya *et al.*, 2021). It will be interesting to test in future studies if the RGS domain of *Snz*/SNX25 might be able to bind to Rab11 and thus directly inactivate this small GTPase, although RGS domain binding to a small monomeric GTPase is unprecedented so it seems unlikely.

Although the physiological function of SNX25, the human ortholog of *Snz*, is currently unknown, its role in vesicular trafficking was reported. SNX25 interacts with TGF- β receptors and enhances receptor degradation via the clathrin-dependent endocytosis and endosome/lysosome degradation pathway (Hao *et al.*, 2011). Human SNX25 is also involved in regulating autophagic flux via VAMP8 trafficking (Lauzier *et al.*, 2022). In recent studies, SNX25 has been associated with diseases such as temporal lobe epilepsy (Du *et al.*, 2013), colorectal cancer (Feng *et al.*, 2022), psoriasis (Vecellio *et al.*, 2021) and chronic kidney disease (CKD) (Kim, Jin and Eom, 2021). In the latter case,

SNX25 mutation is associated with higher activity of indoleamine 2,3-dioxygenase, a proposed CKD marker, and decreased glomerular filtration rate. Given the role of Snz in *Drosophila* nephrocytes, involvement of SNX25 in CKD is particularly interesting, although the effect of SNX25 mutations on human podocyte function has not been studied.

Importantly, our results point to a novel, recycling endosome-specific function of Snz that differs from its role in ER-PM-LD contact sites. In fat cells, the presence of such contacts is highly advantageous as they enable efficient response to altered nutritional conditions through the rapid mobilization of fatty acids. Although nephrocytes also contain LDs, these cells are not specialized for lipid storage and mobilization, hence the absence of ER-PM-LD contacts with fat cell-like functions is quite reasonable. It is obvious that Snz-GFP does not overlap with, and it is not even in the vicinity of, LDs in nephrocytes. LDs are found in the inner regions of these cells, while Snz-GFP localizes to Rab11-positive REs at the cell periphery. Here, an intricate network of lacunar channels is formed by the invaginations of the PM, which is dynamic with continuous vesicular fusion and fission events. The existence of membrane contact sites here that could enable nonvesicular membrane transport is also possible. The peripheral pool of the ER is also present here in the form of fine cisterns, raising the possibility that fat cell-like ER-PM contact sites might form in the periphery of nephrocytes. Although the dMAPPER-Cherry ER-PM contact site reporter does not show intensively fluorescent foci here, we cannot exclude the existence of such contacts based on our results. Accordingly, in some cases we saw partial overlap of Snz-GFP with dMAPPER-Cherry (**Figure 2B**). Based on this observation, one can imagine a scenario in which Snz acts as part of ER-PM contacts in nephrocytes that involves REs instead of LDs as a third organelle. The striking relocalization of REs in *snz* loss-of-function cells may reflect on the role of Snz in stabilizing such contact sites and anchoring REs to the tips of PM channels. However, if ER-PM contacts indeed form in the nephrocyte periphery, one would expect a higher degree of colocalization of Snz with anti-Hrp and anti-Cnx99A that we used as PM and ER markers, respectively. A possible caveat is that although anti-Hrp is widely accepted to stain nephrocyte PM (Paschinger, Rendić and Wilson, 2009; Atienza-Manuel *et al.*, 2021), deeper regions of lacunar channels do not seem to be labeled by this antibody. In the case of the ER, a fine network of peripheral ER cisterns with faint anti-Cnx99A signal is visible (**Figure S2C**), and these ER cisterns occasionally overlap with Snz-GFP. Thus, there is a possibility that ER-PM-RE contact sites form in nephrocytes. These contacts could be involved in non-vesicular membrane transport processes between these organelles, which could also contribute to the intensive membrane remodeling events at the nephrocyte periphery. Such organelle contacts could also tether REs to peripheral ER and to lacunar PM channels to facilitate vesicular trafficking. This model is supported by the striking redistribution of Rab11 vesicles towards the cell interior in *snz* loss-of-function nephrocytes.

Finally, the similar nephrocyte defects arising from Snz loss and Rab11 gain-of-function (seen not only upon expression of GTP-locked mutant Rab11, but also in case of wild type Rab11 overexpression and knockdown of *tbc1d8b* encoding a Rab11 GAP, which inhibits Rab11 by stimulating its GTP hydrolysis), the strongly synergistic effect of Snz loss with Rab11 gain, and that Snz overexpression antagonizes the effects of Rab11 gain-of-function and restores a wild type nephrocyte phenotype, collectively argue that these two proteins function together at recycling endosomes. We would like to emphasize that all the alterations arising upon modulation of Snz levels that we report here can be explained completely by its inhibitory effect on Rab11 activity, both in case of endosomal trafficking and exocytosis. Increased endocytic flux and recycling due to higher Rab11 activity in the absence of Snz likely results in increased exocytosis, which seems to be restored to normal levels in nephrocytes via attenuation of exocyst function, in line with our model as the exocyst is a known Rab11 effector. The recently described Rab11-dependent endocytic contribution to secretory granule maturation that promotes glue release in salivary glands (Ma *et al.*, 2020) is also perfectly in line with the phenotypes that we see upon modulation of Snz levels, because its salivary gland-specific overexpression inhibits glue release (possibly via decreasing Rab11 activity) whereas its loss enhances secretion (probably because of increased endocytic input to secretory glue granules). In conclusion, we

demonstrate here a new role for the sorting nexin 25 homolog Snazarus: its binding to and functional inhibition of Rab11 at recycling endosomes.

Materials and methods

Drosophila strains and genetics

Flies were raised on standard yeast/cornmeal/agar media at room temperature. Fly stocks used in this study were obtained from the Bloomington Drosophila Stock Center (BDSC), from the Vienna Drosophila Resource Center (VDRC), or from FlyORF and were the following: mCherry RNAi (control) [BDSC 35785], snz RNAi 1. [BDSC 39036], snz RNAi 2. [VDRC 105671], vps39 RNAi [VDRC 40425], vha16-1 RNAi [VDRC 49291], sec5 RNAi [BDSC 27526], rab11 RNAi [BDSC 27730], tbc1d8b RNAi [BDSC 32929], UAS-GFP-Rab5 [BDSC BL43336], [UAS-EYFP-Rab11^{Q70L} (Rab11^{CA}) [BDSC 9791], UAS-EYFP-Rab11-3xHA [FlyORF F001035]. The prospero-Gal4 driver was obtained from (Bechtel *et al.*, 2013) and the Glue-dsRed; fkh-Gal4 stock was obtained from (Costantino *et al.*, 2008). The snz^{KO} line, the UAS-dMAPPER-Cherry and UAS-Snz-GFP reporters were described in (Ugrankar *et al.*, 2019). Additional genotypes were the following: hs-Flp; UAS-Dcr2; Actin>CD2>Gal4 UAS-Rab7-GFP and hs-Flp; UAS-Dcr2; Actin>CD2>Gal4 UAS-Lamp1-GFP (Lőrincz *et al.*, 2016). Standard genetic crosses were used to recombine the UAS-Snz-GFP or the UAS-EYFP-Rab11^{Q70L} reporters to the prospero-Gal4-containing or tub-Gal4 containing chromosome. In the case of the rescue experiments, we established a stable stock with the following genotype: snz^{KO}; +; prospero-Gal4, UAS-Snz-GFP/TM6Tb.

Immunolabeling and Lysotracker Red staining

Immunostaining of formaldehyde-fixed nephrocytes was performed as described in (Lőrincz *et al.*, 2016). Briefly, wandering larvae were dissected in PBS and fixed with 4% formaldehyde. Samples were extensively washed with PBS, then incubated in PBTx (0.1% Triton X-100 in PBS) for 30 minutes. After blocking with 5% FCS-containing PBTx, samples were incubated overnight at 4°C in the blocking solution completed with primary antibodies. Samples were then rinsed, extensively washed, and then were incubated in the blocking solution containing the appropriate secondary antibodies for 2 hr at RT. After rinsing and washing, nuclei were stained with DAPI. For Oil Red O staining, at this point we added freshly prepared, filtered 2:3 mixture of distilled water and 0,1% Oil Red O (Sigma-Aldrich #O0625) solution (in isopropanol) to the samples followed by extensive washing with PBS. Samples were mounted in Vectashield. Heat-fixation of nephrocytes was performed as described in (Hochapfel *et al.*, 2017). Briefly, stage L3 larvae were dissected in PBS then the gut segments were put in boiling 0,03% PBTx and immediately microwaved for 20 seconds in water bath. After that, they were washed in TWEEN 20-PBS, then blocked in BSA solution for an hour. Samples were incubated with a-Sns and a-Pyd primary antibodies overnight at 4 °C. After extensive washing, appropriate secondary antibodies were added to the samples for 2 hours at RT, then washed again and mounted. The following primary antibodies were used: polyclonal rabbit a-Rab11 (1:400, (Tanaka and Nakamura, 2008)), polyclonal chicken a-Sns (1:1000, (Hochapfel *et al.*, 2017)), monoclonal mouse a-Sec5 (1:35, (Murthy *et al.*, 2003)), polyclonal rabbit a-Rab5 (1:100, Abcam #31261), monoclonal mouse a-Rab7 (1:10, DSHB), monoclonal mouse a-Cnx99A (1:200, DSHB), monoclonal mouse a-Pyd (1:400, DSHB), polyclonal goat a-Gmap (1:2000, DSHB), polyclonal goat a-Golgin245 (1:2000, DSHB), polyclonal chicken a-GFP (1:400, 2BScientific #AGFP-NM), polyclonal Alexa Fluor 647 goat a-Hrp (1:800, Jackson ImmunoResearch #2338967). Secondary antibodies were: FITC donkey anti-chicken, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 488 goat anti-chicken, Alexa Fluor 568 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 568 donkey anti-goat, Alexa Fluor 568 donkey anti-mouse, Alexa Fluor 647 rabbit anti-mouse (all 1:800, all Invitrogen - Thermo Fisher). For LysoTracker Red staining, larval garland nephrocytes were incubated in medium containing LysoTracker Red (1:1000, Thermo Fisher # L7528) for 5 minutes at RT. Samples were rinsed 3 times, stained with DAPI and imaged immediately.

Tracer uptake and channel diffusion assay

Nephrocytes of wandering stage larvae were dissected in freshly prepared Shields and Sang M3 insect medium (Sigma-Aldrich #S8398). Cells were incubated in M3 medium completed with BSA^{DQ Green} (Thermo Scientific #D12050), BSA^{Alexa-647} (Thermo Scientific #A34785) or Avidin^{TexasRed} (Thermo Scientific #A820) at 80 µg/mL concentration for 5 minutes (pulse), then washed out. Cells containing internalized tracers were then imaged after 20-minute or 1-hour chase intervals. In the case of tracer uptake experiments with short incubation times, nephrocytes were allowed to take up BSA^{Alexa-647} for 1 minute, and after a 9-minute chase they were fixed in 4% formaldehyde, washed extensively with PBS, then DAPI-staining was applied. Channel diffusion assay was carried out as described recently (Milosavljevic *et al.*, 2022).

Co-immunoprecipitation

Approximately 200 mg adult flies of either *snz*^{KO}; tub-Gal4, UAS-Snz-GFP/+, or *snz*^{KO}; tub-Gal4, UAS-Snz-GFP/UAS-Rab11-3xHA genotypes were dounce homogenized in 2 ml of LBW buffer (lysis, binding and wash: 25 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 20 mM KCl, 100 mM NaCl, 0.1x PBS, 10% glycerol, 0.2% Triton X-100). Extracts were clarified by repeated centrifugation taking care to leave as much of the upper fatty layer of the lysate behind as possible at each step. Extracts were further clarified by letting them pass through 300 µl Sephadex-G100 packed into disposable plastic columns by gravity flow. Extracts (750 µl) were incubated with 25 µl anti-HA magnetic beads (Pierce 88837) for 4 hours, washed extensively and eluted by using non-reducing SDS sample buffer. Bound material, along with small amount of the input lysates, were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. Rab11-3xHA and Snz-GFP were detected by anti HA-HRP (Roche 12013819001), anti-GFP (Abcam ab6556) and anti rabbit-HRP (Sigma A0545) antibodies, respectively, using ECL Prime reagent (Cytiva RPN2232) and X-ray film for detection.

EM analysis

Nephrocytes were dissected in ice cold PBS, then were fixed in 3.2% PFA, 0.5% glutaraldehyde, 1% sucrose, and 0.028% CaCl₂ in 0.1 N sodium cacodylate, pH 7.4, overnight at 4°C. Samples were then postfixed in 0.5% osmium tetroxide for 1 h, washed with distilled water (2*5 min at RT) then treated with half-saturated aqueous uranyl acetate for 30 min at RT. Samples were then dehydrated in a graded series of ethanol, and embedded in Spurr resin (Merck) according to the manufacturer's recommendations. 70-nm sections were stained with Reynold's lead citrate and viewed on a JEM-1011 transmission electron microscope (Jeol) equipped with a Morada digital camera (Olympus) using iTEM software (Olympus). Tannic acid impregnation was performed as described (Kosaka and Ikeda, 1983). Briefly, samples were processed for electron microscopy as above, but after the osmification step samples were treated with 1% tannic acid (Merck) in 0.1 M cacodylate buffer (pH 7.4) for 30 min at RT and uranyl acetate treatment was omitted.

Quantification and statistical analysis

We determined the percentage of cell area covered by dots using ImageJ software. In every case we corrected that by the area of the cell nuclei. To measure the late endosome size, we selected the Rab7 positive rings one by one using ImageJ. For colocalization analyses, we measured Pearson's correlation coefficient of red, green and far red channels using ImageJ software. In these experiments the levels of noise- and background staining was reduced using Zeiss Zen. We measured the Rab11 positive structures distance from the plasmamembrane by ImageJ. The Zeiss Zen software was used to measure the DQ Green BSA intensity, corrected in each case with the threshold value. Statistical analysis was performed using the test indicated in Supplementary table S1 and was carried out with GraphPad Prism. A P value of less than 0.05 was considered to be significant; * means $p < 0.05$; ** means $p < 0.01$; *** means $p < 0.001$.

Acknowledgements

This work was supported by the National Research Development and Innovation Office (NKFIH) of Hungary with PD135611 grant to TM, ÚNKP-22-2-III-ELTE-702 grant to DH, FK138851 grant to PL, and Elvonal KKP129797 and the National Laboratory of Biotechnology 2022-2.1.1-NL-2022-00008 grants to GJ. The work was also supported by the Eötvös Loránd University Excellence Fund (EKA 2022/045-P101-2) to PL. This research work was conducted with additional support from the National Academy of Scientist Education under the sponsorship of the Hungarian Ministry of Innovation and Technology (FEIF/646-4/2021-ITM_SZERZ).

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Figure 1. Loss of Snz enhances endocytic activity in nephrocytes

(A-E) Rab5 and Rab7 immunostaining of nephrocytes reveals the enlargement of early endosomes (EEs) and late endosomes (LEs), respectively, in *snz* knockdown (B, C) and knockout cells (D) compared to controls (A) and rescued mutants (E). (F-I) Pulse-chase labeling of nephrocytes after a 1-minute pulse followed by a 9-minute chase using fluorescently labeled BSA as an endocytic tracer. Compared to controls (F), increased amount of tracer is detected in the subcortical region of *snz* knockdown (G), *snz* knockout (H) and Rab5 overexpressing (I) nephrocytes. (J) Quantification of data from medial sections shown in panels S1A-E. (K and L) Ultrastructural analysis of control and *snz* knockdown nephrocytes. LEs, appearing as large, circle-shaped, electron-lucent vesicles with patches of a characteristic electron-dense material, are greatly enlarged upon loss of *snz*. (M and N) Pulse-chase labeling of GFP-Rab7-expressing nephrocytes after a 5-minute pulse followed by a 20-minute chase using fluorescently labeled BSA as an endocytic tracer. (O and P) Quantification of data from panels A-E. (Q) Quantification of data in panels R-T. (R-T) Pulse-chase labeling of nephrocytes using BSA^{DQ Green} and BSA⁶⁴⁷ as endocytic tracers. After a 5-minute pulse followed by a 1-hour chase, the detected green signal represents dequenched, fluorescent products of BSA^{DQ Green} degradation. Dequenching of DQ Green is only observed in patches within enlarged vesicles that accumulate BSA⁶⁴⁷ in *vps39* knockdown nephrocytes due to a block of endocytic progression (S), while the green signal appears both in vesicles and in the cytosol in control (R) and *snz* knockdown (T) cells, indicating that endocytic degradation of the tracer is followed by its release to the cytosol. (U-W) Frames from Supplementary movies V1-V3 show Avidin^{TexasRed}-positive endosomal compartments. Arrowheads point to tracer-positive tubules that are characteristic for actively digesting lysosomes. ER – endoplasmic reticulum, LE – late endosome, LD – lipid droplet, N – nucleus. Scale bars represent 5 μ m in all panels.

Figure 2. Snz colocalizes with and binds to Rab11 in nephrocytes.

(A-D) Snz-GFP expressing nephrocytes stained for various organellar markers. Cortical (A-D) and medial (A'-D') sections are shown in each case. No overlap is seen with the plasma membrane (PM)-marker anti-Hrp (A), the ER-PM contact site reporter dMAPPER-Cherry (B) and lipid droplets (LDs) labeled with Oil Red O (C). Snz-GFP shows extensive colocalization with Rab11-positive recycling endosomes (REs) (D). Insets show the areas marked by yellow rectangles in higher magnification. (E) Quantification of data from A-D and Figure S2A-S2F. (F) Co-immunoprecipitation from fly lysates shows the binding of Snz-GFP to Rab11-3xHA. (G and H) Rab11 immunostaining of control (G) and *snz* knockdown (H) nephrocytes. (H) Quantification of data from G and H. Scale bars represent 5 μ m in the main panels and 1 μ m in insets.

Figure 3. Snz modulates Rab11 activity for the proper maintenance of nephrocyte diaphragm (ND) integrity

ND patterns are visualized by Sns and Pyd immunostaining in heat-fixed nephrocytes. Cortical sections (A-H) show ND pattern at the cell surface and (A¹-H¹) show higher magnifications of the areas in yellow rectangles. Medial sections (A'-H' for Pyd and A''-H'' for Sns) show cross-sectional views of ND units and deeper regions of nephrocytes. Arrowheads show ectopic NDs. Compared to the control (A), knockdown of *snz* (B) cause relatively mild alterations in the distribution of diaphragm components. This effect is similar to those seen upon expression of Rab11^{CA} (C), knockdown of the Rab11 GAP *tbc1d8b* (E), or overexpression of Rab11^{WT} (G). Massive rearrangements of the ND pattern are obvious when *snz* knockdown is combined with Rab11^{CA} expression (D), *tbc1d8b* knockdown (F), or Rab11^{WT} overexpression (H). Scale bars represent 5 μ m in panels (A-H), 2 μ m in panels (A'-H'), (A''-H'') and 1 μ m in panels (A¹-H¹).

Figure 4. Snz and Rab11 control the depth of lacunar channels in nephrocytes

(A–D) TEM images of tannic acid impregnated cells, which highlights the lacunar system of nephrocytes (labeled by dark material). (E and F) Formaldehyde-fixed nephrocytes expressing EYFP-Rab11^{CA} immunostained for Pyd show that constitutively active Rab11 is present on the greatly expanded lacunar system in *snz* knockdown cells. (F¹) Higher magnification of the area in yellow rectangle. (G–J) Channel diffusion assay shows the depth of lacunar channels along the perimeter of whole cells. Note the presence of extremely elongated channels when *snz* knockdown is combined with Rab11^{CA} expression (D, F and J) compared to the control (A, E and G), *snz* knockdown (B and H) or Rab11^{CA} expression (C and I) alone. (K) Quantification of data from panels G–J. Scale bars represent 1 μ m in panels (A–D and F¹), and 5 μ m in panels in panels (E–J). LE – late endosome, N – nucleus. Medial sections are shown in (E–J).

Figure 5. Snz is a negative regulator of exocytosis.

(A–D) Sec5 and Golgin245 coimmunolabeling in nephrocytes visualizes the exocyst complex and the trans-Golgi network, respectively. Expression of Rab11^{CA} together with *snz* RNAi results in massive expansion of the exocyst network and intracellular redistribution of Golgin245 compared to control (A) or either genetic manipulation alone (B, C). (E) Quantification of data from A–D. (F and G) Rab7 and Hrp staining of nephrocytes to label LEs and the PM, respectively. Enlarged endosomes are seen in *snz* RNAi cells (F, compare with the Rab7 structures seen in control cells in Figure 1A). Co-silencing of the exocyst component *sec5* restores LE size to normal (although not their proper distribution, G) in *snz* knockdown cells. (H) Quantification of data from G, H and control cells (not shown). (I – K) Prepupae expressing the Glue-dsRed reporter in salivary glands (SGs). Normally, only a subset of glue protein is secreted from SGs and released to the surface of the animals, while residual glue can be observed in SGs inside the pupal case (I). Overexpression of *snz* increases the amount of released glue (J), while its loss blocks glue release (K). Scale bars represent 5 μ m in panels A–D, F–G and 1 mm in panels I–K.

Figure 6. Snz is a negative regulator of Rab11-mediated recycling in Drosophila nephrocytes

Schematic shows that lacunar channel and diaphragm integrity is maintained by the balance of endocytic and exocytic trafficking in wild type nephrocytes. Loss of Snz alone or combined with Rab11 gain-of-function lead to imbalances in membrane trafficking and prominent functional and morphological changes in the endosomal system. Please see the main text for further details. Created with BioRender.com.

Figure S1. Loss of *snz* enhances endocytic activity in nephrocytes and it does not affect lysosomal degradation.

(A-E) Pulse-chase labeling of nephrocytes after a 1-minute pulse followed by a 9-minute chase using fluorescently labeled BSA as an endocytic tracer – medial sections are shown at the nuclear plane. For quantification, please see Figure 1J. (F-I) Lysotracker Red staining of nephrocytes to label acidic compartments. (J) Quantification of data from panels F-I. (K-N) Analysis of Lamp-GFP fluorescence in nephrocytes. Increased reporter signal intensity indicates impaired degradation. (O) Quantification of data from panels K-N. Note the absence of LTR-positivity and high Lamp-GFP signal in the case of acidification defective *vha16-1* knockdown cells (I and N, respectively) used as a positive control. In contrast, *snz* knockdown or *snz* knockout do not decrease LTR-positivity (G and H, respectively) and do not increase Lamp-GFP fluorescence (L and M, respectively) compared to the wild type situation (F and K, respectively). Scale bars represent 5 μ m in all panels. Medial sections are shown in each case.

Figure S2. Localization of Snz in nephrocytes.

(A - F) Snz-GFP expressing garland nephrocytes are stained for various organellar markers as indicated. Medial sections are shown in each case. See Figure 2E for quantification of colocalizations. Scale bars represent 5 μ m in the main panels and 1 μ m in insets.

Figure S3. Snz and Rab11 control nephrocyte diaphragm (ND) integrity and intracellular ND protein deposits.

Anti-Sns and anti-Pyd immunostainings reveal intracellular deposits of Sns and Pyd upon *snz* knockdown (B), the generation of which is suppressed by *sec5/exocyst* RNAi (E) that inhibits exocytosis. Similar intracellular ND protein deposits are seen in Rab11^{CA} expressing cells (C), which is suppressed by overexpression of *snz* (D). Control cells have no such intracellular deposits (A), while combined knockdown of *snz* and expression of Rab11^{CA} leads to massive accumulation of intracellular Sns and Pyd (F). (G) TEM image showing the cortical region of a *snz* knockdown nephrocyte expressing Rab11^{CA}. Large black arrowheads indicate membrane ingressions (that is, elongated lacunar channels), small red arrowheads indicate ectopic diaphragms and small blue arrowheads indicate normal diaphragms along the cell surface. (H) A *rab11* knockdown nephrocyte showing severe disorganization of the ND in the cortical region without accumulation of Pyd (H') or Sns (H'') in the medial region. Scale bars represent 10 μ m in panels (A-F), 5 μ m in panel (H), 2 μ m in panel (H'-H'') and 1 μ m in panel G. EE – early endosome, LE – late endosome, LCh – lacunar channel.

Supplementary movie V1. Live imaging of control nephrocytes pulse-chase labeled with Avidin^{Texas Red} endocytic tracer.

Supplementary movie V2. Live imaging of *vps39* knockdown nephrocytes pulse-chase labeled with Avidin^{Texas Red} endocytic tracer.

Supplementary movie V3. Live imaging of *snz* knockdown nephrocytes pulse-chase labeled with Avidin^{Texas Red} endocytic tracer.

