VPS13A: shining light on its localization and function
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CHAPTER 5

Timely removal of nurse cell corpses requires a cell-autonomous function of Vps13

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CHAPTER 5

ABSTRACT

Programmed cell death and consecutive timely removal of cellular remnants is essential for development. During *Drosophila melanogaster* oogenesis, the relatively small follicle cells that surround the larger nurse cells, promote nurse cell death, subsequently engulf them and contribute to the timely removal of nurse cell corpses. Players involved in these follicle cell driven processes are starting to emerge. Relatively less is known about endogenous factors expressed by nurse cells required for their complete removal after induction of their cell death by follicle cells. Here we identified a cell-autonomous and nurse cell specific role for Vps13 involved in proper removal of nuclei of nurse cell corpses downstream of developmental programmed cell death. Vps13 is an evolutionary conserved peripheral membrane protein belonging to the VPS13 gene family. It is associated with membrane contact sites and lipid transfer. In *Drosophila melanogaster* Vps13 mutants, induction of cell death occurs normally but remnants of nurse cells persist. Vps13 protein is detected in close proximity to the plasma membrane in nurse cell corpses. Ultrastructural analysis revealed the presence of a Vps13-dependent membranous structure at this subcellular localization in partly degraded nurse cells. The newly identified Vps13-dependent structure and function reveal a cell-autonomous process required for complete degradation of bulky remnants of dying cells. Overexpression of human Vps13A rescues the *Drosophila* Vps13 mutant phenotype, suggestive for a functional conservation of this Vps13-dependent process.

**Key words:** Vps13; *Drosophila melanogaster*; Programmed cell death (PCD); Cell-autonomous; Nurse cells; Oogenesis
INTRODUCTION

During development and tissue remodeling excessive or unnecessary cells undergo programmed cell death (PCD) after which they are removed by phagocytic cells. The most extensively studied form of PCD is apoptosis, which involves the activation of caspases. Apoptosis is characterized by condensation of chromatin and cytoplasm, nuclear fragmentation and membrane blebbing. Other examples of PCD include necrosis and autophagic cell death of which the mechanisms and players involved are starting to be unravelled. In addition to those self-induced mechanisms, cell-death can also be initiated in a non-autonomous manner by surrounding cells. After the induction of cell death, efficient clearance of the dead cell’s remnants is required. Lack of clearance of dead cells has been linked to multiple human diseases including chronic obstructive pulmonary disease (COPD), atherosclerosis and cancer, underscoring the importance of this process.

Both PCD and subsequent clearance of remnants of dying cells play an important role during late stages of oogenesis in Drosophila melanogaster. Drosophila females have two ovaries, each containing 15-17 tubular ovarioles in which the egg chambers develop. Egg chambers consist of 16 germline cells, including 15 nurse cells and 1 oocyte. The germline cells are surrounded by a layer of somatically derived follicle cells. The individual egg chambers are produced by germline and somatic stem cells in the germarium, after which they grow through 14 well-defined developmental stages. During the first stages of oogenesis, nurse cells produce nutrients that are transferred into the oocyte cytoplasm through ring canals. At stage 10-11, the nurse cells deposit their entire cytoplasm in the oocyte in a process called cytoplasmic dumping. This is followed by the initiation of developmental PCD and subsequently removal of dying nurse cells occurs through phagocytosis by the surrounding follicle cells. Failure of either developmental PCD or impairment of subsequent phagocytosis by follicle cells leads to accumulation of persistent nurse cell nuclei during the last stages of oogenesis.

Little is known about the nature of the nurse cell PCD. It is considered to be non-apoptotic as caspases do not play a major role. Autophagy also plays a role in the induction of cell death during late stages of Drosophila oogenesis, because the apoptotic inhibitor dBruce is degraded by autophagy and thereby cell death is triggered. However, autophagy and caspases only play a minor role in PCD during oogenesis because combined inhibition of autophagy and caspases does not largely interfere with developmental PCD, suggesting the presence of other inducers of cell death. Indeed, the stretched follicle cells surrounding the late oogenesis nurse cells also play a role in triggering nurse cell PCD, because after genetic ablation of these cells, PCD is prevented. Moreover, stretched follicle cell specific downregulation of the engulfment gene draper results in impaired DNA fragmentation of nurse cell nuclei, which is an early marker of induced cell death.

The phagocytic gene Ced-12/ELMO, the JNK pathway, integrins and genes associated with lysosomal function and intracellular trafficking are all factors involved in removal of nurse cells, suggesting a major stretched follicle cell-specific and phagocytosis-like function in the process of the timely removal of nurse cells remnants. Combined a model is arising in which stretched follicle cells are required to
induce nurse cell death, provide lysosomes that decorate the nurse cell remnants and cause acidification of the nuclear remnants, allowing for DNAse II activity, and finally engulf the nurse cell corpses, all in concert contributing to efficient removal of the remnants. It is currently not clear for all mentioned follicle cell specific factors whether they solely play a role in nurse cell PCD or whether they also play a role in the following process of removal. In contrast to this emerging picture of the role of follicle cells, nurse cell specific functions in this whole process are less clear. Besides a role for DNAse II, playing a role in the degradation process of DNA and a role for lysosomal genes for the timely removal of cell remnants, cell-autonomous factors involved in the removal of nurse cells are unknown.

Here we demonstrate that the peripheral membrane protein Vps13 (Vacuolar protein sorting 13 homolog), the Drosophila ortholog of human VPS13A, is required for the timely removal of nurse cell corpses during late oogenesis. VPS13-family members are multitasking proteins playing important roles in membrane formation, membrane contact sites and lipid transfer. Mutations in the human VPS13A gene lead to the rare autosomal recessive neurodegenerative disease Chorea-Acanthocytosis (ChAc), which is also characterized by neurodegeneration and the presence of acanthocytes (spiky red blood cells). We have previously reported that Vps13 Drosophila mutants show a neurodegenerative phenotype upon ageing, yet, it is largely unknown why mutations in VPS13A lead to ChAc. While characterizing the phenotype of Drosophila Vps13 mutant flies, we observed a significant accumulation of persistent nurse cell nuclei in mutant ovaries. By using CRISPR/Cas9 we created a Drosophila Vps13 loss of function mutant and a Vps13-GFP expressing Drosophila line to investigate the role of Vps13 during oogenesis. Vps13 is expressed in nurse cells and localized in close association with the plasma membrane at stage 11-12 during late oogenesis. Ultrastructural analysis revealed a Vps13-dependent membrane structure in late stage nurse cells in which PCD has been induced and removal by phagocytosis is ongoing. Our results reveal a Vps13-cell autonomous function in nurse cells required for the timely removal of nurse cell corpses downstream of PCD.

RESULTS

Vps13 mutants have a reduced fecundity

Previously, we have reported that insertion of a piggyBac transposable element in the Vsp13 gene (Vps13PB) leads to a neurodegerative phenotype. Upon further examination we noticed that homozygous female Vps13PB mutants produced less offspring compared to control flies (w1118). Further investigation revealed that the fecundity of Vps13PB females was indeed strongly reduced and a delay and reduction in egg laying compared to control female flies was observed (Figure 1A,B). accordingly, ovaries of homozygous Vps13PB females contained smaller ovaries harboring a smaller amount of mature eggs when compared to ovaries from control females (Figure 1C).

In agreement with the ovary phenotype, Western blot analysis using an antibody against the C-terminal domain of Vps13, revealed the presence of full length Vps13 protein in extracts of control ovaries (Suppl.
Figure 1. Vps13 mutants have a reduced fecundity and the Vps13^{null} mutant resembles the Vps13^{PB} mutant phenotype

A. Egg lay capacity of control (w^{1118}) and Vps13^{3436} mutant flies was recorded for 14 days. The average number of eggs laid per female per day is shown and total egg production is quantified in B. C, F. Dissected ovaries of 4 day old female control (w^{1118}) and Vps13^{PB} (C) or control (w^{1118}) and Vps13^{3436} mutant flies (F). Scale bar indicates 1 mm. D. Schematic representation of the Vps13 gene showing the target sites of the sgRNAs for the generation of a Vps13^{null} mutant using the CRISPR/Cas9 system. Exons are shown as black boxes. Target sites of the qPCR primer set 1 and 2 are indicated. In addition the localization of the PBac insertion site of the Vps13^{PB} mutant is depicted. Sequences of the Vps13 genomic region are indicated of the control (CC control) strain and the Vps13^{null}. The released sequence of the Vps13 gene (flybase.org) is provided as well as the sequence of exon 8. E. Western blot analysis of Vps13 levels in control (w^{1118} and Precise excision line) and both Vps13 mutants using the Vps13 #62 and Vps13 NT antibodies. Tubulin staining was used as a loading control.
Fig. 1). In contrast full length Vps13 was below detection levels in ovary extracts from Vps13\(^{PB}\) homozygous mutants. Conversely, an antibody against the N-terminal domain detected a truncated protein in the mutant extract (Suppl. Fig. 1). This is consistent with Vps13 expression patterns in samples derived from homozygous Vps13\(^{PB}\) fly heads\(^{35}\), and suggests that only the N-terminus of Vps13 is expressed in the mutant in agreement with the position of the piggyBac. Precise excision of the piggyBac element (hereafter called ‘precise excision’, which resembles Excision line 1 from Vonk et al. 2017\(^{35}\)) restored the levels of full length Vps13 protein in ovary extracts (Suppl. Fig. 1).

The presence of a truncated Vps13 product in the Vps13 mutant could lead to a toxic gain of function (dominant negative effect), obscuring the interpretation of the mutant phenotype. Therefore, we created a null Vps13 mutant in which the Vps13 protein is entirely absent. To do this, we used the widely used gene editing system CRISPR/Cas9 that enables precise editing of the genome in various cells, tissues and organisms including Drosophila melanogaster\(^{36–39}\). A Vps13 knockout mutant was generated by targeting exon 4 and exon 8 at the N-terminus of the Vps13 gene (Figure 1D and Methods). Potential mutant lines were analyzed by Western blot using both antibodies recognizing either the Vps13 N- and C-terminus. A null mutant was searched for expressing neither a full length protein nor a N-terminus fragment. Indeed one of the lines fulfilled these characteristics, hereafter called Vps13 null (Figure 1E). Sequencing of the target regions exon 4 and exon 8 of the Vps13\(^{null}\) mutant revealed a 2 bp deletion in exon 8 (Figure 1D), leading to a premature stop codon and consequently the absence of the Vps13 protein.

Analysis of Vps13\(^{null}\) mutant ovaries showed a similar size reduction compared to controls as the Vps13\(^{PB}\) mutant (Figure 1F). qPCR analysis showed a significant reduction in Vps13 mRNA product in the Vps13\(^{null}\) compared to an isogenic CRISPR/Cas9 control line harboring no mutations in the Vps13 gene (CC control) (Suppl. Fig. 2A). To further validate the new Vps13\(^{null}\) mutant, we tested whether one of the hallmarks of the Vps13\(^{PB}\) mutant, a reduction of life span with the majority of mutant flies dying around day 16-20 after eclosion\(^{35}\), was also observed in the Vps13\(^{null}\) mutant. Homozygous Vps13\(^{null}\) mutants indeed had a shortened life span when compared to the isogenic CC control (Suppl. Fig. 2B). Furthermore, Vps13\(^{PB}\) and Vps13\(^{null}\) mutants showed a comparable life span curve (Suppl. Fig. 2B).

Taken together, these results indicate that the Vps13\(^{null}\) CRISPR/Cas9 mutant is a validated Vps13 null (at the genetic, protein and RNA level) mutant that can be used to investigate the role of Vps13 during oogenesis.

**Vps13 mutant ovaries show an accumulation of persistent nurse cell nuclei, a phenotype partly rescued by overexpression of hVPS13A**

To understand why loss of Vps13 leads to a reduction of eggs, we examined oogenesis in control and Vps13\(^{null}\) females. Compared to controls, ovaries of Vps13 mutants exhibited a significant accumulation of persistent nurse cell nuclei at the final stage of oogenesis (stage 14) (Figure 2B,C). As explained in the introduction, after dumping of cytoplasm from the nurse cells into the oocyte compartment, the nurse cells undergo programmed cell death and are fully removed and degraded by the surrounding follicle cells\(^{13}\). When the removal of the nurse cell remnants by the surrounding follicle cells is defective, the phenotype of ‘persistent nurse cell nuclei’ is observed, and this can lead to obstruction and failure of oogenesis and production of mature oocytes\(^{15,22}\) (Figure 2A).
Specific overexpression of human Vps13A in the Vps13 heterozygous mutant background using the UAS/GAL4 system partly abolished the persistent nurse cell nuclei phenotype (Figure 2D), whereas expression of actin-GAL4 or a non-induced hVps13A transgene (as controls) had no effect (Figure 2D). These results demonstrate that Vps13 is required to prevent the formation of persistent nurse cell nuclei and supports a functional conservation between Drosophila Vps13 and human VPS13A in this process.
Vps13 mainly localizes to the nurse cells during late stages of oogenesis.

To further understand this specific Vps13 mutant phenotype, we localized Vps13 during the last stages of oogenesis using previously published antibodies and by using our newly generated Vps13 mutant as a negative control. In addition, using the CRISPR/Cas9 system, we engineered a fly line expressing Vps13 tagged with GFP fusion at the Vps13 endogenous locus (Suppl. Fig. 3). In short, we inserted the GFP coding sequence at the 3’ end of the Vps13 coding region (Suppl. Fig. 3A), which gave rise to a functional fusion protein. The Vps13-GFP fly line is viable, no persistent nurse cell nuclei are visible, and their life span is similar to control flies (Suppl. Fig. 3).

Immunofluorescence staining of w1118 control ovaries with the Vps13 NT antibody revealed a distinct Vps13 expression pattern close to the boundaries of nurse cells around stage 11-12 while a more diffuse staining was visible in the surrounding follicle cells (Figure 3B,B’). The distinct pattern (Figure 3B,B’) was absent in Vps13null ovaries, demonstrating the specificity of this signal (Figure 3D,D’). Furthermore, Vps13-GFP expressing ovaries, showed a similar pattern that overlapped with the Vps13 NT antibody staining near the plasma membrane of the nurse cells (Figure 3C).

To confirm the Vps13 localization close to the plasma membrane, we expressed the plasma membrane marker mCD8-GFP in nurse cells using a nurse cell specific driver (Suppl. Fig. 4A,B). Co-staining with the Vps13 NT antibody revealed that Vps13 localization to late oogenesis nurse cells indeed corresponds to a close association with the plasma membrane, likely at its cytoplasmic face in line with the fact that Vps13 is not an integral membrane protein (Figure 4). Conversely, co-staining with the nuclear envelope marker Lamin-D showed that the Vps13 signal did not co-localize with the nuclear membrane, but rather was visible adjacent to the Lamin-D signal (Suppl. Fig. 5). Of note, at this stage during oogenesis, the nurse cell nuclei are large, the cytoplasm is reduced because of cytoplasmic dumping and the nuclear envelope is very close to the plasma membrane.

Together these results clearly show that Vps13 is expressed in the late ovarian tissue and mainly localized near the cytoplasmic face of the nurse cell plasma membrane. Although a specific Vps13 signal is not clearly visible in the follicle cells, this does not formally exclude that Vps13 is expressed in these cells at very low levels.

Downregulation of Vps13 in the nurse cells, and not in follicle cells leads to the accumulation of persistent nurse cell nuclei

Given the prominent localization of Vps13 in the late oogenesis nurse cells, we hypothesized that Vps13 functions in preventing persistent nurse cell nuclei removal via a cell-autonomous process. To test this, we specifically downregulated Vps13 in the nurse cells or in follicle cells by RNA interference (RNAi) and analyzed the ovaries. Efficacy of general RNAi (using the actin-GAL4 driver) was tested using Western blot analysis that showed that the Vps13 protein level was below detection limit (Suppl. Fig. 6).

Consistent with Vps13 localisation, Vps13 downregulation specifically in follicle cells does not affect the Vps13 specific signal in the nurse cells, a pattern similar to the signal in control cells (Figure 5A). Importantly,
Figure 3. Vps13 is expressed during late stages of oogenesis
A-A’. Schematic representations of a stage 12 egg chamber. A’ is an enlargement of the red box in A. Immunolabeling of stage 12 egg chambers from w1118 (B-B’), the generated Vps13-GFP fly line (C) and Vps13null (D-D’) flies showing endogenous localization of Vps13 as visualized with the Vps13 NT antibody (magenta) and the Vps13-GFP fusion protein in the Vps13-GFP fly line (green). Arrows indicate the Vps13 specific signal. Nuclei of follicle cells are indicated with an arrowhead, nurse cell nuclei are marked with asterisks. B’ and D’ are enlargements of the red boxes in B and D respectively. Scale bars indicate 50 µm.
the resulting ovaries did not display any persistent nurse cell nuclei (Figure 5C). In contrast, knockdown of Vps13 in the nurse cells resulted in the absence of Vps13 staining in the nurse cells (Figure 5B). Furthermore, the nurse cell specific knockdown of Vps13 resulted in a significant increase in the percentage of stage 14 egg chambers with persistent nurse cell nuclei when compared to control (Figure 5C). These results indicate that Vps13 is specifically expressed and required in nurse cells, not in follicle cells, either to induce nurse cell PCD or to facilitate phagocytosis of dead nurse cells.
Cell-autonomous function of Vps13 in nurse cell removal

Vps13 acts downstream of programmed cell death during late stage oogenesis.

Accumulation of persistent nurse cell nuclei can be indicative for a defect in cell death induction or a defect in removal of the dying cells. Nurse cells that undergo PCD display pyknotic nuclei, breakdown of nuclear lamins and acidification visualized by the presence of large lysotracker positive structures.  

Figure 5. Downregulation of Vps13 in nurse cells and not in follicle cells leads to the accumulation of PNCN

Stage 12 egg chamber of Vps13 RNAi/+;GR1-GAL4/+ (targeted Vps13 downregulation in follicle cells; A) and Vps13 RNAi/+;nos-GAL4/+ (targeted Vps13 downregulation in nurse cells; B) stained with DAPI (blue) and Vps13 NT (green). Scale bars indicate 50 µm.

C. Quantification of the percentage of stage 14 egg chambers containing PNCN in various genetic backgrounds; Vps13 RNAi/+;nos-GAL4/+; Vps13 RNAI/+;GR1-GAL4/+; Vps13/act-GAL4 (control) and Vps13 RNAI/+ (control).
To determine whether Vps13 plays a role upstream or downstream of PCD, ovaries were investigated for these markers. Vps13 mutant ovaries show pyknotic nuclei, breakdown of nuclear lamins (Suppl. Fig. 5) and an acidification pattern indistinguishable from control ovaries (Figure 6). These data suggest that Vps13 is not required for PNCD and rather plays a role in efficient removal of remnants of nurse cell corpses.

Figure 6. Nurse cell acidification during developmental PCD is not affected in Vps13 mutants
Stage 12 egg chambers of w¹¹¹⁸ (A), Vps13PB (B), Precise excision (C) and Vps13null (D) were labeled with DAPI (green) and Lysotracker (red) to visualize acidification of nurse cell nuclei. Scale bars indicate 50 µm.
Cell-autonomous function of Vps13 in nurse cell removal

Vps13 localization is independent of the phagocytic receptor Draper and Draper localization is not affected in Vps13 mutants.

The follicle cell dependent phagocytic machinery is required for removing dying nurse cells, a process in which the engulfment gene *draper* plays an essential role\(^2\). To test a possible interaction between Draper and Vps13, we stained late stage ovaries of Vps13-GFP expressing flies with an antibody against Draper. We did not detect any overlap between the signals in the nurse cells (Figure 7A,A’). Draper was detected in the stretched follicle cells surrounding the nurse cells, whereas, Vps13 is mainly detected in the nurse cells (Figure 5, 7A,A’). Furthermore, Draper localization and levels were not affected in the Vps13\(^{null}\) mutant (Suppl. Fig. 7), and conversely, Vps13 localization was not changed in the *draper* mutant (*drprΔ5*) (Figure 7C,D). These results indicate that Draper and Vps13 are not likely to directly interact during the process of timely removal of nurse cell nuclei. The role of Draper is follicle cell-dependent and therefore acts in a non-cell autonomous manner in the nurse cell removal process, whereas our results point to a cell autonomous role of Vps13.

Largescale ultrastructural analysis of *Drosophila* ovaries reveals a novel Vps13-dependent membrane structure during late oogenesis

Vsp13 is localized in close association to the cytoplasmic leaflet of the plasma membrane (Figure 4) and co-localization studies with different organelle markers (for plasma membrane, nuclear envelope and lysosomes) did not reveal specific co-localization with cellular organelles and Vps13 around stage 11-12 of oogenesis (Figure 4; Suppl. Fig. 5 & 8), during which its expression is most pronounced.

In order to further examine the role of Vsp13 in the removal of nurse cell corpses, we analysed the cellular ultrastructure of control and Vps13 mutant ovaries using electron microscopy. To do this, ovaries were embedded, processed, and semi-thin sections of stage 11-12 egg chambers were pre-selected using light microscopy (Suppl. Fig. 9). After identification of the desired stage, ultrathin sections were generated, stained and analysed by electron microscopy. In control nurse cells at this stage, and consistent with our immunofluorescence data (Suppl. Fig. 5) and published data\(^4\) the nucleus is irregular, the nuclear membrane is still intact and shows invaginations, the cytoplasmic part of the nurse cells is reduced and the plasma membrane is in close proximity to the nuclear envelope, vacuoles (large translucent areas) are present representing the acidification process, a degradative process is ongoing characterized by structures containing cellular degradation products and the to-be-degraded nurse cells are surrounded by the engulfing follicle cells (Figure 8A - 8A”). All mentioned ultrastructural characteristics are present in Vps13 mutant ovaries as well at this stage (Figure 8C - 8C”, Suppl. Fig. 10), further indicating that the PCD process and the follicle cell dependent engulfment process were not affected in Vps13 mutants. However, in control cells at stage 11-12, we observed the presence of a structure (Figure 8B - 8B”, black arrowhead) very close to the plasma membrane. This structure appears to be stained by osmium and is likely to be membranous. The structure seems discontinuous, however, examination using 3D analysis is required to conclude this. Three out of three scanned and analysed dying nurse cells of *w*\(^{1118}\) at this stage revealed stretches of the specific membrane structure closely adjacent to the plasma membrane, in close association with and surrounding the nucleus (Figure 8B - 8B”). This structure is not observed in
Figure 7. Vps13 localization and intensity is independent of the phagocytic receptor Draper in late oogenesis

A. Stage 12 egg chambers of Vps13-GFP female flies visualizing Draper with a Draper antibody (red) and Vps13 localization by Vps13-GFP (green). The boxed region represents the enlargement shown in A’. Immunolabeling using a Vps13 antibody to visualize Vps13 in green in stage 12 (B&C) and stage 13 (D) egg chambers in w1118 (B), and in the drprΔ5 mutant background (C&D). DAPI (magenta) was used to visualize nuclei. Scale bars indicate 50 µm.
Figure 8. Vps13-dependent membrane structure during late oogenesis

Large scale electron microscopic images of a nurse cell nucleus (NCN) of a control (w1118; A) and Vps13null (C) stage 12 egg chamber, surrounded by stretch follicle cells (FC). Red arrows indicate structures of the cellular degradative pathway (MVB), black arrows indicate translucent vesicles (TVs) and nurse cell cytoplasm (NC Cyto). A’ & C’. Schematic representation of the dying NC nuclei in A and C where the plasma membrane (red) and nuclear envelope (dark blue) are still largely intact. Structures seen in A and C are indicated as well. A” & C”. Enlargement of the red box in A and C showing a schematic representation of the plasma membrane (pink), the nuclear envelope (blue) with invaginations (asterisks) and the Vps13-dependent membrane (green) found at this stage of PCD in controls. B-B’ & D-D’ Enlargements of the large scale electron microscopic images of A and C. Membrane structures in B and D are indicated in the same colors as A’ and C’ for reference. B” & D” are enlargements of the red boxes in B’ and D’. The plasma membrane is indicated with a black arrow; pink arrows indicate the nuclear envelope, arrowheads indicate the Vps13-dependent membrane. Scale bars in A-A’ and C-C’ indicate 5 µm. Scale bars in B-B’ and D-D’ indicate 500 nm.

nurse cells during earlier stages of oogenesis. Strikingly, this structure was absent or disrupted in six out of six stage 11-12 nurse cells of Vps13 mutants (Figure 8D – 8D”, Suppl. Fig. 10B – 10B”). Our data indicate that this novel membrane is generated in a Vsp13 dependent process and that it is required for the proper completion of the removal of all nurse cell corpses by phagocytosis.

DISCUSSION

Human VPS13A and its orthologs have been implicated to play a role in multiple cellular processes including autophagy42–44, intracellular trafficking45–49, regulation of membrane morphogenesis during sporulation26–28, membrane contact sites25,29,50,51 and protein homeostasis35. Here we report a novel cell-autonomous and nurse cell specific role for Drosophila Vps13 in the timely removal of dying nurse cell nuclei downstream of induced cell death during development. Absence of Vps13 is associated with failure of the formation of a specific membrane structure closely associated with the plasma membrane of dying nurse cells, suggesting a role for this Vps13-dependent novel structure in timely removal of large cellular remnants.

Our results further underscore the variety of cellular processes in which the conserved VPS13 protein family plays a role. Our results are consistent with several previously independently reported functions of Vps13 in other organisms. Firstly, in yeast Vps13 is required for proper formation of prospore membranes26–28. The prospore membrane is a newly formed double membrane structure which forms during meiosis, and while meiosis progresses, the newly formed daughter nuclei are engulfed and surrounded by this newly formed double membrane structure. This membrane structure serves as the plasma membrane of the newly formed spore. In yeast Vps13 localizes to the newly formed prospore membranes and is required for closure of the prospore membrane. Our data suggest that Vps13 in Drosophila is also associated with a membranous structure and in Vps13 mutants this membrane structure is disrupted or not detectable at the ultrastructural level, at least not at the specific position close to the plasma membrane. Together with data published by others26–28 this suggests that Vps13 serves a conserved function in membrane morphogenesis and/or membrane positioning. The conserved function is further strengthened by our results that the Drosophila oogenesis phenotype is rescued by overexpression of human VPS13A in the mutant background. Studies in yeast and mammalian cells showed a close association with Vps13 and VPS13A at contact sites between endoplasmic reticulum (ER) and other organelles and functions as a lipid transfer protein25,29–31,50,51. It is therefore likely that in Drosophila Vps13 plays a role at establishing or maintaining membrane contact sites between our described membranous structure and the plasma membrane, enabling transfer of some cargo, required for timely removal of nurse cell corpse. Interestingly, in Drosophila ER residing proteins, Pretaporter and DmCaBP1 have been identified that become extracellularly exposed upon induction of apoptosis, they serve as ligands for Draper and are required for effective phagocytosis during embryonal development52,53. It is therefore an attractive hypothesis that the Vps13 dependent membranous structure is ER derived and that Vps13 plays a role in tethering the ER to the plasma membrane. This allows extracellular exposure of ER residing factors that are required for effective
phagocytosis of the nurse cell corpses. Those factors could then serve as eat-me-signals or do-not-eat-me-signal inhibitors and as such could play a role in effective phagocytosis.

Mutations in human VPS13A lead to ChAc, a disease with two distinct characteristics: neurodegeneration and acanthocytes, a possible explanation for these two seemingly independent phenotypes is currently missing. Interestingly, ongoing efficient clearance of cell corpses is required both for healthy brain functioning as well as for timely removal of aging erythrocytes\(^{10}\), it may therefore be that both characteristics of the disease can be explained by less efficient clearance of to-be-degraded-cells in the circulation and in the brain.

The conserved role of Vps13 in Drosophila oogenesis, a well-studied system with relatively large cells applicable for a wealth of genetic tools, and our developed tools to visualize and localize endogenous Vps13, could all contribute to further elucidation of the molecular function of Vps13 proteins in health and disease.

**MATERIALS & METHODS**

**Drosophila maintenance**

Drosophila melanogaster stocks and crosses were raised on the standard cornmeal used at the Bloomington Stock Center (Nutri-Fly Bloomington Formulation, Cat# 66-113) at 25°C. For lifespan experiments standard agar food was used. The following stocks were collected from the Bloomington Stock Center (Indiana University, USA): \(w^{1118}\), Actin-GAL4/Tm6B (3954), Actin-GAL4/CyO (4414), nos-GAL4::VP16 (64277, here called nanos-GAL4), UAS-mCD8-GFP (5137), Vps13 RNAi (38270). The Vps13\(^{13+}\) mutant (Vps13\(^{13\text{C03628}}\), precise excision line (Excision line 1) and UAS-hVps13A line are described in and obtained from Vonk et al. (2017). The GR1-GAL4 line was a generous gift from Trudi Schüpbach. The \(w,Sp/CyO,Drpr\Delta5rec9/TM6b\) line, here referred to as \(drpr\Delta5\), was a generous gift from Mark Freeman.

**Drosophila female fecundity assay**

To investigate female fecundity and egg lay, 10 freshly eclosed females were housed on apple juice agar plates supplemented with yeast paste. Three \(w^{1118}\) males were included to ensure mating. Flies were transferred to fresh plates every 24 h and the number of eggs laid in each 24h period was recorded for 14 days.

**Drosophila life span**

One day old male adult CRISPR/Cas9 treated flies were collected with the appropriate control and kept on standard agar food at 25°C. Flies were housed in fly food vials with 10-20 flies each and put into fresh vials every 2 or 3 days. The incidence of dead flies was counted every 2 or 3 days.
CHAPTER 5

Generation of a Drosophila Vps13null mutant and Vps13-GFP line using the CRISPR/Cas9 system

We used the CRISPR/Cas9 system to generate a Vps13 null mutant. In addition we constructed a fly line expressing a Vps13-GFP fusion protein. For the generation of the Vps13 null mutant we designed two sgRNAs targeting either exon 4 or exon 8 of the Vps13 gene (Table 1). For selection of the sgRNA the DRSC Find CRISPR Tool was used. Each of the sgRNAs was cloned into the pU6-BbsI-chirRNA plasmid (Addgene plasmid #45946; gift from Melissa Harrison, Kate O’Connor-Giles & Jill Wildonger) via BbsI restriction sites (Chapter 4 of this thesis). Both constructs were injected simultaneously into transgenic embryos expressing Cas9 in the germline (yw; nos-Cas9(III-attP2)/Tm6C) by BestGene (Chino Hills, California, USA). Potential mutant lines were balanced with a homozygously lethal CyO second chromosome balancer. Offspring homozygous for the potential mutant Vps13 allele was screened for male sterility (data not shown) and used for further study. Fly lines that were male sterile were analyzed on Western blot with antibodies directed to the N-terminal and C-terminal part of the Vps13 protein to identify new Vps13 null mutants.

Table 1. Primers gRNA for Vps13 knockout mutant and Vps13-GFP flies

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<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA Exon 4</td>
<td>CTTCGAAGCCGACTACGGGTTG</td>
<td>AAACCAACGACGGGTAGTCCCTTC</td>
</tr>
<tr>
<td>sgRNA Exon 8</td>
<td>CTTCGTATCATGGCCACCACTAT</td>
<td>AAACATATGGTTGGCGCATCATAC</td>
</tr>
<tr>
<td>sgRNA GFP tag</td>
<td>CTTCCGATTATGAGCCGGATAGCCGA</td>
<td>AAACTGCCCCTATCCCCGGCTGATAATTC</td>
</tr>
</tbody>
</table>

For the creation of the Vps13-GFP line, one sgRNA was designed directed against the 3’ end of the Vps13 gene and cloned into the pU6-BbsI-chirRNA vector (for primers, see Table 1). A HDR plasmid was created in pBluescript II SK+ (Stratagene) with Gibson assembly Cloning kit (New England Biolabs, Ipswich, MA, USA). Briefly a 5’ and 3’ flanking arm of about 1.2 kbp and eGFP were created with PCR, all three products were inserted in pBluescript II SK+ that was linearized with NotI and EcoRV (for primers, see Table 2). Injection of the sgRNA and HDR plasmid and further balancing of potential Vps13-GFP lines was done as described before. Potential Vps13-GFP lines were screened for the presence of GFP using PCR. Vps13null and Vps13-GFP lines were confirmed by sequencing.

Table 2. Primers HDR donor plasmid

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ flanking arm</td>
<td>GCTCCACCCGGGTGGCCAAATCCCTATGGCTTA</td>
<td>ATTAGCCGAGTAGCGACTGTAGCCCTTA ACGGATTG</td>
</tr>
<tr>
<td>GFP sequence</td>
<td>GCGCGGCGCCAATACAACTGAAATTATTC TCCCAAC</td>
<td>GAGGTGACCGGATACGATAAGCTTGCTGCTG TATTGCTCTGACGTC</td>
</tr>
<tr>
<td>3’ flanking arm</td>
<td>CTCACCCGGTCTATGGCCGAGCTGACGGCACAA</td>
<td>GGCGAATATCCATCTGCTTGGCCGCGCCG TTACTCTACGCTGGCCCGCAT</td>
</tr>
</tbody>
</table>

RNA isolation and quantitative Real-Time PCR of CRISPR/Cas9 treated flies

Flies of Vps13null and CC Control were collected and snap frozen in liquid nitrogen. The samples were lysed in TRIZOL (Invitrogen) for RNA extraction and reverse transcribed using M-MLV (Invitrogen) and
random primers (Invitrogen). Relative changes in transcript levels were determined on the CFX Connect (Bio-Rad) using SYBR green supermix (Bio-Rad). Calculations were done using the relative CT method. For each primer set the PCR efficiency was determined. The sequences of used primers are listed in Table 3. The expression levels were normalized for rp49 (house-keeping gene). Primer set 1 was directed to the N-terminus of Vps13 upstream of the gRNA. Primer set 2 was directed to a sequence downstream of the PiggyBac insertion site of the Vps13c03628 mutant.

Table 3. Primers qPCR of new Vps13null mutant

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer set 1</td>
<td>GCCAAGGCACATGTTGTA</td>
<td>CAAGACACACACCTCCTCC</td>
</tr>
<tr>
<td>Primer set 2</td>
<td>AGACGCTGCTGCTGCTCT</td>
<td>AAGGCTCTGCTGACAGCT</td>
</tr>
<tr>
<td>RP49</td>
<td>CCGTCTCCAAGGCCACATATC</td>
<td>GACAATCTCGCTCGCAATC</td>
</tr>
</tbody>
</table>

Genomic DNA isolation and PCR screening of potential Vps13-GFP flies

To isolate Drosophila genomic DNA (gDNA) from the potential Vps13-GFP flies, two different protocols were used:

1) Two flies of the potential Vps13-GFP lines were collected and mashed for 20-30 seconds in 100 µL squishing buffer (10mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl and 400µg/ml proteinase K). After 30 minutes incubation at 37°C the proteinase K was inactivated by heating the samples to 95°C for 3 minutes. The samples were centrifuged shortly and the supernatant was used for PCR.

2) Five flies of the potential Vps13-GFP lines were collected and mashed for 20-30 seconds using a yellow pipet tip with 50 µL solution A (0.1 M Tris-HCl pH 9.0, 0.1 M EDTA and 1% SDS). Then samples were incubated at 70°C for 30 minutes. Afterwards 7 µL of 8M KAc (Merck) was added per sample and samples were incubated on ice for 30 minutes. The samples were centrifuged for 15 minutes at 13,000 rpm at 4°C after which the supernatant was transferred to a fresh tube. 30 µL of isopropanol (Sigma Aldrich) was added to each sample and after some shaking the samples were centrifuged for 5 minutes at 10,000 rpm at 4°C. Afterwards the supernatant was removed and the pellet was washed with 70% EtOH and centrifuged for 5 minutes at 13,000 rpm. After removing the supernatant, the pellet was dried to open air. Finally the pellet was resuspended in 20 µL RNase and DNAse free H₂O (Life Technologies).

The DNA sequences of the potential Vps13-GFP lines were initially screened for presence of GFP (gDNA isolation method 1, data not shown) using the ‘GFP’ primers listed in Table 4. A small selection of lines positive for GFP were then further analyzed by PCR for the flanking regions of the GFP sequence to check whether the GFP was fused to the 3’ of the Vps13 gene (gDNA isolation protocol 2) using the ‘GFP + flanking regions’ primers listed in Table 4. DNA sequences were amplified using Paq5000 Hotstart PCR Master Mix (Agilent), run on an 0.8% agarose gel and visualized with the Chemidoc MP System (Bio-Rad).
Table 4. Primers PCR screening of potential Vps13-GFP lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>GCCGCCGCCCTTGTACAGCTCGTCCAT</td>
<td>AAGGGCGAGGAGCTGTTCAC</td>
</tr>
<tr>
<td>GFP + flanking regions</td>
<td>CTTCTTCGAGCGCGAGTAT</td>
<td>GAAGTTCGTGTACCACAGCCA</td>
</tr>
</tbody>
</table>

Drosophila ovary dissection

Drosophila female flies were collected 0-8 h after eclosion and kept on standard Bloomington food supplemented with yeast paste at 25°C. Three to five w1118 males were added to ensure mating and to stimulate oogenesis. Ovaries of 4 day old females were dissected in PBS and imaged immediately with a Leica M165 FC microscope for size analysis or fixed in 4% formaldehyde (Thermo Scientific) in PBS for further antibody staining.

Antibody staining and microscopy

Ovaries of 4 day old females were dissected in PBS and fixed for 30 minutes in 4% formaldehyde in PBS at RT. The fixed tissue was washed 3 times 10 minutes in PBS + 0.1% Triton X-100 (Sigma-Aldrich) and afterwards permeabilized with PBS + 0.3% Triton X-100 for 1h followed by an optional blocking step with PBS + 5% BSA for 1h. The following antibodies were used: rabbit anti-Vps13 NT (1:500, Vonk et al. 2017), mouse anti-Draper 5D14 (1:100, DSHB), mouse anti-Lamin-D (1:400, DSHB). Appropriate secondary antibodies used were: Alexa 488- or Alexa 594-conjugated antibodies (Invitrogen) used at 1:500. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; 0.2 µg/mL) was performed together with the secondary antibody staining. Lysotracker Red DND-99 (20µM Invitrogen) was used to detect acidification of nurse cells. Freshly dissected ovaries were incubated with Lysotracker for 3 minutes at RT. After a short wash with PBS ovaries were fixed according to normal protocol and stained with DAPI. Ovaries were mounted in Citifluor (Agar Scientific) or 80% glycerol and analyzed on a Zeiss-LSM780 NLO confocal microscope with Zeiss Zen software. Adobe Photoshop and Illustrator (Adobe Systems Incorporated, San Jose, California, USA) were used for image assembly.

Ovary sample electron microscopy processing

Ovaries of 4 day old female flies were dissected and fixed overnight on a rotator at 4°C in freshly prepared fixative containing 2,5% Glutaraldehyde in 0,1 M sodiumcacodylate buffer, pH 7.4. After three short washes with 0.1 M sodiumcacodylate buffer samples were post-fixed with 1% osmiumtetroxide and 1.5% potassiumferrocyanide for 2 hours at 4°C. Then samples were washed with milliQ water at room temperature followed by dehydration in an ethanol series. Finally the samples were embedded in epoxy-resin (EPON). Semi thin sections were stained with Toluidine blue dye and used to select the correct stage of egg chambers after which ultrathin sections (70 nm) were collected on formvar coated copper grids and contrasted with 5% uranyl acetate and Reynolds lead citrate. Images were acquired on a Supra 55 scanning EM (SEM, Zeiss, Oberkochen, Germany) using a scanning transmission EM (STEM) detector with 2.5 nm pixel size using an externa scan generator ATLAS 5 (Fibics, Ottawa, Canada) as described by Kuipers et al. (2016). Individual tiles were stitched and data was exported as an html file. Html files were converted to czi files and areas of interest were selected and exported as TIF images using Zeiss Zen.
software for further image analysis in Adobe Photoshop.

**Western blot analysis**

For samples of fly heads, flies were snap frozen in liquid nitrogen and decapitated by using a vortex. 4 µL of 2x Laemmli buffer (2% SDS, 10% glycerol, 0.004 % bromophenol blue, 0.0625 M Tris HCl pH 6.8) containing 0.8 M urea and 50 mM DTT was added per fly head. For samples of ovaries, 4 day old females were dissected in PBS and ovaries were snap frozen in liquid nitrogen. Per ovary, 4 µL of 2x Laemmli buffer with 0.8 M urea and 50 mM DTT was added. Samples were sonicated 5 times for 5 seconds and boiled for 5 minutes. Protein extracts were run on 8% polyacrylamide gels, transferred onto PVDF membranes overnight using transfer buffer containing 10% methanol. Membranes were blocked with 5% milk in PBS 0.1% Tween-20 and subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were: rabbit anti-Vps13 #62 (1:1000, Vonk et al. 2017), anti-Vps13 NT (1:1000, Vonk et al. 2017), mouse anti-GFP (1:5000, Clontech), mouse anti-alpha-tubulin (1:5000, Sigma). Appropriate secondary HRP-conjugated antibody staining (1:5000, GE Healthcare) was done at room temperature in 5% milk for both Vps13 antibodies and PBS 0.1% Tween-20 for GFP and alpha-tubulin. Detection was performed using ECL or super-ECL solution (Thermo Scientific) with the ChemiDoc Touch (Bio-Rad).

**Quantifications and statistical analysis**

To quantify the percentage of PNCN, ovaries stained with DAPI were analyzed with a Leica fluorescent microscope. Quantification was performed by calculating stage 14 egg chambers with PNCN divided by all stage 14 egg chambers analyzed and presented as a percentage.

Data were analyzed with GraphPad Prism5 statistical software. Statistical significance was determined using Student’s t-tests. Data are represented as mean values ± standard error of the mean (SEM). P-values below 0.05 were considered significant. In the figures, p<0.05 is indicated by a *, p<0.01 by ** and p<0.001 by ***.

All graphs and life span curves were made using GraphPad Prism5.
REFERENCES


37. Housden, B. E., Lin, S. & Perrimon, N. Cas9-Based Genome Editing in Drosophila. The Use of CRISPR/Cas9, TALENs, ZNFs in Generating Site Specific Genome Alterations 546, (Elsevier Inc., 2014).
SUPPLEMENTARY MATERIAL

Supplementary figure 1. Vps13 is expressed in Drosophila ovaries
Western blot analysis of Vps13 protein levels in ovaries of 4 day old Vps13\textsuperscript{PB} mutant and controls (\textit{w}\textsuperscript{1118} and Precise Excision line) using the Vps13 \#62 and Vps13 NT antibody to confirm Vps13 presence in Drosophila ovaries and its absence in mutant lines. α-Tubulin staining was used as a loading control.

Supplementary figure 2. Validation of the Vps13\textsuperscript{null} mutant
A. Relative levels of Vps13 mRNA in the Vps13\textsuperscript{null} mutant and the isogenic CC control adult flies were determined with RT qPCR using two primer sets (primer set 1 and primer set 2). Data are mean ± SEM, n=3. B. Lifespan analysis of the Vps13\textsuperscript{PB} and Vps13\textsuperscript{null} mutant compared with control flies (CC control).
Cell-autonomous function of Vps13 in nurse cell removal

Supplementary figure 3. Creation and validation of a Vps13-GFP fly line using CRISPR/Cas9

A. Schematic representation of the Vps13 gene showing the target site of the sgRNA for the generation of the Vps13-GFP line using the CRISPR/Cas9 system. Exons are shown as black boxes. B. Potential Vps13-GFP lines (g16-7, g16-21, g16-27 and g16-28) were screened by PCR amplification of the flanking regions of the GFP sequence to confirm the incorporation of the ~750 bp GFP sequence. GFP negative lines should give a PCR product of 2820 bp, while Vps13-GFP lines should give a PCR product of 3570 bp. Heterozygous Vps13-GFP flies should show both PCR products. C. Lifespan analysis of the identified Vps13-GFP line (g16-28) and an isogenic control (g16-7) was performed to examine any effects of the inserted GFP sequence on viability. D. Western blot analysis of control (g16-7), homozygous Vps13-GFP and heterozygous Vps13-GFP/CyOdfdYFP fly heads was performed to visualize the Vps13-GFP fusion protein using a GFP antibody that detects the Vps13-GFP fusion protein in homozygous and heterozygous Vps13-GFP flies, but not in the control. Vps13 #62 and Vps13 NT antibodies were used to visualize the Vps13 protein in all samples. Free GFP could only be detected in heterozygous Vps13-GFP/CyOdfdYFP lysates because of the YFP-tagged balancer α-Tubulin was used as a loading control. E. Quantification of persistent nurse cell nuclei in stage 14 egg chambers of 4 day old Vps13-GFP and control (g16-7) females was performed to test functionality of the Vps13-GFP fusion protein in the Drosophila ovary and shows the absence of the Vps13 mutant ovary phenotype of PNCN accumulation in the newly generated Vps13-GFP fly line.
Supplementary figure 4 – Expression patterns of the follicle cell driver GR1-GAL4 and nurse cell driver nanos-GAL4

Overview of the expression patterns of the follicle cell driver GR1-GAL4 (A) and nurse cell driver nanos-GAL4 (B) driving UAS-mCD8-GFP (green) in Drosophila ovaries showing that expression of mCD8-GFP is limited to the cell types in which GAL4 is active. DAPI was used to visualize DNA (blue). Scale bars represent 50 µm.
Supplementary figure 5 – Vps13 does not localize to the nuclear envelope

Stage 12 egg chambers of w1118 (A), Vps13PB (B), precise excision (C) and Vps13null (D) stained with Lamin D (LamD, red) to visualize the nuclear envelope, Vps13 NT (green) and DAPI to visualize DNA. The panels in A’-D’ are zoom-in pictures of A-D to illustrate that Vps13 NT and Lamin D do not co-localize. An intense Vps13 signal is found when the nuclear envelope and Lamin D are still intact, indicated with arrows. Degradation of the nuclear envelope is associated with loss of Vps13 staining (arrowheads). Scale bars represent 50 µm.
Supplementary figure 6 – Validation of the Vps13 RNAi fly line using Western blot analysis

To test the efficiency of the Vps13 RNAi fly line, Vps13 protein levels were analyzed using Western blot in fly head samples of Vps13 RNAi/act-GAL4 and control (4414 and Vps13 RNAi). In addition, fly head samples of w1118, precise excision and Vps13PB mutant lines were loaded. Vps13 levels were detected with both the Vps13 #62 and Vps13 NT antibody. α-Tubulin was used as a loading control.
Supplementary figure 7 – Absence of Vps13 does not affect Draper localization and intensity in ovaries during late stages of oogenesis

A-E Immunolabeling of stage 12 egg chambers with a Draper specific antibody (green) in w^{118} (A), Drpr^{Δ5} mutant (B), Vps13^{PB} (C), precise excision (D) and Vps13^{null} (E) to visualize Draper in Vps13 mutant ovaries. DAPI (magenta) was used to visualize nuclei. Scale bars represent 50 µm.
Supplementary figure 8 – Vps13 does not co-localize with different organelle markers during late oogenesis

A & A'. Different planes of a control stage 12 egg chamber labeled with Vps13 (green) and Lysotracker (red). Arrows indicate an acidified nurse cell nucleus. Acidic compartments, most likely lysosomes, are indicated with arrowheads. Scale bars represent 50 µm.
Supplementary figure 9 – Scheme to select areas for ultrastructural analysis of Drosophila ovaries

A. Bright-field microscopic image of a semi thin section of a w^{1118} ovary stained with Toluidine blue which was used to select the right stage of the egg chamber after which ultrathin sections were processed for electron microscopy. B. Large scale electron microscopic image of the selected egg chamber and region, represented by the boxed area in A, containing nurse cells and follicle cells. C. Enlarged image of the boxed area in B showing an individual nurse cell nucleus at the correct stage of cell death.
Supplementary figure 10– Localization of Vps13-dependent membrane structures during late oogenesis

A. Large scale electron microscopic images of a dying NC during developmental PCD in Vps13<sup>PB</sup> ovaries. A'. Schematic representation of A with a nurse cell nucleus (NCN), follicle cells (FCs), multivesicular bodies (MVBs), translucent vesicles (TVs) and nurse cell cytoplasm (NC cyto). Plasma membrane is pictured in pink, nuclear envelope in blue. The red boxes indicates the area that are depicted in A". B & B'. Enlargement of the boxed area in the large scale electron microscopic image in A. Membrane structures in B are indicated in the same color as A' and A" for reference. B". Enlargement of the boxed area in B'. The plasma membrane is indicated with a black arrow, pink arrows indicate the nuclear envelope. Scale bar in A indicates 5 µm. Scale bar in B indicates 500 nm.