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### VPS13A: shining light on its localization and function

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# SUMMARIZING DISCUSSION

After the initial discovery of *VPS13A* as the causative gene of the neurodegenerative disease Chorea-Acanthocytosis (ChAc) 17 years ago, research and knowledge about its function progressed very slowly. Desired progression in management and treatment of ChAc demands better understanding of the underlying disease mechanisms and affected cellular processes. Therefore we aimed to shed more light on *VPS13A* function and localization by focusing on a multicellular *Drosophila* disease model, as well as by studying mammalian cell models.

## **The fruit fly as a model organism to study neurodegenerative diseases and Chorea-Acanthocytosis in particular**

The investigation of human diseases is largely dependent on the use of model systems and organisms, including different cell lines for *in vitro* studies and *in vivo* models like yeast, fruit flies and mice. *Drosophila melanogaster*, or the fruit fly, proved to be of high importance in the study and understanding of many human diseases. Their maintenance is easy and relatively cheap, they can be produced in large numbers with an identical genetic background and the possibilities for genetic and pharmacological manipulation are extensive. Research using the versatility of the fruit fly has already contributed to knowledge about various neurodegenerative diseases, which we reviewed in Chapter 2<sup>1</sup>. For our research we aimed to utilize the fruit fly to investigate *Vps13*, the *Drosophila* ortholog of *VPS13A*. Because most data available on *VPS13A* function has been acquired with the use of unicellular eukaryotic organisms like yeast and ciliates, the urgency for a multicellular model to investigate the function of *VPS13A* and the consequences of *VPS13A* depletion or absence is high. A mouse model for ChAc was developed, but the neurodegenerative phenotypes are variable and depend heavily on the genetic background of the mice strain in which the *VPS13A* mutation is generated<sup>2</sup>, further increasing the need for a solid multicellular model organism. To address this issue, we established and characterized a *Drosophila melanogaster* model for ChAc as described in Chapter 3 of this thesis. With the use of this *Vps13* fly mutant we assessed the consequences of *Vps13* dysfunction on the central nervous system and found that it is associated with a reduced life span and neurodegeneration<sup>3</sup>. We further demonstrated that *Vps13* mutant flies are sensitive to proteotoxic stress and show impaired protein homeostasis, which leads to the accumulation of ubiquitinated proteins in the central nervous system of both larvae and adult flies. Rescue of some of these phenotypes by the overexpression of human *VPS13A* in the *Vps13* mutant flies is suggestive of functional conservation of both human and *Drosophila* genes and proteins<sup>3</sup>.

Protein synthesis, folding, trafficking and degradation are kept in tight balance and defects in protein homeostasis have been associated with several neurodegenerative diseases for quite some time now<sup>4,5</sup>. Aggregation of misfolded proteins is a hallmark of diseases like Alzheimer, Parkinson and Polyglutamine diseases, usually as a consequence of a gain-of-function mutation. Since ChAc is caused by homozygous loss-of-function mutations it seems improbable that absence of *VPS13A* directly and actively promotes protein aggregation, it is therefore more likely that *VPS13A* plays a role in the elimination of misfolded or defective proteins through mechanisms like autophagy to maintain a stable and functional

proteome. Autophagy is a cellular degradation mechanism that disassembles unnecessary and defective cytoplasmic content. VPS13A has been implicated to play a role in the autophagic pathway<sup>7</sup> and it was shown that autophagic flux and autolysosomal degradation is impaired in cells of ChAc patients<sup>8</sup>. Our co-fractionation experiments revealed that Vps13 is a peripheral membrane protein associated with Rab7 positive membranes<sup>3</sup>. Rab7 is a GTPase associated with late endosomes and lysosomes known to play an important role in endosomal membrane trafficking and autophagosome formation<sup>9,10</sup>. Dysfunction of Rab7 leads to the accumulation of autophagosomes<sup>11</sup>. Interestingly, VPS13A-depleted human cells accumulate autophagosomal markers<sup>7</sup>. In addition, yeast VPS13 plays an important role in transport of membrane proteins to the vacuole, the yeast version of the lysosome<sup>12-14</sup>. Our data suggest localization and function of Vps13 in the autophagic degradative pathway, possibly in the transport or fusion of autophagic organelles and lysosomes.

### A role for Vps13 in apoptotic cell removal

In Chapter 5 we identified a cell-autonomous role for Vps13 during developmental programmed cell death (PCD) in the *Drosophila* ovary. The processes of cell death induction and the consecutive removal of the dying nurse cells (NCs) and their enormous nuclei are still poorly understood. Currently, most attention is paid to non-autonomous cell death induction during developmental PCD in late oogenesis, where surrounding follicle cells (FCs) promote the death of NCs. The phagocytic receptors Draper and Ced-12 were identified as crucial factors in this process, as well as the integrin subunit PS3<sup>15,16</sup>. It was suggested that Draper functions in a parallel pathway from integrins and Ced-12, where integrins probably act upstream of Ced-12<sup>16</sup>. Combined inhibition of both pathways failed to completely block NC removal<sup>16</sup> which suggests the presence of another pathway or redundant mechanism necessary for the complete removal of all NCs. Absence of Vps13 in the NCs specifically causes defects in the clearance of dying NCs, leading to the accumulation of persistent nurse cell nuclei (PNCN), while Vps13 depletion in the FCs did not lead to PNCN accumulation (Chapter 5). Using antibody staining and expression of endogenous Vps13-GFP we localized Vps13 in close proximity to the plasma membrane and the nuclear envelope of NCs in late oogenesis. The Vps13 signal surrounding NC nuclei was most intense when nuclei were not yet completely fragmented and degraded (Chapter 5). Furthermore we showed that acidification of the NC nuclei is not affected in *Vps13* mutants, which suggests that Vps13 functions downstream of cell death induction by Draper and integrins, but is required for the proper removal of dying NCs. Co-localization studies of Vps13 with multiple organelle markers in late oogenesis did not show any overlap (unpublished data) pointing to a possible novel underlying Vps13-dependent structure. To investigate this structure, we performed electron microscopy of wild type and *Vps13* mutant ovaries to identify structural differences as a consequence of the absence of *Vps13*, focusing on NC nuclei in late oogenesis that started but not yet completed the nuclear fragmentation. In ovaries of control flies we observed a special membrane adjacent to the plasma membrane that was absent in *Vps13* mutants, suggesting that this Vps13-dependent membrane structure is involved in the proper removal of dying NCs in developmental PCD.

Selective removal and degradation of portions of the nucleus is under specific conditions necessary to promote viability of a cell<sup>17,18</sup>. This degradation occurs via the process of nucleophagy<sup>19</sup>. It often requires

the core autophagy genes<sup>20</sup> and is described in yeast<sup>21</sup>, filamentous fungi<sup>22</sup> and the ciliate *Tetrahymena thermophila*<sup>23,24</sup>. Usually nucleophagy involves only damaged and non-essential nuclear components, including parts of the nuclear envelope and the granular nucleolus, and is mainly crucial to promote viability of a cell<sup>17,25</sup>. Encapsulation of nucleus-derived cargo by autophagosomes that later fuse with lysosomes leading to degradation is referred to as macronucleophagy<sup>26,27</sup>, while during micronucleophagy the nucleus is in direct contact with lysosomes or the yeast vacuole that engulf small portions of the nucleus<sup>20,21,28</sup>. Nuclear vacuole junctions (NVJ) in the yeast *Saccharomyces cerevisiae* are known to promote micronucleophagy using two key proteins Vac8 and Nvj1<sup>17,29</sup>. Interestingly, in yeast VPS13 was found at NVJs<sup>30,31</sup> and loss of VPS13 increases the length of individual NVJs, possibly to compensate for the lack of VPS13-containing NVJs<sup>31</sup>. Although the exact function of VPS13 at NVJs still has to be resolved, VPS13 is currently thought to play a role in lipid transport at different membrane contact sites, including NVJs. In combination with our results in *Drosophila* it is tempting to speculate that VPS13 in yeast plays a role in nucleophagy at NVJs. More research is necessary to investigate this hypothesis.

Autophagy is involved in developmental PCD in the *Drosophila* ovary, although its role is only minor<sup>32,33</sup>. However, it has been shown that apoptotic vesicles containing nuclear material of dying NCs are engulfed by follicle cells<sup>33,34</sup> suggestive for nucleophagy. The origin of the membranes that enclose the nuclear material is unknown and needs to be studied in further detail. Vps13 could be involved in the process of nucleophagy, both in micronucleophagy via its localization at the NVJs in lower eukaryotes, as well as in macronucleophagy where Vps13 supports the formation of specialized membranes that enclose parts of the nucleus that need to be degraded. Further studies are required for the investigation of a function for nucleophagy during developmental PCD in the *Drosophila* ovary and the involvement of Vps13 herein.

We demonstrated a functional conservation of human VPS13A in the removal of dying NCs during late oogenesis, as overexpression of human VPS13A in the *Vps13* mutant background significantly reduced the accumulation of PNCN (Chapter 5). Unfortunately we cannot conclude yet whether the removal of dying cells is generally affected in ChAc patients. It would therefore be very interesting to investigate whether removal of dying neurons for example is impaired in the *Vps13* *Drosophila* mutant, which might provide a link to the neurodegeneration that characterizes ChAc. Interestingly, it was reported that erythrocytes of ChAc patients show reduced exposure of phosphatidylserine (PS) upon stimulation with LPA<sup>35</sup>. PS is a highly conserved phospholipid that is exposed on the surface of apoptotic cells and is recognized as a universal 'eat-me' signal<sup>36-38</sup>. Vps13 might play a role in the exposure of PS in dying NCs and thereby induce their removal. Absence of Vps13 would then cause a delay or deficiency in the proper removal of dying NCs. Involvement of PS exposure during developmental PCD has not been shown to date, but due to the universality of PS as an eat-me signal it is likely that PS plays a role in the removal of dying NC during late oogenesis as well. Unfortunately, detection of PS in ovaries suffers from technical difficulties as stainings with Annexin V, the reporter often used to visualize PS exposure, is unable to penetrate the FC layer to reach the dying NCs (preliminary results).

## VPS13A function in membrane contact sites and lipid droplet homeostasis

We and others found that Vps13 is a peripheral membrane protein associated with different membrane structures depending on the organism<sup>313</sup> (Chapter 6). In Chapter 6 we showed that human VPS13A is dynamically associated with the Endoplasmic Reticulum (ER) and mitochondria via different binding domains. Association with mitochondria is dependent on the C-terminus of VPS13A, although it is unclear whether this is a direct or indirect binding. Direct binding of VPS13A to the ER protein VAP-A is dependent on the FFAT motif present in VPS13A. Furthermore we showed that an increase in lipid content causes translocation of VPS13A from mitochondria to lipid droplets (LDs) (Chapter 6). Presence of VPS13A on many organelle membranes and at the interface between ER and mitochondria, its response to increased lipid content and regulation of lipid droplet (LD) mobility implicates a function in lipid homeostasis and membrane contact sites.

Movement, transport and exchange of lipids is crucial to maintain the proper composition of the different organelles in a cell<sup>39</sup>. Communication and exchange of ions, lipids and other metabolites between organelles can occur through vesicular trafficking pathways and membrane contact sites (MCSs), which are defined as regions where two organelles come closely together (10-30 nm)<sup>39-41</sup>. In *Saccharomyces cerevisiae*, VPS13 was shown to bypass defects in the ER-mitochondria tethering complex ERMES through its localization at vacuole-mitochondria contact sites, while combined loss of ERMES and VPS13 is lethal<sup>30,42</sup>, suggestive for a crucial role for VPS13 in MCSs. VPS13 localization to different MCSs is dependent on nutritional status and growth conditions of the yeast<sup>30,31</sup>. Very recent work of Bean and colleagues (2018)<sup>43</sup> characterized a binding motif that can be found in multiple VPS13-adaptor proteins, with slight differences depending on the protein. VPS13 localization to different membranes and MCSs is regulated through competition of those adaptor proteins that all bind to a conserved six-repeat region in VPS13<sup>43</sup>. The exact function of VPS13 at the different MCSs still remains elusive.

We provide evidence that mammalian VPS13A localizes at membrane contact sites and plays a role in lipid homeostasis (Chapter 6). Most ER MCSs are mediated by VAP proteins<sup>44</sup> that bind other proteins containing an FFAT domain<sup>45</sup>. Time-lapse imaging reveals simultaneous overlap of VPS13A signal with both mitochondria and ER, and the binding of VPS13A to VAP-A is dependent on its FFAT motif since a VPS13A FFAT-deletion mutant no longer localizes to the mitochondria-ER interface but solely localizes to mitochondria (Chapter 6). Increased cytoplasmic calcium levels caused an increase in VPS13A and VAP-A binding, which suggests VPS13A involvement in ER-mitochondria contact sites, as it is known that cellular calcium levels regulate the assembly of MCSs<sup>46,47</sup>.

Induction of intracellular LD formation by exogenous addition of oleic acid (OA) causes VPS13A to shift from ER to LDs where it uniformly encircles the individual LDs. Furthermore, presence of VPS13A on LDs causes a slowdown in their movement while downregulation of VPS13A causes an increase in both numbers and size of LDs. Interestingly, the *Drosophila* Vps13 mutant shows accumulation of LDs in glia cells of the central nervous system (Chapter 6). Together this strongly implies a role for VPS13A in LD homeostasis, which might as well apply to *Drosophila* Vps13 as we reported the accumulation of LDs in glia of the central nervous system of Vps13 mutant flies.

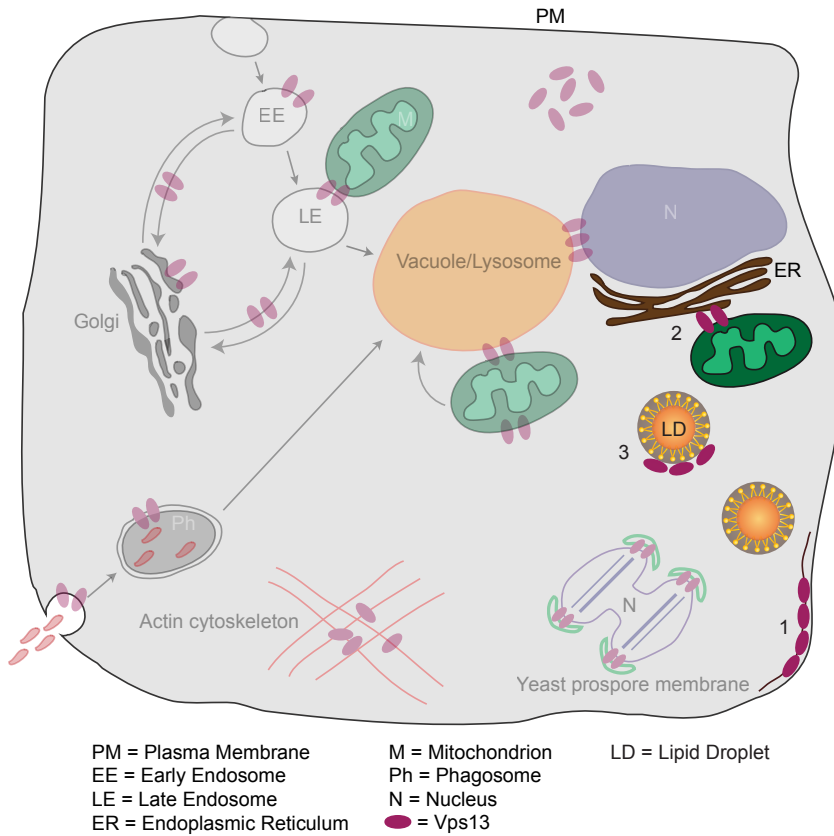
LDs were found in close association with the newly identified membrane structures in the *Drosophila* ovary (Chapter 5). It is known that LDs are important for formation of membrane structures, including autophagosomes<sup>48,49</sup>. In addition, autophagosomes derive their membrane from multiple other organelles including ER and mitochondria and form at ER-mitochondria contact sites<sup>41,49,50</sup>. Together this might connect the presence of VPS13A at MCSs, the formation of a Vps13-dependent membrane structure during developmental PCD and the accumulation of LD in the absence of Vps13, although a role for Vps13 in MCSs in *Drosophila* has not been shown.

### **CRISPR/Cas9 as the technique of the future?**

The CRISPR/Cas9 technique that we adapted (Chapter 4) appeared to be of high value in our research and enabled us to study the localization of *Drosophila* Vps13 in a well-controlled manner. However, a critical note must be placed about the generation of off-target mutations when applying this technique. While generating the *Vps13<sup>null</sup>* mutant using CRISPR/Cas9 we observed multiple lines with various phenotypes that turned out to be unrelated to a mutation in *Vps13*. It is therefore of importance to be aware of the possibility that off-target mutations are created, even though multiple prediction programs might have predicted otherwise. In our study we thoroughly verified that the phenotypes we observed in our CRISPR/Cas9 generated *Vps13<sup>null</sup>* mutant are due to the particular mutation in *Vps13*, rather than them being the consequence of an off-target event (Chapter 4). A very recent publication of Kosicki *et al.* (2018)<sup>51</sup> reports that large deletions and other mutations can occur several kilobases from the target site, which can be missed when only a relatively small genomic region surrounding the target site is screened. It might have potential pathogenic consequences when those events take place in stem cells or progenitor cells and go unnoticed<sup>51</sup>. So although CRISPR/Cas9 has many benefits and might be very promising for future applications in health and disease, potential pitfalls of the technique should not be underestimated.

### **Concluding remarks and future perspectives**

The data presented in this thesis expands the body of knowledge about VPS13A function and localization by identifying its localization in both mammalian cells and a multicellular organism, all summarized in Figure 1. The versatility and dynamic localization define VPS13A as a so-called “Swiss army knife” [R. Fuller, Ann Arbor, Michigan, 2016], whereby its function depends on the cellular localization and binding partners. The VPS13 adaptor binding (VAB) domain identified in yeast is conserved in human VPS13A<sup>43</sup> and *Drosophila* Vps13 (unpublished data). Mass Spectrometry analysis of human VPS13A and *Drosophila* Vps13 will be of high value in identifying new adaptor proteins that interact with the VAB domain and thereby opening new roads for the investigation of VPS13A function. The *Drosophila* models that we generated and characterized in this thesis will be of significant value for further studies on the pathophysiology underlying ChAc.



**Figure 1. Overview of VPS13A localization and function identified in this thesis and by others.**

Schematic representation of localization and functions of VPS13 that we identified in this thesis (dark purple), in addition to previously found localization and function of VPS13 (faint structures, light purple), which are discussed in Chapter 1. *Drosophila* Vps13 was found in close proximity to the plasma membrane and is involved in formation of a specialized membrane structure during developmental programmed cell death in oogenesis (1). Human VPS13A localizes to the ER-mitochondria interface (2) and translocates to LDs upon an increase of cellular lipid content (3).



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