Deciphering the role of VPS13A in
Chorea acanthocytosis
INTRODUCTION

Neuroacanthocytosis syndromes and Chorea acanthocytosis

Neuroacanthocytosis is a general term for a group of rare progressive disorders. Acanthocytosis originates from the Greek word *acantha*, meaning thorn. Acanthocytes are star-shape red blood cells present in most of the patients having these syndromes. The main causes of the neuroacanthocytosis syndromes are genetic mutations which produce many clinical features, in particular neurodegeneration of the basal ganglia. The diseases are hereditary\textsuperscript{1,2}.

Neuroacanthocytosis was first described in 1950 as Bassen-Kornzweig syndrome, an autosomal recessive and childhood-onset disease. The affected patients fail to produce low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicrons. The main symptoms are ataxia, retinitis pigmentosa, peripheral neuropathy and different forms of nerve dysfunction\textsuperscript{3}.

Afterwards, Irvine M. Levine in 1960\textsuperscript{4} and MacDonald Critchley in 1968 described a second form of Neuroacanthocytosis, the Levine-Critchley syndrome. This syndrome combines the presence of acanthocytes with neurological disorder but the levels of serum lipoprotein are not affected\textsuperscript{5}. Typical symptoms are movement disorders, difficulty in swallowing, tics, poor coordination, chorea, hyporeflexia and seizures\textsuperscript{5}.

Nowadays four distinct disorders are usually classified as the "core" neuroacanthocytosis syndromes: chorea-acanthocytosis (ChAc), McLeod syndrome (MLS), Huntington's disease-like 2 (HDL2) and pantothenate kinase-associated neurodegeneration (PKAN). ChAc, MLS and PKAN are recessive disorders caused by mutations in the *VPS13A*, *XK* and *PANK2* genes respectively\textsuperscript{6-8}. HDL2 is a dominantly inherited disease caused by a CAG/CTG repeat expansion in the *JPH3* gene\textsuperscript{9}.

This thesis is focused on one of these core neuroacanthocytosis syndromes, ChAc and the protein Chorein or VPS13A, that is affected in this group of patients. ChAc is an autosomal recessive neurodegenerative disorder characterized by neurological features and abnormal red blood cells\textsuperscript{10,11}. 
Chorea refers to the involuntary movements performed by people affected by this disorder. Acanthocytes are spiky red blood cells present in most of the patients. The onset of this disease starts in early to mid-adulthood and unavoidably leads to premature death. Common symptoms are movement abnormalities like dystonia, dysarthria, orofacial dyskinesias, involuntary vocalizations, and involuntary lip- and tongue-biting, cognitive and behavior changes like schizophrenia or obsessive compulsive disorder (OCD) and seizures\textsuperscript{12,13}. Typical clinical findings in addition to the presence of acanthocytes are: atrophy of human basal ganglia (especially the head of the caudate nuclei, which are structures of the basal ganglia), elevated concentration of creatinekinase (CK) in the serum. The disease is caused by homozygous mutations that occur in the \textit{VPS13A} (Vacuolar protein sorting 13A) gene, which most of the time lead to lower levels of the VPS13A protein in ChA patients\textsuperscript{14}. The diagnosis of Chorea-Acanthocytosis is first suspected upon identification of the characteristic symptoms. Subsequently, blood tests can reveal the presence of acanthocytes, the increased levels of CK and finally the absence of VPS13A or Chorein protein will be determined by Western-blot analysis\textsuperscript{1}. Imaging techniques such as computerized tomography (CT) scanning and magnetic resonance imaging (MRI) are used to detect the typical atrophy of the basal ganglia. In addition, the gene sequencing may help in further confirming the diagnosis.

\textbf{Vps13 gene and protein structure}

Vps13 family genes are present in the genome of many eukaryotic organisms, both unicellular and multicellular. The human genome contains four \textit{VPS13} genes, encoding four distinct proteins: \textit{VPS13A}, \textit{VPS13B}, \textit{VPS13C}, \textit{VPS13D}\textsuperscript{15}. Mutations in three of these genes, \textit{VPS13A}, \textit{VPS13B} and \textit{VPS13C} are responsible for the onset of human neurodegenerative diseases, Chorea-Acanthocytosis (ChAc), Cohen syndrome (CS)\textsuperscript{16} and Young-onset Parkinson disease (YOPD)\textsuperscript{17} respectively.

The human \textit{VPS13A} gene is localized on chromosome 9q21-q22 and its transcript has a full-length sequence of 11,262 bp with an open reading frame of 9,525 nucleotides organized in 73 exons. Two different transcripts were described: variant A containing exons 1-68 and 70-73 encoding a 3,174 amino
The molecular weight of VPS13A is about 360 kDa. The protein contains several conserved domains: the domain, closest to the N-terminal of the protein is called the chorein domain. The exact function of this domain is not yet known. Downstream of this, there is the VPS13 domain, the function of this domain is also unknown. More towards the middle of the protein there are two VPS13_mid_rpt domains, both are repeating coiled regions of VPS13A containing a well-conserved P-x4-P-x13-17-G motif. Interestingly between the amino acid 842-848 there is a putative FFAT domain located. The C-terminal region of the protein harbors four conserved domains: a SHR_BD domain, a APT1 domain, a ATG_C domain and a PH domain. The SHR_BD domain, SHORT-ROOT transcription factor-binding domain, previously called DUF1162, in vitro, interacts equally well with all phosphoinositides, phosphatidic acid and lysophosphatidic acid. The APT1 domain, Golgi-body localization domain of maize (Zea mays) aberrant pollen transmission 1 (APT1) protein, is conserved from plants to humans. In vitro, the APT1 domain of Saccharomyces cerevisiae Vps13 almost exclusively binds phosphatidylinositol 3-phosphate PI3P. The ATG_C domain, autophagy-related protein C-terminal domain, is also present in the c-terminal of ATG2, a protein required for autophagy and cytoplasm to vacuole trafficking. The PH domain, pleckstrin homology domain, is present in a wide range of proteins and can bind different phosphatidylinositol lipids. This domain plays a role in recruiting and targeting proteins to different membranes and cellular compartments (Fig.1).

Until now, almost all of the information about function and localization of Vps13 was discovered using S. cerevisiae as a model. S. cerevisiae has a single intron-less Vps13 (YLL040C) gene encoding a Vps13 protein. The Vps13 of S. cerevisiae is a large protein (358 kDa) and it is most similar to the amino acid sequence of human VPS13A and contains several common domains (Fig.1).

In Drosophila melanogaster, one of the models used in our research, three different genes encoding Vps13 proteins are present, orthologues of human VPS13A, VPS13B and VPS13D. VPS13A is very similar to the Drosophila
orthologue called Vps13 (Fig.1), a large protein containing 3321 amino acids. *Drosophila* and mammals share many similarities, including comparable neurotransmitters and cellular pathways\textsuperscript{23}. In addition, about 77% of the genes known to cause, when mutated, a disease in humans, have a close orthologue in *Drosophila*\textsuperscript{24}. Thus, *Drosophila* is a very suitable model to investigate the function of proteins involved in human diseases. Another advantage of *Drosophila* as a model organism is the possibility to study its complex behavior in combination with the availability of easy and straightforward genetics and pharmacological tools for manipulation.

**Figure 1.** Schematic representation of Vps13 structures in different organisms. Vps13 from *Homo sapiens* (upper), *S. cerevisiae* (middle) and *D. melanogaster* (bottom) are shown with the conserved domains.

### Vps13 function in Yeast and other lower organisms

Vps13 protein was described for the first time in 1986 in *S. cerevisiae*. Vps13 was identified as a result of a screening in which mutants, unable to deliver vacuolar protease carboxypeptidase Y (CPY) to the vacuole, were selected\textsuperscript{25}.

Afterwards Vps13 was found to have an important role in the vesicle transport between TGN (Trans-Golgi Network) and PVC (Pre Vacuolar Compartment). Brickner et al. proved a double function for Vps13: at TGN, Vps13 is promoting the entry of these proteins into vesicles targeted to the PVC, while at the PVC,
Vps13 promotes entry of proteins into retrograde transport vesicles\textsuperscript{26,27}. Vps13 forms a complex with Cdc31p that is crucial for TGN-PVC transport and for homotypic and heterotypic vesicles fusion\textsuperscript{28} (Fig. 2).

Under conditions of stress, \textit{Saccharomyces cerevisiae} cells can enter meiosis and produce four haploid spores, this process is called sporulation\textsuperscript{29}. \textit{Saccharomyces cerevisiae} Vps13 mutants show defective sporulation\textsuperscript{30}. It was shown that Vps13 is involved in membrane expansion and closure of the prospore. This defect most likely is due to the reduced pool of PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} to the prospore membrane\textsuperscript{30}. The interaction between Vps13 and Spo71 is essential for the localization of Vps13 at the prospore membrane and both proteins are necessary to regulate prospore membrane morphogenesis\textsuperscript{31}. Supporting the idea that Vps13 is able to influence or is influenced by specific phospholipid levels. At the c-terminal of the protein two important domains are present: SHR\_BD and APT1. These domains were described to be important for binding to phospholipids. In vitro, APT1 has exclusive affinity for PI3P while SHR\_BD interacts equally well with all phosphoinositides, phosphatidic acid and lysophosphatidic acid\textsuperscript{18}. Additionally, in \textit{Saccharomyces cerevisiae}, Vps13 was found to interact with actin and \textit{Saccharomyces cerevisiae} Vps13 mutants show defective actin cytoskeleton polarization\textsuperscript{18}.

Recent experiments show that Vps13 can bypass endoplasmic reticulum-mitochondrial encounter structure (ERMES) mutants. ERMES is a complex consisting of both ER and mitochondrial proteins, located at the interface between the two organelles\textsuperscript{32}. This complex is crucial for the stability of mitochondria, probably regulating ER-mitochondria lipid exchange\textsuperscript{32}. Dominant Vps13 mutations are able to rescue mitochondrial phenotypes present in ERMES mutants\textsuperscript{33,34}. Presumably Vps13 is involved in the formation of vacuole-mitochondrial contact sites compensating the absence of ER-mitochondrial contact sites in ERMES mutants\textsuperscript{35} (Fig. 2). Because ERMES seems to be lost during evolution in metazoans, it is also tempting to speculate that Vps13 could take over some functions of ERMES\textsuperscript{36}.

In \textit{Tetrahymena thermophila}, the VPS13A orthologue TtVps13A is associated with phagosomal structures. TtVps13A depleted cells show several
Deciphering the role of VPS13A in Chorea acanthocytosis

Phagocytosis related phenotypes, as slow growth, delayed phagosomal contents digestion and reduced phagosome formation\textsuperscript{37}. \textit{Dictyostelium discoideum} cells lacking TipC, a VPS13A orthologous protein, exhibit a decreased number of autophagosomes and an impaired autophagic degradation due to a defect in autophagosome formation\textsuperscript{38}.

**Figure 2.** Pathways in which Vps13 might be involved in yeast cells. NVJ, ER-derived nuclear envelope-vacuole junctions; vCLAMP, vacuole and mitochondria patch; EMJ, endosome-mitochondria junctions; ERMES, endoplasmic reticulum-mitochondria encounter structure; CW, cell wall; EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; Mito, mitochondrion; PM, plasma membrane. Figure printed from Rzepnikowska et al. “Yeast and other lower eukaryotic organisms for studies of Vps13 proteins in health and disease”, Traffic (2017).

**Vps13A in multicellular organisms and Human**

VPS13A is conserved among many different species. Homologous proteins are present in multicellular organisms as \textit{M. musculus}\textsuperscript{39}, \textit{D. melanogaster}\textsuperscript{40}, \textit{C. elegans}\textsuperscript{15} among others. Until now the real mechanisms and causes that lead to ChAc are still unknown. In support of this, many model organisms were created and patients samples were used\textsuperscript{41,42}.

The ChAc mouse model, containing homozygous deletion alleles of \textit{VPS13A}, shows a marginal phenotype\textsuperscript{43}. Differences in survival, involuntary movements
or clear behavioral changes are not present. On the other hand, VPS13A deficient mice show acanthocytes and osmotic fragility of red blood cells, typical hematological phenotype seen in human patients. Additionally, in the striatum and hippocampus of VPS13A deficient mice expression of the GABAA receptor γ2-subunit and Geplrin are increased. Subcellular analysis of brain lysates in control mice showed high level of VPS13A in the microsomal and synaptosomal fraction.

Due the presence of acanthocytes, ChAc patient’s erythrocytes were the subject of numerous studies. Compromised cytoskeletal architecture has been proposed as the cause of acanthocytes. Red blood cells of ChAc patients show a reduced level of cytoskeletal proteins β-adducin isoform 1 and β-actin and Chorein was found to interact with these two proteins. In erythrocytes and fibroblasts of ChAc patients, actin filaments are depolymerized while microtubular network and intermediate filament of desmin and cytookeratin show diminished levels and disorganized network structure. The origin of the cytoskeletal disorganization might be attributed to an increased Lyn kinase activity observed in ChAc red blood cells. Lyn phosphorylates Band-3, a plasma membrane protein in red blood cells, which subsequently binds β-adducin, a component of the cytoskeleton. Presumably the rearranged interactions between plasma membrane and cytoskeletal proteins could be responsible for the different shape of red blood cells in ChAc.

Another hypothesis proposed to explain the cytoskeletal disorganization is the down-regulation in phosphorylation of PI3K-p85 subunit. In fact, VPS13A depleted K562 cells, a red blood progenitor cell line, show decreased levels of phosphorylated PI3K-p85 subunit. Consequently a decreased activation of the downstream proteins Rac1 and PAK1, which are involved in the actin polymerization process, is observed. Thus, the alterations of the actin polymerization described in VPS13A deficient K562 cells may be caused by a decreased activity of Rac1 and PAK1.

The down-regulation in phosphorylation of PI3K-p85 subunit is also proposed to activate apoptosis via two different mechanisms. The first mechanism involves the reduced activity of PAK1. PAK1 is known to phosphorylate BAD, avoiding the binding and inactivation of the anti-apoptotic protein Bcl2.
Hence, in Chorein silenced K562 and rhabdomyosarcoma cells, increased levels of dephosphorylated BAD are observed and consequently an increment of BAD-Bcl2 dimer is present, which in turn activates apoptosis\textsuperscript{49,50}. The second mechanism involves the capacity of PI3K to regulate the store operated Ca\textsuperscript{2+} entry (SOCE) complex\textsuperscript{51}. SOCE is composed mainly by two kinds of proteins: the plasma membrane proteins ORAI which form channels and the transmembrane proteins of the endoplasmic reticulum STIM that can sense the concentration of Ca\textsuperscript{2+} inside the ER\textsuperscript{52}. When the concentration of Ca\textsuperscript{2+} inside the ER becomes low, STIM proteins aggregate and interact with ORAI proteins activating the channels\textsuperscript{52}. PI3K is able to regulate ORAI1 expression via an PI3K/SGK1/NF\kappa B pathway\textsuperscript{53}. This might explain lower levels of ORAI1 and STIM1 proteins in cortical neurons differentiated from induced pluripotent stem cells (iPSC) produced from fibroblasts of ChAc patients\textsuperscript{53}. Thus, SOCE is less efficient and this may be the cause of an increased percentage of apoptotic cells observed in ChAc neurons compared to control neurons.

Interestingly, Chorea acanthocytosis red blood cells show also impaired autophagy, with an accumulation of autophagy related proteins Ulk1 and Atg7, and reduced clearance of lysosomes (accumulation of Lamp1 structures) and mitochondria\textsuperscript{54}. A link between accumulation of active Lyn and autophagy dysfunction has been proposed based on Lyn coimmunoprecipitation with Ulk1 and Atg7\textsuperscript{54}. Atg7 was also found to interact with Chorein in healthy erythrocytes\textsuperscript{54}. Besides, VPS13A depleted Hela cells show accumulation of autophagic markers and lower autophagic flux\textsuperscript{38}. In addition, blood platelets from ChAc patients have reduced level of VAMP8, a protein essential for fusion of cellular membranes and required for dense-granule secretion in platelets. Chorein absence also leads to decreased degranulation and aggregation of blood platelets\textsuperscript{55,56}.

Most of the knowledge concerning Vps13 from yeast to human cells has been summarized above. These results suggest that Vps13 can influence multiple pathways such as cellular trafficking, membrane contact sites, autophagy, apoptosis, cytoskeleton assembling and others. However, so far there are no data that clearly indicate a specific localization or conserved function of Vps13, which can be considered responsible for the onset of ChAc.
AIM OF THE THESIS

ChAc is a rare human neurodegenerative disease caused by the absence of VPS13A protein. Until now no treatment is available and many questions about this disease are still unanswered. In order to understand how mutations in VPS13A gene lead to ChAc and how this can be prevented by a specific therapy a suitable model organism for this disease is required. Vps13 mutant organisms show multiple phenotypes but only few binding interactors are known. The aim of this project is to establish a *Drosophila melanogaster* model for ChAc and try to find new binding partners in *Drosophila* to gain insight into processes and pathways compromised in Vps13 mutants. Another important enigma to solve is VPS13A localization. Discovering VPS13A localization might be crucial information to learn more about cellular functions and dynamics of the human VPS13A protein.


In this chapter we established and validated a suitable model organism to study ChAc. *Drosophila melanogaster* Vps13 mutants showed shortened life span, decreased climbing ability and the presence of vacuoles in the brain. Furthermore, Vps13 mutant flies were sensitive to proteotoxic stress and accumulated ubiquitylated proteins. Many of these phenotypes could be rescued by the overexpression of human VPS13A in the Vps13 mutant background, indicating a partially conserved function of the protein in these two species and making *Drosophila melanogaster* a suitable organism to study ChAc.

Chapter 3: Mass spectrometry identified Galectin as a Vps13 interacting protein in *Drosophila*.

In this chapter we aimed to determine Vps13 interactors to predict novel functions and roles in ChAc. We performed an immunoprecipitation coupled to mass spectrometry (IP-MS) in fly heads using control and Vps13 mutant flies to obtain a list of possible Vps13 interactors. As one of the hits, Galectin was identified. Interaction with Galectin was validated via immunoprecipitation in S2 cells.
Chapter 4: Human VPS13A is associated with multiple organelles and required for lipid droplet homeostasis.

Cellular functions and the localization of human VPS13A are still unknown. The discovery of VPS13A localization may be crucial to gain insight into the mechanisms of the disease and to find a possible therapeutic treatment for the patients. In this chapter, we show that VPS13A is associated with mitochondria and, through its FFAT domain, with the ER protein VAP-A. These results suggest a role in the formation of ER-mitochondria membrane contact sites. Interestingly, in cells treated with fatty acid, VPS13A translocates from mitochondria to newly synthesized lipid droplets influencing their motility and size.

Chapter 5: Solving the VPS13A puzzle: conclusion and future perspectives.

VPS13A may have several independent functions, because of this, the challenge is not only to discover processes and pathways in which the VPS13A protein plays a role but also try to identify which of these disturbed processes are responsible for the disease in order to find a treatment. Here, we offer an overview of the research accomplished in this thesis and possible perspectives and future developments of the work are presented.
REFERENCES


