CHAPTER 1

Introduction and aim of the thesis
INTRODUCTION

Chorea-Acanthocytosis

Chorea-Acanthocytosis (ChAc) is one of the core neuroacanthocytosis (NA) syndromes together with McLeod syndrome (MLS). NA syndromes also include Huntington’s disease-like 2 (HDL2) and panthothenate kinase-associated neurodegeneration (PKAN)\(^1\)\(^-\)\(^3\). NA syndromes are a genetically and clinically heterogeneous group of rare neurodegenerative diseases that are characterized by neurological abnormalities, degeneration of the basal ganglia, and spiky formed red blood cells called acanthocytes\(^3\)\(^-\)\(^5\).

Since presence of acanthocytes can be variable it is not required for diagnosis of one of the NA syndromes\(^4\). All four NA syndromes are caused by genetic mutations in genes that have been identified over the last decades. ChAc, MLS and PKAN are caused by loss-of-function mutations in \(\text{VPS13A}\) (\(\text{Vacuolar Protein Sorting 13A}\))\(^6\)\(^,\)\(^7\), \(\text{XK}\)\(^8\)\(^,\)\(^9\), and \(\text{PANK2}\) (\(\text{Panthotenate Kinase 2}\))\(^10\) respectively. The autosomal dominant disease HDL2 is caused by an expansion mutation of three nucleotides in the \(\text{JPH3}\) (\(\text{Junctophilin 3}\)) gene\(^11\).

NA syndromes are extremely rare with around 1000 cases of ChAc worldwide\(^6\).

In this Chapter we give a description of Chorea-Acanthocytosis and provide an overview of previous data on the affected cellular processes of the disease. Furthermore we summarize the current knowledge on the localization and function of VPS13 in different organisms. Finally we highlight the \(\text{Drosophila melanogaster}\) ovary that is used for further study in this thesis and serves as a powerful and versatile system to investigate a broad number of cellular processes.

Clinical manifestations and aetiology of Chorea-Acanthocytosis

The progressive autosomal recessive neurodegenerative disorder ChAc usually presents between ages 20-40 with a mean age of onset around 35 years of age\(^1\). The disease is characterized by a variety of movement abnormalities including chorea, mostly of the limbs, and dystonia. The latter mainly affects the oral region and the tongue in particular, making orofacial dystonia the most distinctive feature of ChAc\(^1\). Dyskinesias of eyes, mouth, tongue and vocalizations are common, and tongue protrusion and feeding dystonia are highly specific hallmarks for ChAc\(^12\). These symptoms cause severe problems with feeding resulting in weight-loss. Although ChAc usually presents with hyperkinetic movement disabilities, some patients present with parkinsonism\(^4\). Cognitive and psychiatric symptoms are common among ChAc patients and may include anxiety, depression and obsessive behavior\(^13\). Seizures can be a predominant feature of ChAc and affect at least one third of the patients, where they usually precede the appearance of movement abnormalities\(^4\)\(^,\)\(^12\)\(^,\)\(^14\). Elevated levels of creatine phosphokinase (CK) in serum are found in the majority of ChAc patients and muscle weakness is commonly reported\(^4\). Neuroimaging shows atrophy of the striatum and caudate nucleus in particular\(^14\)\(^,\)\(^15\)\(^,\)\(^16\). Histopathological analysis of post-mortem material is consistent with those findings, showing neurodegeneration and astrogliosis in the striatum\(^17\)\(^-\)\(^19\), although the exact extend of the neuronal loss and neuropathology in patients is still poorly understood.
Patients are usually diagnosed based on clinical symptoms, presence of acanthocytes and elevated serum CK levels. ChAc can be confirmed by detecting loss of VPS13A protein using Western Blot analysis of red blood cells. The current treatment of ChAc is purely symptomatic to temporarily relieve the chorea and dystonia. Deep brain stimulation might be helpful and was shown to temporarily improve the motor symptom severity in about two third of the patients treated. Progression of the disease inevitably leads to premature death.

ChAc is caused by mutations in the gene encoding vacuolar protein sorting associated protein 13A (VPS13A) leading to the absence of VPS13A protein (also called Chorein) in patients. Various mutations, distributed throughout the VPS13A gene have been identified in different ChAc patients including nonsense, frameshift, splice-site mutations and deletions. VPS13A belongs to the VPS13 protein family that contains three additional homologs: VPS13B-D, which are all associated with different neurological disorders. The rare autosomal recessive Cohen syndrome is caused by mutations in VPS13B while mutations in VPS13C lead to autosomal recessive Parkinson’s disease. Only recently it was discovered that mutations in VPS13D are associated with a novel recessive ataxia and childhood onset movement disorder.

**VPS13 function and localization**

In 2001 the human CHAC gene (later renamed VPS13A) was discovered to be the causative gene for ChAc. The gene codes for a transcript consisting of 11,263 base pairs (bp), which is widely expressed in different tissues of the body, encoding a protein of 3,174 amino acids (aa) that are organized into 73 exons. Multiple splicing variants exist with slight differences in transcript and protein size. Many different species contain an orthologous gene and protein of VPS13A, including *M. musculus*, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, *T. thermophila* and *D. discoideum*. The highest conservation of the protein is found in the terminal domains and all proteins contain a chorein domain at the N-terminus.

The exact function of VPS13A, and the mechanism underlying the pathogenesis of ChAc are still largely unknown. Most of the knowledge that is currently available comes from unicellular organisms and yeast in particular. Figure 1 summarizes the proposed localization and functions of VPS13A and its orthologs. VPS13, the yeast ortholog of the human VPS13A, is a peripheral membrane protein localized to endosomes and plays a role in intracellular trafficking of membrane proteins from the trans-Golgi network (TGN) to the prevacuolar compartment (PVC) and recycling back to the TGN. In addition, it regulates membrane morphogenesis during sporulation in *Saccharomyces cerevisiae*, where it is translocated from endosomes to the prospore membrane. Absence of VPS13 leads to defects in prospore formation that are caused by reduced levels of PI(4)P and PI(4,5)P₂ at the prospore membrane. VPS13 functions redundantly with ERMES, a complex that connects the endoplasmic reticulum and mitochondria, and dynamically localizes to contact sites between mitochondria and the vacuole and nuclear-vacuole junctions. Furthermore, VPS13 is important for mitochondrial integrity and function, mitophagy, homotypic fusion of TGN membranes, endosomal recycling and its function in prospore membrane formation, TGN-PVC transport and TGN homotypic fusion involves or depends on binding to various phospholipids.
In the ciliate *Tetrahymena thermophila* it was found that the ortholog of VPS13A, TtVPS13A, is necessary for efficient phagocytosis and associates with the phagosome membrane during the entire cycle of phagocytosis. In addition, growth speed of a TtVPS13A knockout strain was significantly reduced in conditions where phagocytosis is required. A role for VPS13 in autophagy was implicated in *Dictyostelium discoideum*. Mutants defective for the VPS13A-related protein TipC show autophagic dysfunction, which was also found in HeLa cells by downregulation of VPS13A. VPS13A is further implicated in a number of cellular processes, including actin polymerization, autophagy, apoptosis, regulation of phospholipids and dopaminergic vesicle release.

A ChAc mouse model was established which carries a deletion-mutation also found in Japanese ChAc patients. Those mice display acanthocytes and disturbances in motor function at old age, but there is no reduction in life span. Later it was found that the genetic background of the mice strain is of influence on the phenotype suggestive for genetic modulators that influence ChAc phenotypes. This is in line with ChAc patients who also exhibit a range of symptoms. However, for the proper study of VPS13A function it is beneficial to reduce individual genetic background variation to a minimum and therefore the need for an additional solid multicellular disease model is of high importance.
The *Drosophila melanogaster* ovary system

Model organisms are of high importance to understand neurodegenerative diseases and to develop future treatments. The fruit fly (*Drosophila melanogaster*) has made a substantial contribution to the understanding of many neurological and neurodegenerative diseases as well as providing a foundation for therapies and intervention strategies (Chapter 2).

When investigating a neurodegenerative disease in fruit flies, the most obvious choice is to study the *Drosophila* central nervous system. However, different organ systems can be of great help to gain insight in the cellular function of a certain gene and protein of interest. For example, the *Drosophila* gene *wingless* (*wg*) was discovered to play an important role in wing development\(^{59,60}\) and only later it was found that *wg* is the homolog of the mammalian proto-oncogene *Wnt-1* that regulates cell proliferation\(^{61}\). Wnt signaling is now recognized to play a pivotal role in development of human cancers\(^{61}\), which illustrates the significant role different organ systems can play in research.

In addition to the central nervous system, we focus on the *Drosophila* ovary in this thesis. The *Drosophila* ovary system and the process of oogenesis is an excellent system to study a wide variety of biological processes\(^{63}\). *Drosophila* females have a pair of ovaries, each of these ovaries contains 15-17 tubular ovarioles in which the egg chambers develop\(^{64}\) (Figure 2A). Each ovariole contains a series of developing egg chambers that are connected via stalk cells\(^{65}\). The germline and somatic stem cells that produce the egg chambers are located in the germarium at the tip of the ovariole (Figure 2B). The individual egg chambers bud off from the germarium to travel down the ovariole and grow through 14 well-defined developmental stages until mature eggs pass into the oviduct where they are fertilized and then deposited\(^{64,66}\) (Figure 2B). Asymmetrical division of the germline stem cell creates another stem cell and a daughter cell: the cystoblast. The 16 germline cells of an egg chamber all originate from this single daughter cell through four rounds of division. Because cytokinesis is not complete during this process all germline cells remain connected via special cytoplasmic bridges called ring canals while developing into a mature egg\(^{63,65}\). One of the 16 germline cells develops into the oocyte at the posterior end of the egg chamber. The other 15 cells differentiate into polyploid nurse cells (NCs) that have an important role in production and supply of nutrients for the oocyte. A layer of somatically derived follicle cells (FCs) surrounds the 16 germline cells, together they form an individual egg chamber\(^{63,64}\). The 14 developmental stages of the developing individual egg chamber can be distinguished based on morphology and size. Egg chambers in stage 1-5 are characterized by their spherical shape, while during stage 6-9 the egg chamber elongates and acquires a more oval shape (Figure 2B). In addition, the posterior FCs that are in contact with the oocyte start to take a more columnar shape while the anterior FCs flatten and stretch over the NCs. The oocyte compartment increases in size taking up a volume of about one third of the entire egg chamber by the end of stage 9. During stage 10 and 11 a massive dumping of NC cytoplasm into the oocyte compartment takes place which drastically increases the size of the oocyte. This is also the start of formation of the dorsal appendages (DA) that are thought to play a role in respiration of the future egg\(^{64-66}\). In stage 12-14 the remaining NC nuclei are degraded and removed by the stretch FCs that surround them. Additionally, the DA are completed, the oocyte maturates and the eggshell, or chorion, is secreted by the FCs followed by their programmed cell death. Finally the layer of dying FCs covering the egg slides off and a finished egg is produced\(^{64,66}\).
King was the first to elaborately describe the process of *Drosophila* oogenesis in 1970, and since then many areas of research took advantage of the versatility of the *Drosophila* ovary making it one of the most studied systems of the fruit fly. One of the greatest benefits of this system is of practical nature being that the ovary is the largest organ in the female fly, easily accessible, and manipulation of the ovary is possible without affecting survival of the female fly itself. The large egg chambers and individual cells facilitate the study and visualization of many cellular processes using different imaging techniques that have contributed to the current knowledge of molecular, cellular and developmental biology. Processes that are studied include stem cell maintenance, cell differentiation, morphogenesis and a large number of common cell biological functions.

Another advantage for studying the *Drosophila* ovary is the availability of a wide range of tools for oogenesis research. These tools include the specific control of gene expression in either germline

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**Figure 2. The Drosophila ovary and oogenesis**

A. Schematic representation of the *Drosophila* ovary consisting of multiple ovarioles that contain different developmental stages of individual egg chambers (green). Nuclei are in blue. B. Oogenesis begins in the germarium located at the tip of an ovariole where germline (GSC) and somatic stem cells (FSC) continuously generate new egg chambers. An individual egg chamber consists of 15 nurse cells (NCs): light green; one future oocyte: dark green, surrounded by a layer of follicle cells (FCs): purple. Each individual egg chamber grows through 14 well defined developmental stages.
Introduction

or somatic cells using the GAL4/UAS system (Chapter 2, Figure 1), expression of fluorescently tagged proteins at endogenous levels with the use of protein trap lines and the possibility to generate genetically mosaic ovaries via different techniques. Together this makes the Drosophila ovary a convenient and advantageous system to utilize and answer questions from various fields of research, including the investigation of pathophysiology underlying human diseases, localization of specific proteins or cellular consequences of specific gene dysfunction.

AIM AND OUTLINE OF THE THESIS

The research in this thesis was aimed to provide understanding about the underlying pathophysiology of ChAc by gaining insight in the function and localization of VPS13A and its ortholog in Drosophila melanogaster: Vps13. The main focus of investigation was on Drosophila melanogaster as a model organism and the initial objective was to characterize a Drosophila Vps13 mutant that can serve as a model for ChAc. Next we implemented the gene editing technique CRISPR/Cas9 and created an additional Vps13 knockout mutant and Vps13-GFP flies to enable the investigation of Vps13 localization and function. We then continued the study of Vps13 function and localization in the well described multicellular system of the Drosophila ovary. Finally we tried to understand the versatile role of human VPS13A at a molecular level to unravel the subcellular localization, dynamics, binding partners and various domains of VPS13A in mammalian cells to eventually verify this in the Drosophila Vps13 mutant.

Chapter 2: Modelling in Miniature: using Drosophila melanogaster to study human neurodegeneration

Model organisms are of extreme importance when studying the pathophysiology underlying many human diseases. In Chapter 2 we provide an overview about the contribution of Drosophila melanogaster as a model organism in the field of neurodegeneration. The review shortly summarizes the history of the fruit fly in research. Furthermore, the versatility and the extensive toolbox that make Drosophila a powerful model are discussed followed by examples of how the fruit fly has been utilized in the study of several neurodegenerative diseases and genetic and pharmacological screening. Finally we highlight some findings from Drosophila that were validated in other model organisms and are now further developed for applications in the clinic.

Chapter 3: Drosophila Vps13 is Required for Protein Homeostasis in the Brain

Loss-of-function mutations in the Vacuolar Protein Sorting 13 homolog A (VPS13A) gene lead to the rare neurodegenerative disease Chorea-Acanthocytosis (ChAc). The disease is characterized by movement disabilities and spiky morphology of erythrocytes (acanthocytes). Knowledge about the function of VPS13A and the consequences of VPS13A impairment is limited and urge the development of models to investigate underlying disease mechanisms of ChAc. Therefore, in Chapter 3 we characterized a Drosophila Vps13 mutant. We demonstrated that Vps13 mutants have a shortened lifespan, impaired
climbing capacity and vacuoles in the brain, which are all typical for neurodegeneration in flies. Furthermore we found accumulation of ubiquitinated and aggregated proteins in the brain, suggestive for impaired protein homeostasis. Overexpression of HvPS13A in the Vps13 mutant background partially rescued some of the phenotypes, which indicates the functional conservation of both proteins and underscores the relevance of this Drosophila disease model.

Chapter 4: CRISPR/Cas9 based genome editing of Drosophila for the generation of a Vps13 knockout mutant and Vps13-GFP flies

The genome editing technique CRISPR/Cas9 has gained extreme popularity in many fields of research over the last couple of years because of the easy implementation, high specificity and low costs. Initially discovered as an adaptive immune system in bacteria, CRISPR/Cas9 is now applied as a targeted mutagenesis technique but can also be used to endogenously tag genes of interest with a fluorescent marker like a Green Fluorescent Protein (GFP). In Chapter 4 we elaborately describe the implementation of the versatile CRISPR/Cas9 technique to generate a Vps13 knockout mutant and a Vps13-GFP fly line. We showed that by injection of two sgRNAs into cas9 expressing Drosophila embryos it is possible to introduce a double stranded break in the Vps13 gene. This led to the creation of a Vps13 knockout mutant that does not express the Vps13 gene, leading to the complete absence of Vps13 protein. With the simultaneous injection of a sgRNA and a donor plasmid containing the GFP sequence flanked by two homologous arms of the C-terminus of Vps13 we were able to create a Vps13-GFP fly line with proper expression of Vps13-GFP and presence of Vps13-GFP protein. We also discussed our most striking observations about the application of the CRISPR/Cas9 technique for the creation of both lines. Further validation and application of both lines is described in Chapter 5.

Chapter 5: Timely removal of nurse cell corpses requires cell-autonomous function of Vps13

The Drosophila ovary system is widely used to study biological and cellular processes because of its easy accessibility and the availability of many genetic tools. Cell death and removal of superfluous Nurse Cells (NCs) during late oogenesis is a poorly understood mechanism, of which mainly non-autonomous factors in Follicle Cells (FCs) have been discovered. In Chapter 5 we identified Vps13 as a cell-autonomous player during developmental programmed cell death in the Drosophila ovary using the Vps13null and Vps13-GFP fly lines we created in Chapter 4. Vps13 mutant females have a deficit in egg lay and produce lower numbers of offspring. A striking accumulation of persistent nurse cell nuclei (PNCN) in late stage egg chambers of mutant females was observed. Absence of Vps13 in NCs specifically led to PNCN accumulation, while knockdown of Vps13 in FCs does not. Antibody staining and endogenous Vps13-GFP expression showed a specific signal in close proximity to nuclei of dying NCs in late-stage oogenesis. Large scale electron microscopy revealed a novel Vps13-dependent membrane structure adjacent to the plasma membrane of NCs undergoing cell death in control flies that was almost entirely absent in Vps13 mutants. Together these data implicate a cell-autonomous function of Vps13 in proper egg development and removal of cells that undergo programmed cell death.
Chapter 6: Human VPS13A is associated with multiple organelles and required for lipid droplet homeostasis

Previous research about the function of VPS13A indicates a versatile role for the protein in a wide range of cellular processes, including autophagy, protein homeostasis and actin polymerization. In Chapter 6 we investigated the subcellular localization, dynamics, binding partners and individual domains of VPS13A to provide more understanding about its molecular function. We demonstrated that VPS13A is associated with mitochondria and interacts with VAP-A, thereby establishing membrane contact sites between mitochondria and endoplasmic reticulum (ER). Altering levels of fatty acids induces a dynamic shift in VPS13A localization from mitochondria to lipid droplets (LD). When VPS13A is localized to LDs their movement is temporarily paused while absence of VPS13A leads to increased LD number and size that show faster directional mobility. Finally we showed that Drosophila Vps13 mutant flies accumulate LDs in the central nervous system using large scale electron microscopy which indicates functional conservation of VPS13 in LD homeostasis.

Chapter 7: Summarizing discussion and future perspectives

Chapter 7 summarizes the main results presented in this thesis. Furthermore it provides a general discussion and future perspectives that follow from the data presented here.
REFERENCES
