Unraveling VPS13A pathways: from Drosophila to human

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CHAPTER 5

Solving the VPS13A puzzle: conclusion and future perspectives

GENERAL DISCUSSIONS

The Vps13 protein was already discovered in 1986 in *S. cerevisiae*[1] but only in 2001, it was found that mutations in the human *VPS13A* gene are associated with the onset of the neurodegenerative disease ChAc[2,3]. Until now the molecular function(s) of VPS13A protein and the mechanisms that lead to ChAc are still not well understood. *VPS13A*, together with *VPS13B, C* and *D*, are the four *VPS13* genes present in the human genome[4]. Mutations in *VPS13B* and *VPS13C* are responsible for the onset of human disorders, Cohen syndrome[5] (CS) and Young-onset Parkinson disease[6] (YOPD) respectively. Recent studies also showed that mutations in *VPS13D* cause a recessive form of spinocerebellar ataxia[7,8]. It is yet inconclusive whether the various VPS13 proteins have similar, redundant, complementary or independent functions but the fact that mutations in these genes lead to the onset of different diseases suggest at least the presence of gene-specific functions or gene specific expression patterns. In this thesis we used different approaches and different organisms to gain insight into the cellular and molecular function of VPS13A.

Most of the knowledge concerning the function of Vps13 was discovered using *S. cerevisiae* as a model. *S. cerevisiae* has a single intron-less *Vps13 (YLL040C)* gene encoding a Vps13 protein. In *S. Cerevisiae* the single Vps13 protein is required for proper sporulation[9] and it is involved in trans-Golgi network (TGN) -pre-vacuolar compartment (PVC) trafficking[10–12]. Furthermore, recent experiments show that Vps13 can bypass endoplasmic reticulum-mitochondrial encounter structure (ERMES) mutants[13,14]. ERMES is a complex consisting of both ER and mitochondrial proteins, located at the interface between the two organelles[15]. Because yeast only harbors one *VPS13* gene, studies in additional models, possessing more *VPS13* genes have to be done to uncover the function of the separate VPS13 family members. Especially multicellular model organisms are valuable to study how loss of VPS13A is causing a neurodegenerative disorder like ChAc, the main disease of interest of this thesis. A *VPS13A* knock out mouse has been established, however it shows only a mild phenotype[16]. Differences in survival, involuntary movements or clear behavioral changes are absent in this model[16]. On the

other hand, VPS13A deficient mice do show acanthocytes[16]. The role of acanthocytes is still not understood and it is still under debate if there is a link between the neurodegeneration and the acanthocytes. Because a multicellular model with a brain is valuable to study ChAc and the *VPS13A* knock-out mouse model only shows a mild or no neurodegenerative phenotype, depending on the background of the used strain, in **chapter 2** we established and validated *Drosophila melanogaster* as a new model organism to study ChAc. The *Drosophila* genome encodes for three *Vps13* genes, orthologues to *VPS13A*, *B* and *D*[4]. The *Drosophila* orthologue of VPS13A referred to as Vps13, is ~29 percent identical to human VPS13A and contains several common domains of the human VPS13A protein (see chapter 1). *Drosophila melanogaster Vps13* mutants showed shortened life span, decreased climbing ability and age associated neurodegeneration, typical phenotypes indicative for neurodegeneration in *Drosophila*[17]. Progressive features, such as locomotor abnormalities which accumulate with age and a reduced life span, are also observed in ChAc patients. In addition, *Drosophila Vps13* mutants accumulate ubiquitylated proteins in the central nervous system and are more sensitive to proteotoxic stress. Levels of Ref(2)P, the *Drosophila* orthologue of human p62, are also increased in *Vps13* mutants[17]. Our data suggest also that Vps13 is a peripheral membrane protein, enriched in fractions containing Rab7 vesicles. Overexpression of human VPS13A in the Vps13 mutant background rescues some of these phenotypes, proving a partially conserved function of the protein in these two species and making *Drosophila melanogaster* a suitable organism to study ChAc. The proteotoxic stress, together with the accumulation of ubiquitylated proteins and Ref(2)P, suggest impairments in one or more of the cellular degradation pathways in *Vps13* homozygous mutants. Consistently, human VPS13A depleted cells have been reported to accumulate autophagosomes and consequently to have an impaired autophagic degradation[18]. Autophagy is a well-regulated cellular pathway that allows degradation and recycling of cellular components. Defects in the autophagy pathway are responsible for the onset of many neurodegenerative diseases[19]. In fact, mutations in genes coding for other vacuolar sorting proteins such as VPS35 cause a dominantly inherited form of Parkinson's disease[20,21] (PD) and mutations in CHMP2B, orthologous to yeast VPS2, cause frontotemporal dementia (FTD)[22]. Interestingly, both *VPS35* and

CHMP2B mutations lead to accumulation of autophagosomes as a result of impairment in autophagic degradation[23,24]. A mutation in the *ATG5* gene has been found to lead to a familial form of ataxia[25]. *Drosophila* Vps13 is associated with Rab7 enriched membranes (chapter 2). Rab7 is a Rab GTPase localized to late endosomes/lysosomes and its dysfunction leads to a defect in autophagosome to lysosome fusion[26]. Therefore, it may also be possible that VPS13A is involved in autophagosome fusion with endosomal compartments. Mutations in *Rab7* gene have been identified and associated with Charcot-Marie Tooth type 2B (CMT2B)[27]. CMT2B is a peripheral neuropathy with onset in the second or third decade of life. Common symptoms include severe sensory loss, weakness that develops mostly in the legs, reduced tendon reflexes in the ankles and the loss of muscle tissue (muscle atrophy). Data demonstrate that increased levels of Rab7 in the activated form are responsible for CMT2B disease[27]. These results could indicate that VPS13A has some role in autophagy and its impairment leads to a specific form of neurodegeneration. However, the exact molecular function of VPS13A in autophagy is largely unclear.

In order to discover specific interactors of Vps13, in **chapter 3** we performed immunoprecipitation assays coupled to mass spectrometry (IP-MS) in fly heads to determine Vps13 binding partners. Identification of these binding partners may give us clues about possible specific cellular functions in which VPS13A is involved. Despite no proteins involved in autophagy were found, interestingly, Galectin was one of the top hits. Interaction with Galectin was validated via immunoprecipitation of GFP-Galectin in *Drosophila* S2 cells. Recent studies showed the importance of human VPS13C-Galectin-12 interaction. In fact, this interaction is stabilizing Galectin-12, preventing lysosomal degradation and Galectin-12 stability is crucial for adipocyte differentiation[28]. Because of this interaction and our results in **chapter 3**, here we will discuss implications of an interaction of *Drosophila* Vps13 and Galectin. DIOPT (DRSC Integrative Ortholog Prediction Tool) analysis showed that *Drosophila* Galectin is most similar to human Galectin-4, Galectin-8, Galectin-9 and Galectin-3. In humans 10 Galectin proteins are described and they are expressed in a wide range of tissues. Galectin proteins have been reported to be involved in many pathways including neurodegeneration processes. Recent studies prove also that Galectin-3 has an anti-apoptotic

effect in cells, the mechanism is still not well understood but it was shown that Galectin-3 prevents mitochondrial damage, cytochrome c release and consequently capsase-3 activation[29,30]. Interaction with Synexin protein is crucial for the anti-apoptotic activity of Galectin-3[29]. In addition, Galectin-3 has also been reported to decrease the progression of Amyotrophic lateral sclerosis (ALS)[31]. In a mouse model of amyotrophic lateral sclerosis it is shown that deletion of Galectin-3 does not change the onset of the disease but it increases the progression of the disorder, probably via an activation of microglia cells which consequently accelerates neuroinflammation through release of TNF-alpha[31]. The increase of neuroinflammation is associated with oxidative damage which is the cause of faster progression of the disease in *Galectin-3* mutant mice[31]. In contrast, a recent study shows an opposite function of Galcetin-3 in mice 24 h after traumatic brain injury[32]. In fact, after traumatic injury Galectin-3 is released and can bind TLR-4 present on microglia cells surface, increasing the expression of inflammatory molecules as IL-1beta, IL-6, TNF-alpha, NOS2[32]. Consequently the absence of Galectin-3 in the first phase, after brain injury, is reducing neuroinflammation promoting neuroprotection[32]. Despite conflicting results, these data suggest that Galectin-3 plays a role in the modulation of inflammation in the brain influencing neurodegeneration.

Galectin-1 has been reported to have a protecting function against inflammation-induced neurodegeneration in Multiple sclerosis (MS)[33]. MS is a chronic disease that affects the central nervous system, caused by demyelination in brain and spinal cord neurons. The myelin sheath that covers nerve fibers is crucial to insulate and conduct electrical signals efficiently. In a mouse model of experimental autoimmune encephalomyelitis (EAE), Galectin-1, released from astrocytes, can bind the protein CD45 on microglial cell surface. This interaction activates CD45 which in turn activate p38MAPK, CREB, NF-KB signaling pathways that suppress downstream release of pro-inflammatory mediators[33].

Galectin-8 has also been reported to have a protecting function specifically against tau aggregation in Alzheimer's disease (AD)[34]. Alzheimer's disease (AD) is a chronic neurodegenerative disease that usually starts with short-term memory loss and worsens over time until the patients show severe cognitive

and physical impairments. Multiple genes are involved in AD and the mechanism of the disease is still far from being understood. One hypothesis states that increased phosphorylation of tau protein can promote tau aggregation and accumulation in neurons[35,36]. These aggregates can be the cause of malfunctions in neurons, which can lead to cell death[36]. Galectin-8 is able to sense damaged endosome membranes and activate autophagy recruiting the protein NDP52 (nuclear dot protein 52)[34]. NDP52 is an autophagy receptor able to bind to ubiquitinated intracellular pathogens via an ubiquitin-binding domain and brings them to nascent autophagosomes via interaction with LC3, an autophagosomal membrane protein[37]. It has been reported that autophagy activated by Galectin-8, as a result of damaged endomembranes, has a protective function not only against pathogens but also against tau aggregation[34]. The mechanism is not completely understood but the main hypothesis is that autophagy triggered by Galectin-8 reduces the entry of tau aggregates, at early stages, into the cytosol and thereby further aggregation is prevented[34].

Above, possible roles of Galectins in various neurodegenerative phenotypes have been summarized. Interestingly Galectin-3 and Galectin-1 have been reported to have a protective function against ALS and MS respectively. The general mechanism of action is the decrease of neuroinflammation that normally occurs during the progression of the neurodegenerative diseases. It would be interesting to test if Vps13 depletion is able to influence a Galectin-mediated anti-inflammation effect. Intriguingly, Galectin-8 has been shown to trigger autophagy, as a result of damaged endomembranes, and consequently to have a protective function against tau aggregation in AD. Considering that in *Vps13 Drosophila* mutants we observed accumulation of ubiquitynated proteins/aggregates and an autophagy phenotype was described in Hela cells[18], it would be interesting to verify the presence of a possible link between these phenotypes and the Vps13-Galectin interaction. *Drosophila* is a suitable model organism to investigate a possible genetic interaction between Vps13 and Galectin. It will be of interest to test if downregulation of Galectin via an RNAi or CRISPR/CAS9 approach in flies will lead to similar phenotypes as *Vps13* mutant flies, such as accumulation of ubiquitilated proteins or accumulation of lipid droplets in glia cells. It could also be tested whether a simultaneous downregulation of *Galectin* and *Vps13* will aggravate these

phenotypes and whether overexpression of Galectin in the *Vps13* mutants background can rescue some of the *Vps13* mutant phenotypes and hereby compensate the absence of Vps13. In summary, a possible link between VPS13A and Galectins is interesting and opens new research directions.

In light of all the previous findings in Drosophila, in **chapter 4** we investigated the role of VPS13A in human cells trying to understand the cellular function of this protein. In **chapter 4** we showed the presence of VPS13A at the interface of ER and mitochondria. VPS13A interacts with the ER protein VAP-A, via the FFAT domain and this interaction is modulated by calcium levels. Upon increased fatty acid uptake, VPS13A translocates from mitochondria to newly formed lipid droplets (LDs) influencing their size and motility. These results are also supported by the mass spectrometry data of the chapter 3, in which many mitochondria and lipids droplet proteins were found, indicating that probably similar pathways are involved in *Drosophila* and humans. In addition, **chapter 4** suggests that VPS13A plays a role in the formation or functionality of ER-mitochondria Membrane Contact Sites (MCS). ER-mitochondria MCSs were described for the first time in 1956 when close membranes between ER and Mitochondria were observed[38]. ER-mitochondria MCSs are involved in many processes such as phospholipid exchange between ER and mitochondria, sterol metabolism, autophagy, mitophagy, mitochondrial dynamics, calcium homeostasis, protein folding, energy metabolism and others[39]. Interestingly, ER-mitochondria MCSs are present in brain tissue, including neurons and synapses, and they are crucial in synaptic activity, probably via regulation of calcium signals[40,41]. ER-mitochondria MCSs have also been associated with the onset of different disorders including neurodegenerative diseases such as Frontotemporal Dementia (FTD), Parkinson's Disease (PD), Alzheimer's Dementia (AD), Charcot-Marie-Tooth disease (CMT), Amyotrophic Lateral Sclerosis (ALS)[39]. Multiple genes are involved in the onset of the familiar forms of ALS, including VAP-B (ALS type 8)[42]. The mechanism is still not well understood. VAP-B interacts with PTPIP51, an outer mitochondrial membrane protein, forming ER-mitochondria contact sites[43]. The loss or modulation of this interaction can perturb mitochondria Ca^{2+} uptake from ER stores and this may be the cause of the onset of ALS type 8[43]. It is tempting to speculate that VPS13A, with its interaction with VAP-A, acts in a similar way influencing Calcium uptake by mitochondria.

In **chapter 4** we also describe the influence of VPS13A on lipid droplets size and motility and we observed an accumulation of lipid droplets in glia of *Vps13* mutants. A correlation between the accumulation of lipid droplets in glia and neurodegeneration has previously been reported. In *Drosophila* the downregulation in glia cells of ND23 protein, an orthologue of the human NDUFS8 (a subunit of the mitochondrial complex I), lead to lipid droplet accumulation in glia and is associated with neurodegeneration[44]. One of the causes triggering accumulation of lipid droplets in glia is the elevated levels of ROS, as a consequence of mitochondrial impairment. Elevated ROS levels subsequently promote transfer of lipids from neurons to glia where they form lipid droplets[45,46]. It has been shown that ROS induce oxidation and peroxidation reactions that can damage lipids, protein and nucleic acids[47]. Recent studies support now that lipid droplets in glia have a protective effect against neurodegeneration by avoiding the accumulation of peroxidated lipids in neurons[46]. In **chapter 4** we show the presence of VPS13A at the ER-mitochondria interface and an accumulation of lipid droplets in glia cells of *Vps13* mutant *Drosophila*. The presence of Vps13/VPS13A at the ER-mitochondria contact sites is consistent with studies in yeast and COS-7 cells[48,49]. Interestingly, new studies also proved VPS13A to be a lipid transport protein[49]. Thus, the accumulation of lipid droplets in glia cells in *Vps13* mutant brains can be an indicator of a neurodegenerative process due to a possible impairment of mitochondria or lipids transport between neurons and glia.

In conclusion, many different phenotypes observed in various Vps13 mutant model organisms or cells are reported and we discussed how our findings could explain how loss of Vps13 can lead to the onset of ChAc. It is yet not possible to build a model in which all these observations are linked together, also because our results further confirm that Vps13 has multiple functions in various cells and organisms.

FUTURE PERSPECTIVES

Progress in VPS13A research is slowly moving forward and one of the main questions remains unanswered: how can loss of VPS13A lead to the onset of Chorea Acanthocytosis. VPS13A may have several independent functions, therefore, the challenge is not only to discover processes and pathways in which VPS13A protein plays a role but also try to identify which of these are associated with the disease. This identification is required to design a possible treatment. Future research may take advantage of the *Vps13* mutant *Drosophila* model established in this thesis, which can contribute greatly to the understanding of the role of VPS13A in the onset of ChAc. Screens to verify which genes can improve or aggravate the phenotypes observed in *Vps13 Drosophila* mutants can be very useful to get an indication about pathways involved. In addition, further investigation of the identified hits in chapter 3 may provide clues of Vps13 dependent cellular processes. Additionally, in the future, screening compound libraries or FDA approved drugs in the *Drosophila* model may identify compounds with rescuing potential. Additional information is also required to further delineate the role of VPS13A in autophagy and how defective autophagic degradation could lead to the neurodegeneration observed in ChAc. Further research needs to be done to understand the exact role of VPS13A in ER-mitochondria membrane contact sites and if VPS13A loss can affect mitochondrial functions in a pathological way. For the first time we showed the interaction of Vps13 with Galectin in *Drosophila melanogaster*. It would be interesting to verify if the Vps13-Galectin interaction is conserved in humans and to determine which of the Galectins interact with VPS13A. It may be possible that VPS13A, via its interaction with Galectins, can be involved in modulation of neuroinflammation influencing the progression of the disease. Additionally, autophagy impairment seen in absence of Vps13 can also be linked to abnormalities in Galectin-8 functioning. Although functions of VPS13A at the molecular level are still not completely understood, the work presented in this thesis provides new information about VPS13A, its subcellular localization and identified possible binding partners. These new insights, could possibly lead towards a future treatment of ChAc.

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