CHAPTER 6

Human VPS13A is associated with multiple organelles and required for lipid droplet homeostasis

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Human VPS13A is associated with multiple organelles and influences mitochondrial morphology and lipid droplet motility.
ABSTRACT

Human VPS13A is highly conserved among eukaryotes and associated with the autosomal recessive neurodegenerative disease Chorea-Acanthocytosis (ChAc). The cellular function, localization and dynamics of the VPS13A protein are unknown. Here, we show that VPS13A is associated with mitochondria and establishes membrane contact sites with the endoplasmic reticulum (ER) by interacting with VAP-A, via a FFAT domain, a binding influenced by calcium levels. Upon increased fatty acid uptake, VPS13A translocates from mitochondria to newly formed lipid droplets (LDs). Localization of VPS13A to LDs pauses their movements whereas loss of VPS13A induces increased LD motility and size. Accordingly, VPS13A plays a conserved role in LD homeostasis in Drosophila, as in its absence these organelles accumulate in glia cells of the central nervous system, a phenotype associated with neurodegeneration, impaired locomotor function and decreased life span. Altogether, our data link VPS13A with multiple organelles and its dysfunction induces an alteration in LDs homeostasis.
INTRODUCTION

The vertebrate VPS13 protein family consists of four closely related proteins, VPS13A, VPS13B, VPS13C and VPS13D. The VPS13D gene variants are linked with embryonic lethality, higher IL-6 production and septic shock lethality. Mutations in VPS13A and VPS13B are associated with the onset of neurological and developmental disorders. Mutations in the VPS13A gene are causative for a specific autosomal recessive neurological disorder, Chorea-Acanthocytosis (ChAc). Most reported VPS13A mutations in ChAc patients result in low levels or absence of the protein. ChAc patients display gradual onset of hyperkinetic movements and cognitive abnormalities. In addition to neuronal loss, the presence of circulating spiky red blood cells (acanthocytes) implies that the function of VPS13A is not restricted to the brain but also to other tissues. In line with this, VPS13A mRNA is ubiquitously expressed in human tissues.

Despite their medical relevance, the molecular and cellular function of VPS13 proteins remains largely unknown. The limited knowledge is largely derived from studies about yeast Vps13. Vps13 is a peripheral membrane protein. It is involved in the transport of membrane bound proteins between the trans-Golgi network and prevacuolar compartment (PVC) and from endosome to vacuole. Vps13 is also required for prospore expansion, cytokinesis, mitochondrial integrity, membrane contacts and homotypic fusion. Last, the influential role of Vps13 in these processes is postulated to be dependent on the availability of phosphatidylinositides. It is currently unclear whether the four mammalian VPS13 proteins share similar functions with yeast Vps13. The cellular localization and function of VPS13A is largely unknown.

The VPS13A gene is located at chromosome 9q21 and encodes a high molecular weight protein of 3174 amino acids. In various model systems, loss of VPS13A is associated with diverse phenotypes, such as impaired autophagic degradation, defective protein homeostasis, delayed endocytic and phagocytic processing, actin polymerization defects and abnormal calcium homeostasis. These studies indicate that VPS13A plays a role in a multitude of cellular functions and its loss of function is associated with a wide range of cellular defects in eukaryotes. Here, to understand the versatile role of VPS13A at the molecular level, the subcellular localization, dynamics, binding partners and the role of the domains of VPS13A were determined in mammalian cells. Our results demonstrate that VPS13A is a protein with unique properties since it is associated to multiple organelles, its localization dynamically shifts upon altered levels of fatty acids and VPS13A influences lipid droplet homeostasis. These findings were further substantiated in a validated neurodegenerative Drosophila model for ChAc using a large scale scanning electron microscopy (nanotomy) approach enabling identification of affected cells in large cross sections of the central nervous system. We discuss how these findings can explain neurodegeneration in ChAc.
RESULTS

Human VPS13A is a peripheral membrane protein

To determine the subcellular localization of endogenous human VPS13A, we first separated the membrane and cytosolic fractions of HeLa cells by high-speed centrifugation. VPS13A was enriched in the pellet, which contained the transmembrane epidermal growth factor receptor (EGFR) and relatively little of α-tubulin, a cytosolic marker protein (Figure 1A). To further investigate the membrane association of VPS13A, a detergent based subcellular fractionation was performed in HEK293T cells [31]. Following digitonin treatment and centrifugation, more than 80% of VPS13A, remained in the fraction containing membrane associated proteins such as EGFR and the ER integral protein VAP-A, and little VPS13A was detected in the cytosolic non-membrane bound and GAPDH containing fraction (Figure 1B and B'). The type of membrane association of VPS13A was further investigated by assessing its dissociation from lipid bilayers after treatment with different chemical agents. Similar to ATP5A, a peripheral membrane associated protein of mitochondria, part of VPS13A was solubilized by alkaline and urea-containing solutions. In contrast, the integral membrane protein EGFR remained in the membrane pellet irrespective of the treatment (Figure 1C and D). Altogether, these analyses revealed that VPS13A is a peripheral membrane-associated protein.

To determine to which intracellular membranes endogenous VPS13A is associated to, we next performed subcellular fractionation experiment on a sucrose gradient. This experiment showed that VPS13A was predominantly detected in fractions containing VAP-A, Rab7 and ATP5A, which are marker proteins of the ER, endosomes and mitochondria respectively (Figure 1E and F).

VPS13A localization to mitochondria is mediated via the C-terminal end

To characterize the subcellular localization of VPS13A in more detail, GFP- and Myc-tagged VPS13A was expressed in HEK293T cells. This yielded a high molecular weight band, corresponding to full-length tagged VPS13A (Supplementary Figure 1A and B). Under normal growth conditions, VPS13A-GFP showed two main subcellular distribution patterns. In approximately 90% of the cells, VSP13A displayed a reticular pattern (Figure 2A). In the remaining cells, VPS13A localized to vesicular structures (Figure 2A'). To identify these compartments, we co-localized VPS13A with a variety of organelle marker proteins. Although not co-localizing with the endosomal and lysosomal marker proteins Rab5, Rab7, LAMPI and FYCO1 (Supplementary Figure 1C-F), VPS13A-GFP strongly decorated the circumference of nearly all mitochondria stained with mitotracker (Figure 2B and B', and Supplementary movie 1). The striking VPS13A localization to the mitochondrial surface prompted us to determine the VPS13A domain that mediates this localization. To do so, we expressed GFP-tagged truncated forms of VPS13A (Figure 2C and Supplementary Figure 2) in different cell lines. Whereas most of the constructs were cytosolic, the C-terminal region of VPS13A (aa 2615-3174) strongly localized to the mitochondria in the cell lines tested (Figure 2D and Supplementary Figure 3A-C). The localization of the VPS13A C-terminal domain to the surface of mitochondria was identical to the full-length VPS13A localization (Figure 2E). This strongly suggests that the C-terminal region of VPS13A is involved in targeting the protein to a close vicinity of the mitochondrial membrane.
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Figure 1. VPS13A is enriched in membrane fractions and is peripherally associated to membranes. 
(A) Light membrane fractions from HeLa cell homogenates were separated by centrifugation in a cytosolic and a membrane fraction. Equal amounts of proteins were processed for immunoblotting against VPS13A, EGFR and α-tubulin. (B) Digitonin extraction of cytosolic proteins in HEK293T cells. HEK293T cells were treated with digitonin on ice and the cell free fraction containing cytosolic proteins was collected after centrifugation. The remaining pellet containing all membranes was lysed in buffer containing NP-40. Extracted fractions were immunoblotted for the indicated proteins (left panel). The amount of protein was quantified using image J and presented as a percentage of the total (B’). (C) Membrane fractions were prepared as in A and subjected to different chemical agents to extract proteins from membranes. Equal amount of proteins were processed for immunoblotting using antibodies against VPS13A, EGFR and ATP5A. (D) The amount of protein was quantified as above and presented as a percentage of the total. (E) Sucrose gradient fractionation from HeLa cells. HeLa cells were lysed in detergent free buffer and separated in 5-55% sucrose gradients by high speed centrifugation. After TCA precipitation, fractions were processed for immunoblotting using antibodies against VPS13A, VAP-A, RAB7 and ATP5A. (F) Densitometric quantification of protein band intensities in E was performed using image J and plotted as percentage of the total. The plot of VPS13A is highlighted in green.
Figure 2. Vps13A is localized at mitochondria via its C-terminal.

(A) HEK293T cells were transfected with VPS13A-GFP and the GFP signal was visualized using confocal microscopic examination. White arrowheads show reticular structures (A) and pink arrowheads show vesicular structures (A'). Cell borders are marked by white dots,
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VPS13A localizes to the mitochondrial/ER interface

Furthermore, VPS13A also largely overlapped with the ER marker protein VAP-A (Figure 3A). However, not all ER membranes were positive for VPS13A-GFP (Figure 3B and C). To further investigate the co-localization of VPS13A with the membranes of these organelles, we conducted time-lapse imaging of live cells expressing VPS13A-GFP and stained with mitotracker or co-transfected cells with mCherry-VAP-A. This analysis showed that VPS13A-GFP was closely associated to the ER, and largely followed the ER dynamics (Figure 3D and Supplementary Movie 2). VPS13A positive regions of the ER co-localized with the mitochondrial marker (Figure 3C).

Given the complete decoration of mitochondria with VPS13A-GFP and its association to the ER, these results indicate that VPS13A was enriched at the interface between these two organelles.

VSP13A directly binds VAP-A through its FFAT motif

We then asked what mediated the VPS13A association to the ER. Several membrane-associated proteins bind to the ER resident VAP-A through a 7 amino acids FFAT motif. Interestingly, VPS13A also contains a putative FFAT motif, which is located between the amino acids 842-848 (Figure 4A). To test whether VPS13A indeed interact with VAP-A, we performed a co-immunoprecipitation experiment with endogenous proteins. In line with this hypothesis, VAP-A was enriched in immunoprecipitates of endogenous VPS13A (Figure 4B). Similarly, VPS13A was present in the VAP-A immunoprecipitates (Figure 4B').
To test whether VSP13A and VAP-A interact via the putative VSP13A FFAT motif, we conducted a set of in vitro pull-downs. We generated GST-tagged recombinant VPS13A fragments (Figure 4C) that were incubated with bacterially expressed 6x-His tagged VAP-A. We found that all the constructs containing the VPS13A FFAT motif were efficiently binding VAP-A (Figure 4D), including the FFAT motif itself (Figure 3. Vps13A is localized at ER-mitochondrial interface.

(A) Hek293T cells were co-transfected with VPS13A-Myc and GFP-VAP-A. Cells were stained with anti-myc (red) and DAPI (blue). (B) Representative single stack image of Hek293T cells expressing BFP-Sec61B and VPS13A-GFP. Mitochondria labeled using mitotracker Red. White arrowheads indicate the enrichment of VPS13A at the ER-mitochondria interface. Pink arrows indicate ER tubule without mitochondrial contact and not marked by VPS13A-GFP. Scale bar=2 µm. (C) Representative single stack image of Hek293T cells expressing mCherry-VAP-A and VPS13A-GFP Mitochondria labeled using anti-Tom20 antibody. White arrowheads indicate the enrichment of VPS13A at the ER-mitochondria interface. Scale bar=5 µm. (D) Representative time-lapse images of Hek293T cells expressing VPS13A-GFP and mCherry-VAP-A for 48 hours (Supplementary Movie 2). White arrowheads show continuous dynamic associations of VPS13A-GFP and mCherry VAP-A. Scale bar=10 µm, insets= 2 µm.
Figure 4. Direct interaction of VPS13A and VAP-A.
(A) Amino acid sequence alignment of VPS13A-FFAT and 4 other FFAT containing proteins. The FFAT containing region of each protein was selected and aligned using ClustalW multiple alignment tool. (B) Endogenous VPS13A was immunoprecipitated from
Importantly, the introduction of the D845A point mutation in this motif, which is known to affect VAP-A binding in other FFAT-containing proteins \(^{32,35}\), strongly reduced its association to VAP-A (Figure 4D, Lane 6). Similar results were obtained when GST-tagged recombinant VPS13A truncated proteins were incubated with HeLa cell lysates. Following GSH pull down, endogenous VAP-A from HeLa cells was found to be enriched together with GST-VPS13A variants in a FFAT-dependent manner (Supplementary Figure 4). We concluded that VPS13A interacts with VAP-A via a FFAT domain.

To investigate whether the FFAT motif is required for the localization of VPS13A to the ER, we generated a VPS13A FFAT-deletion mutant (VPS13A \(^{\Delta FFAT}\)) tagged with GFP. Analysis of confocal images showed that VPS13A\(^{\Delta FFAT}\) localized to mitochondria but no longer to the ER-mitochondria interface indicating that the FFAT domain is the main hub for ER targeting of VPS13A (Figure 4E). The FFAT domain was not sufficient for an \textit{in vivo} association with the ER because FFAT containing truncated VPS13A constructs remained cytosolic and did not co-localize to the ER (Figure 2D).

The assembly of membrane contact sites is regulated by cellular calcium levels \(^{36,37}\). Calcium levels are mainly regulated through the activity of sarcoendoplasmic reticulum calcium ATPase (SERCA), which can be pharmacologically inhibited with thapsigargin (TG), leading to an increase in cytosolic calcium. In order to understand the effect of cellular calcium on VPS13A-VAP-A interaction, we treated cells with different concentrations of TG. Following TG treatment, we found an increased amount of VPS13A bound to VAP-A (Figure 4F and G) and such increase was proportional to the concentration of TG applied. The calcium mediated VPS13A-VAP-A interaction suggests that VPS13A plays a role in ER-mitochondria contact sites.

In conclusion, our data support a model in which VPS13A can associate simultaneously with mitochondria and ER via its C-terminus and FFAT domain, respectively. The FFAT domain is required but not sufficient for an ER association, suggesting that a full-length mitochondrial associated VPS13A is required to establish the VPS13A-ER connection.

**VPS13A is associated with lipid droplets independent of the FFAT motif**

As shown in Figure 2A', VPS13A-GFP also brightly decorates vesicular structures in a small percentage of the cells, which were not mitochondria (Supplementary movie 1). We noticed that when this localization is observed, VPS13A-GFP is absent from the reticular ER-mitochondrial interface (Figure 2A'). Using confocal microscopy with lipid droplets (LDs) specific dyes, LipidTox red or Nile-red, we found that these vesicular structures were LDs (Figure 5A-B).
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To test this further, cells were cultured under conditions that elicit LD biogenesis. For instance, exogenous addition of oleic acid (OA), a fatty acid, known to induce intracellular LD formation. Cells expressing VPS13A-GFP were therefore visualized at different times after OA induction. Before the addition of OA, the majority of the VPS13A-GFP signal was present in the reticular pattern reflecting its distribution at the mitochondria-ER contact sites (Figure 5C, left panel). After 2h of exposure to OA, VPS13A-GFP was entirely re-localized to the FA-BODIPY-positive LDs. Line scan analysis of individual LDs revealed that VPS13A-GFP uniformly encircled LDs (Figure 5D), suggesting enrichment of VPS13A at the membrane and not at the interior of LDs. Live-cell imaging showed that LDs that were VSP13A-GFP negative gradually acquired VPS13A from the reticular pool (Figure 5C). This indicated that VPS13A is not always associated with newly formed LDs and it can be recruited to already formed LDs.

To corroborate these observations, we next investigated whether endogenous VSP13A was also recruited to LDs. We thus analyzed the subcellular distribution of endogenous VPS13A by sucrose gradient fractionation of cells grown under normal conditions, starved for serum or exposed to OA for 24 hours (Supplementary Figure 5A). Western blot analysis of sucrose gradient fractions revealed that VPS13A was mainly enriched in the heavier fractions under starvation and normal growth conditions, and only a small portion (~4%) appeared in the fraction corresponding to LDs that floated on top of the sucrose gradient, which was identified using Perilipin2 (PLIN2) as the specific LD marker protein. Part of PLIN2 was sequestered in the fractions with high density organelles that contained marker proteins such as VAP-A, EGFR and ATP-5A (Figure 6A, B and B', Supplementary Figure 5 C, D and D'), consistent with previous work showing that very minimal amount of LDs are formed under starvation conditions. Induction of LD formation after incubation of cells with OA for 24 hours leads to a shift in the distribution of endogenous VPS13A towards enrichment at the LD fraction. As expected, PLIN2 was enriched in the top fraction consistent with the fact that LDs are formed in response to OA induction (Figure 6C, D and D', Supplementary Figure 5E, F and F'). The distribution of the plasma membrane protein, EGFR and the lysosomal protein-LAMP1 was not affected upon OA induction or serum starvation (Figure 6A-D', Supplementary Figure 5 C-F'). In addition, comparison of the amount of VPS13A in the LD fraction showed that VPS13A was highly concentrated in the LD fractions of OA fed cells. Addition of 250 µm OA to starved
cells doubled the amount of VPS13A in the LDs fraction and 500 µm OA increased in the amount of LD associated VPS13A by about four fold (Figure 6E and F, Supplementary Figure 5B). Taken together, these data confirmed our observation that VPS13A is associated with lipid droplets and this association can be enhanced by oleate induced LDs formation.

We then questioned whether the ER localization through VAP-A binding was important for the LD localization of VPS13A. To do so, we expressed VPS13AΔFFAT in OA fed cells and showed that it was recruited to LDs similar to WT VPS13A (Figure 6G). This indicates that the FFAT motif of VPS13A is not required for its localization...
VPS13A is associated with multiple organelles recruitment to LDs.

In conclusion, our results showed that the subcellular localization of VPS13A-GFP is metabolically regulated. Surplus amount of cellular lipid leads to LDs biogenesis and directs VPS13A from mitochondria to LDs independently from its binding to ER-localized VAP-A.

**VPS13A negatively affects lipid droplet size and motility**

We further studied the role of VPS13A on LDs by investigating their motility and size under VPS13A overexpressing and downregulation conditions. We downregulated VPS13A in U2OS cells and analyzed LD number and dynamics. Under normal culturing conditions, VPS13A downregulated cells showed increased numbers of bigger LDs and a reduction in small (<5µm) LDs (Figure 7A and B). In addition, fluorescent activated cell sorting (FACS) quantification of the total Nile red intensity showed a significantly increased intensity in the absence of VPS13A (Figure 7C). The largest LDs in both mock and VPS13A siRNA treated cells were generally immobile. However, smaller and peripheral LDs moved faster in VPS13A depleted cells compared to the controls (Figure 7 D and D').

Live tracking of individual LDs showed that VPS13A-GFP positive LDs slowly and randomly oscillated for a longer time. Nonetheless, when these LDs were briefly dissociated from VPS13A-GFP, they directionally traveled fast and such mobility was interrupted when VPS13A-GFP was again associated with LDs (Figure 7E, Supplementary movie 3). Quantitatively, when the mean intensity of VPS13A-GFP at LDs was measured in each frame and correlated to the distance it traveled, there was an inversely proportional amount of...
Figure 6. Endogenous VPS13A is partially recruited to LDs.

(A) FCS starved HeLa cells were processed as described in Supplementary Figure 5A. Fractions with equal amounts of proteins were processed for Western blotting and detected by immunoblotting against antibodies for VPS13A, LAMP1, EGFR, PLIN2, VAP-A and ATP5A. (B) Densitometric quantification of protein band intensities in A was performed using Image J and plotted as percentage of the total. B’ shows a zoomed in values of the top 3 light sucrose density fractions. (C) FCS starved Hela cells were further incubated with 500 µM OA and processed as A. Fractions with equal amounts of proteins were processed for Western blotting and detected by immunoblotting against antibodies for VPS13A, LAMP1, EGFR, PLIN2, VAP-A and ATP5A. (D) Densitometric quantification of protein band intensities was performed as in B. D’ shows a zoomed in values of the top 3 lowest sucrose density fractions. (E) HeLa cells were either grown in complete medium (Ctr), FCS starved (as in A) or further incubated with 250µM or 500µM OA and processed as described in A. LDs were isolated from the top fraction. Equal amounts of Proteins were resolved by Western blotting and detected by immunoblotting against antibodies for VPS13A, LAMP1, EGFR, PLIN2, VAP-A, ATP5A and α-tubulin. (F) Densitometric quantification of proteins in LD fraction in E was performed using Image J and normalized to inputs of each condition. (G) Representative single stack image of HEK293T cells expressing and VPS13A-GFP or VPS13A-GFP ∆FFAT. Cells were incubated with 500µM OA for 3 hours. LDs stained with LipidTox red. Scale bar=10 µm
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Figure 7. VPS13A negatively regulates LD mobility.

(A) Confocal image of U2OS cells treated with either Mock or VPS13A siRNA for 72 hours. Cells were fixed and LDs were stained with LipidTox red. Nucleus stained with DAPI. Scale bar=10 µm. (B) Size distribution of LDs in A. Single stacks confocal images were captured at the middle of cells and LDs were categorized based on their sizes and plotted as percentage of the total. Student’s t-test, *p<0.05, ** p<0.01, *** P<0.001. (C) U2OS cells were treated with siRNA as in A. Cells were stained with Nile red and intensity was measured using FACS. Student’s t-test, *p<0.05, ** p<0.01, *** P<0.001. (D) U2OS cells were treated with siRNA as in A. Cells were incubated with Lipidtox red 20 minutes before imaging. Time lapse images were taken every second. Two consecutive images were superimposed to see the mobility of LDs. D’ Colocalization of LDs in consecutive frames was manually counted and normalized to the total number of LDs and plotted as percentage of overlap. (E) Representative montage of HEK293T cells expressing VPS13A-GFP. Cells were incubated with 500µM OA for 3 hours. LDs stained with LipidTox red. Time lapse images were taken every 600 milliseconds. Scale bar=10 µm. (F) The intensity of VPS13A signal on the LD was quantified in every frame and plotted against the speed of the LD.
VPS13A-GFP signal to the speed of LDs (Figure 7F).

In summary, the presence of VPS13A on LDs negatively influence their motility and when LDs temporarily escape from VPS13A, they showed faster directional mobility. In the absence of VPS13A, increased numbers and larger LDs are present, strongly indicating a role of VPS13A in LD homeostasis.

**Lipid droplets accumulate in glia cells of Drosophila Vps13 mutants**

Given the influential role of VPS13A on LDs in cell culture, we investigated whether the previously established *Drosophila vps13*−/− model for ChAc exhibits abnormal LD characteristics in brain tissue. *Drosophila* Vps13 is a structural and functional orthologue of human VPS13A. Homozygous Vps13 mutants express low levels of a small truncated Vps13 protein and serve as a model for ChAc. Similarly, in most of the reported ChAc cases, VPS13A protein cannot be detected in patient material. Vps13 mutant flies show a decreased life span, normal locomotor function at young age, reduced locomotor function and brain vacuoles upon ageing (17 days and older), indicative for neurodegeneration.

Visualization of lipid droplets using fluorescent markers in combination with light microscopic analysis in the *Drosophila* brain did not show a positive signal, most likely due to limited tissue penetration of the dyes. Therefore, we used a large scale electron microscopy approach enabling analysis of entire brain areas and to identify possible LDs and affected cells at the ultrastructural level (see materials and methods to access data set). As previously reported, in contrast to wild type brains, large vacuoles are apparent in neurodegenerative brains of Vps13 homozygous mutants (Figure 8 A and B). In addition, electron microscopic analysis showed an accumulation of LDs throughout the (17 day old) Vps13 mutant brain. The central complex (an area with a dense network of neurons and glia, involved in locomotor function) was selected for ultrastructural visualization and quantification of the lipid droplets. No LDs were observed in central complex of control brains (Figure 8 C). Only 1 LD was observed in an adjacent area (Figure 8E). >30 LDs were observed in the central complex of Vps13 mutants (N=2, complete central complexes were analyzed) (Figure 8D) in cells reminiscent of glia. LD were also observed in glia in other areas of the mutant brain (Figure 8 F-J). Glia are electron dense, non-neuronal cells, surrounding neuronal cells in the central nervous system, with a supportive and protecting function. The LDs varied in size and occasionally occupied the complete cytoplasm of the glia cells. LDs were absent in aged matched wild type brains (N=2) (for access to the full data set see materials and methods).

These data indicate that, comparable to mammalian cells, Vps13 could also play a role to regulate LD homeostasis in the *Drosophila* central nervous system.
Figure 8. LDs accumulate in glia of Drosophila ChAc model

(A-J) Large scale electron microscopic images. (A) 17 day old Drosophila wild type brain. Insert depicted in A is enlarged in C visualizing the central complex of wild type brain. 1 indicates the location of one lipid droplet visualized in E. (B) 17 day old Vps13 mutant brain. Insert depicted in B is enlarged in D visualizing the central complex of Vps13 brain. 2,4,5,6 indicate the location of lipid droplets visualized in F, H, I and J. (C) Central complex of the wild type brain (link and access codes are provided in materials and methods allowing the user to zoom). (D) Central complex of Vps13 mutant brain (link and access codes are provided in materials and methods allowing the user to zoom in). Insert depicted in D is visualized in G. Central complex area is indicated in red; Blue droplets indicate positions of LDs; blue triangles indicate LDs in higher magnifications; N=nucleus; M=mitochondria, red stars indicate electron dense cytoplasm characteristic for glia.
DISCUSSION

We show that VPS13A is at the interface between mitochondria and ER under normal culturing conditions. When cellular lipid content is increasing VPS13A dynamically shifts from its mitochondrial-ER interface to the membrane of LDs where it influences their mobility (Figure 9). Large LDs are also observed in glia cells of an aged Drosophila ChAc model suggesting altered LD homeostasis as a possible explanation for neurodegeneration in ChAc.

Our results are partly consistent with previous findings of other VPS13 family members, in addition, unique characteristics and functions of VPS13A are being revealed. The peripheral membrane characteristics of VPS13A are shared by human VPS13B and C, yeast VPS13p and Drosophila VPS13. In addition, we identified ER and mitochondrial membranes to be in close association with VPS13A and VPS13A directly binds to the ER protein VAP-A through the FFAT motif. VAP-A/B proteins have been extensively characterized as a hub when the ER establishes membrane contacts with other organelles including endosomes, mitochondria, peroxisomes, plasma membrane (PM) and Golgi. In combination with published data, our results suggest a function for VPS13A in tethering mitochondria and ER and thereby building membrane contacts sites facilitating exchange of molecules between these organelles under normal culturing conditions. The reported phenotypes of VPS13A depleted cells, impaired autophagic flux and mitochondrial clearance may result from abnormal mitochondria-ER contact sites. Interestingly, in contrast to VPS13A depleted cells, increased mitochondrial clearance is observed in VPS13C depleted cells.

Under conditions of increased LD formation, an additional organelle is targeted and VPS13A translocates from the mitochondrial-ER interface to the surface of LDs. Vps13p in yeast also relocates in response to a metabolic shift, although to different interfaces, from vacuole-mitochondria to vacuole-nucleus. The VPS13A specific association with ER, mitochondria and LDs reveals the presence of a directional pathway from the ER-mitochondrial interface to LDs.

LDs have long been considered as inert lipid inclusions and studies of their biology were constrained. Evidence is now accumulating that LDs are far from being only fat depots as they are decorated by a large number of proteins that regulate their formation, destruction and communication with other organelles. VPS13A influences the mobility of LDs a feature reminiscent of identified proteins regulating dynamics of endosomal vesicles. Endosomal movement is halted when endosomes make contacts with the ER and, movement of peroxisomes is increased upon loss of the VAP-ACBD5 tethering complex. Consistent with this, we show that VPS13A negatively influences LD motility. LDs are more steady when they are decorated by VPS13A and move faster in a directional manner when they briefly lose the VPS13A signal.

In addition to a role of VPS13A in mitochondria-ER contact sites, this reflects an additional possible function of VPS13A in mediating the formation of contacts between LDs and other organelles. We therefore predict that loss of VPS13A affects cellular processes that require mitochondria-ER contact sites and exchange of cargo between LDs and other organelles. So far, no other ER-mitochondria interface
proteins were identified that also influence the dynamics and thereby contact events of LDs with other organelles. Interestingly, we found that VPS13A depletion leads to increase number and size of LDs. A phenotype that is again opposite to VPS13C loss of function 65, demonstrating that the human VPS13 family members possess different functions, which is consistent with the fact that all 4 members are associated with unique diseases.

The question remains why loss of VPS13A leads to ChAc, a movement disorder, mostly presenting in the third decade of the patient’s life. LD abnormalities are associated with several neurodegenerative diseases such as Hereditary spastic paraplegias 66, Huntington’s disease 67, and Parkinson’s disease 68. In ageing Vps13 mutant flies, LDs are observed in glia at the age when locomotor function declines and brain vacuoles are present. The role of LD in the adult central nervous system is largely unknown, however, LD are transiently formed in Drosophila glia cells surrounding photoreceptors in young flies in response to oxidative stress 69. It is currently not clear whether in the absence of VPS13 LD formation is enhanced or degradation/turnover decreased. It may be possible that due to aging, oxidative stress builds up, LDs are being formed and accumulate because of a compromised turnover due to decreased contact sites with their target organelles. Gradually increasing numbers of large LDs in an aging organism may form physical obstructions and thereby hampering the cellular functions of glia and their neighboring neuronal cells. Glia cells contribute to increasing numbers of brain diseases 70. Glia cell functions have not been studied in ChAc models nor in ChAc patient derived material, leaving this field largely open for future research.

Our study reveals the dynamic localization of VPS13A and highlights a conserved importance of VPS13A to regulate LD dynamics and homeostasis in mammalian cells and the central nervous system. VPS13A’s movement from a mitochondrial residence to LDs upon metabolic changes reveals an inter-organelle directional pathway in which VPS13A plays a central role.
Figure 9. Proposed model for VPS13A function.

(A) Under normal growth conditions VPS13A is localized at the ER-mitochondria contact sites where it is anchored to VAP-A through its FFAT domain to facilitate cargo exchange between ER and mitochondria. (B) Inhibition of SERCA with thapsigargin increases cytosolic calcium and simultaneously increases VPS13A/VAP-A interaction thereby stabilizes ER-mitochondria contacts. (C) Upon increased cellular fatty acid intake, VPS13A translocates to LDs where it possibly regulates LD contact sites formation and stabilization thereby limiting LD mobility.
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Author contributions
O.S., W.Y., and S.I. conceived the project, designed the experiments and wrote the paper. M.Z., W.Y., F.P. and A.F. performed biochemical and cellular studies. W.Y. and R.G. performed the in vivo imaging. L.L. and N.G. performed the studies with Drosophila melanogaster. L. L. performed the electron microscopy analysis.

Competing interest statement
All authors declare no competing financial interest.

MATERIALS AND METHODS

Immunoblotting
Cells were homogenized by sonication in 2x laemmli buffer that contains urea (sigma) and DTT (Sigma) to a final concentration of 0.8M and 50mM respectively. After the homogenates were boiled at 99 °C for 5 minutes, the indicated proteins were resolved with polyacrylamide gel and transferred to PVDF membrane using the Trans-Blot Turbo System (Bio-Rad) or overnight wet transfer. Membranes blocked in 5% fat free milk for 1 hour at room temperature, rinsed in PBS-Tween 20. Incubations with primary antibodies were done overnight at 4 °C followed by incubations with secondary antibodies for 1.5 hours at room temperature. The following primary antibodies were used: anti-VPS13A (Sigma, 11000), anti-VPS13A (N-terminal) (Santacruz Biotechnology, 1500), anti-myc (11000), anti-GFP (Clontec 11000), anti VAP-A (Santacruz Biotechnology, 11000), anti-EGFR (Santacruz Biotechnology, 11000), anti-α tubulin (Sigma, 15000), anti-GAPDH (Fitzgerald 110,000), anti-LAMPI (abcam, 11000), anti ATPSA (abcam, 11000), anti-CST (Santacruz Biotechnology, 11000), anti Rab7 (abcam, 11000). Membrane was developed using ECL reagent (Thermoscientific) and signal was imaged using the ChemiDoc imager (Biorad), images exported as Tiff files and densitometric analysis of band intensities was performed using image J software.

Cell culture and transfection
Hela, U2OS and Hek293T cells were cultured in DMEM (Gibco or Sigma) containing 10 % FCS and Penicilin/streptomycin (Gibco) in 5 % CO2 at 37 OC. Plasmid transfections of Hela and U2OS cells were done using polyethylenimine (PEI) transfection reagent. Cells were analysed after 24 or 48 hours of transfection. Medium was refreshed 24 hours post transfection. For procedures that require overexpression of full length VPS13A-GFP or VPS13A-Myc, HEK293T cells were transfected using Calcium Phosphate precipitation method. Cells were analysed 24 or 48 hours after transfection. Medium was refreshed 24 hours after transfection.
**SiRNA**

SiRNA transfection was performed using lipofectamine 2000 (Invitrogen) according to manufacturer’s direction. SiRNA transfected cells were analysed 72 hours after transfection. VPS13A SmartPool SiRNA was purchased from Dharmacon. Non targeting SirNA pool was used as a control throughout.

**Immunoprecipitation**

Hela cells were washed once with ice cold PBS and scrapped in ice cold PBS. After removal of PBS following brief centrifugation, cells were resuspended in immunoprecipitation buffer (50mM Tris HCl, 150mM NaCl, 1mM EDTA, 1.5mM MgCl2, 10mM KCl, 1% Triton x-100, PH 7.6) supplemented with protease inhibitor cocktail (Roche). Cells were snap frozen in liquid nitrogen and passed through 26 gauge needle. The homogenate was spun down at 10000 xg for 10 mins, the supernatant was recovered and subjected to overnight immunoprecipitation using indicated antibodies or control IgG of the same host. Immunoprecipitates were enriched on agarose beads (santacruz) at 4°C for 1.5 hrs. Agarose beads were gently washed with buffer and resuspended in 2x laemmli buffer containing DTT and urea and processed for immunoblotting as described above. Co-immunoprecipitation using GFP-Trap beads (Chromo Tek) was done according to manufactures directions.

**Digitonin extraction**

Digitonin extraction of cytosolic proteins was performed according to 31. Briefly, HEK293T cells were cultured in 5cm dishes. When about 70% confluent, cells were collected by trypsinization, washed with ice cold PBS and resuspended in 5ml of digitonin buffer (150mM Nacl, 50mM HEPES PH=7.4, 25ug/ml digitonin, protease inhibitor coctail). After rolling the suspension for 10 minutes at 4°C, the tube was centrifuged at 2000xG for 5 minutes. The supernatant was collected as cytosolic fraction. The pellet was washed once with cold PBS and resuspended in 5 ml of NP-40 buffer (150mM NaCl, 50mM HEPES PH=7.4, 1% NP-40, protease inhibitor coctail). After rolling the suspension for 30 minutes at 4 degrees, the tube was centrifuged at 7000xG for 5 minutes. The supernatant was collected as membrane fraction. Both the cytosolic and membrane fractions underwent TCA precipitation and equal amounts of proteins were processed for immunoblotting as described above.

**Immunofluorescence**

Fixed cells

Cells grown on poly L-lysine coated coverglasses were transfected with the respective SiRNA. 72hours after SiRNA, cells were fixed in 4% paraformaldehyde. For LipidTox staining, cells were quenched for 10 minutes in 50mM NH4Cl in PBS and permeablized for 5 minutess with 0.1% Triton x-100 in PBS followed by incubation with LipidTox dye(1:200). Cells were mounted using citifluor mounting medium (Agar Scientific) and imaged immediately. Confocal images of cells were collected either using Leica sp8 confocal laser scanning microscope fitted with 63x oil immersion objective or DeltaVison confocal microscope (applied precision) fitted with 60x or 100x oil immersion objective. Images from the Delta vision were deconvolved by the SoftwoRx software (Applied Precision). Mitotracker (Invitrogen) was added for 20 minutes in serum.
free medium at a concentration of 100nM (for live) and 200nM (for fixed) cells.

Live cell
For Live imaging procedures, cells were seeded in 35mm glass bottom dishes coated with poly-d-lysine (Mat Tek). Live cell recordings were made using a DeltaVision confocal microscope. Prior to imaging, the cage was allowed to reach 37°C and cells were supplemented with 5% CO₂ throughout the entire recording. Images were deconvolved by the softworx software and stored as movies.

Ultrastructural analysis
Fixation, embedding of Drosophila brain tissue and processing for ultrastructural analysis of large images was performed as previously described.²¹

For full access of the wildtype brain
Copy and paste the following link in a Firefox browser
username: Fly
password: butter
fill in pixel size 1 upon request

For full access of the Vps13 mutant brain
Copy and paste the following link in a Firefox browser
username: Fly
password: butter
fill in pixel size 1 upon request

In vitro Protein-protein interaction
GST-tagged protein coding plasmids were transformed in E.coli BL21 and bacteria was grown overnight. Protein expression was induced using IPTG (final concentration of 1mM). Cells were pelleted by centrifugation and broken down by sonication in lysis buffer (50mM Tris HCl, pH 7.5, 150mM NaCl, 5% glycerol, 0.1% Triton x-100 and 1mM PSMF) supplemented with protease inhibitor cocktail (Roche). Debris was removed by centrifugation and the clean supernatant was mixed with glutathione beads. For protein-protein interaction assays, a bacterial lysate that contains His-VAP-A or HeLa cell lysate was added to the GST-VPS13A enriched beads and incubated at 4°C.

Cytosol and Membrane fractionation
Around 4-5, 90% confluent, T75 flasks of HeLa cells were resuspended in 1ml homogenization buffer HB (50mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, Protease inhibitor). The cell suspension was lysate through 2 freeze-thaw cycles and 20 strokes using a 27g needle. The nuclei and intact cells were pelleted by centrifugation 5 min at 800 g, and the resulting postnuclear supernatant (PNS) was applied to ultracentrifugation at 100,000 g, 4 C, for 1 h using a TLA 100.3 rotor to generate the cytosol (C) and the membrane fraction (M). Membrane fraction was washed in 1ml of HB and centrifuged 1h at 100000g. Laemmli sample buffer was added to the cytosol and membrane fractions, samples were quantified and
20µg of proteins of each sample were loaded on SDS-gel and processed for Western blot analysis.

Membrane extraction
The membrane fractions were resuspended in HB (control), 1 M KCl, 0.2 M sodium carbonate (pH 11), or 6 M urea for 45 min shaking on ice, and then centrifuged at 4°C, 100,000 g for 1 h obtaining soluble (supernatant) and insoluble (pellet) fractions. Laemmli sample buffer was added to the insoluble and soluble fractions, samples were quantified and 20µg of proteins of each sample were loaded on SDS-gel and processed for Western blot analysis.

Subcellular fractionation
For subcellular fractionation around S-6, 90% confluent, T75 flasks of HeLa cells were resuspended in 1 ml of homogenization buffer HB (50mM Tris HCl pH 7.5, 150mM NaCl, 1mM EDTA, Protease inhibitor, 0.25 M sucrose). The cell suspension was homogenized as previously described to obtain PNS. The PNS was then loaded onto a 10 ml continuous sucrose gradient containing 5%-55% (w/v) in HB, and the gradient was spun at 4°C at 274 000 g for 4 h using a swinging bucket SW41 rotor in a Sorvall Discovery 90se. Gradient fractions of 0.5 ml were collected from top to bottom. The proteins in each fraction were concentrated using TCA precipitation and resuspended in 75-100 µl of sample buffer. All the procedures were performed on ice. Equal volume of each fraction was loaded on SDS-gel and processed for Western blot analysis.

LD fractionation
HeLa cells were collected by trypsinization and washed once with PBS. After the PBS was removed following brief centrifugation, cell pellets were resuspended in detergent free homogenizing buffer (50mM Tris Hcl, 150mM NaCl, 1mM EDTA, 1.5mM MgCl2, 10mM KCl, PH 7.6) supplemented with protease inhibitor cocktail. Cells were snap frozen in liquid nitrogen and passed through 26 gauge needle. Nucleus and unbroken cells were removed by spinning down at 1600 xg for 5 mins. The supernatant was recovered and mixed with equal volumes of 0.25M sucrose in homogenizing buffer. After saving an input, the sample was loaded on top of a discontinuous sucrose gradient prepared by layering 1 ml of 30%, 20%, 10% and 5% sucrose in SW55 ultracentrifuge tube. The gradient was centrifuged for 3 hours at 40000 RPM using an ultracentrifuge (Beckman coulter). Tubes were carefully removed and 8 fractions of 600 ul were collected from top to bottom. 600ul of the top fraction containing LDs was collected using a 20ul pipette with a tip cut off. The refractive index of each fraction was measured and correlated to the linearity of the sucrose concentration throughout the tube. The bottom part containing the pellet was resuspended with buffer to a final volume of 600ul and was neither included in the refractive index measurement nor in the quantification of protein distribution among gradients. Proteins from each fraction were precipitated using the trichloroacetic acid precipitation method. Equal amounts of proteins were processed for immunoblotting as described above. The amount of protein in each fraction was calculated as a ratio of the densitometric signal in each fraction to the sum of the total protein in fractions 1-8 (Protein per fraction = densitometric signal of a fraction/ sum of total densitometric signal (1-8) x 100%).
Plasmids and constructs

Human VPS13A-GFP and VPS13A-Myc plasmids were kind gifts from A. Velayos-Baeza. To generate GFP-VPS13A (2-854), GFP-VPS13A (835-1700), GFP-VPS13A (855-1700), GFP-VPS13A (2003-2606) and GFP-VPS13A (2615-3174), the fragments were amplified by PCR from the full length VPS13A and inserted in to pEGFP-C1 (Clontech) via BamHI and XhoI restriction sites. To generate GST-VPS13A (4-113), GST-VPS13A (114-855), GST-VPS13A (2-835), GST-VPS13A (2-854), GST-VPS13A (835-1600), GST-VPS13A (855-1600), GST-VPS13A (2003-2606) and GST-VPS13A (2615-3174), the fragments were amplified by PCR from the full length VPS13A and inserted into PGEX5x2 (GE Healthcare) with Sall and NotI restriction sites. To produce GST-VPS13A (2-854/ D845A), a mutagenesis was performed on GST-VPS13A (2-854), with the QuickChange Site Directed mutagenesis kit (Agilent) according to the protocol. To construct CST FFAT, oligonucleotides encoding the FFAT domain in human VPS13A (AA 842-848) and flanked with Sall and 3’ NotI were synthesized, annealed and inserted into pGex5x2 via Sall and NotI. To construct His-VAP-A, Human VAP-A was amplified with PCR from HEK293 cDNA and inserted into pET28a (EMD Biosciences) with NdeI and BamHI restriction sites. To construct GFP-VAP-A, VAP-A was amplified with PCR from His-VAP-A and inserted into pEGFP-C1 (Clontech) with EcoRI and BamHI. To construct mCherry-VAP-A, tubulin in pcDNA3.1 mCherry tubulin (kind gift from B. Giepmans) was replaced by VAP-A by using PCR and subcloned with BspEI and XhoI. BFP-Sec61B (Addgene plasmid #49154 ) and mCherry-Sec61B (Addgene plasmid #49155) were kind gifts from Gia Voeltz. GFP-Rab5 Q79L (Addgene plasmid #28046) and GFP-Rab7 Q67L (Addgene plasmid #28049) were kind gifts from Qing Zhong. Lamp1-mGFP (Addgene plasmid # 34831) was a kind gift from Esteban Dell’Angelica. mCherry Fyco1 was a kind gift from Harald Stenmark.
REFERENCES

34. Murphy, S. E. & Levine, T. P. VAP, a Versatile Access Point for


60. Bi, J. et al. Seipin Promotes Adipose Tissue Fat Storage through the ER Ca2+-ATPase SERCA. Cell Metab. 19, 861–871 (2014).


CHAPTER 6


**SUPPLEMENTARY FIGURES**

**A** 48 hours after transfection with either VPS13A-myc or VPS13A-GFP, HEK293T cells were processed for immunoblotting using antibodies against VPS13A, α-tubulin, Myc and GFP. GFP transfected or non-transfected (NT) cells were used as controls. Note the enrichment of VPS13A in both VPS13A-myc or VPS13A-GFP lanes. (B) Densitometric quantification of protein bands in A. The ratio of VPS13A to α-tubulin was normalized to NT cells. (C) HEK293T cells were co-transfected with VPS13A-Myc and GFP-Rab5 Q79L. Cells were stained with anti-myc (red) and DAPI (blue). (D) HEK293T cells were co-transfected with VPS13A-Myc and GFP-Rab7 Q67L. Cells were stained with anti-myc (red) and DAPI (blue). (E) HEK293T cells were co-transfected with VPS13A-Myc and LAMP1-GFP. Cells were stained with anti-myc (red) and DAPI (blue). (F) HEK293T cells were co-transfected with VPS13A-Myc and mCherry FYCO1. Cells were stained with anti-myc (green) and DAPI (blue).

**Supplementary Figure 1. VPS13A does not colocalize with endocytic compartment.**
Supplementary Figure 2. VPS13A truncated constructs.
CGBP-VPS13A constructs represented in Figure 2C and used in figure 2D, 2E and Supplementary figure 3 were overexpressed in Hek293T cells for 24 hours. Western blot samples were resolved on SDS PAGE and probed for an anti-GFP antibody. GFP alone was used as a control. The stain free scan of the gel is shown as a loading control.
Supplementary Figure 3. The C-terminal VPS13A localizes to mitochondria in different cell lines. 

(A) GFP-VPS13A (2003-2606) and GFP-VPS13A (2615-3174) constructs were Co-expressed with mCherry Sec61B in HEK293T. Scale bar=25 µm. 
(B) GFP-VPS13A (2003-2606) and GFP-VPS13A (2615-3174) constructs were Co-expressed with mCherry Sec61B in HeLa cells. Scale bar=25 µm. 
(C) GFP-VPS13A (2003-2606) and GFP-VPS13A (2615-3174) constructs were Co-expressed with mCherry Sec61B in U2OS. Scale bar=25 µm.
Supplementary Figure 4. VPS13A interacts with VAP-A.
GST-fusion proteins of VPS13A fragments expressed in E. Coli were enriched on Sepharose beads and incubated with equal amounts of HeLa cell lysate. GST alone used as a control. Samples were immunoblotted against VAP-A, GST and N-terminal VPS13A (an antibody that is directed against amino acids 73-174 of VPS13A).
Supplementary Figure 5. Endogenous VPS13A is recruited to LDs upon OA induction.

(A) Workflow of LD isolation and sucrose gradient fractionation. FCS starved HeLa cells and HeLa cells further incubated for 24 hours with 500μM OA in FCS free medium were lysed and fractionated in 5-30% sucrose density gradients. Proteins in fractions were concentrated by TCA precipitation and subsequently separated by SDS-PAGE. (B) The ratio of VPS13A in the LD fraction of Figure 6A and 6C. The amount of VPS13A before OA (Stv) is set to 1. (C) HeLa cells grown in complete medium were fractionated on sucrose gradient and processed as described in Supplementary Figure 5A. Equal amounts of proteins were resolved by western blotting and detected by immunoblotting against antibodies for VPS13A, LAMP1, EGFR, PLIN2, VAP-A and ATP5A. (D) Densitometric quantification of protein band intensities in B was performed using image J and plotted as percentage of the total. D’ shows a zoomed in values of the top 3 fractions. (E) FCS starved HeLa cells were further incubated with 250μM OA and processed as described in B. Equal amounts of proteins were resolved by western blotting and detected by immunoblotting against antibodies for VPS13A, LAMP1, EGFR, PLIN2, VAP-A and ATP5A. (F) Densitometric quantification of protein band intensities in E was performed using image J and plotted as percentage of the total. F’ shows a zoomed in values of the top 3 fractions.
Supplementary Figure 6. Original blots
VPS13A is associated with multiple organelles.
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