

ORP-3 rescues ER membrane expansions caused by the VAPB-P56S
mutation in familial ALS

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ABSTRACT

A mutation in ER membrane protein VAPB is responsible for causing a familial form of ALS (ALS8). The VAPB-P56S mutation causes protein aggregation and a nuclear envelope defect, where retrograde transport is disrupted. Over-expression of a FFAT peptide from OSBP1 reduces the size of VAPB-P56S aggregates and restores retrograde transport. A screen was performed on FFAT-motif containing ORPs to determine if any could rescue the mutant phenotype. ORP3 successfully reduced aggregate size and restored transport to the nuclear envelope. ER membrane protein Sac1, a PI4P phosphatase cycles between the ER and Golgi and becomes trapped in expanded ERGIC compartments with VAPB-P56S. Loss of Sac1 in the ER leads to an increase in intracellular PI4P. ORP3 may increase Sac1 phosphatase activity by acting as a lipid sensor. We propose that VAPB, Sac1 and ORP3 are interacting partners that together modulate levels of PI4P. Disruptions in the gradient of PI4P may result in the vesicle trafficking defects observed in VAPB-P56S cells.

Table of Contents

ABSTRACT	I
LIST OF TABLES	IV
LIST OF FIGURES	V
LIST OF ABBREVIATIONS	VI
ACKNOWLEDGEMENTS	VIII
INTRODUCTION	1
AMYOTROPHIC LATERAL SCLEROSIS IS A LATE-ONSET NEURODEGENERATIVE DISEASE	1
VAPB-P56S MUTATION (ALS8) CAUSES FAMILIAL ALS	2
CHARACTERISTICS OF ALS8.....	2
THE VAP PROTEINS.....	3
STRUCTURE OF THE VAPB DOMAINS	7
VAPB EXPRESSION AND LOCALIZATION	7
VAPB MRNA EXPRESSION IS LOW IN SALS CASES.....	9
CONSEQUENCES OF THE VAPB-P56S MUTATION	9
<i>Conformational change in the MSP domain exposes hydrophobic residues and causes aggregation.....</i>	9
VAPB-P56S CAUSES A NUCLEAR ENVELOPE DEFECT	11
THE FFAT MOTIF FROM OSBP RESCUES THE VAPB-P56S PHENOTYPE.....	11
POSSIBLE FUNCTIONS OF VAPB.....	12
TRAFFICKING BETWEEN ER-ERGIC	12
VAPB ASSOCIATES WITH MICROTUBULES	13
VAPB INVOLVEMENT IN LIPID METABOLISM	14
<i>ER is the site for Lipid synthesis</i>	14
VAPB IS INVOLVED IN PHOSPHOLIPID METABOLISM	14
VAPB INTERACTS WITH LIPID TRANSFER PROTEINS	14
THE OSBP/ORP FAMILY OF PROTEINS	16
FUNCTION OF OSBP	16
STRUCTURE OF ORPs	16
ORPs REGULATE PHOSPHATIDYLINOSITOL LEVELS IN CELLS	17
TABLE 2. FAMILY OF ORPs	19
HYPOTHESIS AND AIMS	20
METHODS AND MATERIALS	21
DNA PLASMID CONSTRUCTS	21
HELA CELL CULTURE	22
TRANSIENT DNA TRANSFECTION	22
PRIMARY ANTIBODIES.....	22
SECONDARY ANTIBODIES.....	22
IMMUNOCYTOCHEMISTRY	23
STATISTICAL ANALYSIS.....	23
EXPERIMENTS	23
SCREENING OF ENDOGENOUS ORPs FOR EFFICACY IN REDUCING VAPB-P56S PHENOTYPE	23
SOLUBILITY OF VAPB-P56S IN TRITON X-100 IN CELLS TRANSFECTED WITH FFAT MOTIF OR OSBP, ORP2, ORP3, ORP5, ORP6, ORP9, ORP10 OR ORP11	24
ORP3 RESCUES TRANSPORT OF EMERIN TO THE NUCLEAR ENVELOPE	26

ACTIVATION OF ORP3 WITH 25-HYDROXYCHOLESTEROL.....	26
CO-LOCALIZATION OF VAPB AND SAC1	26
VAPB KNOCKDOWN AFFECTS LOCALIZATION OF SAC1	27
EXPRESSION OF ORP3 IN HeLA CELLS	27
DETERMINATION OF KNOCKDOWN EFFICIENCY FOR SAC1	27
LEVELS OF PI4P IN HeLA CELLS	28
<i>ORP3 can partially rescue Emerin defect in VAPB knockdown cells</i>	28
RESULTS.....	30
ORP3 REDUCES SIZE AND NUMBER OF MEMBRANE EXPANSIONS IN HeLA CELLS	30
ORP3 SHIFTS VAPB-P56S FROM THE TRITON X-100 INSOLUBLE TO SOLUBLE FRACTION	34
EXPRESSION OF ORP3 RESTORES RETROGRADE TRANSPORT TO NUCLEAR ENVELOPE (EMERIN)	37
ACTIVATION OF ORP3 WITH 25-HYDROXYCHOLESTEROL FURTHER REDUCES SIZE OF MEMBRANE EXPANSIONS.....	40
TABLE 3. SIZE OF ER MEMBRANE EXPANSIONS IN CELLS TREATED WITH 25-HYDROXYCHOLESTEROL FOR 4 HOURS.	41
CO-LOCALIZATION OF VAPB AND SAC1	45
VAPB KNOCKDOWN CAUSES MIS-LOCALIZATION OF SAC1	48
ORP3 EXPRESSION IN HeLA CELLS	49
EFFICIENCY OF SAC1 KNOCKDOWN	50
PI4P LEVELS ARE INCREASED IN MAMMALIAN VAPB AND SAC1 KNOCK-DOWN CELLS	51
OVEREXPRESSION OF ORP3 PARTIALLY RESCUES EMERIN TRANSPORT DEFECTS IN VAPB KNOCKDOWN CELLS.....	55
DISCUSSION.....	57
ORP3 REDUCES SIZE AND NUMBER OF ER MEMBRANE EXPANSIONS IN VAPB-P56S CELLS	57
ORP3 AND THE FFAT MOTIF INCREASE SOLUBILITY OF THE VAPB-P56S PROTEIN	58
ORP3 RESTORES TRANSPORT OF EMERIN TO THE NUCLEAR ENVELOPE.....	59
ACTIVATING ORP3 WITH 25-HYDROXYCHOLESTEROL FURTHER REDUCES SIZE OF MEMBRANE EXPANSIONS IN VAPB-P56S CELLS	60
VAPB AND SAC1 CO-LOCALIZE IN MAMMALIAN CELLS.....	62
PI4P LEVELS ARE INCREASED IN CELLS LACKING VAPB AND SAC1.....	64
OVEREXPRESSION OF ORP3 PARTIALLY RESCUES THE EMERIN TRANSPORT DEFECT IN VAPB KNOCKDOWN CELLS	66
DYSLIPIDEMIA IN ALS	67
VAPB-P56S ERGIC PI4P MODEL	68
REFERENCES	75

List of Tables

Table 1. Genes currently associated with ALS and other motor neuron diseases....	2
Table 2. Family of ORP proteins.....	19
Table 3. Size of ER membrane expansions in cells treated with 25- hydroxycholesterol for 4 hours.....	41

LIST OF FIGURES

FIGURE 1. Overview of the secretory pathway.....	4
FIGURE 2. VAPB co-localizes with ERGIC marker.....	5
FIGURE 3. VAPB Structure.....	6
FIGURE 4. PyMOL Structure of human MSP domain.....	8
FIGURE 5. Structure of the Oxysterol Binding Protein family.....	18
FIGURE 6. Endogenous ORP screen.....	31
FIGURE 7. Co-expression of ORP3 reduces size of ER membrane expansions in VAPB-P56S cells.....	32
FIGURE 8. Co-expression of ORP3 reduces number of ER membrane expansions in VAPB-P56S cells.....	33
FIGURE 9. Expression of ORP3 increases solubility of VAPB-P56S.....	35
FIGURE 10. Solubility of VAPB-P56S protein when co-expressed with FFAT motif or one of the eight ORPs.....	36
FIGURE 11. Expression of ORP3 rescues VAPB-P56S-induced disruption of nuclear envelope trafficking.....	38
FIGURE 12. Expression of ORP3 restores Emerin at the nuclear envelope.....	39
FIGURE 13. 25-hydroxycholesterol treatment significantly reduces aggregate size in VAPB-P56S cells.....	43
FIGURE 14. Number of aggregates per cell after treatment with 25-OH.....	44
FIGURE 15. Sac1 co-localizes with VAPB-WT and VAPB-P56S.....	46
FIGURE 16. Sac1 colocalization quantification.....	47
FIGURE 17. Knockdown of VAPB disrupts Sac1 localization in the ER.....	48
FIGURE 18. Expression of ORP3 in HeLa cells.....	49
FIGURE 19. Efficiency of Sac1 knockdown in HeLa.....	50
FIGURE 20. Intensity of endogenous PI4P in HeLa cells.....	52
FIGURE 21. PI4P intensity quantification.....	53
FIGURE 22. SAC1-WT-GFP co-localizes with ERGIC-53.....	54
FIGURE 23. ORP3 overexpression partially rescues Emerin transport defect in VAPB knockdown cells.....	56
FIGURE 24. Structure of Sac1 phosphatase.....	63
FIGURE 25. Model in VAPB-WT cells.....	71
FIGURE 26. Model in VAPB-P56S cells.....	72
FIGURE 27. Model in VAPB knockdown cells.....	73
FIGURE 28. Model in VAPB-P56S cells overexpressing ORP3.....	74

LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
BSA	Bovine Serum Albumin
CCD	Coiled-Coil Domain
cDNA	Complimentary Deoxyribonucleic Acid
CERT	Ceramide Transfer Protein
COP	Cytosolic Coat Protein
DHE	Dehydroergosterol
dVAP	<i>Drosophila</i> VAP
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ERG30	Endoplasmic Reticulum and Golgi 30 kDa protein
ERGIC	ER-Golgi Intermediate Compartment
fALS	Familial ALS
FBS	Fetal Bovine Serum
FFAT	Two phenylalanines in an acidic tract
FTD	Frontotemporal Dementia
FTDP	Frontotemporal Dementia with Parkinson's disease
GFP	Green Fluorescent Protein
HeLa	Henrietta Lacks
iPSC	Induced Pluripotent Stem Cells
INO1	Inositol-1-Phosphate synthase
MND	Motor Neuron Disease
MSP	Major Sperm protein
NE	Nuclear Envelope
Nups	Nucleoporins
OBD	Oxysterol Binding Domain
ORPs	Oxysterol Binding Protein-Related Protein
OSBP	Oxysterol Binding Protein
PH	Pleckstrin Homology domain
PI	Phosphatidylinositol
PI4KII α	PI4 kinase II alpha
PI4P	Phosphatidylinositol-4-Phosphate
PM	Plasma Membrane
Sac1	Suppressor of Actin 1
sALS	Sporadic ALS
SCS2	Suppressor or Choline Sensitivity 2
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering RNA
SREBP	Sterol Regulatory Element Binding Protein

TMD	Transmembrane domain
VAMP	Vesicle-Associated-Membrane-Protein
VAP 33	VAP of 33 kDa
VAPB	Vesicle-Associated-Membrane-Protein-associated protein B
VPR-1	VAP orthologue in <i>c. elegans</i>
25-OH	25-Hydroxycholesterol

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INTRODUCTION

Amyotrophic Lateral Sclerosis is a late-onset neurodegenerative disease

Amyotrophic lateral sclerosis (ALS) was first described by the French neurobiologist Jean-Martin Charcot between 1865-1869. Charcot and colleague Joffroy discovered lesions in the lateral column of the spinal cord in patients with a late-onset progressive muscle paralysis, and subsequently named the disease “Amyotrophic lateral sclerosis” literally translating into “no muscle nourishment and hardening in the lateral corticospinal tract” (Kumar et al., 2011).

ALS is a progressive neurodegenerative disease affecting motor neurons in the brainstem and spinal cord. The course of the disease typically ranges from 2-5 years from symptom onset to respiratory failure. As motor neurons die, individuals lose their ability to speak (dysarthria), swallow (dysphagia), and move voluntary muscles. Cognition is not affected in the majority of cases, so patients are fully aware of their deterioration as they become locked inside their body. ALS commonly affects those in their 4th-6th decade of life, and is more prevalent in males than in females (Boillee et al., 2000). The incidence of ALS is approximately 2.4 per 100,000 people in Canada (Wolfson et al., 2009), which translates into two or three Canadians losing their lives to ALS every day (MND Association).

The majority of all ALS cases are sporadic (sALS) in nature with an unknown etiology, while 5-10 percent of cases are inherited in a dominant manner (familial ALS or fALS). Sporadic and familial ALS are similar in presentation, including symptoms such as muscle weakness, spasticity, and fasciculations (Boillee et al., 2000). Currently over 10 genes have been identified as causes of fALS, or contributors to sALS (Table 1).

Disease	Gene	Onset	Heredity
ALS1	SOD1	Adult	Dominant
ALS2	Alsin	Juvenile	Recessive
ALS3	Unknown	Adult	Dominant
ALS4	Sentaxin	Juvenile	Dominant
ALS5	Unknown	Juvenile	Recessive
ALS6	Unknown	Adult	Dominant
ALS7	Unknown	Adult	Dominant
ALS8	VAPB	Adult	Dominant
ALS-FTD	C9ORF repeat	Adult	Dominant
ALS-FTDP	MAPT (Tau)	Adult	Dominant

Table 1. Genes currently associated with ALS and other motor neuron diseases. Frontotemporal dementia (FTD), Frontotemporal dementia with Parkinson's disease (FTDP).

VAPB-P56S mutation (ALS8) causes familial ALS

In 2004, Nishimura et al. discovered a proline to serine missense mutation in the Vesicle Associated Membrane Protein (VAMP)/synaptobrevin-Associated Protein-B (VAPB) was the cause of familial ALS in a large Brazilian family of Portuguese decent (Nishimura et al., 2004a). The group mapped a new locus responsible for producing an atypical form of ALS (ALS8) to 20q13.3 (Nishimura et al., 2004b). This dominant missense mutation has been described in eight Brazilian families affecting over 200 individuals (Nishimura et al., 2004a), an unrelated German family (Funke et al. 2010), and subsequently another mutation in VAPB (T46I) has been identified in the United Kingdom (Papiani et al., 2012).

Characteristics of ALS8

ALS8 is a heterologous disease with age at onset varying from 25-55 years of age. Both sexes are equally affected with no clinical anticipation reported. Onset symptoms include “muscular cramps, fasciculations, progressive limb and trunk

weakness with decreased or absent deep-tendon reflexes” (Nishimura et al., 2004a). Three ALS phenotypes are observed in these families: Typical ALS, atypical ALS, and late-onset spinal muscular atrophy. Individuals with atypical ALS have a disease progression ranging from 2-60 years and experience essential tremor, which is not observed in typical ALS (Nishimura et al., 2004a).

The VAP proteins

There are two VAP genes in humans, designated VAPA and VAPB. The VAP proteins are type II membrane proteins that localize to the ER and ER Golgi intermediate compartment (ERGIC)(Figure 1 and 2) (Soussan et al., 1999). The VAP protein was initially identified in *Aplysia californica* as VAP-33 and was found to interact with vesicle associated membrane protein (VAMP), SNAP-25 and syntaxin on the plasma membrane (Skehel et al., 1995). VAP-33 is evolutionarily conserved with homologues found in *Saccharomyces cerevisiae* (SCS2), *Caenorhabditis elegans* (vpr-1), *Arabidopsis thaliana*, *Drosophila melanogaster* (DVAP) and humans (Soussan et al., 1999). In humans there are three isoforms of the VAP protein: VAPA, VAPB, and VAPC, the latter of which is a splice variant of VAPB (Nishimura et al., 2004a). Both VAPA and VAPB are ubiquitously expressed (Nishimura et al., 1999) and share 76% identical amino acid residues, with an N-terminal Major Sperm Protein domain (MSP) in the first 120 amino acids. This is followed by a predicted amphipathic coiled-coil structure and a 20 amino acid long C-terminal transmembrane domain, which anchors into the ER membrane (Gkogkas et al., 2008)(Figure 3). Both VAPA and VAPB contain an MSP domain, a coiled-coil region and a transmembrane domain, while VAPC contains only the N-terminal half of the MSP domain of VAPB followed by 29 unique amino acids (Kukihara et al., 2009). Human VAPA is 242 amino acids long, and is identical to VAP-33 with the exception of two amino acid residues. VAPB is 243 amino acids long and shares 48% sequence similarity with VAP-33 (Nishimura et al., 1999).

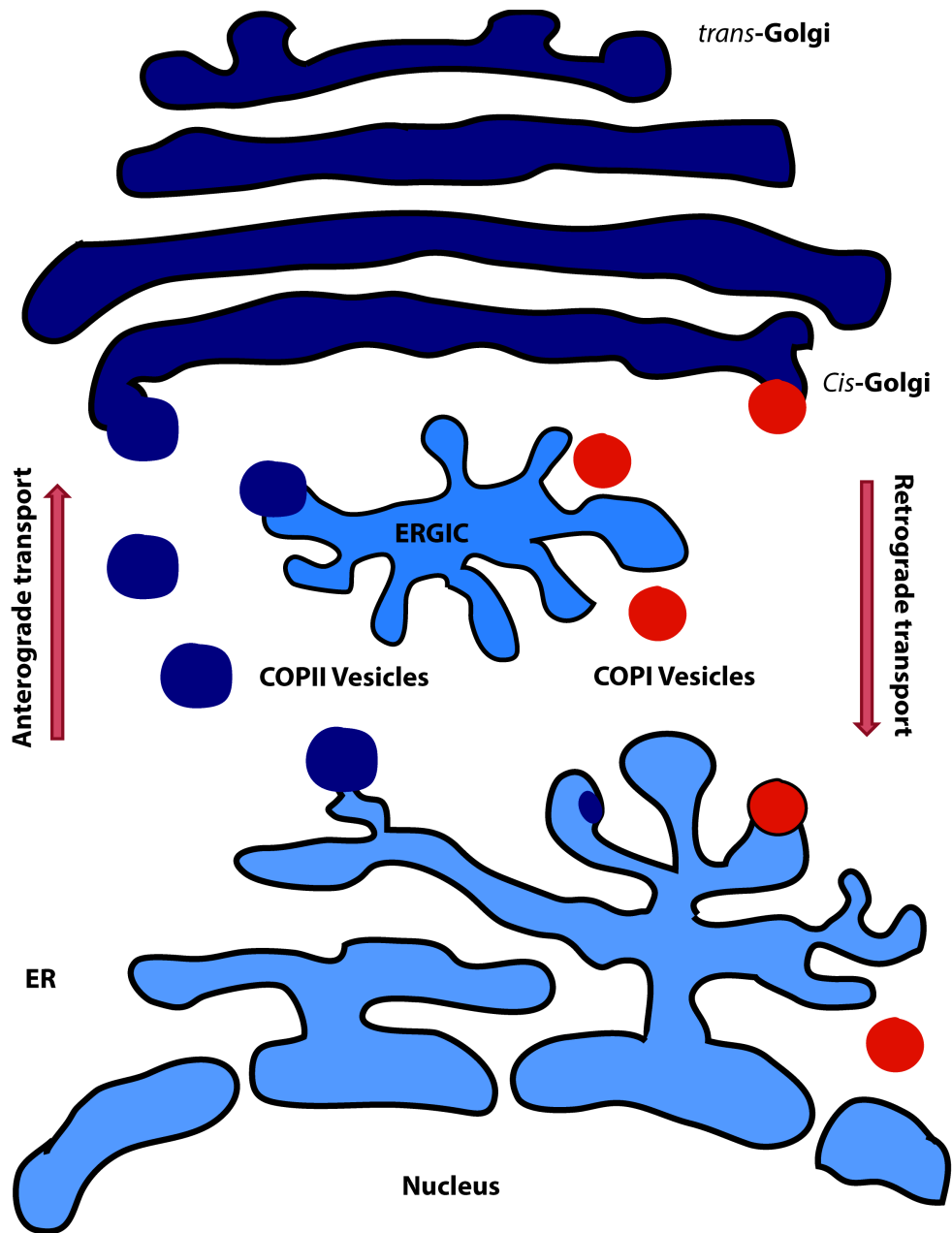


Figure 1. Overview of secretory pathway. Proteins synthesized in the ER are transported to the ERGIC and Golgi compartments for sorting via COPII vesicles. Proteins move from the *cis*-Golgi to the *trans*-Golgi where they are further sorted for final transport to the plasma membrane or other membrane compartments. This process describes anterograde transport. Retrograde transport utilizes COP1 vesicles and shuttles proteins from the Golgi back to the ERGIC compartments, ER and nuclear envelope.

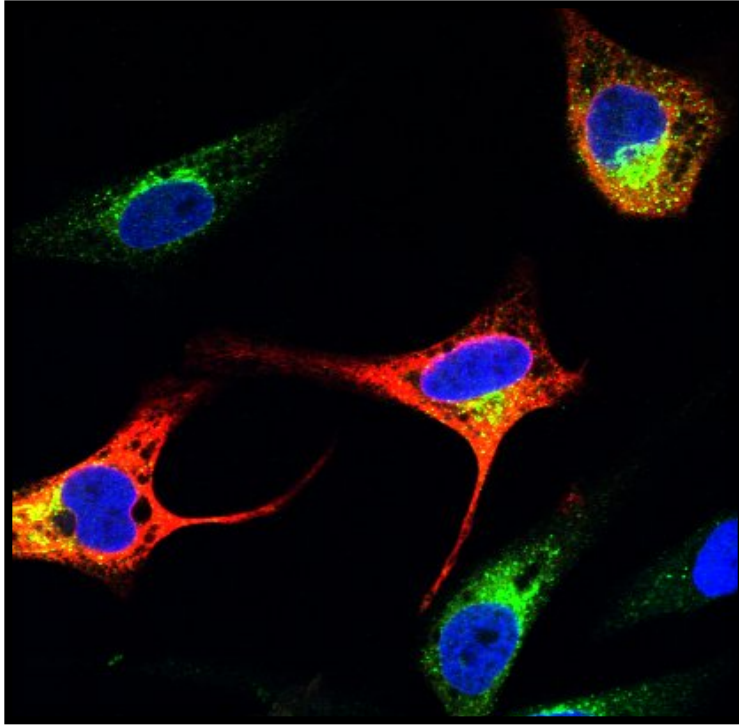


Figure 2. VAPB co-localizes with ERGIC-53 marker.

Flag-tagged VAPB-WT (red) localizes in the ER and co-localizes with ERGIC marker (ERGIC-53 green) in HeLa cells.

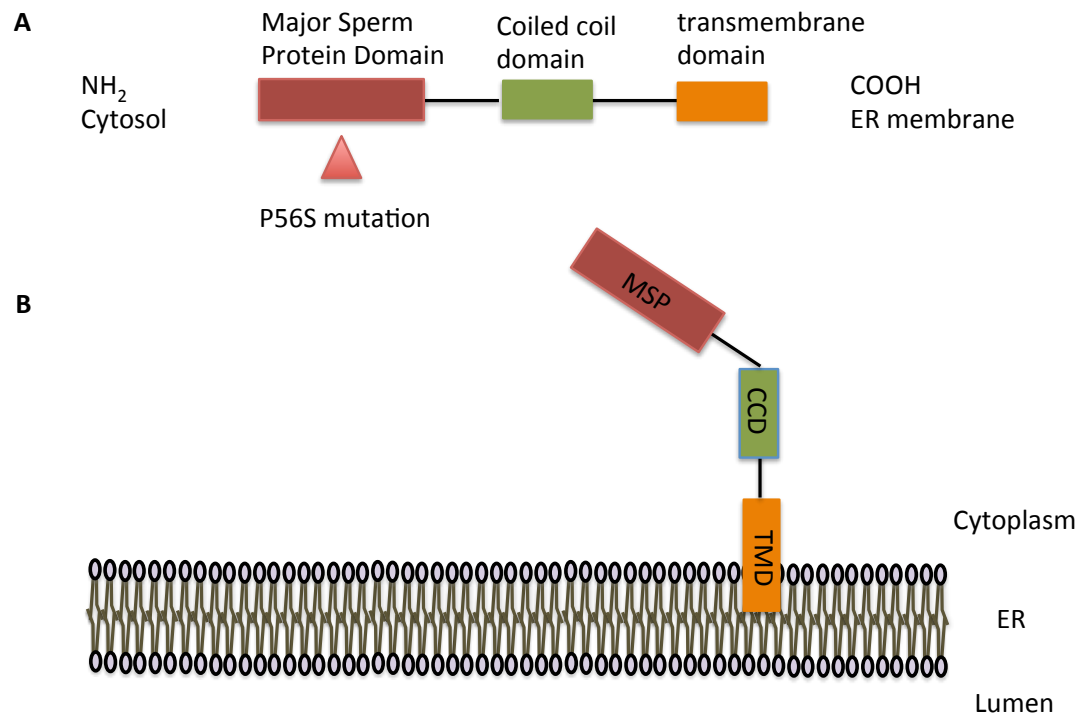


Figure 3a. Structure of VAPB. The VAPB protein consists of an N-terminal Major Sperm protein domain (MSP), a central coiled-coil and a transmembrane domain. **Figure 3b.** VAPB is a type two integral membrane protein with the C-terminal domain embedded in the ER. The MSP domain faces the cytosol to interact with soluble lipid transfer proteins.

Structure of the VAPB domains

The MSP domain of VAPB binds proteins that contain a “FFAT motif” consisting of two phenylalanines in an acidic tract with a consensus sequence of EFFDAxE. Many cytoplasmic lipid-binding proteins contain a FFAT motif, including the oxysterol binding protein-related protein family (ORP), ceramide transfer protein (CERT), and the NIR family of proteins. The FFAT motif targets these proteins to the cytosolic face of the ER, where they aid in lipid transfer between the ER and other organelles such as the Golgi, endosomes and plasma membrane (Shi et al., 2010).

It is within the highly conserved MSP domain that the proline to serine substitution occurs at position 56. The VAPB MSP domain is composed of seven-stranded immunoglobulin-like β sandwiches with an s-type topology (Shi et al., 2010). Two S-shaped loops are stabilized by the cis-peptide bond conformation of proline 12 and proline 56 (Figure 4). It has been proposed that the S-loops are responsible for stabilizing the MSP domain, and a serine substitution at proline 56 would result in mis-folding and exposure of hydrophobic residues creating insolubility. In fact, in VAPB-P56S the beta strand structure of the VAPB-P56S MSP domain is completely eliminated and replaced by a non-native helical conformation that has been found to associate with lipid membranes (Qin et al., 2013). The coiled-coil domain of VAPB is 40 amino acids long and is thought to promote protein-protein interactions. The transmembrane domain anchors into the ER and is responsible for homo and heterodimerization between VAPB and VAPA (Hamamoto et al., 2005; Soussan et al., 1999).

VAPB Expression and Localization

Western blot analysis has shown that VAPB is present in all cell lines examined and is highly expressed in kidney, heart, skeletal muscle, liver, spleen, lung and all tissues in the nervous system (Teuling et al., 2007). VAPB

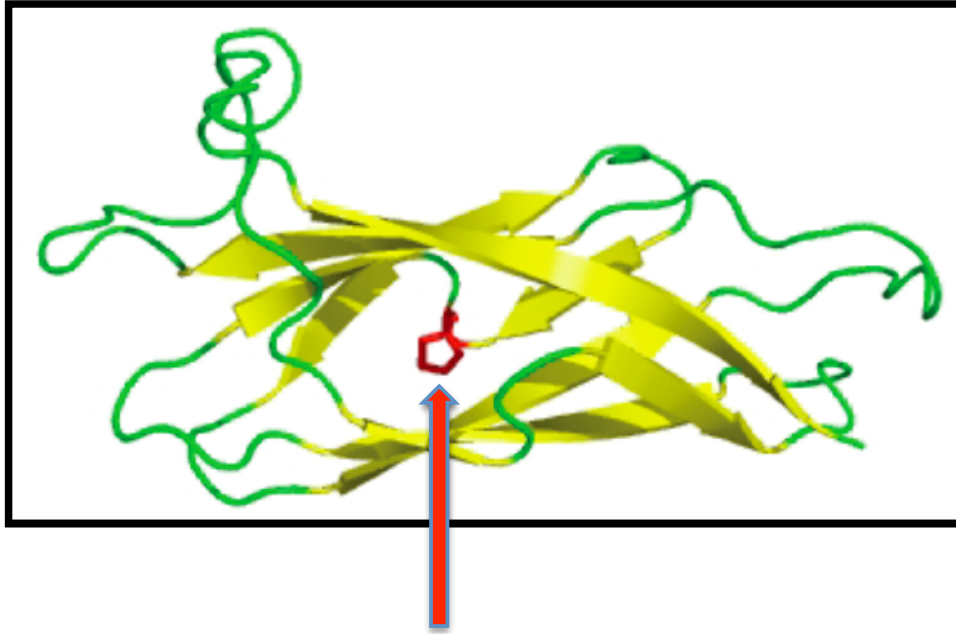


Figure 4. PyMOL Structure of the human MSP domain. The human VAPB MSP domain consists of immunoglobulin-like β -sheets stabilized by the proline 56 residue (red arrow). The proline to serine missense mutation disrupts the native conformation and exposes hydrophobic residues. The result is a highly aggregate-prone MSP domain.

immuno-reactivity is found predominantly in neuronal cell bodies and dendrites, and rarely in glial cells. The highest levels of VAPB expression are detected in motor neurons of the spinal cord and caudal brainstem (Teuling et al., 2007).

VAPB is known to localize on the cytoplasmic face of the rough ER and on the outer membrane of the nucleus, both of which are sites of protein synthesis. VAPB is present in pre-Golgi intermediate compartments and at membrane contact sites between closely opposing organelles (Loewen et al., 2003), but is not found in the Golgi or the synapse (Soussan et al., 1999).

VAPB mRNA expression is low in sALS cases

Though VAPB mRNA is found throughout the grey matter of the spinal cord, it is most abundant in the anterior horn of human spinal cord sections (Anagnostou et al., 2010). The protein is expressed in a similar fashion, being highly abundant in the large motor neurons of the anterior horn. A study examining the spinal cords of sALS patients found no change in gene expression levels of VAPB, however they reported a significant decrease in VAPB protein levels. The reduction was most apparent in individuals with a rapid progression who died within 3 years from symptom onset (Anagnostou et al., 2010). Similarly, levels of VAPB protein are reduced in ALS8-induced pluripotent stem cells (iPSCs) derived from ALS8 fibroblasts. Because mRNA levels remain unchanged, it has been concluded that the VAPB-P56S mutation causes a down-regulation of the VAPB protein via a post-translational mechanism (Mitne-Neto et al., 2011).

Consequences of the VAPB-P56S mutation

Conformational change in the MSP domain exposes hydrophobic residues and causes aggregation

The S-shaped loops responsible for stabilizing the MSP domain are held in an energetically unfavorable cis-bond conformation by the proline 56 residue. The serine substitution at position 56 causes a change in conformation, which exposes hydrophobic residues from the MSP domain core (Teuling et al., 2007). The aberrant VAPB structure is highly aggregate prone, forming stable aggregates that are insoluble in non-ionic detergent (Kanekura et al., 2006). The P56S substitution has no effect on the transmembrane domain of VAPB, and therefore the mutant protein can still dimerize with the VAPB-WT. VAPB-P56S causes a dominant negative effect by binding the VAPB-WT and trapping it in detergent insoluble aggregates originating from endoplasmic reticulum membranes (Kanekura et al., 2006; Prosser et al., 2008). Interestingly, over-expression of VAPB-P56S causes large aggregated ER membrane expansions in cell cultures, but these aggregates are not observed in induced pluripotent stem cells (iPSC) from patients with ALS8 (Mitne-Neto et al., 2011). One potential explanation for this discrepancy came from work by Papiani et al. (2012). They showed that the VAPB-P56S mutant protein is inserted into the ER where it rapidly clusters and coalesces into large para-nuclear structures. These aggregates are cleared efficiently and rapidly by the proteasome system involving the ER associated degradation (ERAD) machinery (Papiani et al., 2012).

Conflicting results have been reported in mouse models of ALS8. Tudor et al. (2010) reported no advertent phenotype in ALS8 mice, in which there were no motor defects or aggregates found within the cells. In contrast, Aliaga et al. (2013) found ALS8 mice developed motor defects and accumulated VAPB-P56S mutant protein in neuronal cells. The conflicting results observed in these studies may be due to the use of different promoters. Aliaga et al. used a Thy1.2 promoter, which drives expression in corticospinal tract and spinal motor neurons and some glial cells (Belle et al., 2007). In contrast Tudor et al. used a modified prion promoter, which expresses protein in the brain, spinal cord and heart of mice (Borchelt et al., 1996). Regardless, it is currently unknown how these aggregates affect the neurodegeneration process.

VAPB-P56S causes a nuclear envelope defect

In addition to causing aggregation of ER-derived membranes, the VAPB-P56S mutation also causes a nuclear envelope defect. The ER is continuous with the nuclear envelope, therefore, any defect in ER structure may also affect the nuclear envelope. Tran et al., (2012) discovered the inner and outer nuclear membranes of CHO cells overexpressing VAPB-P56S were dilated and separating apart (Tran et al., 2012). Because nucleoporins making up the nuclear pore complex bridge the inner and outer membranes of the nuclear envelope, they contribute to the close apposition of the two membranes (Tran et al., 2012). Mis-localization of nuclear pore complex proteins then may lead to a separation of the inner and outer nuclear envelope. The distribution of two Nups were investigated: an integral membrane protein GP210, along with Nup214, which is a soluble nucleoporin that forms the structural scaffold (Tran et al., 2012). In cells expressing the mutant VAPB, both GP210-GFP and Nup214-GFP were mis-localized from the nuclear envelope to cytoplasmic membrane puncta. The Nups were sequestered in mutant VAPB expanded ER membranes that co-localized with an ERGIC marker (ERGIC-53). This phenomenon was also observed in cells that had endogenous VAPB knocked down with shRNA. This suggests that VAPB is required for the transport of nucleoproteins from the ERGIC compartment back to their final destination at the nuclear envelope.

The FFAT motif from OSBP rescues the VAPB-P56S phenotype

Co-expression of an N-terminal myc-tagged FFAT motif from OSBP was previously found to significantly reduce the number of VAPB-P56S cells with large aggregates, perhaps by preventing further aggregation of the MSP domain, or by increasing solubility of the mutant protein (Prosser et al., 2008). In contrast, a C-terminal GFP-tagged FFAT motif from Nir2 failed to rescue the VAPB-P56S phenotype in another study (Teuling et al., 2007). The reason for this remains unclear, but may be due to steric hindrance by the large GFP moiety. Co-expression

of the myc-tagged FFAT motif from OSBP was also able to restore transport of nucleoporins to the nuclear envelope (Tran et al., 2012). This suggests that VAPB-P56S is still able to bind to FFAT motif-containing proteins, as was recently described by (Kuijpers et al., 2013). In contrast, Teuling et al. (2007) were unable to pull down FFAT motif containing proteins with the mutant VAPB. The ability of VAPB-P56S to bind FFAT motif containing proteins may have implications for future treatments.

Possible Functions of VAPB

In 1999, Soussan et al. described a protein then called ERG30 that localized to the ER and ERGIC. This protein was initially identified to play a role in COPI dependent vesicle transport of proteins between the Golgi and ER, and was subsequently renamed VAP-33. Later work identified that VAPB interacts with components of the microtubule cytoskeleton (Skehel et al., 2000). Perhaps most prominently, VAPB is implicated in lipid metabolism, lipid synthesis and lipid homeostasis (Forrest et al., 2013).

Trafficking between ER-ERGIC

Proteins move between organelles of cells by means of coated vesicles that bud from one membrane and fuse with another (Rothman and Weiland, 1996). Cytosolic coat proteins (COP) form on the donor membrane and their budding is regulated by small GTPases. COPII vesicles are involved in transport from the ER to the Golgi apparatus (anterograde transport), while COPI vesicles mediate transport from the Golgi back to the ERGIC and ER (retrograde transport) (Malhotra et al., 1989). Proteins and lipids are synthesized in the ER and are transported via COPII vesicles to the ERGIC compartment and *cis*-Golgi network for modifications (Dancourt and Barlowe, 2010). Forward transport is balanced by retrograde trafficking to retrieve any ER resident proteins that are necessary for ER function.

Proteins move through the *cis*-Golgi network to the *trans*-Golgi network where they undergo sorting before reaching their final destinations.

Soussan et al., (1999) used anti-ERG30 (VAPB) antibodies to detect where in the secretory pathway VAPB acted. They found that COPI vesicles accumulated, indicating that VAPB is active in the early secretory pathway, most likely in the retrograde direction. In addition, the anti-ERG30 antibodies prevented COPI un-coating. It is possible that VAPB is involved in triggering ADP-ribosylation factor (ARF)-GTPase activating protein (GAP), which stimulates the vesicle un-coating process. Work by Tran et al., (2012) also supports the view that VAPB acts in the retrograde pathway, delivering nucleoporins from the ERGIC compartments to the nuclear envelope. Knockdown of VAPB blocked transport of Nups to the nuclear envelope, causing them to accumulate in cytoplasmic puncta, which co-localized with an ERGIC-53 marker. This disruption in retrograde transport to the nuclear envelope may be contributing to the pathogenesis of ALS8, a late onset neurodegenerative disease.

VAPB associates with microtubules

Previous work in mice (Skehel et al., 2000) and *Drosophila* (Pennetta et al., 2002) has shown that VAPA, and DVAP-33A associates with microtubules at sites where vesicles are closely opposing microtubules. DVAP-33A is responsible for normal microtubule organization in presynaptic terminals. It has been proposed that DVAP-33A may act as a bridge between membranes and the microtubule cytoskeleton network (Pennetta et al., 2002). It was then determined that VAPB also plays a role in stabilizing microtubules, acting as a bridge that connects the ER membrane with the cytoskeleton (Amarilio et al., 2005). Over-expression of VAPB and Nir3, a FFAT motif-binding partner of VAPB, caused a structural change in the ER, with bundling of microtubules along altered ER membranes (Amarilio et al., 2005). This work suggests that the interactions between VAPB and FFAT motif-containing proteins play a role in the organization and positioning of the peripheral ER network.

VAPB involvement in lipid metabolism

ER is the site for Lipid synthesis

The ER consists of an extensive membrane network that is continuous with the nuclear envelope and extends out to the cell periphery. The ER makes membrane contact sites with endosomes, lysosomes, mitochondria and the plasma membrane (Voeltz, Rolls and Rapoport, 2002) The ER is also the primary site for lipid synthesis. Many lipid binding or lipid transfer proteins are then responsible for the non-vesicular transport of lipids through out the cell.

VAPB is involved in phospholipid metabolism

VAPs involvement in phospholipid metabolism was first discovered in the yeast VAP homologue Scs2 (suppressor of inositol auxotrophy of the choline-sensitive dominant mutation CSE1). Scs2 was found to interact with Opi1p via its FFAT motif. Opi1p is a transcription factor in yeast that negatively regulates phospholipid synthesis by repressing the expression of inositol-1-phosphate synthase (INO1). Yeast lacking Scs2 display reduced levels of INO1 expression, reduced phosphatidylinositol levels and increased levels of phosphatidylcholine (Lev et al., 2008). This study determined that Scs2 recruits Opi1p to the ER by interactions with the FFAT motif and therefore prevent nuclear translocation of Opi1p to activate INO1 activity (Lev et al., 2008). Thus, VAPs can interact with FFAT containing proteins to control phospholipid metabolism. Whether this function is conserved through evolution remains unclear because there is no human orthologue of Opi1. It is possible that phospholipid homeostasis is regulated by other mechanisms in mammals (see below).

VAPB interacts with lipid transfer proteins

The crystal structure of the rat VAPA MSP domain-FFAT interaction revealed a 2:2 complex, where the FFAT motif binds a highly conserved positively charged

region on VAP. The first phenylalanine of the FFAT motif binds a hydrophobic pocket in the MSP domain, and has extensive hydrophobic van der Waals contacts with the VAP protein (Kaiser et al., 2005).

FFAT motif containing proteins include the oxysterol binding proteins (OSBPs), OSBP-Related Proteins (ORPs), the Ceramide transport protein (CERT), and phosphatidylinositol/phosphatidylcholine transfer proteins Nir1, Nir2 and Nir3 (Lev et al., 2008). The EFFDAXE consensus sequence was first discovered to interact with VAP homologues in yeast, where Scs2 was co-precipitated with eight different proteins: Fks1p, Num1p, Opilp, Osh1, Osh2, Prn10p, Stt4p and YGR086Cp (Loewen et al., 2003). Proteins with a FFAT motif are targeted to the ER and nuclear envelope where Scs2 resides. Binding is ablated upon mutation of the FFAT motif to AAAT, indicating it is the two phenylalanines that are required for recruitment to the ER (Loewen et al., 2003). In yeast, Osh2 and Osh3 are localized to the peripheral ER, suggesting that Scs2 positions them in the ER adjacent to the sites they target.

In mammals, VAP proteins have been known to interact with lipid binding proteins such as Nir1-3, CERT, and the OSBP family (Wyles et al., 2002). Proteins in the OSBP family in general localize to the cytoplasm, but can be recruited to the ER upon binding to the MSP domain of VAPB (Perry and Ridgway 2006). The OSBP-related proteins (ORP) contain a highly conserved oxysterol-binding domain at the C-terminal end, which bind to oxysterols, cholesterol, and phosphatidylinositol-4-phosphate. Oxysterols are 27-carbon products of cholesterol oxidation, and have the ability to suppress cholesterol synthesis (Lehto and Olkkonen, 2003). Proteins known to modulate cholesterol synthesis, uptake and efflux are controlled tightly by positive and negative feedback mechanisms by interacting directly with cholesterol or oxysterols (Ngo et al., 2010). Regulators that interact with cholesterol and oxysterols include SREBP (Sterol-regulatory-element-binding protein), the Insig complex, the liver X receptor and HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA). These regulator proteins are localized in cholesterol poor regions of the cell, such as the ER and therefore are able to detect slight changes in

cholesterol/oxysterol levels (Ngo et al., 2010). Lipid binding/transfer proteins are thought to be the major delivery mechanism for transferring cholesterol/oxysterols to the regulatory elements. One major group of lipid binding proteins is the OSBP family.

The OSBP/ORP Family of proteins

The first protein in this family to be described was OSBP, a soluble cytoplasmic protein that trans-locates to Golgi membranes when bound with 25-hydroxycholesterol (Lehto and Olkkonen, 2003). There are currently twelve OSBP proteins (See table 2) OSBP, ORP1, ORP2, ORP3, ORP4, ORP5, ORP6, ORP7, ORP8, ORP9, ORP10 and ORP11, which can be further split into six subfamilies based on amino acid homology.

Function of OSBP

The OSBP protein reportedly increases the mRNA levels of the LDL receptor, HMG-CoA reductase, HMG-CoA synthase, and boosts cholesterol synthesis by 80% (Lehto and Olkkonen, 2003). OSBP is translocated to the Golgi upon sterol depletion and regulates the transcriptional control of regulatory genes by inducing the expression of SREBP1-c (Yan et al., 2007).

Structure of ORPs

Most of the Oxysterol binding protein-Related Protein family contain an N-terminal pleckstrin homology (PH) domain, which bind to phosphatidylinositols in non-ER membranes. The PH domain consists of β -sandwich modules, which assist in binding membranes rich in phosphatidylinositols (Lehto and Olkkonen, 2003). ORPs containing a PH domain are termed “long (L) ORPs”, while those lacking an N-terminal PH domain are termed “short (S) ORPs” (Weber-Boyvat et al., 2013). Many of the ORPs contain a FFAT motif, and all contain a highly conserved C-terminal lipid-binding domain (Figure 5), which binds to cholesterol, oxysterols and sometimes phosphatidylinositol phosphates (PIPs) (Saint-Jean et al., 2011).

Each ORP is targeted to a different membrane upon activation by cholesterol/oxysterols or PIPs (Table 2). The binding of PIPs in the oxysterol-binding domain of ORPs is important for membrane targeting (Weber-Boyvat et al., 2013). For example upon activation by 25-hydroxycholesterol (25-OH), OSBP1 undergoes a conformational change, exposing the PH domain, which allows translocation and binding to Golgi membranes (Banerji et al., 2010). Until recently, it was unknown which of the ORPs are recruited to VAPB. Recent research has shown that ORP3, ORP6 and ORP9 are interacting partners with VAPB (Kuijpers et al., 2013), but it remains unknown if any of the others interact with VAPB in the ER.

ORPs regulate phosphatidylinositol levels in cells

A 2011 study by de Saint-Jean et al. found that the yeast OSBP homologue Osh4 was able to catalyze in vitro exchange of dehydroergosterol (DHE) for PI4P. This study proposes a model in which Osh4 transports sterols from the ER to compartments containing PI4P, and transports PI4P backwards. The authors determined the crystal structure of Osh4 bound to PI4P and found PI4P inserts its two acyl-chains into the central tunnel of the sterol-binding pocket. OSBP has also been reported to regulate the levels of PI4P by activating the PI4 kinase II alpha (PI4KII α) in response to 25-OH treatment (Banerji et al., 2010).

Studies in yeast have recently shown that mammalian ORP3 orthologue Osh3 regulates levels of PI4P by activating the ER membrane protein PI4P phosphatase Sac1 at ER-PM contact sites (Stefan et al., 2011). Sac1 reportedly interacts with VAP in the ER and is bridged to opposing membranes by ORPs to dephosphorylate PI4P.

The primary aim of this study is to investigate if any of the endogenous ORPs can rescue the VAPB-P56S defect similar to that of the FFAT motif in HeLa cells.

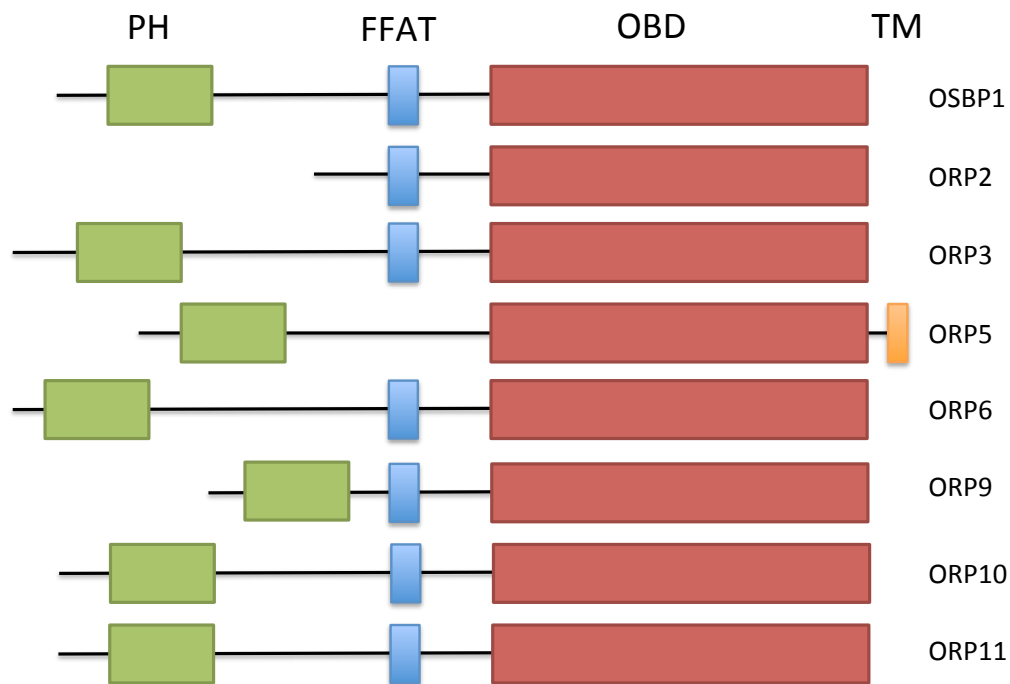


Figure 5. Oxysterol Binding Protein-Related Protein (ORP) Structure

Most of the ORPs contain a pleckstrin homology domain (PH domain, green) at the N-terminal end, a FFAT motif (blue) and a highly conserved oxysterol binding domain (OBD) at the C-terminus (red). ORP5 contains a transmembrane domain, which anchors into the ER. This figure represents the ORPs used in this study.

Protein	Family	FFAT motif	Target membrane for the PH domain	Tissue Expression	Role
OSBP		EFFDAPE	Golgi (PI4P)		Translocates to Golgi to increase PI4 kinases, boosts cholesterol synthesis, negatively regulates ABCA1
ORP1	I	EFYDALS			Regulates vesicle transport from Golgi
ORP2	II	EFFDAVT	Surface of lipid droplets	Expressed in central nervous system	Inhibitory role in phospholipid metabolism and cholesterol ester synthesis
ORP3	III	EFFDAQE	PM PI3P, PI4P, PI(3,4)P2, PI(4, 5)P2	Kidney, lymph nodes, thymus, nervous system	Moves sterols between the ER and PM (yeast)
ORP4	I			Brain, retina, heart, kidney	
ORP5	1V	N/A	Localizes to the ER		Contains a transmembrane domain
ORP6	III	EFFDAQE	PM	Brain, skeletal muscle	Upregulated in cholesterol loaded macrophages
ORP7	III	EFFDACE	PM	Stomach, intestinal tract	
ORP8	IV			Contains a trans-membrane domain	Negatively regulates ABCA1 expression in macrophages (deletion of ORP8 increased ABCA1 expression through LXR)
ORP9	V	EFYDADE	Golgi (PI4P)		
ORP10	VI			Liver, kidney and lung	
ORP11	VI			Lung and spleen	

Table 2. Family of ORPs

Hypothesis and Aims

I hypothesize that the full length endogenous ORPs may have the ability to rescue the mutant VAPB-P56S phenotype by decreasing aggregation of VAPB-P56S by preventing further aggregation of MSP domain. Reducing aggregation will allow VAPB to function in retrograde transport of nuclear envelope membrane proteins such as Emerin. In addition I hypothesize that VAPB, ORP3 and Sac1 are interacting partners, which regulate levels of PI4P in the cell, particularly in the ER, ERGIC and *cis*-Golgi compartments.

To test this hypothesis a morphological screen was devised in order to assess the effect of each of the endogenous ORPs on the VAPB-P56S membrane expansions. Once the successful ORP was identified, further experiments were performed to determine if its ability to rescue the VAPB-P56S phenotype utilize similar mechanisms as the FFAT motif. Next, it was determined if mutant VAPB influences another ER resident protein Sac1 (PI4P phosphatase). VAPB, ORP3 and Sac1 have been shown to be interacting partners in yeast, which together regulate levels of PI4P. Lastly it was to assess whether the three proteins interact to regulate levels of PI4P in mammalian cells.

METHODS AND MATERIALS

DNA plasmid constructs

The VAPB-WT and VAPB-P56S were cloned into a pFlag-CMV2 plasmid as described in Prosser et al. (2008).

The FFAT motif from rabbit OSBP (residues 347-468) was cloned into a pcDNA3.1 (+)-Myc plasmid as described by Prosser et al. (2008).

Plasmids

TRC Lentiviral pLKO.1 plasmids were used for all knockdown experiments. All were obtained from Open Biosystems (Huntsville, AL, USA). An empty pLKO.1 plasmid was used as a control in all knockdown experiments.

Lentiviral pLKO.1 plasmids used in these experiments include: **shVAPB** (TRCN0000152888), and **shSac1** (TRCN0000062788, TRCN0000062790, TRCN0000062791, TRCN0000062789, TRCN0000062792).

OSBP/ORP family plasmids

ORP plasmids were obtained from Open Biosystems:

Mouse OSBP accession number bc0003443

Mouse ORP2 accession number bc031794

Human ORP3 accession number bc017731

Human ORP5 accession number bc032646

Mouse ORP6 accession number bc022908

Mouse ORP9 accession number bc026927

Human ORP10 accession number nm_017784

Human ORP11 accession number bc065213

The Sac1-GFP Plasmid was a generous gift from Dr. Peter Mayinger (Oregon Health and science University).

HeLa Cell Culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen. Cells were supplemented with 10% fetal bovine serum (FBS) from Invitrogen, and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were maintained at 37°C with 5% CO₂.

Transient DNA transfection

HeLa cells were seeded into a 24 well plate onto 12 mm coverslips (Fisher) at a density of 0.8×10^5 one day prior to transfection.

Transient transfections were performed using Effectene Transfection Reagent (QIAGEN, Valencia, CA, USA) and used as per the manufacturers protocol.

Primary Antibodies

Flag-tagged VAPB-WT and VAPB-P56S proteins were visualized using a mouse anti-Flag antibody (Applied Biological Materials, Richmond, BC, Canada). Anti-Flag was used at a dilution of 1:500. To visualize the ERGIC compartments, an Anti-ERGIC-53 polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) produced in rabbit was used at a dilution of 1:100. To visualize the nuclear envelope, a mouse anti-Emerin antibody was obtained from the Developmental Studies Hybridoma Bank (Iowa City IA, USA) as MANEM1 and was used at a dilution of 1:100. A mouse anti-PI4P IgM antibody was obtained from Echelon Biosciences (Salt Lake City, UT, USA) and was used at a dilution of 1:100.

Secondary Antibodies

Alexa-Fluro Goat-anti mouse or goat-anti rabbit 488 (green) or 594 (red) were used (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:500. For PI4P staining, a biotinylated goat-anti-mouse IgM antibody was purchased from Jackson ImmunoResearch labs (1:2000) and a Streptavidin-AlexaFluor 488 (Molecular Probes) was used at a dilution of 1:2000.

Immunocytochemistry

Forty-eight hours post transfection, cells were washed with 1X PBS (Fisher Scientific, Waltham, MA, USA) and coverslips were fixed with 4% paraformaldehyde (Cedarlane) in PBS for 20 minutes. Cells were then washed 3 times for 5 minutes each in PBS containing 0.1 M glycine (Roche). Cells were permeabilized in block buffer containing 0.4% saponin (Sigma), 1% bovine serum albumin (Sigma), 2% normal goat serum (Invitrogen) and 0.01% Sodium azide/ NaN_3 for 30 minutes at room temperature. Cells were then incubated in primary antibody for 1 hour at room temperature, or overnight at 4°C in the dark. Coverslips were washed 3 times each for 5 minutes, followed by block buffer for 30 minutes. The secondary antibody was applied in block buffer for 1 hour at room temperature. Cells were washed 3 times, and an equilibration buffer was applied (30% Glycerol in PBS) before coverslips were mounted in SlowFade Gold Reagent with DAPI (Invitrogen). Coverslips were fixed into place on 3x1x1 mm glass microscope slides (Fisher) with nail polish.

All images were taken using a Zeiss LSM510 META laser scanning confocal microscope. The 60x oil immersion objective was used for all images and modified using the LSM510 software program Zen.

Statistical analysis

Statistical comparisons of these data were performed either using unpaired Student's t test or One-way anova followed by post-hoc Tukey test, using the GraphPad Prism software (version 5.0a). Significance was considered sufficient when $p < 0.05$.

EXPERIMENTS

Screening of endogenous ORPs for efficacy in reducing VAPB-P56S phenotype

To observe the phenotype of the VAPB-P56S mutant protein when co-expressed with each of the ORPs, HeLa cells were transfected with either pFlag-VAPB-WT or P56S and one of: pcDNA3.1 (+) Myc-FFAT motif, SPORT6-OSBP, ORP2, ORP3, ORP5, ORP6, ORP9, ORP10, ORP11 or empty vector in a 1:2 ratio of VAPB:ORP. Cells were fixed and stained with the mouse anti-Flag primary antibody (Applied Biological Materials) (1:500) and the Alexa fluoro-goat-anti-mouse 594 secondary antibody (Invitrogen) (1:500).

To determine whether co-expression of any of the ORPs could reduce the size or number of membrane expansions in the VAPB-P56S cells, confocal images were analyzed using Image J software. The size of VAPB-P56S ER membrane expansions were measured using Image J. The nucleus counter function was used to detect membrane expansions (smallest pixel size = 1, largest pixel size = 500), which is equivalent to anything > 1 μ m in size being measured. The threshold was set to 80. Feret diameter size was determined by Image J, and data was exported to an excel file. The Feret diameter size was converted from pixels to μ m in excel, using the resolution of each individual image. The size and number of each membrane expansion per cell for each set was exported to Prism GraphPad for further analysis.

Solubility of VAPB-P56S in Triton X-100 in cells transfected with FFAT motif or OSBP, ORP2, ORP3, ORP5, ORP6, ORP9, ORP10 or ORP11

VAPB-WT is soluble in Triton X-100, however VAPB-P56S is insoluble. To determine if any of the ORPs could shift the VAPB-P56S mutant protein into the Triton X-100 soluble fraction, the VAPB-P56S protein was expressed with each of the ORPs. HeLa cells were seeded onto 10 cm plates, followed by transfection with: pFlag-VAPB-WT or P56S + one of: pcDNA3.1 (+) Myc-FFAT motif, SPORT6-OSBP, ORP2, ORP3, ORP5, ORP6, ORP9, ORP10, ORP11 or empty vector.

Cell Harvest

48 hours after transfection, plates were washed two times with cold PBS, followed by the addition of 500 μ l of Triton X-100 Lysis buffer (50 mM Tris-HCl, 150mM NaCl, 2 mM EDTA, 1% Triton X-100, 5mM DTT and 1 protease inhibitor cocktail tablet (Roche). Cells were scraped off plate into a 1.5 ml microcentrifuge tube and were left on ice for 30 minutes. Cells were then spun at 53,000 RPM (120,000 x g) in an Optima TLX Ultracentrifuge (120.2 TLA rotor) at 4°C for 30 minutes. The supernatant was removed and transferred to a new 1.5 ml tube. This is the Triton X-100 soluble fraction. Sample was immediately placed in the -80°C freezer for storage. The remaining pellet was solubilized in 250 μ l of SDS lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% SDS, and 5 mM DTT). Cells were sonicated briefly (<10 seconds) and then spun at 53,000 RPM at 4°C for 30 minutes. The remaining supernatant is the SDS soluble fraction. Samples were moved to -80°C freezer for storage.

SDS Page and Western Blot

Protein concentration was quantified using a Bio-Rad DC protein assay (Bio Rad Hercules, CA, USA). Equal amounts of each sample (Triton X-100 and SDS) were loaded onto an SDS polyacrylamide gel (25 μ g). The SDS gel consisted of 12.5% acrylamide separating layer and a 4% acrylamide stacking layer. Following SDS-PAGE, proteins were transferred from the gel onto a nitrocellulose membrane. Following transfer, the membrane was incubated in block buffer (5% skim milk, 150 mM NaCl, 10 mM Tris-HCl pH 7.5 and 0.1% tween) for 30 minutes, followed by incubation with mouse anti-Flag (1:1000) in block buffer for 1 hour at room temperature. Membrane was washed 3 times for 5 minutes each in wash buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5 and 0.1% tween) and then incubated with secondary antibody goat-anti-mouse 488 in block buffer for 1 hour. Membrane was washed 3 times for 5 minutes each and then imaged on a Typhoon 8600 variable mode imager (Molecular Dynamics).

Image J was used to measure the intensity of the bands on the western blot. Intensity values were exported to Prism GraphPad for analysis and graphing.

ORP3 rescues transport of Emerin to the nuclear envelope

Cells with the VAPB-P56S mutation have a defect in transport to the nuclear envelope, which is restored upon expression of the FFAT motif (Tran et al., 2012). In order to determine if ORP3 could cause a similar rescue, HeLa cells were transfected with: pFlag-VAPB-WT or P56S plus one of: pcDNA3.1 (+) Myc-FFAT motif, SPORT6-OSBP, ORP2, ORP3, ORP5, ORP6, ORP9, ORP10, ORP11 or empty vector. Cells were fixed and stained with rabbit anti-Flag (Sigma) (1:500), and mouse anti-Emerin (1:100) primary antibodies. Alexa Fluoro goat-anti-mouse 488, and goat anti-rabbit 594 secondary antibodies (Invitrogen) were used. Confocal images were scored in Image J. The endogenous Emerin signal was scored as being either: 1) in the nuclear envelope > cytoplasm, 2) Cytoplasmic puncta > nuclear envelope, 3) no nuclear signal. After three replicates were completed, counts were imported into Prism GraphPad for graphing.

Activation of ORP3 with 25-Hydroxycholesterol

To determine if activation of ORP3 further reduces the size and number of VAPB-P56S membrane expansions, HeLa cells were treated with 25-hydroxycholesterol. One-day post transfection, regular media was changed to de-lipidated media (Sigma) and left overnight. The next day, 25-hydroxycholesterol in de-lipidated media was added to HeLa cells for 4 hours at a concentration of 2.5 µg/ml. Cover slips were processed as in first experiment.

Co-localization of VAPB and Sac1

In order to investigate if Sac1 and VAPB co-localize in the ER, HeLa cells were transfected with Sac1-GFP and VAPB-WT or VAPB-P56S.

Two days post-transfection, cells were fixed and stained with anti-Flag and Alexa Fluoro-594 to visualize VAPB. Co-localization analysis was done using Image J to determine the Pearson's Correlation Coefficient. A value of 1 indicates perfect co-localization, 0 indicates random co-localization, and -1 no co-localization.

VAPB knockdown affects localization of Sac1

It has been reported that VAPB and Sac1 are interacting partners in the ER. To investigate whether or not VAPB affects the localization of Sac1, VAPB was knocked down using short hairpin RNA. HeLa cells were transfected with shRNA VAPB, or pLKO.1 control and Sac1-GFP (described above).

Expression of ORP3 in HeLa cells

Primers specific to the PH domain of ORP3 were designed for PCR: forward GCTGCATTGATGTCGGG, reverse GAAGAAGTCTTTGATGAG (119 base pair amplicon). RNA was extracted from both HeLa and HEK293 cells using a Qiagen RNeasy Plus Micro kit (QIAGEN, Valencia, CA, USA). RNA was reverse transcribed for use in PCR using a Qiagen OneStep reverse transcription-PCR kit. PCR product was run on a 1% agarose gel stained with ethidium bromide and imaged on a ImageQuant LAS 4000 gel doc system.

Determination of Knockdown efficiency for Sac1

To determine which of the shRNA plasmids knocked down Sac1 expression most effectively, HeLa cells were transfected with each of the 5 Sac1 shRNA plasmids. Two days post transfection, HeLa cells were harvested with a RIPA buffer containing 10 mM Tris-HCL (PH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholates, 140 mM NaCl and 1mM PMSF and scraped

off 10 cm plate into a 1.5 ml microcentrifuge tube. The cell suspension was placed on ice for 5 minutes and then sonicated briefly. Cells were centrifuged at 14,000 x g for 15 minutes at 4°C and supernatant was transferred to a new tube. A Bio-Rad DC protein assay (Bio Rad Hercules, CA, USA) was used to quantify protein, and equal concentrations of protein were loaded onto an SDS page gel, followed by western blotting.

Levels of PI4P in HeLa cells

Previous work has shown that PI4P levels are increased in yeast cells lacking VAP and the Sac1 PI4P phosphatase. To address whether mammalian cells behave in a similar fashion, VAPB and Sac1 were knocked down with shRNA (described above). The clone used to knockdown Sac1 was TRCN0000062792, as it demonstrated the highest efficiency of knockdown (see below). HeLa cells were co-transfected with either shVAPB or shSac1 or a pLKO.1 control and a mCherry-C1 vector to identify transfected cells. Cells were fixed and stained with an anti-mouse PI4P IgM antibody (Echelon Biosciences) as described in the immunocytochemistry section, followed by a biotinylated goat-anti-mouse IgM antibody (Jackson ImmunoResearch Labs) and a Streptavidin-AlexaFluor 488 antibody (Molecular Probes). Slides were examined under the Zeiss confocal microscope at 60X. The gain intensity settings were set to the pLKO.1 + mCherry slides to capture the intensity of the PI4P antibody. The shVAPB + mCherry and shSac1 + mCherry slides were imaged under the same conditions. Image J was used to measure the intensity of the PI4P signal in the pLKO.1 control cells versus the shVAPB or shSac1 cells. The mean intensities from three experiments were plotted using GraphPad.

ORP3 can partially rescue Emerin defect in VAPB knockdown cells

HeLa cells were transfected with shVAPB plus mCherry and empty pCMV vector, or ORP3, OSBP1 or ORP9. Cells were fixed and stained with anti-Emerin antibody and imaged. Image J was used to determine the intensity of the Emerin signal in the nucleus and the Emerin intensity in the total cell. The ratio of Emerin

in the nucleus/total was determined for each of the conditions and graphed in Prism GraphPad.

RESULTS

ORP3 reduces size and number of membrane expansions in Hela cells

The expression of Flag-tagged VAPB shows that VAPB is localized to the ER and ERGIC compartments. In contrast, the VAPB-P56S protein is aggregated around the nuclear envelope in membranes derived from the ER (Prosser et al., 2008). Previous work has shown that co-expression of an N-terminal tagged FFAT motif from OSBP can successfully resolve the VAPB-P56S ER membrane expansions (Prosser et al., 2008 and Tran et al., 2012), restoring cells to the WT phenotype, where VAPB is distributed throughout the ER periphery. This suggests that one or more of the endogenous ORPs could be recruited to VAPB to rescue the VAPB-P56S phenotype similar to the FFAT motif. To determine whether this is the case, I co-transfected the VAPB-P56S mutant protein with a series of ORPs. After the cells were fixed and stained and probed for Flag-VAPB, the size and number of the membrane expansions were measured using Image J Software. An expansion was defined as anything $>1\ \mu\text{m}$ in size. The mean size of the VAPB-P56S expansions were $1.66\ \mu\text{m} \pm 0.01\ \text{S.E.M.}$ ($n=283$), and were reduced to $1.13\ \mu\text{m} \pm 0.075\ \text{S.E.M.}$ ($n=69$), $p= <0.05$ in cells co-expressing ORP3 and to $1.08\ \mu\text{m} \pm 0.087\ \text{S.E.M.}$ ($n=51$), $p= <0.05$ in cells expressing the FFAT motif. The VAPB-P56S cells had a mean of $5.42 \pm 0.68\ \text{S.E.M.}$ ($n=52$) membrane expansions per cell, while cells co-overexpressing ORP3 had a mean number of $0.86 \pm 0.22\ \text{S.E.M.}$ ($n=81$), $p=<0.05$, and $1.55 \pm 0.41\ \text{S.E.M.}$ ($n=36$), $p=<0.05$ in the FFAT expressing cells (Figure 6, 7, 8). Co-overexpressing the other ORPs did not show this effect, suggesting that ORP3 in particular is recruited to VAPB in the ER membrane and can overcome the effects of mutant VAPB. Recent work by Kuijpers et al. (2013) has shown in a pull-down assay that VAPB interacts with ORP3, ORP6 and ORP9 in particular, however, ORP6 and ORP9 did not reduce the size or number of membrane expansions. Therefore, the rest of the experiments focused on the interaction between ORP3 and VAPB.

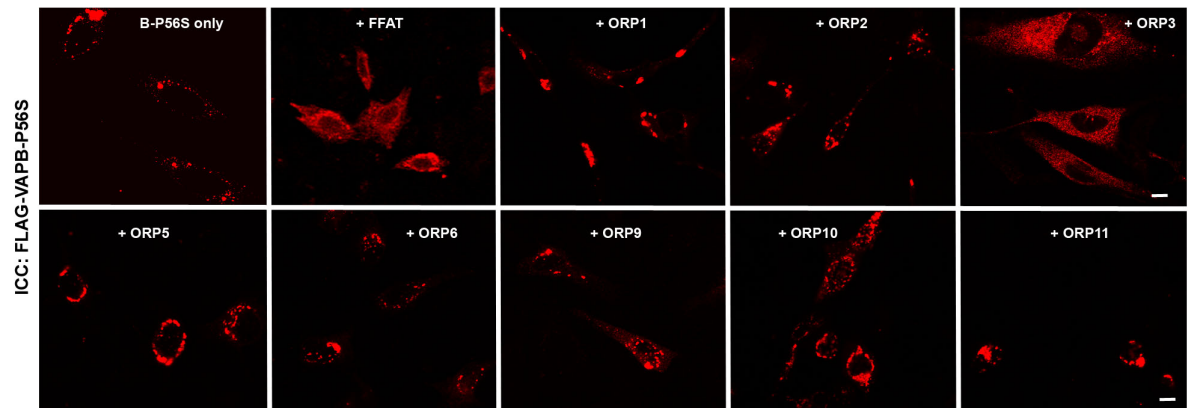


Figure 6. ORP3 rescues VAPB-P56S phenotype. Co-expression of the B-P56S protein with either the FFAT motif or ORP3 resulted in a reduction in the size of aberrant ER membrane expansions. Expression of ORP1, 2, 5, 6, 9, 10, or 11 had no effect on the size of membrane expansions in HeLa cells overexpressing the VAPB-P56S mutant protein.

ORP3 reduces size of membrane expansions

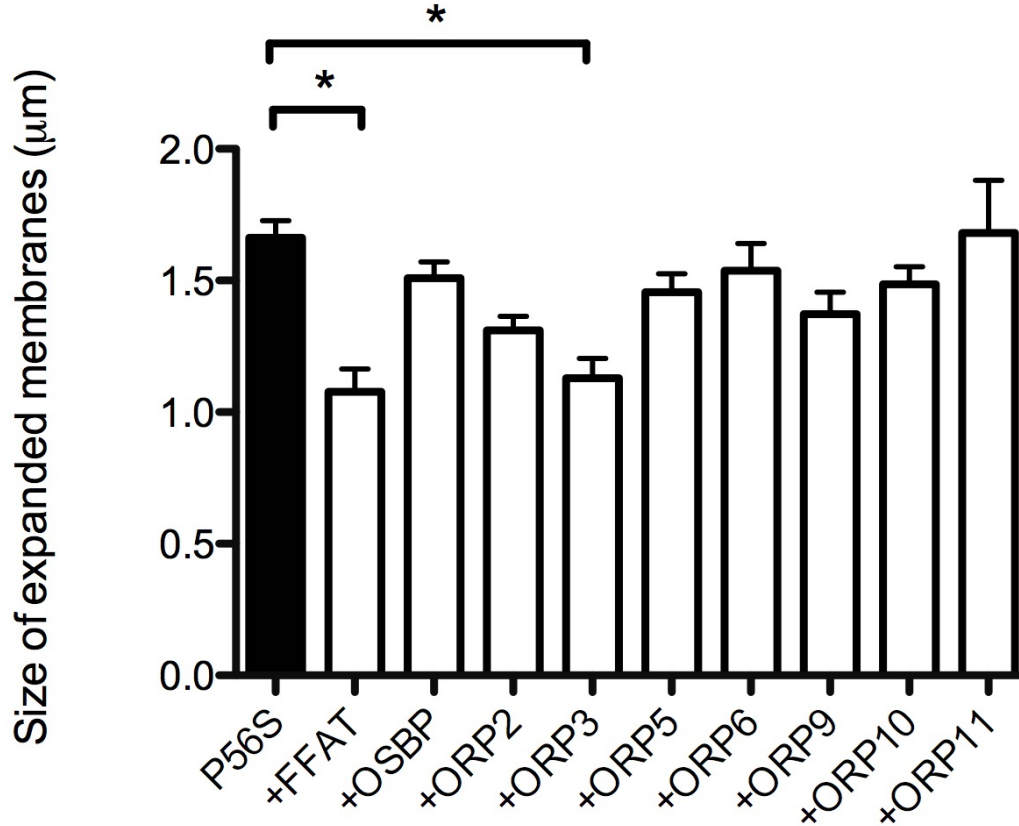


Figure 7. Co-expression of ORP3 reduces size of ER membrane expansions in VAPB-P56S cells. HeLa cells were transfected with a Flag-tagged VAPB-P56S plus one of the endogenous ORPs or the FFAT motif. The size and the number of ER membrane expansions were measured in Image J. An aggregate was defined as anything larger than 1 μ m in size. VAPB-P56S membrane expansions had a mean size of 1.66 μ m $\pm$ 0.006 S.E.M. (n=283), and an average of 5.42 membrane expansions per cell $\pm$ 0.68 S.E.M. (n=52). Co-expression of the FFAT motif reduced the mean size significantly to 1.08 μ m $\pm$ 0.0087 S.E.M. (n=51) p=0.0002, while ORP3 expression reduced the sizes to 1.13 μ m $\pm$ 0.075 S.E.M. (n=69), p<0.05.

ORP3 reduces the number of membrane expansions per cell

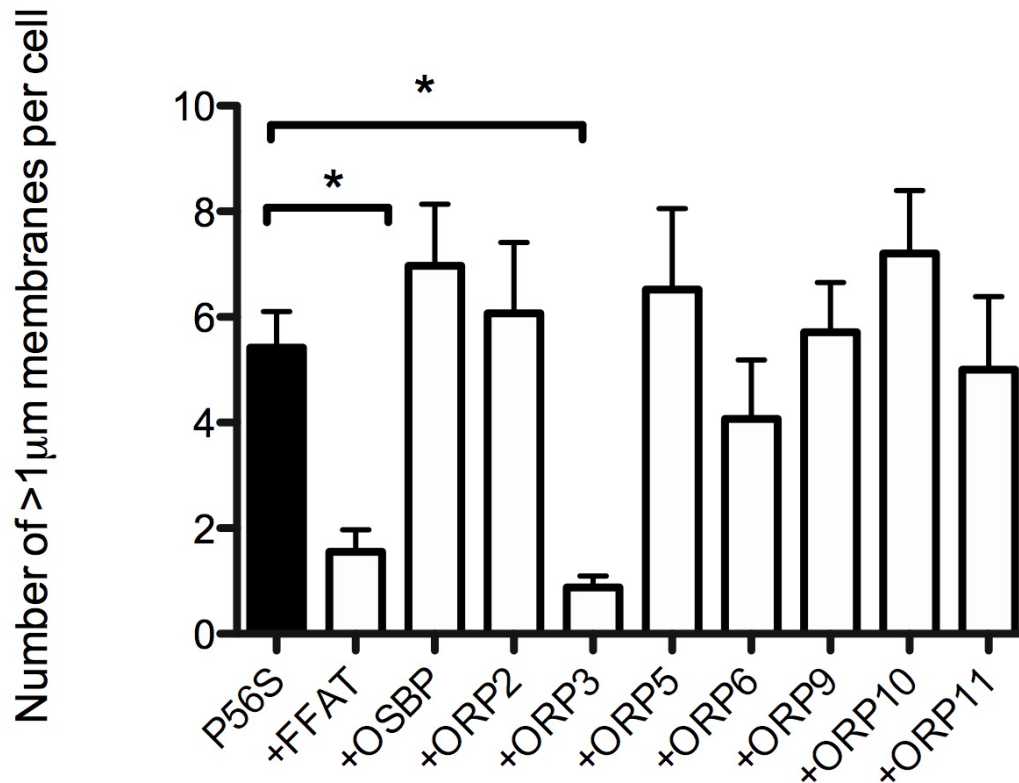


Figure 8. Co-expression of ORP3 reduces number of ER membrane expansions in VAPB-P56S cells. The number of membrane expansions per cell was reduced to 1.55 ± 0.41 S.E.M. (n=36), $p < 0.051$ in FFAT motif expressing cells and 0.86 ± 0.22 S.E.M. (n=81), $p < 0.05$ in cells overexpressing ORP3. This phenomenon was not observed with the co-expression of the other ORPs.

ORP3 shifts VAPB-P56S from the Triton X-100 insoluble to soluble fraction

VAPB is an ER membrane protein that is soluble in non-ionic detergents such as Triton X-100. VAPB-P56S is prone to misfolding and the aggregated mutant protein is insoluble in non-ionic detergents (Kanekura et al., 2006; Teuling et al., 2007). Insoluble aggregated VAPB-P56S can lead to a dominant negative effect by aggregating VAPB-WT, leading to a loss of VAPB function (Suzuki et al., 2009). Previous unpublished work in the Ngsee lab has shown that co-expression of the FFAT motif can shift the mutant VAPB protein from the Triton X-100 insoluble to soluble fraction, similar to the distribution of the VAPB-WT. Thus, the FFAT motif is able to change the solubility of the mutant protein, perhaps by binding to the misfolded MSP domain to prevent further aggregation. In order to determine if any of the ORPs had this same effect, the VAPB-P56S mutant was co-transfected with each of the ORPs, and then fractionated with Triton X-100 (Moumen et al., 2011). Eighty percent of the VAPB-WT is soluble in Triton X-100 compared to only 23% of the mutant VAPB-P56S. Co-expression of the FFAT motif or ORP3 with VAPB-P56S increased the solubility of the mutant protein in Triton X-100 to 63% and 61%, respectively. Overexpression of the ORPs had no effect on the WT protein (data not shown), and none of the other ORPs were able to shift VAPB-P56S into the Triton X-100 soluble fraction (Figure 9, 10). Together, this suggests that the presence of the FFAT motif or ORP3 changes the conformation of mutant VAPB-P56S, rendering it more soluble in Triton X-100.

Fraction of VAPB present in the Triton or SDS soluble fraction

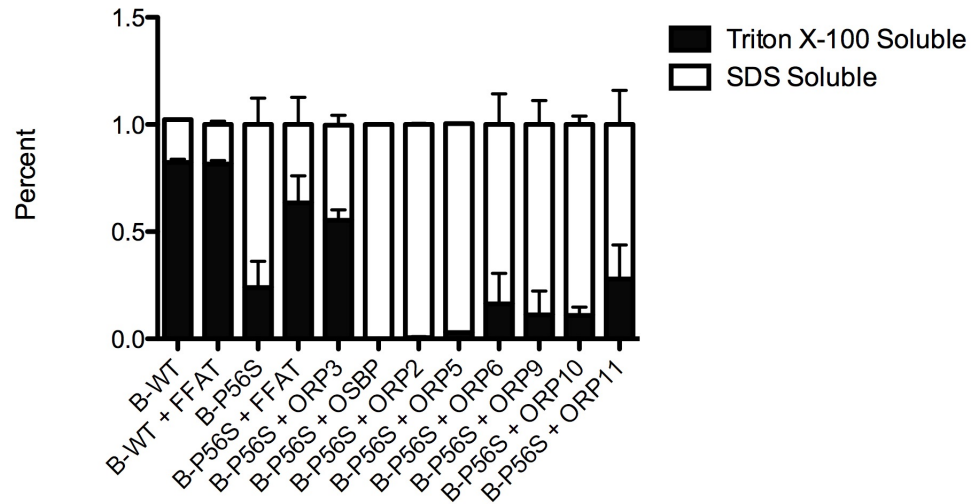
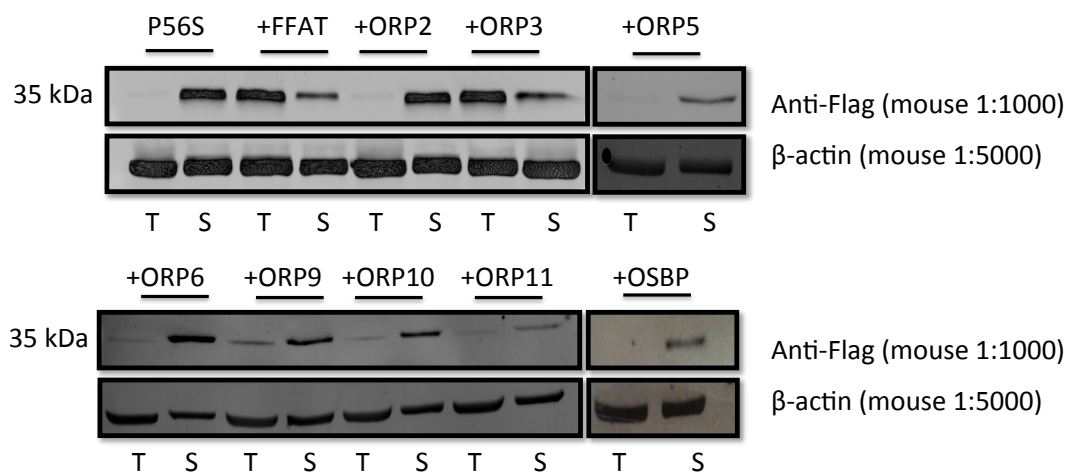


Figure 9. Expression of ORP3 increases solubility of VAPB-P56S.

The VAPB-P56S mutant was co-transfected with each of the ORPs, and then fractionated into either Triton X-100 or SDS soluble fractions as described in the methods section. The VAPB-WT protein was 80% in the Triton X-100 fraction, while 23% of the VAPB-P56S protein was in the Triton X-100 fraction. Co-expression of the FFAT motif shifted 63% of the VAPB-P56S cells into Triton X-100 fraction while ORP3 shifted 61% of the protein into the Triton X-100 fraction. Overexpression of the ORPs had no effect on VAPB-P56S solubility. Error bars represent S.E.M.



T = Triton x 100 soluble fraction
S = SDS soluble fraction

Figure 10. Solubility of VAPB-P56S protein when co-expressed with FFAT motif or one of the eight ORPs. Representative western blot showing solubility of the VAPB-P56S protein when co-transfected with the ORPs. Only the FFAT motif and ORP3 were able to shift VAPB-P56S into the Triton X-100 soluble fraction significantly. β -actin was used as a loading control.

Expression of ORP3 restores retrograde transport to nuclear envelope (EMERIN)

Previous work in the Ngsee lab has shown that nuclear transport is disrupted in cells overexpressing VAPB-P56S. Overexpression of VAPB-P56S causes membrane expansions that trap nucleoporins like Emerin in the insoluble aggregates. Cells expressing shRNA against VAPB do not contain membrane expansions, however, they do result in a loss of transport to the nuclear envelope. Nucleoporins like Emerin are mis-localized in the cytoplasm, indicating that VAPB is necessary for their transport from the ERGIC compartment to the nuclear envelope (Tran et al., 2012). Co-expression of the FFAT motif disrupts the membrane aggregates to restore transport to the nuclear envelope. It is currently unknown whether any of the ORPs have this same effect. I used endogenous Emerin as a marker to assess whether any of the full-length ORPs can rescue the VAPB-P56S phenotype. Emerin is a membrane protein that localizes to the inner nuclear membrane where it associates with lamins to maintain structure of the nuclear envelope. In VAPB-WT cells, Emerin appears as a ring around the nuclear envelope. In VAPB-P56S cells, Emerin is either absent from the nuclear envelope (loss of signal) or it accumulates in cytoplasmic puncta. To determine if the endogenous Emerin signal is restored to the nuclear envelope in VAPB-P56S cells co-expressing the different ORPs, the VAPB-P56S mutant was co-transfected with each ORP into HeLa cells. The cells were fixed and stained for anti-Flag and Emerin and the intensity of the Emerin signal was scored as being nuclear > cytoplasmic, cytoplasmic > nuclear, or no nuclear signal.

Figure 11 and 12 show the Emerin distribution in the HeLa cells. All VAPB-WT cells had a nuclear only signal, while the VAPB-P56S cells showed the majority of Emerin in cytoplasmic puncta. Co-expression of ORP3 shifted Emerin back to the nucleus, while co-expression of the other ORPs did not (data not shown).

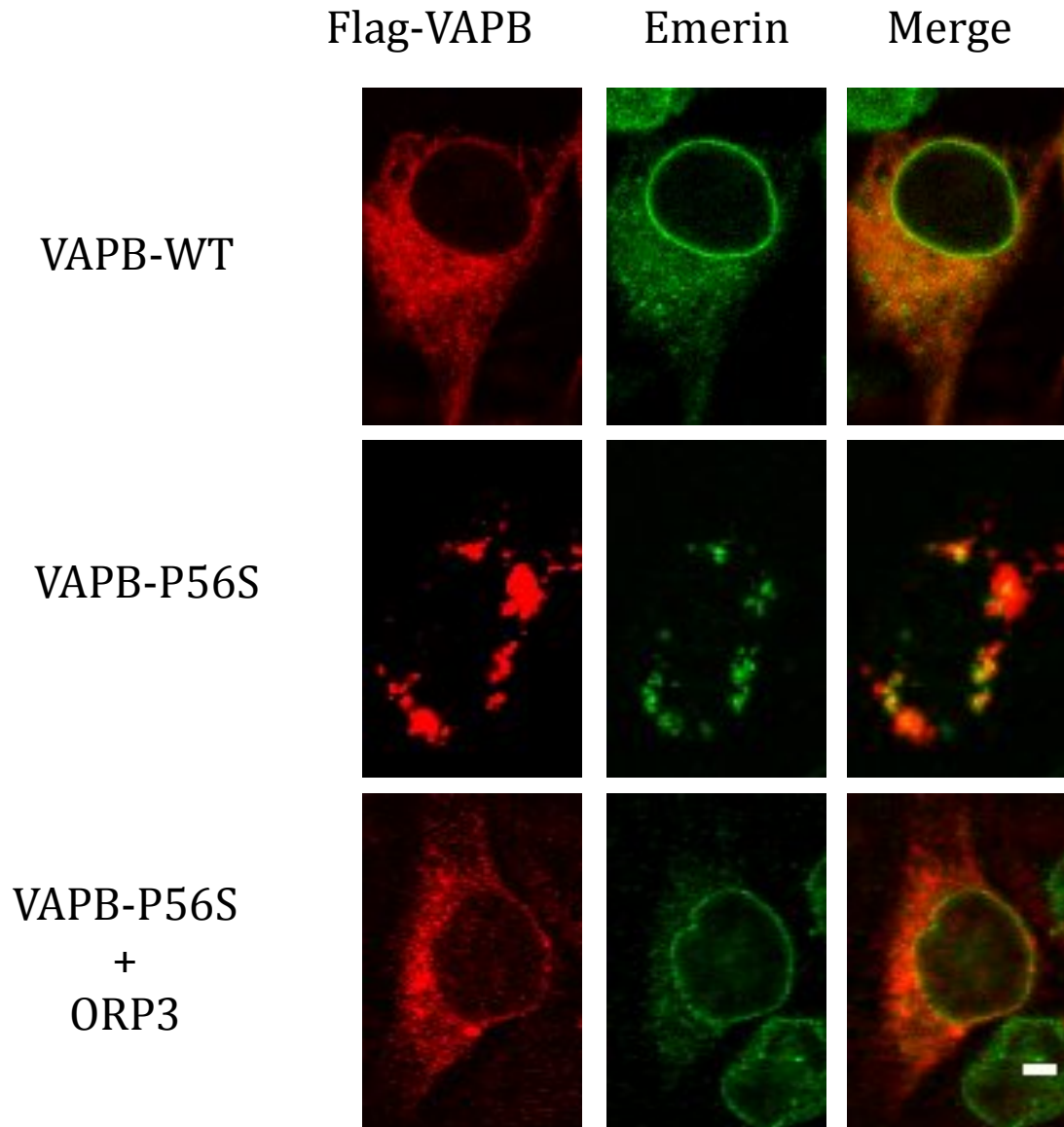


Figure 11. Expression of ORP3 rescues VAPB-P56S-induced disruption of nuclear envelope trafficking. Endogenous Emerin staining in VAPB-WT cells shows a nuclear ring (top panel). HeLa cells transfected with VAPB-P56S mutant show a defect in transport to the nuclear envelope (middle panel). Co-expression of the VAPB-P56S protein with ORP3 rescues the defect in transport of Emerin to the nuclear envelope (bottom panel). Scale bar = 10 μ m

Endogenous Emerin signal distribution in HeLa cells transfected with VAPB-WT, VAPB-P56S or VAPB-P56S + ORP3

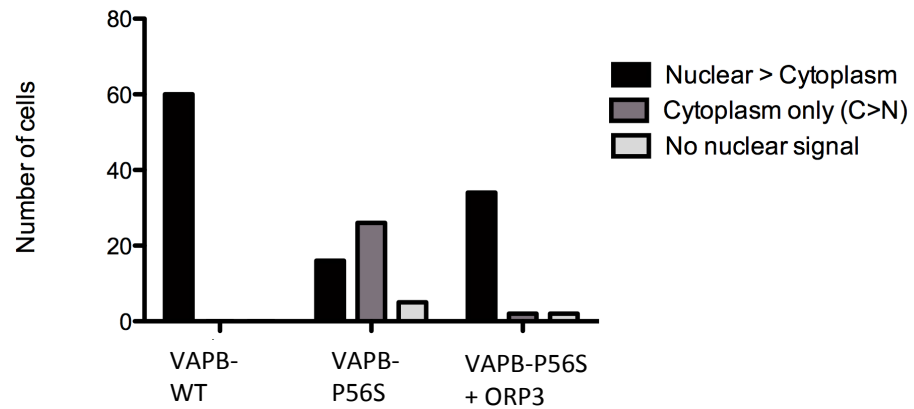


Figure 12. VAPB-P56S disrupts nuclear transport-Expression of ORP3 rescues phenotype. All VAPB-WT cells had a nuclear only signal (60/60 cells), while VAPB-P56S expressing cells showed the majority of Emerin signal in cytoplasmic puncta (26/50) and a small number with no ring present (5/50 cells). Co-expression of ORP3 shifted the signal back to the nucleus (34/38), while co-expression of the other ORPs did not (data not shown).

Activation of ORP3 with 25-Hydroxycholesterol further reduces size of membrane expansions

Binding of oxysterols such as 25-hydroxycholesterol has been suggested to cause a conformational change in OSBP that exposes the PH domain, allowing it to bind phosphatidylinositols in non-ER membranes. Each ORP has a different target membrane (TABLE 2), with ORP3 being targeted to membranes rich in PI4P. ORPs are able to modify lipid content and therefore affect morphology of membranes, which may change the function of a membrane compartment. In order to test if oxysterol/ligand-bound “activated” ORP3 can further reduce the size of the ER membrane expansions, HeLa cells transfected with the VAPB-P56S mutant together with the FFAT motif, OSBP1, ORP3, ORP9, ORP11 or empty pCMV2 control plasmid were stimulated with 2.5 µg of 25-hydroxycholesterol (25-OH) for 4 hours (Ridgway and Lagace, 1995), or DMSO (0.2%) as a control. Results are shown in Table 3 below.

Cell	Mean size of expansions in cells treated with DMSO (Mean $\pm$ SEM)	Mean size of expansions in cells treated with 25-Hydroxycholesterol (Mean $\pm$ SEM)	p-value between DMSO and 25-Hydroxycholesterol treatments
P56S + CMV2 vector	1.55 $\mu\text{m} \pm 0.09$	1.25 $\mu\text{m} \pm 0.05$	0.003
P56S + FFAT	1.07 $\mu\text{m} \pm 0.09$	1.39 $\mu\text{m} \pm 0.05$	0.013
P56S + ORP3	1.15 $\mu\text{m} \pm 0.06$	0.90 $\mu\text{m} \pm 0.03$	0.002
P56S + OSBP1	1.60 $\mu\text{m} \pm 0.09$	1.62 $\mu\text{m} \pm 0.07$	0.89
P56S + ORP9	1.46 $\mu\text{m} \pm 0.08$	1.28 $\mu\text{m} \pm 0.04$	0.045
P56S + ORP11	1.62 $\mu\text{m} \pm 0.17$	1.26 $\mu\text{m} \pm 0.014$	0.013

Table 3. Size of ER membrane expansions in cells treated with 25-Hydroxycholesterol for 4 hours.

The size of membrane expansions were reduced significantly when VAPB-P56S overexpressing cells were stimulated with 25-OH. This may be due to activation of endogenous ORPs, or due to inhibition of cholesterol synthesis. There was a similar reduction in aggregate size when ORP9 and ORP11 were overexpressed, but no reduction with OSBP1 or the FFAT motif. The FFAT motif may be competitively inhibiting other ORPs from binding to the MSP domain of VAPB. Cells overexpressing OSBP1 did not show any change in the size of aggregates compared

to the DMSO control. Size of membrane expansions was significantly reduced in cells overexpressing ORP3 upon stimulation by 25-OH (Figure 13), however the number of aggregates were unchanged or slightly increased after treatment with 25-OH (Figure 14).

Size of membrane expansions after 4 hrs of 25-Hydroxycholesterol treatment in de-lipidated media

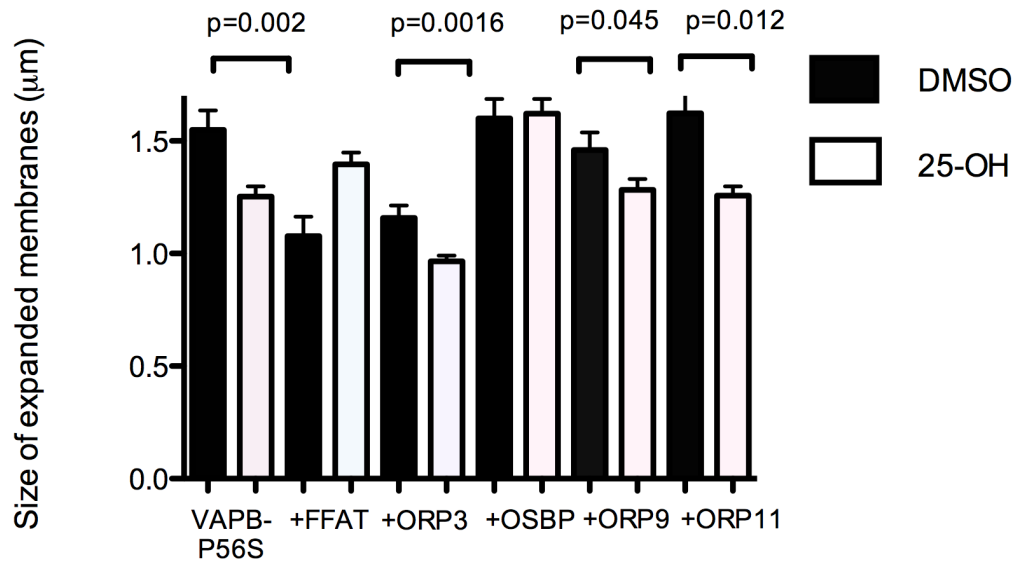


Figure 13. 25-hydroxycholesterol treatment (2.5 µg/ml) for 4 hours significantly reduced the size of membrane expansions in VAPB-P56S cells. Treatment with 25-OH reduced the size of membrane expansions in VAPB-P56S cells from 1.55 µm ± 0.09 S.E.M. to 1.25 µm ± 0.05 S.E.M., p=0.0026. Cells co-transfected with VAPB-P56S and FFAT had larger expansions after treatment than in the DMSO control with an mean size of 1.07 µm ± 0.09 before treatment and 1.39 µm ± 0.05 after treatment with 25-OH. Cells co-transfected with either OSBP1, ORP9 or ORP11 had sizes of 1.60 µm ± 0.09; 1.46 µm ± 0.08 and 1.62 µm ± 0.17 in DMSO and 1.62 µm ± 0.07, 1.28µm ± 0.04 and 1.26 µm ± 0.014 after treatment with 25-OH respectively. Only ORP3 showed a further reduction in membrane expansion size after treatment with 25-OH. Mean size of aggregates was 1.15 µm ± 0.06 with DMSO treatment and 0.90 µm ± 0.03 after treatment with 25-OH, p = 0.002.

**Number of VAPB-P56S membrane expansions per cell
after treatment with 25-OH or DMSO**

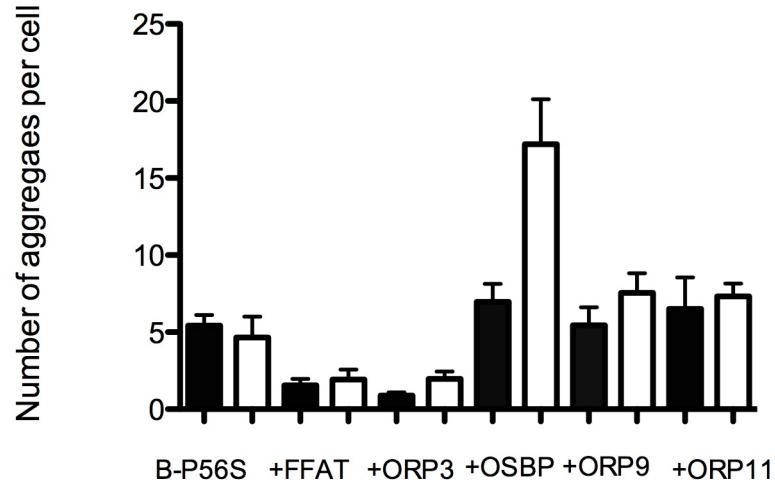


Figure 14. Number of membrane expansions per cell after treatment with 25-OH or DMSO. Cells transfected with B-P56S + empty vector had a mean of 5.4 ± 0.68 aggregates per cell ($>1 \mu m$) in the DMSO treated and 4.66 ± 1.33 in the 25-OH treatment. There was a significant increase in aggregate number in the 25-OH group in cells co-transfected with ORP3 and OSBP; $p = 0.02$ and 0.004 respectively.

Co-localization of VAPB and Sac1

A previous yeast two-hybrid screen identified the conserved phosphoinositide phosphatase Sac1 as an interacting partner with VAPB in the ER (Giot et al., 2003). This work was confirmed by a co-immunoprecipitation assay (Forrest et al., 2013) using a Myc-tagged Sac1, and a Flag-tagged VAPB. The two ER membrane proteins interact via the transmembrane domain of VAPB and the C-terminal transmembrane of Sac1 (Forrest et al., 2013). These researchers also confirmed that the MSP domain of VAPB was dispensable for Sac1 binding, suggesting that mutant VAPB-P56S could still interact with Sac1. To determine whether VAPB-WT and VAPB-P56S co-localize with Sac1 in mammalian cells, a Sac1-GFP plasmid was co-transfected with VAPB-WT and VAPB-P56S as described in the methods section. Co-localization analysis shows that VAPB-WT co-localizes with Sac1 in the ER with a Pearson's correlation coefficient of 0.73, and 0.82 for VAPB-P56S. Co-expression of the FFAT motif and ORP3 resolved many of the membrane expansions, but Sac1 continues to co-localize with a Pearson's correlation coefficient of 0.83 and 0.76 respectively (Figure 15). This data indicates that Sac1 does co-localize and possibly interact with VAPB in mammalian cells. Restoring VAPB distribution to WT-like patterns with the FFAT motif or ORP3 promotes localization with Sac1.

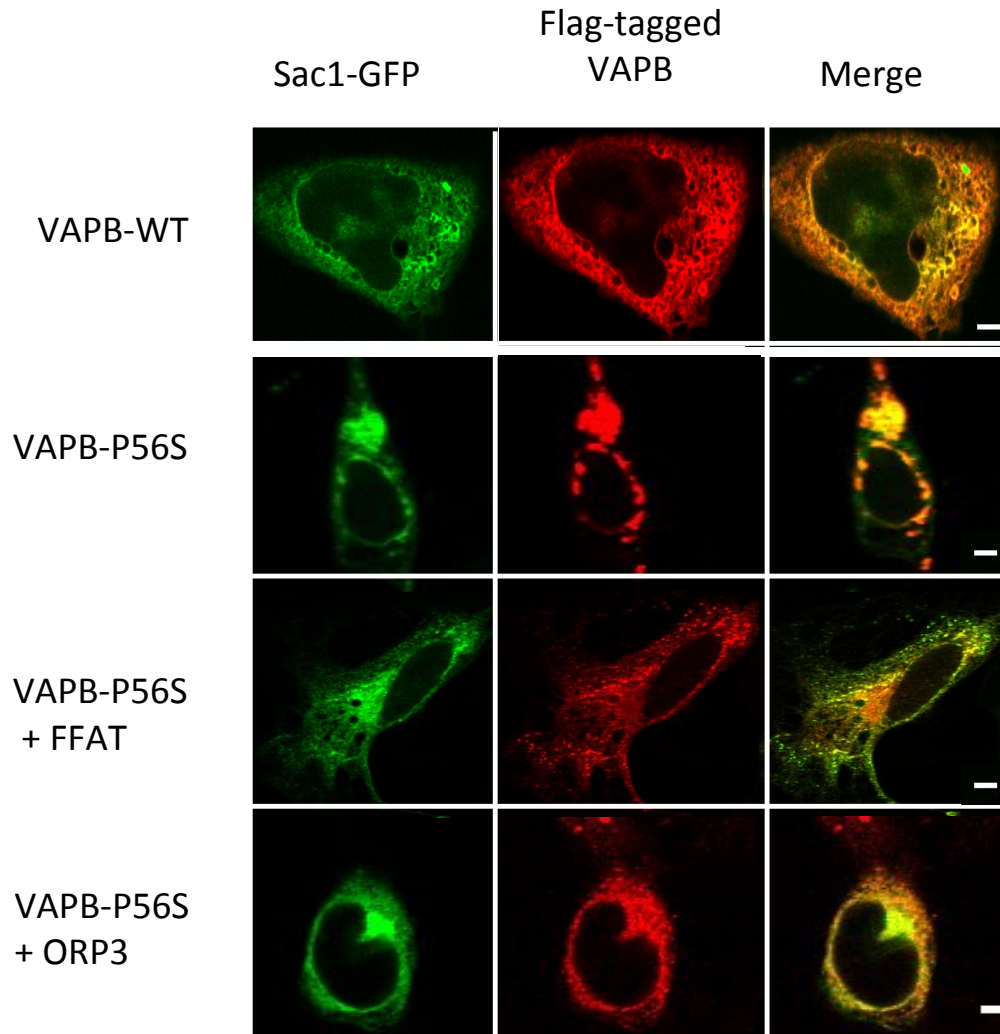


Figure 15. Sac1-GFP co-localizes with VAPB.

HeLa cells were co-transfected with Sac1-GFP and VAPB-WT, VAPB-P56S, or VAPB-P56S plus FFAT or ORP3. Sac1-GFP is shown in green (left panel), VAPB-P56S is shown in red (middle panel), and a merge of the two channels on the right. Scale bar = 10 μ m.

Sac1-GFP co-localization with FLAG-tagged VAPB

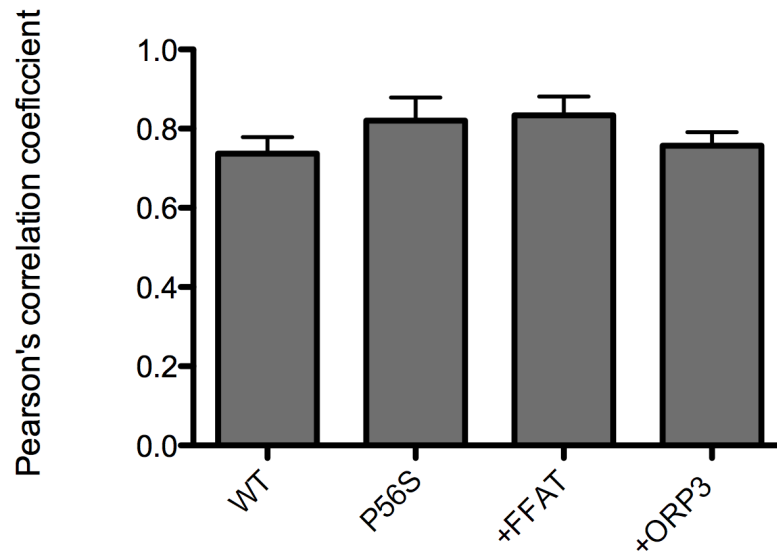


Figure 16. Pearson's correlation coefficient was performed using Image J for co-localization analysis. A value of 1 indicates perfect co-localization, zero is random co-localization and -1 indicates no co-localization. Sac1-GFP + VAPB-WT had a value of 0.73, while Sac1-GFP + VAPB-P56S, FFAT and ORP3 had values of 0.82, 0.83 and 0.76 respectively, indicating co-localization.

VAPB knockdown causes mis-localization of Sac1

In order to determine if the VAPB-P56S membrane expansions were non-specifically sequestering the Sac1 phosphatase, VAPB was knocked down in HeLa cells as described in the methods section. Instead of the normal ER/Golgi distribution (Figure 17a), Sac1- GFP was clustered around the nuclear envelope in the Golgi/ERGIC compartments (Figure 17b and c). Sac1- GFP co-localizes with an ERGIC marker (ERGIC-53), indicating that Sac1 also localizes to the ERGIC (Figure 17d and 22).

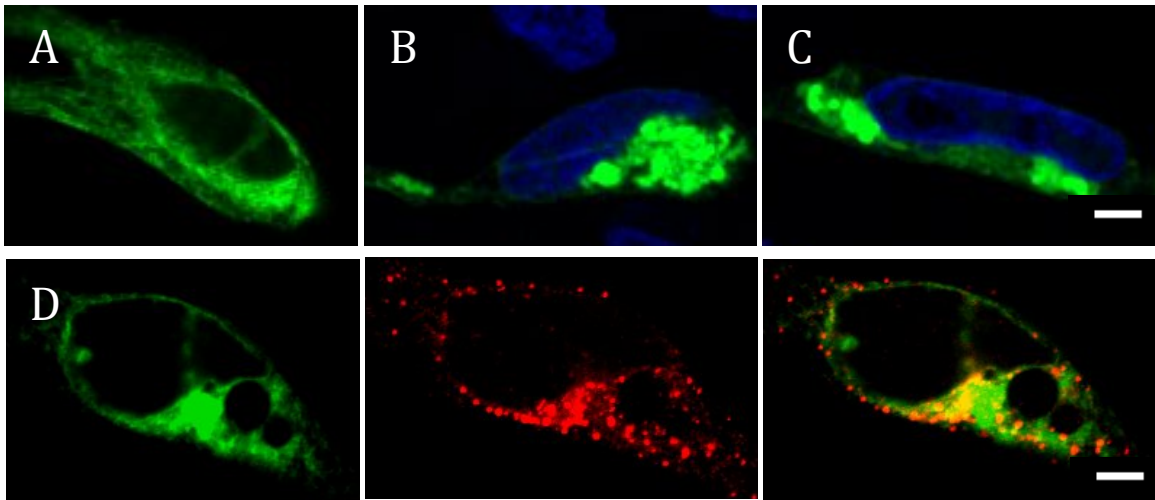


Figure 17. VAPB knockdown disrupts localization of Sac1.

Panel A shows HeLa cell co-transfected with Sac1-GFP plus empty pLKO.1 vector. Sac1-GFP is localized to the peripheral ER and Golgi. In cells lacking VAPB (Panels B and C) Sac1-GFP is no longer in the peripheral ER, but clusters in the ERGIC region around the nuclear envelope (panel D). This indicates that VAPB is necessary for the distribution of Sac1-GFP in the ER. Scale bar = 10 μ m.

ORP3 expression in HeLa cells

As ORP3 appears to play a role in VAPB function, in future experiments shRNA against ORP3 may be used. I therefore checked for expression of ORP3 in HeLa and HEK293 cells. The results shown in Figure 18 indicate that ORP3 is expressed in both HeLa and HEK293 cells.

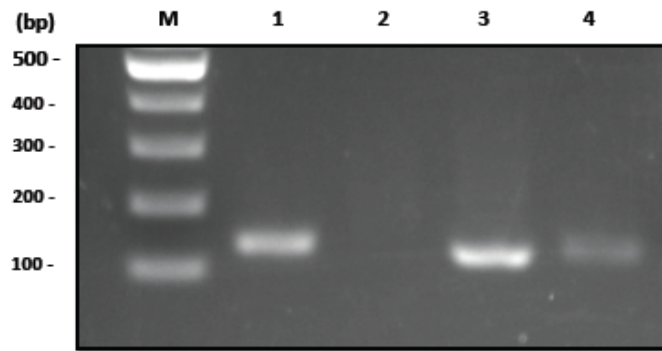


Figure 18. Expression of ORP3 in HeLa cells. RNA was extracted from HeLa and HEK 293 cells for reverse transcription and subsequent amplification with an ORP3 specific primer in PCR. Expected amplicon size is 119 base pairs in size. Lane 1, the positive control shows the expected amplicon from the pCMV-SPORT6-ORP3 plasmid. Lane 2, the negative control is HeLa cell cDNA amplified with just the forward primer. Lane 3 shows amplification of ORP3. In HeLa cells, while lane 4 shows ORP3 amplification in HEK293 cells.

Efficiency of Sac1 knockdown

A set of shRNA cDNA plasmids against Sac1 were obtained from Open Biosystems. To determine which of the shRNA clones was most effective, HeLa cells were transfected with Sac1-GFP and each of the shRNA clones or an empty pLKO.1 vector. Unfortunately there is currently no Sac1 antibody so Sac1-GFP was used. Cells were harvested and protein was run on an SDS PAGE gel, followed by western blotting and probing with an anti-GFP antibody. For Sac1, clones 62788, 62791 and 62792 were most effective at reducing the GFP signal and therefore 62792 was used in all cell culture experiments (Figure 19).

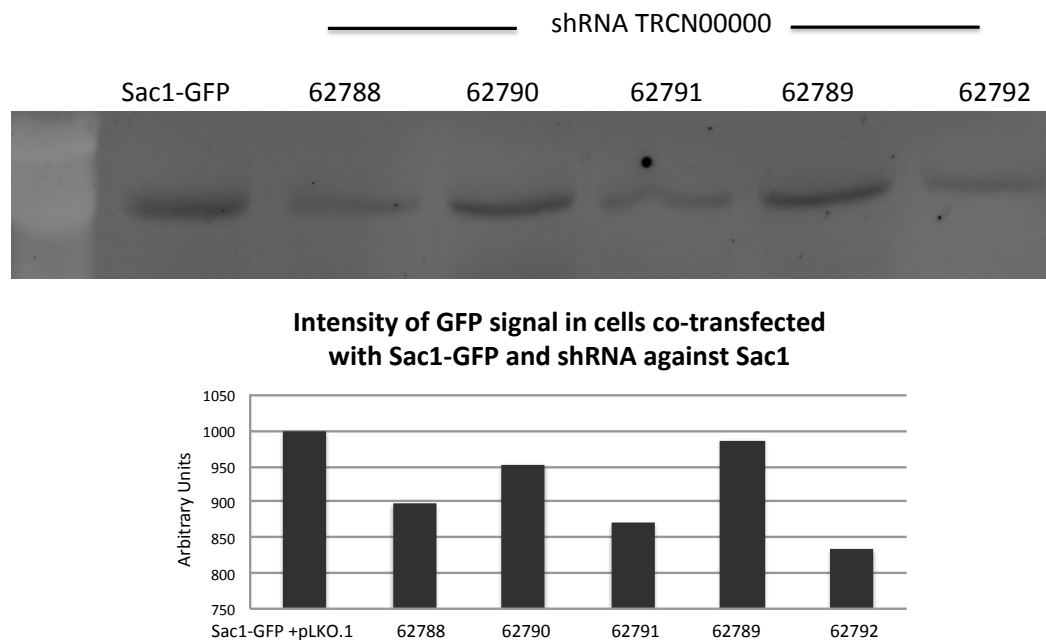


Figure 19. Efficiency of Sac1 Knockdown.

HeLa cells were transfected with Sac1-GFP and each of the shRNA clones or an empty pLKO.1 vector. Cells were harvested and protein was run on an SDS PAGE gel, followed by western blotting and probing with an anti-GFP antibody. Clones 62788, 62791 and 62792 were most effective at reducing the GFP signal. These results show that shRNA against Sac1 does target the sequence, however, we cannot conclude what the endogenous levels are after the knockdown.

PI4P levels are increased in mammalian VAPB and Sac1 knock-down cells

Previous work in yeast has shown that the VAP homologues are important ER/PM tethering proteins, which bring the ER in close apposition to the PM (Manford et al., 2012). It has been proposed that in yeast VAPs bridge the ER to the PM so ER membrane protein Sac1 can dephosphorylate PI4P to regulate levels in non-ER membranes (Stefan et al., 2011). Indeed, Manford et al, found that knocking down either VAP or Sac1 resulted in dramatically increased levels of PI4P in the cell due to reduced Sac1 activity. When the VAP orthologues Scs2 and Scs22 in yeast were deleted, the peripheral ER collapsed and aggregated around the nucleus. Therefore, Sac1 was unable to reach the PM to modulate levels of PI4P. In order to determine if levels of endogenous PI4P are increased in mammalian cells, HeLa cells were transfected with shRNA against either VAPB or Sac1 and stained with a PI4P antibody. mCherry was used as a transfection control. Images were captured with a fixed gain, and PI4P pixel intensities of individual cells were measured. The same pattern was observed in mammalian cells, with a significant increase in PI4P in cells lacking either VABP or Sac1 (Figure 20 and 21). The mean intensities and fold increases are shown in the table below. shVAPB and shSac1 cells had significantly increased levels of PI4P, compared to control pLKO.1 values $p < 0.0001$ in both cases.

	Mean Intensity $\pm$ SEM	Fold increase
pLKO.1	35649 $\pm$ 2310 (n=32)	0
mCherry	16825 $\pm$ 988 (n=52)	0
shVAPB	52200 $\pm$ 2445 (n=63)	1.5
shSAC1	74110 $\pm$ 7193 (n=30)	2.1

Intensity of PI4P antibody in transfected HeLa cells

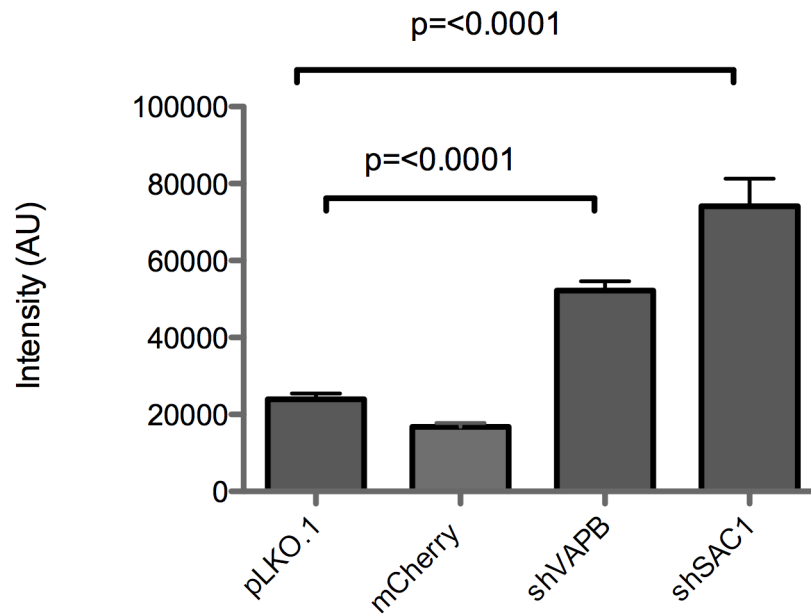


Figure 20. Intensity of endogenous PI4P signal in HeLa cells.

HeLa cells were transfected with empty pLKO.1 vector, shVAPB or shSAC1 and then stained with an anti-PI4P antibody. Cells lacking VAPB or Sac1 have significantly increased levels of PI4P compared to the pLKO.1 control.

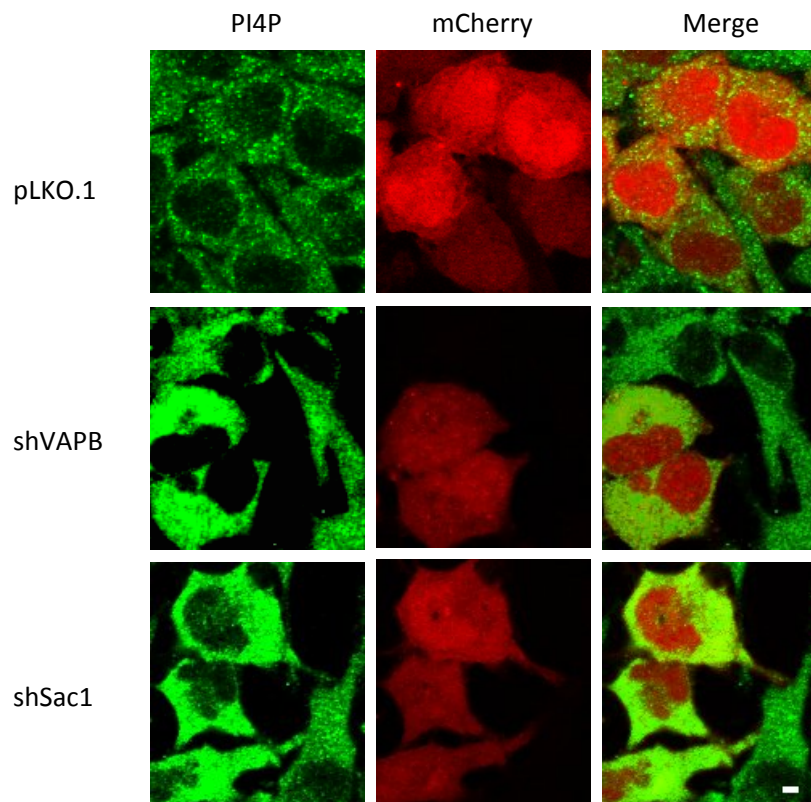


Figure 21. Intensity of endogenous PI4P signal in HeLa cells lacking VAPB or Sac1. Cells transfected with either shVAPB or shSac1 are mCherry positive (red). The endogenous PI4P signal is increased in the mCherry positive cells, compared to neighbouring untransfected cells and the empty pLKO.1 plasmid. Scale bar =10 μ m.

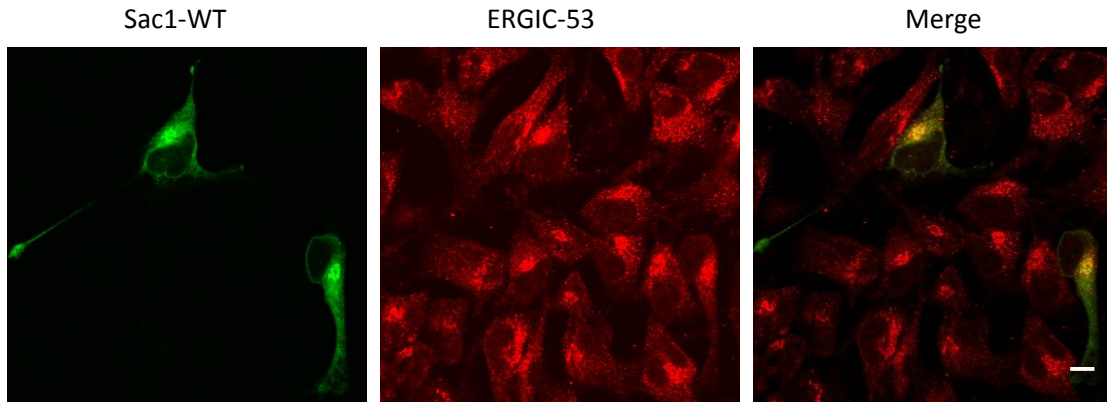
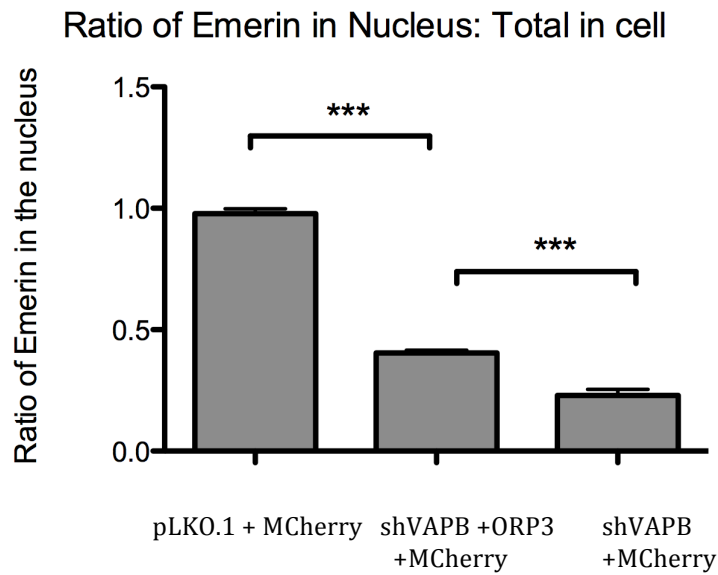


Figure 22. Sac1-WT-GFP co-localizes with ERGIC marker. Sac1-GFP (shown in left panel) was transfected into HeLa cells. Cells were fixed and stained with anti-ERGIC-53 antibody (shown in red, middle panel). Right panel shows merge of two channels. Sac1 co-localizes with the ERGIC-53 marker. Scale bar = 10 μm .

Overexpression of ORP3 partially rescues Emerin transport defects in VAPB knockdown cells

To investigate whether ORP3 overexpression could rescue the effects of the VAPB knockdown, HeLa cells were transfected with shRNA against VAPB plus ORP3. Untransfected HeLa cells show 100% of Emerin signal in the nucleus. Cells lacking VAPB show a defect in Emerin localization, with large puncta in the cytosol and a loss of the nuclear signal. Cells transfected with shRNA against VAPB show only 20% of Emerin in the nucleus, and 80% in the cytoplasm. VAPB knockdown cells overexpressing ORP3 have 40% in the nucleus, indicating a partial rescue (Figure 23).

A



B

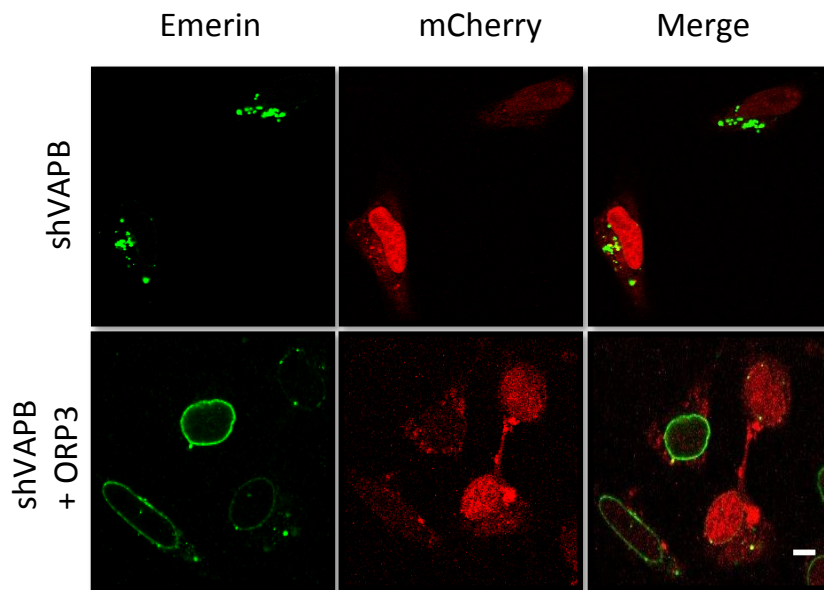


Figure 23a. ORP3 Partially rescues Emerin transport defect in VAPB knockdown cells. Untransfected HeLa cells show 100% of Emerin signal in the nucleus. Cells transfected with shRNA against VAPB show only 20% in the nucleus (top panel), and 80% Emerin in the cytoplasm. **Figure 23b.** VAPB knockdown cells show clustered Emerin distribution in the cytosol (top panel). Overexpressing ORP3 partially rescues the Emerin distribution around the nuclear envelope, with 40% in the nucleus (bottom panel). Scale bar = 10 μ m

Discussion

ORP3 reduces size and number of ER membrane expansions in VAPB-P56S cells

The primary goal of this study was to find an effective way to reduce the detrimental effects of the mutant VAPB aggregates. Insoluble aggregates not only contain VAPB-P56S, but also the WT VAPB, further leading to a loss of VAPB function. The FFAT motif from OSBP1 alone is able to reduce the size and number of ER membrane expansions in mutant cells (Prosser et al., 2008).

This raises the possibility that one of the endogenous FFAT-motif-containing proteins may have a similar effect at reducing the aggregates in mutant cells *in vitro*. The oxysterol binding protein family represents a large group of proteins, which contain a FFAT motif known to bind the MSP domain of VAPB (Mikitova and Levine, 2012). Our ORP screen results show that ORP3 in particular is most effective at reducing the size and number of ER membrane expansions in cell culture (Figure 6 - 8). Though other proteins not involved in this study may contain a FFAT motif, the ORP family is the largest group of FFAT-motif-containing proteins, and therefore was the focus of this study. Previous groups have reported that ORP3, ORP6, ORP9 and the Nir2 protein interact with VAPB via the FFAT motif (Teuling et al., 2007; Kuijpers et al., 2013). Interestingly, ORP3 was confirmed as an interacting partner with VAPB by pull down methods and mass spectrometry in HeLa cells, where it was given the highest coverage score (Kuijpers et al., 2013). ORP6 and ORP9 did not show a significant reduction in size of VAPB aggregates or number of aggregates per cell, suggesting ORP3 is the most likely ORP to regulate VAPB function. Though all the ORPs were expressed using the same promoter, expression levels may vary due to copy number, protein stability and other factors. However, the lack of suitable antibodies precluded accurate comparisons. In the future, different concentrations of ORP proteins could be expressed to determine if any others exhibit these benefits if expression levels were higher. In all subsequent experiments, the efficacy of ORP3

was compared to the FFAT motif, as Prosser et al. (2008) found it rescued the VAPB-P56S phenotype and restored transport of Nups to the nuclear envelope (Tran et al., 2012). Other researchers have reported conflicting results with the use of the FFAT motif. The Ngsee group uses an N-terminal myc-tagged FFAT motif from OSBP1, whereas Teuling et al. (2007) used a FFAT motif from Nir2 fused to the C-terminus of GFP and saw no rescue when co-expressed with VAPB. This phenomenon may be due to the large C-terminal placement of the GFP tag, which may interfere with binding to the MSP domain of VAPB. The N-terminal Myc-tag is small and may not interfere with VAPB binding. Further, Teuling et al. (2007) expressed an ORP3-GFP construct with VAPB-P56S and reported no co-localization or rescue of the VAPB phenotype. They concluded that VAPB-P56S was unable to bind FFAT-motif containing proteins, although it was later shown that VAPB-P56S has the ability to bind to FFAT-containing proteins (Kuijpers et al., 2013). These results suggest that the GFP moiety may profoundly affect the overall structure of ORP or accessibility to the FFAT motif.

ORP3 and the FFAT motif increase solubility of the VAPB-P56S protein

The aberrant VAPB-P56S structure is highly aggregate prone, forming stable aggregates that are insoluble in non-ionic detergent (Kanekura et al., 2006; Teuling et al., 2007). Thus, solubility in non-ionic detergent is an indicator of mutant VAPB aggregation. To further understand how ORP3 may be rescuing the phenotype of the VAPB-P56S mutant, I investigated the solubility of VAPB-P56S when co-expressing the FFAT motif or the ORPs (Figure 9 and 10). Co-expression of either the FFAT motif or ORP3 shifted the VAPB-P56S from the Triton X-100 insoluble fraction to the soluble fraction. Since the FFAT motif is known to bind to the MSP domain of VAPB (Prosser et al., 2008), these results suggest that the FFAT motif and ORP3 may be binding to the MSP domain to prevent further aggregation and increase solubility of newly synthesized protein. It is likely that binding to the FFAT motif or ORP3 stabilizes the MSP conformation and prevents aggregate formation rather than breaking up already formed aggregates since the MSP domain is inaccessible to

FFAT in aggregated mutant VAPB (Teuling et al., 2007). None of the other ORPs were able to shift the VAPB-P56S protein into the Triton X-100 soluble fraction. In particular, OSBP1 from which the FFAT motif was derived did not cause a solubility shift. This suggests that OSBP1 behaves differently as a full-length protein, perhaps due to influences from the PH domain or oxysterol-binding domain, which targets OSBP1 to the Golgi. The results of the solubility experiments matched the immunocytochemistry in that co-expression of OSBP1 actually increased the number of aggregates per cell causing large insoluble ER membrane expansions. The role of OSBP1 will be discussed further below. On the other hand, ORP3 and the FFAT motif decreased the size and number of ER membrane expansions, likely by increasing their solubility.

ORP3 restores transport of Emerin to the nuclear envelope

The VAPB-P56S mutant causes a disruption in transport to the nuclear envelope (Tran et al., 2013). Both soluble (Nup-214) and membrane (Emerin and GP210) Nups were found to accumulate in expanded ERGIC compartments when expressed with the VAPB-P56S protein. This was accompanied by a loss of signal at the nucleus. This phenomenon was also observed in cells lacking VAPB, where Nups accumulated in cytoplasmic puncta (Tran et al., 2013). Prosser et al. (2008) previously identified that the VAPB-P56S mutation has little effect on anterograde transport, however retrograde transport from the Golgi to the ER or nuclear envelope may be affected (Tran et al., 2012). Ngsee's group proposes that nucleoporins travel from the ER to the ERGIC/Golgi for assembly and then back to the nuclear envelope, which is continuous with the ER. If VAPB is necessary for retrograde transport from the ERGIC to the nuclear envelope, then a loss of VAPB either by shRNA knockdown or due to its inclusion in large aggregates will result in Nups becoming trapped in expanded ERGIC compartments.

Co-expression of the FFAT motif was previously found to rescue defective transport to the nuclear envelope. In a similar manner, expression of ORP3 allowed transport of Emerin to the nuclear envelope (Figure 11 and 12). This again may be

due to the ability of ORP3 and the FFAT motif to competitively bind the mutated MSP domain, preventing aggregation of some of the VAPB protein to allow transport to occur. Interestingly, overexpression of ORP3 in cells transfected with shRNA against VAPB show a partial rescue of Emerin to the nuclear envelope. The VAPB knockdown with RNAi may not be complete and any residual VAPB remaining in the cells will be acted upon by ORP3. Because ORP3 does not fully rescue the Emerin transport defect, it suggests that ORP3 may act in the same pathway as VAPB, and both need to be present for the transport defect to be corrected.

Activating ORP3 with 25-Hydroxycholesterol further reduces size of membrane expansions in VAPB-P56S cells

Due to the observation that the full length OSBP1 did not shift the solubility of VAPB-P56S or reduce the size or number of ER membrane expansions, the function of the full-length protein was investigated. 25-hydroxycholesterol is known to activate OSBP1 and other ORPs (Lehto and Olkkonen, 2003). ORPs are cytoplasmic proteins that upon stimulation with 25-OH are translocated to intracellular membranes, where the PH domain binds phosphatidylinositols (Lehto and Olkkonen, 2003). I next wanted to determine if stimulating the ORPs with 25-OH would alter the VAPB-P56S aggregation phenotype. In HeLa cells transfected with VAPB-P56S, activation with 25-OH significantly reduced the size of the membrane expansions, but had no effect on the number of aggregates per cell. This is likely due to stimulation of all endogenous ORPs including ORP3, which is expressed in HeLa cells (Figure 18). When cells transfected with VAPB-P56S and the FFAT motif were stimulated with 25-OH, the size of the membrane expansions were larger than in the DMSO control treated cells (Figure 13 and 14). This suggests that the FFAT motif is competitively inhibiting binding of endogenous ORPs and therefore interferes with recruitment of activated ORP recruitment to VAP. In VAPB-P56S cells co-transfected with either ORP9 or ORP11, activation with 25-OH had no further impact on aggregate size compared to the VAPB-P56S cells alone (Figure 13). Interestingly, in cells co-transfected with OSBP1, the size of membrane

expansions were slightly larger than the VAPB-P56S aggregates alone and the number of aggregates were significantly increased after activation with 25-OH (Figure 14). One interesting possibility is that activation of OSBP1 by 25-OH increases activation of PI4 KinaseII α (PI4KII α), which increases the levels of PI4P in the Golgi apparatus to recruit the ceramide transfer protein and increase sphingomyelin synthesis (Banerji et al., 2010). This is in contrast to ORP3, which is known to activate the Sac1 PI4P phosphatase to decrease levels of PI4P in the Golgi and at membrane contact sites (Stefan et al., 2011). When ORP3 was activated with 25-OH, size of the membrane expansions were reduced further compared to the DMSO treated cells, which suggests that activated ORP3 reduces the VAPB-P56S membrane expansions. It is thought that upon activation with 25-OH, ORP3 is recruited to membranes rich in PI4P, primarily the PM, Golgi, and pre-Golgi compartments (Liu et al., 2008) The PH domain of ORP3 is also known to bind PI(3,4)P₂, PI(3,4,5)P₃, and PI(3,5)P₂ to a lesser extent (Lehto et al., 2005), all of which are localized primarily in the PM. In yeast, the ORP3 homologue Osh3 is known to activate Sac1 via the oxysterol-binding domain (Stefan et al., 2011) to negatively regulate Golgi levels of PI4P, which attenuates anterograde transport to the plasma membrane (Rohde et al., 2003). VAPB and Sac1 are known binding partners in *Drosophila*, and it has been hypothesized that Osh3 binds VAP and bridges Sac1 to the plasma membrane, or other non-ER membranes, where it dephosphorylates PI4P (Stefan et al., 2011; Forrest et al., 2013). Indeed, it has been shown in *Drosophila* that DVAP and Sac1 together regulate the levels of PIs in neurons, and that expression of VAPB-P58S or down-regulation of either dVAP or Sac1 causes an increase in phosphoinositide levels, which causes neurodegeneration in a dose dependent manner (Forrest et al., 2013). This degeneration could be reversed by down-regulation of the PI4P kinases in *Drosophila*, the *PI4KIII α* and *Four-wheel drive (Fwd)*. Thus, disruptions in the PI4P gradient may lead to unregulated ERGIC expansion. The activation cascade that starts with activation of ORP3 by 25-OH to activation of Sac1 may help restore the gradient of PI4P in the cell (Figure 25 - 28).

VAPB and Sac1 co-localize in mammalian cells

We next investigated the distribution of Sac1 in cells transfected with the VAPB-P56S mutant. Forrest et al. (2013) confirmed that Sac1 is a binding partner of DVAP in *Drosophila*. DVAP and Sac1 are both ER membrane proteins that interact via their transmembrane domains. The MSP domain of DVAP was dispensable for binding to Sac1, and therefore the proline to serine 58 mutation in *Drosophila* has no effect on binding. They also found that both DVAP-P58S and Sac1 were mis-localized from their normal distribution throughout the ER network, and were contained in cytosolic aggregates that co-localized with DVAP-P58S. This mis-localization leads to an increase in PI4P, which lead to neurodegeneration in the eye sac and defects in the synaptic microtubule cytoskeleton (Forrest et al., 2013). In addition, down-regulation of DVAP or Sac1 showed accumulations of proteins and vesicles along the axons, and disruptions in synaptic morphology characterized by abnormal localization of post-synaptic markers such as β -spectrin.

To determine if VAPB-WT and VAPB-P56S co-localized with Sac1 in HeLa cells, a Sac1-GFP plasmid was co-transfected with VAPB-WT or P56S. Sac1 co-localized with VAPB-WT in the ER, and was present in the Golgi and ERGIC compartments (Figure 23). Sac1 was found in large cytosolic aggregates that co-localized with VAPB-P56S in mutant cells (Figure 15). These large cytoplasmic aggregates containing VAPB and Sac1 are consistent with a model in which VAPB and Sac1 are mis-localized from their normal position in the ER, which leads to the inability of Sac1 to maintain levels of PI4P in the ER. The aggregate prone VAPB-P56S leads to a loss of VAPB function in a dominant negative manner. By sequestering Sac1, it may also lead to a loss of its normal function of decreasing PI4P levels in the ER, thus leading to an increase in ER PI4P (will be further discussed below). By co-expressing the FFAT motif and ORP3, the VAPB-P56S aggregates were resolved, restoring the normal distribution of Sac1 in the peripheral ER (Figure 15).

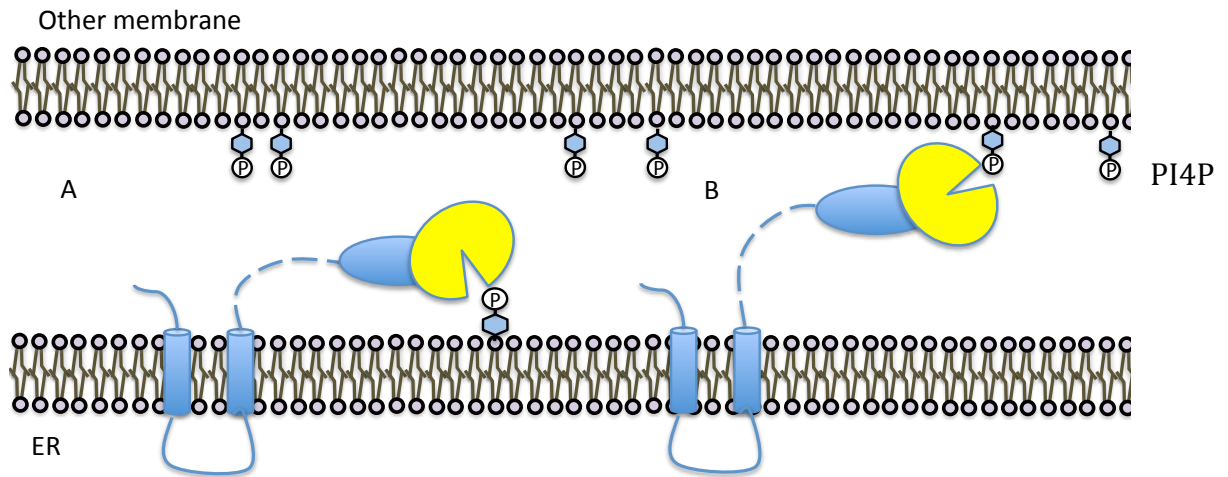


Figure 24. Sac1 PI4P phosphatase resides in the ER

The Sac1 phosphatase is a type II membrane protein which resides in the ER and Golgi. The catalytic domain consists of a C-terminal CX₅R(T/S) motif, which is essential for activity (yellow). The N-terminal domain (blue) is conserved throughout the Sac family, and is thought to be necessary for protein-protein interactions. Mutations in the N-terminus of Sac3 (FIG4 in yeast) can cause familial forms of ALS and Charcot-Marie-Tooth disease type 4 (Manford et al., 2010). The long flexible linker between the catalytic domain and the transmembrane anchor allow Sac1 to hydrolyze its substrate in **A. cis** (at the ER) or in **B. trans** (non-ER membranes). Sac1 has only been shown to act in *trans* in the yeast model.

Because mutant VAPB is aggregate prone, it is possible that Sac1 is sequestered non-specifically by the aggregated protein. To determine whether Sac1 localization is dependent on VAPB, Sac1 was expressed in cells transfected with shRNA against VAPB. Sac1 distribution was affected in the VAPB knockdown cells, where it was clustered around the nucleus instead of spread out in the peripheral ER (Figure 17). This data indicates that VAPB is required for proper ER localization of Sac1, and in cells lacking VAPB, Sac1 is retained in Golgi/ERGIC-like compartments. Sac1 is known to cycle between the ER and Golgi depending on nutrient status (Blagoveshchenskaya et al., 2008). Sac1 is translocated from the ER to the Golgi in quiescent cells or under nutrient stress. In the Golgi, Sac1 de-phosphorylates PI4P to down-regulate anterograde transport, as PI4P is necessary in the trans-Golgi for anterograde trafficking (Blagoveshchenskaya et al., 2008). Upon stimulation by mitogens, Sac1 is transported back to the ER via COPI vesicles. Catalytically inactive mutant Sac1 is trapped in the Golgi, indicating that phosphatase activity is required for interactions with COPI vesicles (Rohde et al., 2003). The observation that Sac1 is trapped in the ERGIC/Golgi region in VAPB knockdown cells supports the idea that VAPB is required for retrograde transport to the ER. Our results indicate that VAPB and Sac1 co-localize in the ER in both VAPB-WT and VAPB-P56S mutant cells. Sac1 is mis-localized and sequestered into ER membrane expansions with the VAPB-P56S protein, likely leading to a loss of ER function of both proteins. A loss of Sac1 function in the ER would then lead to an intracellular increase of PI4P, and a decrease in PI4P in the ERGIC and *cis*-Golgi compartments.

PI4P levels are increased in cells lacking VAPB and Sac1

ER-PM contact sites have been observed in numerous cell types and organisms (Manford et al., 2012). In yeast, the ER is associated with 20-45% of the PM, with an average of 33 nm between the two opposing membranes. Stefan et al. (2011) described a pathway for the regulation of phosphoinositide (PI) lipid turnover at the plasma membrane, catalyzed by an ER membrane protein phosphatase Sac1. Sac1 interacts with VAP in the ER membrane and is brought in

close opposition to adjacent membranes containing PI4P by the ORP family of proteins, in particular ORP3 (Stefan et al., 2011). Thus, junctions between the ER and opposing membranes appear to be sites of lipid metabolism regulation and lipid transport. Manford et al. (2012) found that the VAP homologues in yeast were important tethering proteins, which connect the ER to the PM. Knocking out the VAP homologues in yeast resulted in a collapsed ER that no longer was able to make contacts with the PM. Also, VAP knockdown cells exhibited a 2-fold increase in the levels of PI4P, likely due to the fact that the PM-ER contact sites were disrupted and Sac1 no longer had access to its substrate. Similarly, a loss of Sac1 resulted in a 10-fold increase in PI4P in the plasma membrane of yeast cells (Manford et al., 2012). Interestingly, knockdown of Osh3 in yeast had the greatest effect of PI4P levels, with cells exhibiting an 18-fold increase in PI4P (Manford et al., 2012). Forrest et al. (2013) have recently shown that like yeast, a loss of DVAP or Sac1 in *Drosophila* leads to a significant increase in intracellular PI4P. Sac1 is sequestered into large cytosolic aggregates with the VAPB-P56S mutant, which prevents normal Sac1 distribution and activity, leading to increases in PI4P (Forrest et al., 2013). The increased levels of PI4P lead to neurodegeneration in a dose dependent manner and the loss of Sac1 resulted in the same phenotype seen in DVAP null flies. A loss of both DVAP and Sac1 leads to a decrease in the number of synaptic boutons and show microtubule disorganization. Sac1, DVAP double knockdowns do not show a more severe phenotype, indicating that they function in a common pathway (Forrest et al., 2013). If Sac1 is trapped in Golgi/ERGIC compartments due to a loss of VAPB, it may decrease the levels of PI4P in the trapped compartment in a *cis* configuration (Figure 24), and subsequently increase in other compartments due to loss of Sac1 activity. Decreased PI4P in the Golgi has previously been shown to decrease COPII vesicle fusion with the *cis*-Golgi, as PI4P is essential for vesicle fusion (Lorente-Rodriguez and Barlowe, 2010). Vesicles not able to fuse with the Golgi would return to the ERGIC and cause its expansion, as is seen in VAPB-P56S cells. Or conversely, COPII vesicles may still fuse with the *cis*-Golgi, but do so at a slower rate (discussed below). While this decrease in transport may not have immediate lethal effect possibly due to alternative transport routes or compensatory mechanisms, it may

affect cell viability over the span of a life-time as these compensatory mechanisms decrease with ageing, leading to the late onset motor neuron degeneration characteristic of ALS. It has been proposed that the large ER membrane expansions observed in VAPB-P56S cells may be due to increased lipid production in the ER. Both mice and humans with ALS have been reported to have alterations in lipid metabolism (Ikeda et al., 2012; Yang et al., 2013; Bouteloup et al., 2009; Marques et al., 2006; Paganoni and Wills, 2013; Dupuis et al., 2008; Wang et al., 2012). One therapeutic avenue is to increase expression of ORP3, which stimulates Sac1 to reduce PI4P levels in the ER, and therefore reduce the size of the membrane expansions. Activation with 25-OH is known to inhibit cholesterol synthesis (Nishimura et al., 2005; Adams et al., 2004) and may also act to further reduce the expansions. Unfortunately, due to the toxic profile of 25-OH, it would not be a candidate for treatment (Morin and Peng, 1989).

Overexpression of ORP3 partially rescues the Emerin transport defect in VAPB knockdown cells

In *Drosophila*, Forrest et al. (2013) determined that Sac1 and DVAP function in a common pathway, as Sac1 and DVAP double mutants do not show a more severe phenotype than single mutants. To investigate where ORP3 may act in this pathway, VAPB was knocked down using shRNA and the ratio of the Emerin signal in the nucleus to total cell was measured. Cells lacking VAPB had 20% Emerin in the nucleus and 80% in cytoplasmic puncta (Figure 23). Overexpression of ORP3 resulted in a two-fold increase in nuclear Emerin signal. The knockdown of VAPB may not be completely effective, and any residual may be acted upon by ORP3, thus attenuating the phenotype. These results suggest that ORP3 and VAPB may function in the same pathway; otherwise, overexpression of ORP3 would expectedly have stronger effects on rescuing defective Emerin transport. It remains possible that VAPB, ORP3 and Sac1 all act in a common pathway, and therefore, knocking down any of the three may result in nuclear transport defects. In fact, preliminary work in the Ngsee lab has shown that knockdown of ORP3 or Sac1 results in a similar

Emerin defect as seen in VAPB knockdown cells. Future work will be needed to elucidate this further.

Dyslipidemia in ALS

Alterations in metabolism have previously been described in both familial and sporadic ALS cases, where an increased metabolic rate has been observed at symptom onset (Dupuis et al., 2008). Hyperlipidemia has been identified as one possible reason for the increased energy expenditure in ALS patients. Increased lipid levels and peripheral lipid clearance have been observed in the blood of French patients, where two-thirds had abnormally high LDL-HDL ratios (Dupuis et al., 2010). Patients with ALS8 have a similar lipid profile and exhibit increased cholesterol levels, reduced HDL levels and hypertriglyceridemia (Marques et al., 2006). Hyperlipidemia has been positively associated with survival of ALS patients, whereas weight loss and malnutrition are associated with a faster decline (Dupuis et al. 2010). A recent study by O'Reilly et al. (2012) found that a higher body mass index (BMI) is associated with better survival in ALS patients. Individuals with a low/healthy BMI throughout adulthood were more likely to develop ALS than overweight or obese individuals. Similar results were found in SOD1 mice, which are the most commonly used mice to model ALS. SOD1 mice have a mutation in the superoxide dismutase 1 gene, which accounts for the majority of fALS cases (Paganoni and Wills, 2013). SOD1 mice fed a diet consisting of 38% carbohydrates, 47% fats, and 15% protein, had an increased mean survival time of 90% (Paganoni and Wills, 2013). SOD1 mice live on average 128 days. Another high fat diet consisting of 21% butterfat and 0.15% cholesterol extended survival time of SOD1 by 20 days. In contrast, caloric restriction reduced survival time (Paganoni and Wills, 2013). SOD1 mice fed a regular chow diet exhibited reduced cholesterol levels. Low cholesterol levels are also reported in male ALS subjects, but not females (Yang et al., 2013). In this study, total cholesterol, triacylglyceride and LDL/HDL levels were significantly lower than control subjects. Fergani et al. (2007) found that lipids supplied by the diet are quickly cleared from the plasma and

metabolized by skeletal muscle in SOD1 mice. Feeding with a high fat diet protected against motor neuron loss in these hypermetabolic mice. It is interesting to note that increased lipid levels correlate with survival time. ALS8 patients who have a tendency to live beyond the average 2-5 year diagnosis exhibit increased serum cholesterol and triglyceride levels (Marques et al., 2006). Together, these studies suggest that metabolic dysfunctions and alterations in lipid metabolism may be an underlying feature of both sporadic and familial ALS. Motor neurons have a high-energy demand and increased lipid synthesis, which may leave them selectively vulnerable to degeneration. Another form of fALS is caused by a loss of function mutation in the phosphatase FIG4. FIG4 de-phosphorylates PI(3,5)P₂ at the 5 position on the inositol ring. FIG4 also contains a Sac1 homology domain (Figure 24), which suggests that defects in PI phosphatases may be important in the pathogenesis of ALS. Therefore, elucidating VAPB's role in lipid metabolism may have importance for individuals with both sALS and fALS. Low levels of the VAPB protein have also been reported in sALS (Anagnostou et al., 2010), indicating that VAPB might play a much larger role in regulating lipids, in particular phosphatidylinositol in motor neuron disease.

VAPB-P56S ERGIC PI4P Model

The current study proposes that VAPB and Sac1 are interacting partners, which co-localize in the ER and ERGIC compartments. ORP3 is a soluble FFAT-motif containing protein that is recruited to VAPB in the ER. Stefan et al. (2011) show that in yeast, the oxysterol-binding domain was sufficient to activate the Sac1 phosphatase, to decrease levels of PI4P at membrane contact sites. This study has shown that increased expression of ORP3 abrogates the mutant VAPB-P56S phenotype. We indeed demonstrate here that ORP3 reduces the size and number of ER membrane expansions in HeLa cells. Activation with ligand 25-OH further reduces the aggregate size. Co-overexpression of ORP3 or the FFAT motif changes the solubility of the VAPB-P56S mutant protein, shifting it into the Triton X-100

soluble fraction. This suggests that ORP3 and FFAT may prevent aggregation of newly synthesized VAPB proteins by binding to the aggregation prone mutant MSP domain, stabilizing its native conformation. By preventing aggregation of VAPB-P56S and VAPB-WT, the native VAPB, will be available for normal housekeeping functions in the cell such as retrograde trafficking to the nuclear envelope, which is restored upon co-expression of ORP3. More work will be needed to elucidate whether or not these proteins act in a common pathway, as has been shown in yeast, since some phenotypes are yeast-specific.

In VAPB-WT cells, the PI4P gradient is low in the ER due to Sac1 activity, and increases through the ERGIC and *cis*-Golgi compartments (Figure 25). This PI4P gradient may be disrupted in VAPB-P56S cells (Figure 26). One potential model to explain the VAPB-P56S phenotype may be as follows: Sac1 cycles between the ER-ERGIC and Golgi compartments in response to nutrient availability and is primarily needed in the ER for housekeeping functions (Liu et al., 2008). In VAPB-P56S mutant cells, trafficking between the ER and ERGIC is impaired, and COPI contained cargo are trapped in the ERGIC. This may be due to a vesicle fusion defect in which the COPI coatamer does not uncoat as described by Soussan et al., (1999). The Sac1 phosphatase relies on COPI vesicles to return from the Golgi to the ER (Liu et al., 2008), and any defect in COPI trafficking would result in Sac1 being trapped in the ERGIC and *cis*-Golgi compartments, reducing PI4P in these compartments. We would expect to see a lower PI4P intensity in areas where Sac1/VAPB-P56S are trapped together. Subsequently, PI4P would be increased in the ER and other intracellular compartments due to Sac1 not being able to access the substrate. However, this detail remains unclear. If PI4P is decreased in the *cis*-Golgi, it would be expected that fusion of COPII vesicles would decrease, as PI4P is required for the fusion step. Work in the Ngsee lab has shown that VAPB-P56S mutant cells do show a slight delay in trafficking of VSVG from the ER to the Golgi apparatus, but trafficking from the *trans*-Golgi to the plasma membrane is not affected (Prosser et al., 2008). Thus, the experimental evidence so far indicates that the rate-limiting step is ER to Golgi transport. In VAPB knockdown cells, Sac1 is redistributed and

scattered throughout the ERGIC, which, disrupt the PI4P gradient similar to the VAPB-P56S cells (Figure 27).

Overexpression of ORP3 (Figure 28) may activate any available Sac1, and help bridge it to membranes rich in PI4P to reduce the levels. It remains unclear if Sac1 has the ability to act in *trans* in mammalian cells. If Sac1 acts in *cis*, overexpression of activated ORP3 may prevent VAPB aggregates from forming, allowing COPI transport of Sac1 back to the ER to reduce PI4P levels in *cis*. If Sac1 also acts in *trans* in mammalian cells, activation of ORP3 may bridge VAPB and Sac1 to the ER, which would have abnormally high PI4P to reduce the levels. Thus, Sac1 appears to need VAPB for proper targeting, and ORP3 for activation.

In VAPB and Sac1 knockdown cells, intracellular levels of PI4P are dramatically increased (Figure 22) suggesting high levels in the ER network. Any change in PI4P levels in the ERGIC compartment will likely result in defective vesicle fusion and budding processes, as PI4P is needed for vesicle fusion at the *cis*-Golgi. It has been reported that high PI4P at the *cis*-Golgi increases fusion of COPII vesicles and conversely, increases budding of COPI vesicles (Carvou et al., 2010). Any disruption in this PI4P gradient may cause anterograde and retrograde vesicles to become unbalanced. In the VAPB-P56S case, high levels of PI4P in the ER may cause COPI vesicles to continually bud off exit sites when they are supposed to fuse, creating an expanded ERGIC compartment with Nups and Sac1 not being able to return to the ER/nuclear envelope.

Increasing evidence suggests that widespread alterations in phospholipid metabolism may play a key role in the age-dependent onset of ALS8.

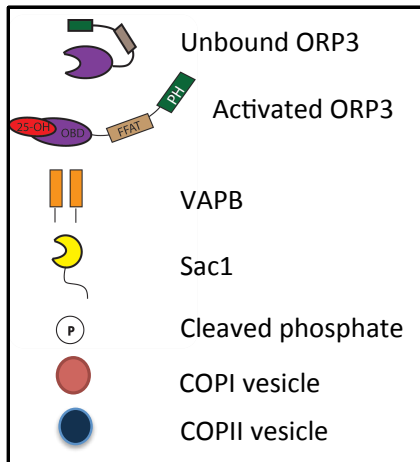
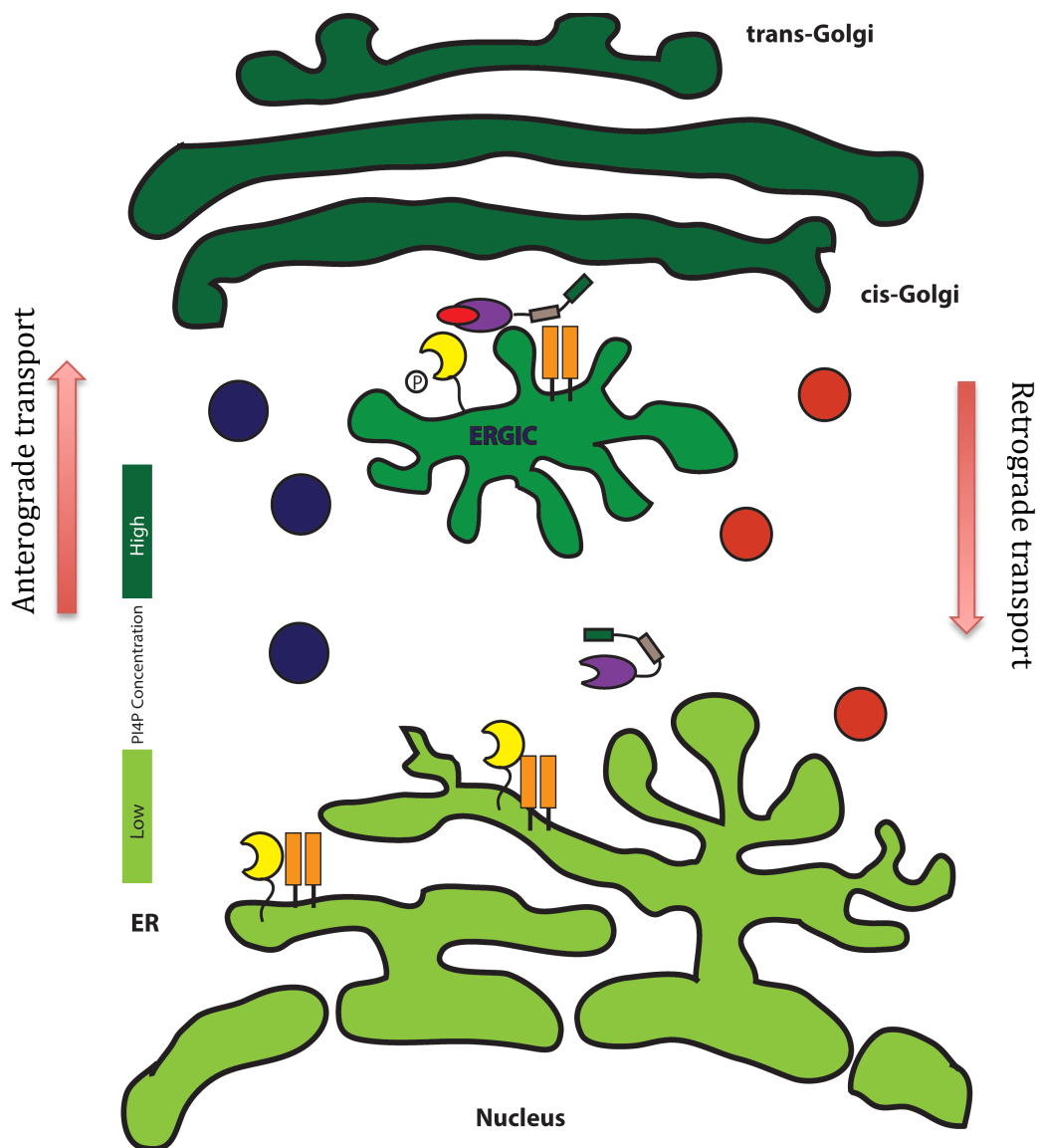


Figure 25. Model in VAPB-WT cells.

1. PI4P levels are low in the ER due to Sac1 activity, and increase through the ERGIC and *cis*-Golgi compartments. 2. ORP3 with bound oxysterol is recruited to VAPB. The oxysterol-binding domain with bound oxysterol stimulates Sac1 activity.



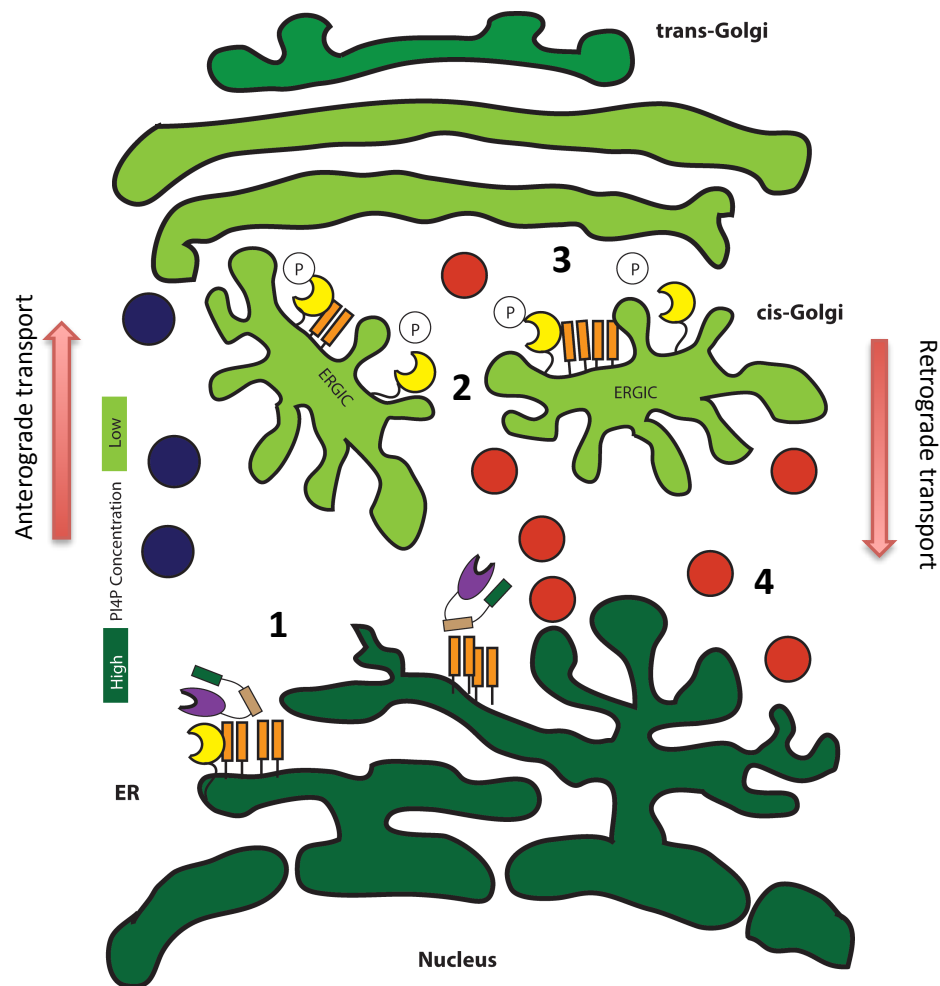


Figure 26. Model in VAPB-P56S cells.

1. PI4P levels are elevated in the ER due to Sac1 and VAPB sequestration into insoluble aggregates (2).
3. Sac1 remains active in trapped compartments, reducing PI4P levels in the ERGIC and *cis*-Golgi.
4. Retrograde trafficking is impaired and cargo in COPI vesicles build up.

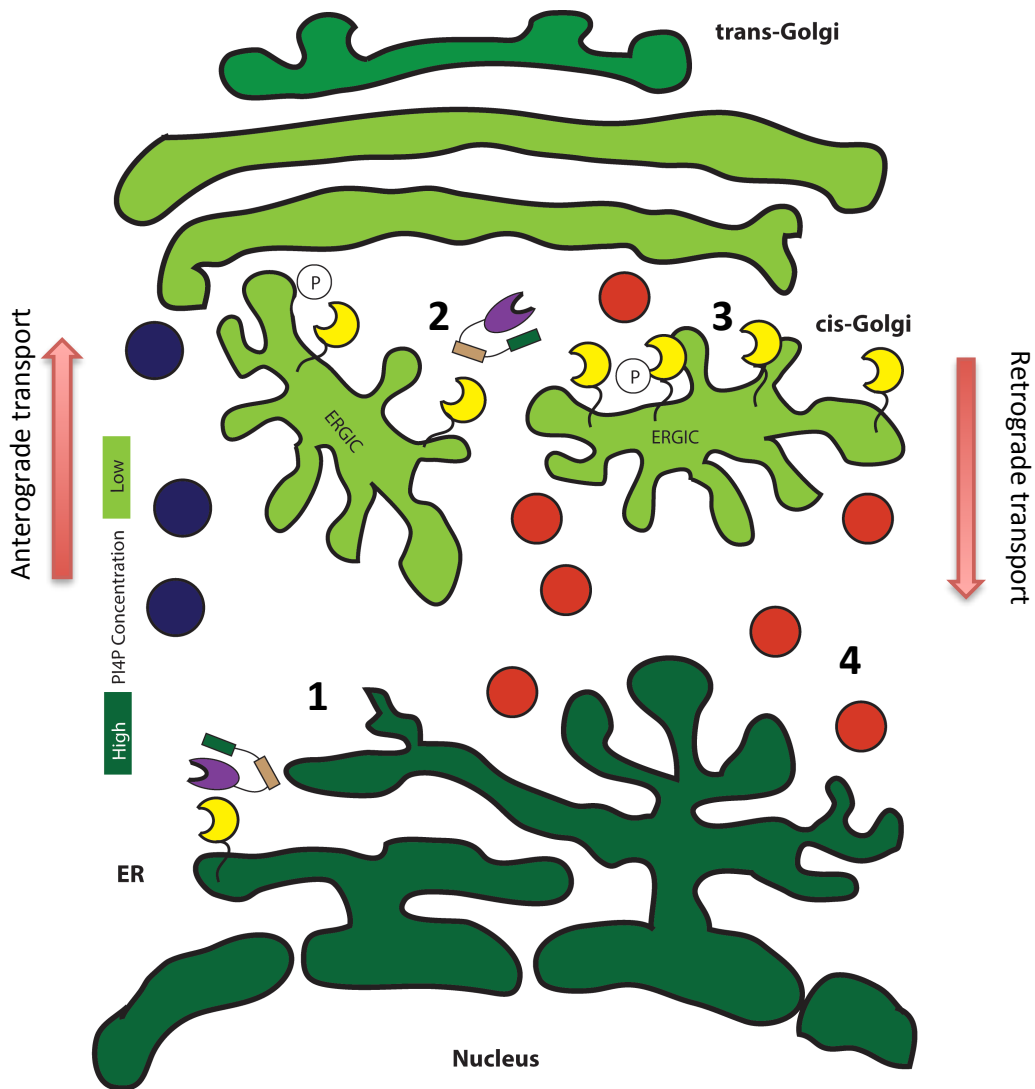


Figure 27. Model in VAPB knockdown cells.

1. Loss of VAPB causes increased PI4P levels in the ER due to Sac1 mislocalization to ERGIC (2). 3. Sac1 remains active in trapped compartments, reducing PI4P levels in the ERGIC and *cis*-Golgi. 4. Retrograde trafficking is impaired due to loss of VAPB and cargo in COPI vesicles build up.

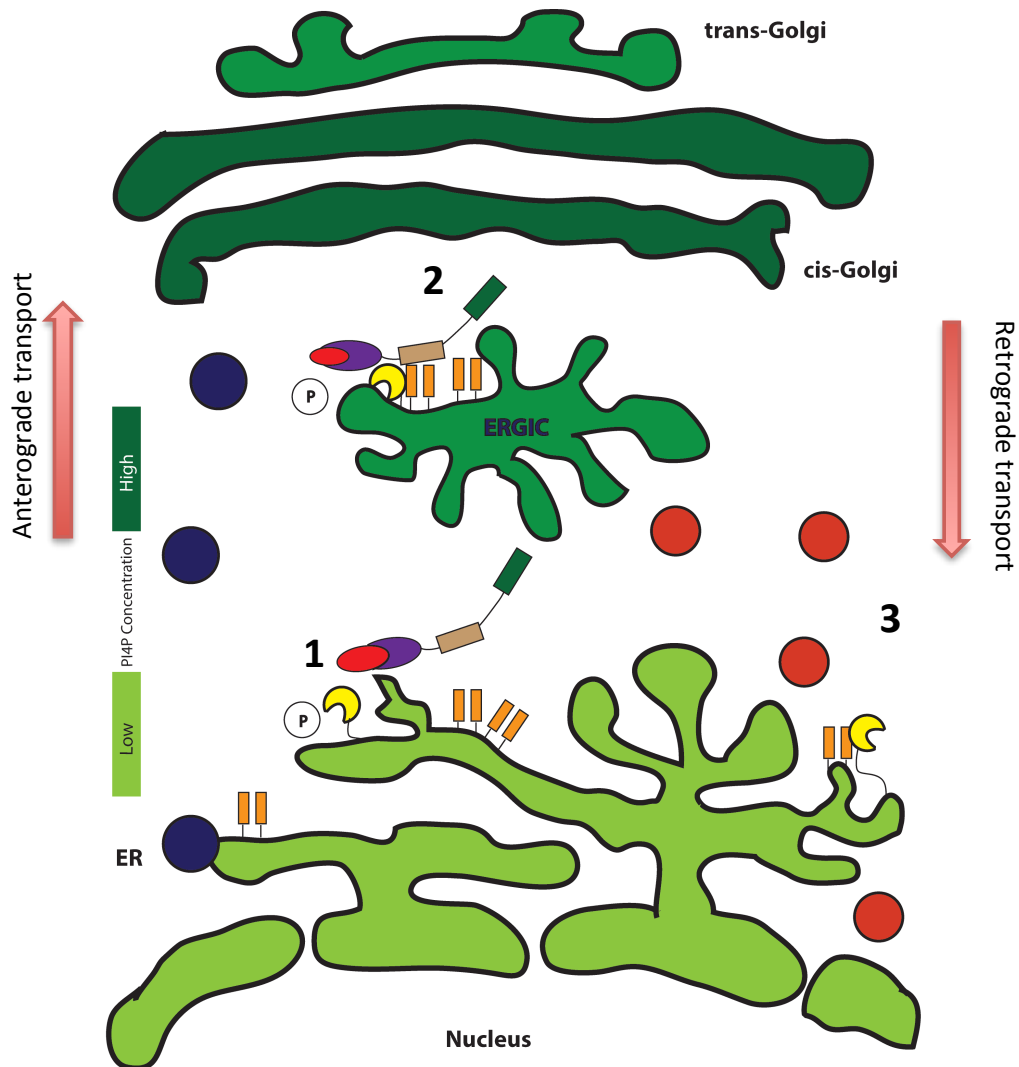


Figure 28. Model in VAPB-P56S cells overexpressing ORP3.

1. ORP3 is recruited to VAPB-P56S MSP domain to prevent further aggregation. ORP3 with bound oxysterol activates Sac1 to maintain low levels of PI4P in ER and ERGIC **(2)**. **3.** Retrograde trafficking is restored and Sac1 and Nups are able to transit to ER and nuclear envelope.

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