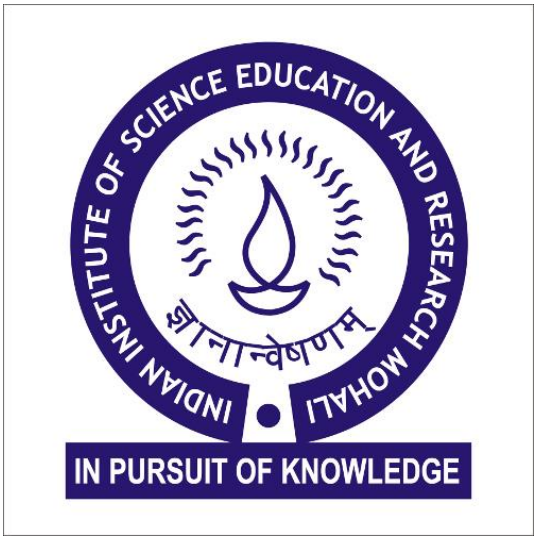
Characterizing the interaction of a novel autophagy regulatory protein with multisubunit tethering factor HOPS complex

Gyana Lipsa Parida

A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science



Indian Institute of Science Education and Research Mohali
April, 2021

Certificate of Examination

This is to certify that the dissertation titled "Characterizing the function of a novel autophagy regulatory protein with multisubunit tethering factor HOPS complex" submitted by Ms. Gyana Lipsa Parida (Reg. No. MS16037) for the partial fulfillment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Indranil Banerjee Dr. Sharvan Sehrawat Dr. Mahak Sharma

(Supervisor)

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Mahak Sharma at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship, to any other university or Institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussion. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Gyana Lipsa Parida

(Candidate)

Dated: 21/04/2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr Mahak Sharma

(Supervisor)

Acknowledgement

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Notations

TE buffer	**Tris-EDTA buffer**
LiAc buffer	**Lithium Acetate**
PEG	**PolyEthylene Glycol**
3-AT	**3-amino-1,2,4-triazole**
PBS	**Phosphate Buffer Saline**
FBS	**Fetal Bovine Serum**
PHEM	**Pipes Hepes EDTA Magnesium chloride**
LAMP1	**Lysosomal-associated membrane protein 1**
PFA	**Paraformaldehyde**

CONTENTS

Abstract

Eukaryotic cells maintain constant communication among the organelles by uptaking cargos from extracellular and intracellular spaces and sorting them to their correct functional location. External cargos like growth factors and intracellular cargos such as misfolded proteins and damaged organelles are delivered to lysosomes by endocytic and autophagic pathways, respectively. Key molecular players such as the small GTPases and tethering factors regulate these cargo trafficking pathways. HOPS (**HO**motypic fusion and vacuole **P**rotein **S**orting) complex is a multisubunit tethering factor that mediates the fusion of autophagosomes and late endosomes with lysosomes. This hexameric multisubunit complex consists of subunits including Vps11, Vps16, Vps18, Vps33a, Vps39, and Vps41 that are conserved across evolution from yeast to mammals. A previous report indicates that a protein belonging to the TECPR family of proteins, TECPR2 (Tectonin beta-propeller repeat containing 2), interacts with HOPS complex. We confirmed TECPR2 interaction with multiple HOPS subunits by yeast-two-hybrid assay. Next, we have constructed the domain-deletion mutants of TECPR2 and set up yeast two-hybrid assays with HOPS subunits. We found that the C-terminal TECPR domains of the protein are important for binding with HOPS subunits. Furthermore, confocal microscopy imaging of overexpressed TECPR2 showed mostly cytosolic distribution of the protein with few punctae colocalizing with lysosomal protein LAMP1. Future work is required to decipher the minimal region required for TECPR2 interaction with the HOPS complex.

Chapter 1: Introduction

1.1 Lysosomes: The digestive bags

Lysosomes are single membrane-bound; spherical, vesicular organelles discovered by Christian De Duve in the early 50s. These organelles contain more than 50 hydrolytic enzymes in their lumen responsible for the degradation of various biological macromolecules, including proteins, lipids, carbohydrates, and nucleic acids, into simpler compounds that are subsequently reutilized for the cellular metabolic process. The pH of the lumen is maintained around 4.5-5.0, which is optimal for the enzymes involved in hydrolysis, and this acidification is mediated by the proton-pumping vacuolar ATPase complex [1]. The lysosome also includes a specific set of integral-membrane, luminal and peripherally associated proteins, among which the most abundant lysosomal membrane proteins are LAMP1, LAMP2 that protects the membrane of the lysosome from degradation by its own acid hydrolases [2]. Cargo delivery to lysosomes occurs via various routes, including endocytic, phagocytic, and autophagic pathways.

Lysosomes are often known as the "garbage-disposal system" of the cell as they regulate the ultimate degradation process. Apart from their traditional role of degradation, recent studies have revealed that lysosomes also regulate various pathways, including plasma membrane repair, the release of exosomes, clearance of intracellular pathogen, and bone resorption [3]. Notably, defects in proteins involved in lysosomal function may lead to a category of diseases called Lysosomal Storage Disorders (LSDs). Such defect may result in the accumulation of autophagic substrates, protein aggregates which may progress to various neurodegenerative disorders [4]. Owing to all these reasons, it is imperative to study the physiology and functions of lysosomes.

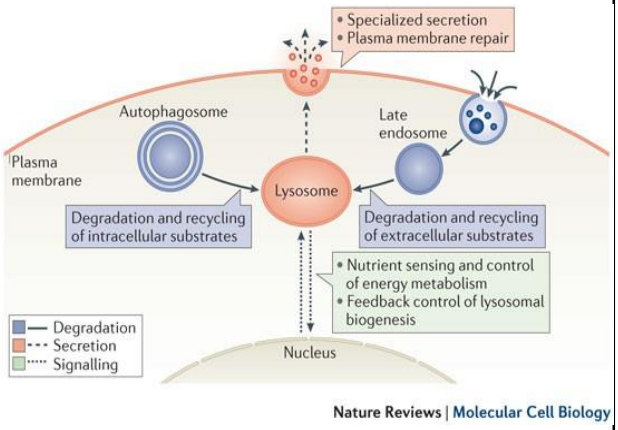

Figure 1: Schematic representation of functions of lysosomes.

1.2 Pathways directing cargo towards lysosomes: The Endocytic and Autophagic pathway

The two conventional pathways that mediate cargo trafficking towards lysosomes are the endocytic pathway and the autophagic pathway. The endocytic pathway is fundamental to all eukaryotic cells, in which cells uptake nutrients, growth factors, and plasma membrane components from extracellular space and internalize them into small vesicles called endosomes. These vesicles fuse with early endosomes where efficient cargo sorting occurs. The receptors generally recycle back to cell surface either directly from early endosomes or they traffic first to the recycling endosomes and subsequently to the plasma membrane for another round of ligand binding. The nutrients or ligands or signaling receptors are transported to late endosomes, which undergo frequent transient or complete fusion with the lysosome to form hybrid compartments termed as endolysosomes. Final sorting of cargo occurs in late endosomes, and cargo is terminally degraded in endolysosomes [5].

The autophagic pathway regulates the degradation of misfolded proteins, damaged organelles, and pathogens in lysosomes. Apart from maintaining cellular homeostasis by cellular turnover, it is also induced in response to cellular stress and upon nutrient starvation [6]. The process initiates by the formation of a cup-shaped isolation membrane called phagophore around the cargo to be degraded, wherein the membrane is derived from endoplasmic reticulum or Golgi. The phagophore then elongates and engulfs the cargo to be degraded to form a double-membrane sealed structure called autophagosome[7]. At the early stages of autophagosome formation, the cytosolic microtubule-associated protein light chain 3 (LC3B), also known as LC3B-I, is conjugated phosphatidylethanolamine to LC3B-II which then recruits to both inner and outer autophagosomal membrane for

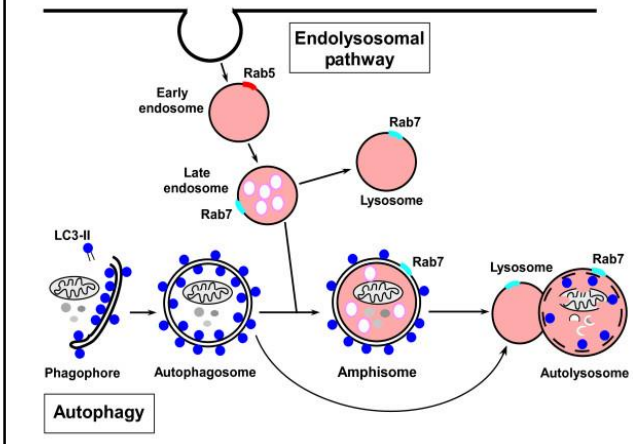

Figure 2: Pathways leading to lysosomes for cargo degradation

selecting cargo for degradation. The outer membrane eventually fuses with lysosomes to form an autolysosome where the contents of autophagosomes are degraded by the lysosomal enzymes [6].

1.3 Key regulators of cargo trafficking pathways

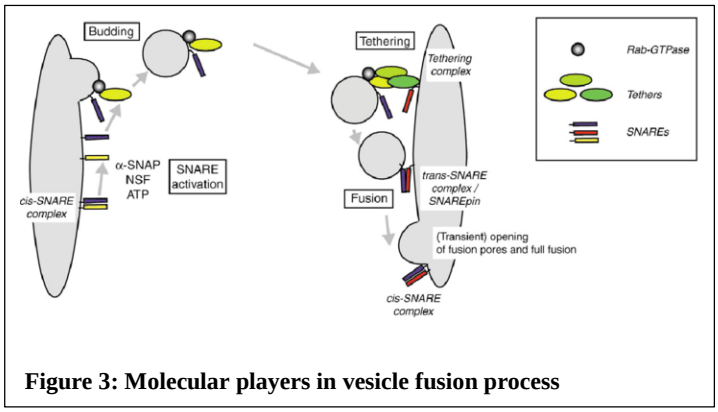

Figure 3: Molecular players in vesicle fusion process

Various regulatory proteins such as small GTPases, tethering factors, SNAREs, coat, and motor proteins regulate the transport of cargo through the endolysosomal and Autophagic pathways. The process involves the budding of the vesicle from the donor compartment by means of coat proteins, which then travels via motor proteins to come in close proximity to their acceptor compartment. The small GTP-binding proteins (G) in their GTP-bound active state localize to a specific compartment and recruit the downstream effector proteins, for instance, named tethering factors. Tethering factors bring the donor vesicle in close proximity to the acceptor membrane for the SNARE proteins to facilitate the final step of fusion. The regulatory factors involved in vesicle fusion with lysosomes are discussed below.

1.4 Small GTP-binding proteins

Small GTP binding (G) proteins are monomeric low molecular weight proteins that act as molecular switches and regulate various steps of vesicular trafficking. They cycle between their cytosolic GDP-bound inactive forms to membrane-anchored GTP-bound active forms, wherein the G protein recruits downstream effectors to mediate vesicular transport. The active GTP bound form undergoes GTP hydrolysis by GTPase Activating protein (GAP), and this GDP-bound inactive form remains in its inactive state by binding to Guanine Dissociation Inhibitor (GDI). The G protein again switches back to its active form with the help of a Guanine Exchange

Factor (GEF). The eukaryotic endomembrane system is decorated with multiple G proteins such as Rab5 and Rab7 on the early and late endosomes, respectively, and Arl8b on the lysosomal membrane.

1.5 Tethering factors

Tethering factors are protein complexes recruited to the organelle membrane by small GTPases and act as a bridge between acceptor and donor membranes. Structurally, tethering factors can be broadly divided into two types: they can either be long coiled-coil proteins or multisubunit complexes. Some of the coiled-coil tethering factors are associated with organelles like Golgi (Golgins, GARP, COG); others like EEA1 are recruited by Rab GTPases in the early endolysosomal pathway [8]. The multisubunit tethering complexes that regulate the endocytic trafficking pathway are CORVET (class **C** c**ORe** **V**acuole /**E**ndosome **T**ethering) and HOPS (**HO**motypic fusion and vacuole **P**rotein **S**orting). While CORVET mediates early homotypic fusion, the HOPS complex regulates the tethering of late endosomal and lysosomal compartments [9]. In yeast, HOPS complex is recruited to late endosomes/lysosomes by small GTPase Rab7 [10], whereas in mammalian cells, Arl8b is the primary G protein required for membrane localization of HOPS complex [11]. HOPS complex recruit SNAREs and proof-read SNARE complex formation for correct vesicular tethering and fusion, as shown for the yeast HOPS complex[12]. The structural and functional detail of the HOPS complex is described in detail in section 1.8.

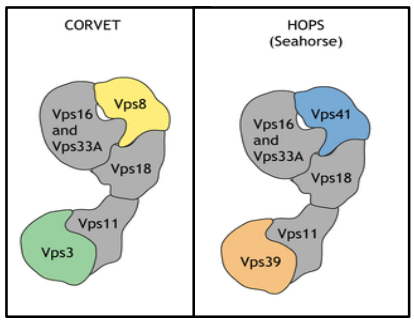

Figure 4: Schematic representation of tethering complex CORVET and HOPS.

1.6 SNAREs

SNAREs (Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor) are small, highly conserved membrane-bound proteins that mediate vesicular fusion events. More than 60 mammalian SNAREs have been discovered, such as Syntaxin1A and VAMP2 neuronal SNAREs being the first. All SNAREs in their membrane-proximal regions contain conserved heptad repeat sequences forming

coiled-coil structures. SNAREs are classified into v-SNAREs and t-SNAREs depending upon their presence on the vesicular membrane or target membrane, respectively [13]. Based on the presence of amino acid residues in the zeroth ionic layer in the SNARE domain, SNAREs are categorized into R-SNAREs (arginine containing SNAREs) and Q-SNAREs (glutamine-containing SNAREs) [14]. The coiled-coil SNARE motif forms a zipper-like helical structure that narrows the gap between transport vesicles and target membrane and mediates their fusion. Proteins belonging to SM(Sec1/Munc18) family, such as Vps33, regulate the assembly of SNARE complexes [15]. In *Saccharomyces cerevisiae*, the Vps33 subunit of HOPS is known to bind Vam3p and Vam7p SNARE proteins [16]. In mammalian cells, Vps33a subunit of HOPS interacts with syntaxin17, SNAP29 and VAMP8 during autophagosome-lysosome fusion [17][12]. HOPS-dependent fusion of autophagosomes with lysosomes is mediated by the interaction of autophagosomal SNARE syntaxin 17 and lysosomal SNARE VAMP8 [18].

1.7 HOPS complex is a multisubunit tethering factor that tethers late endosome-lysosome and autophagosome-lysosome

HOPS (Homotypic fusion and vacuole protein sorting) is a multimeric tethering factor that tethers late endosomes and autophagosomes with lysosomes and also regulates their fusion. It is a six-subunit complex with four subunits forming the core, namely, Vps11, Vps16, Vps18, Vps33a, and two accessory subunits Vps39 and Vps41, respectively. First discovered in the yeast saccharomyces cerevisiae, the HOPS complex is conserved from yeast to mammals. The mammalian HOPS also assemble as a hexameric complex similar to its yeast counterpart. hVps41 subunit of HOPS complex interacts with small G protein of

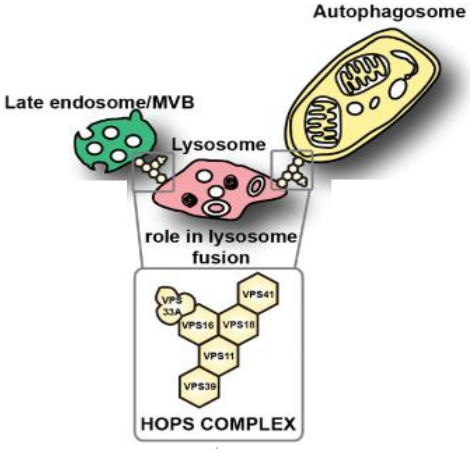

Figure 5: HOPS complex tethers late endosome and autophagosome with lysosome (Wartosch, Gunesdogan et al. 2015)

lysosome, Arl8b, which regulates lysosomal assembly of the human HOPS complex. [19]. HOPS complex also interacts with late-endosomal G protein Rab7 and its effector RILP. RILP regulates the recruitment of HOPS complex on the endosomal membrane [20]. Structural analysis of yeast HOPS by EM combined with single-particle analysis, and tomography studies suggest a sea-horse-like shape of HOPS complex with a bulky lobe at the tail end and deeper cavity in the head end [21]. HOPS subunit Vps33a interacts with SNAREs and has an α-helical structure. Apart from Vps33a, the rest of the subunits have a secondary structure with a possible C-term α-solenoid and N-term β-propeller domains [22], [23].

Defects in HOPS functioning lead to inhibition of vesicle fusion and cargo retention, which causes severe abnormalities in various organisms [24]. Recessive mutation in Vps41 subunit of HOPS complex inhibits HOPS functioning and signaling of mTORC1 and causes diseases like ataxia and dystonia with mental retardation [25].

Previous studies show the interaction of HOPS subunits with the TECPR domain-containing protein TECPR2 [26]. We discuss below this novel interaction partner of the HOPS complex.

1.8 TECPR2: A member of the tectonin-β-propeller repeat-containing Family

A previous study has shown that tectonin beta-propeller repeat-containing family member, TECPR2 interacts with certain subunits of the HOPS complex, including Vps18, Vps16, Vps33a, and Vps41. The protein has an N-terminal WD40 (tryptophan-aspartic acid) repeats, and C-terminal TECPR (tectonin-β-propeller) repeats followed by a C-terminal LC3 interacting region (LIR domain). The TECPR consensus sequence was first discovered in proteins of slime mold *Physarum polycephalum*. The tectonin domain of TECPR2 comprises tandem

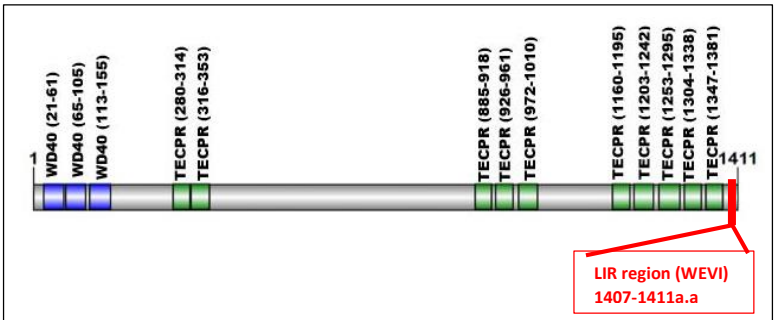

Figure 6: Domain architecture of TECPR2 protein: TECPR2 protein has WD40 repeats in its N-terminal with several Tectonin-β-propeller

repeats of about 35 amino acids, each forming four-stranded anti-parallel β sheets [27]. The blade-shaped beta-sheets are arranged symmetrically around a central axis in a toroidal manner to form a funnel-like active site. The clinical significance of this protein relies on the study that a point mutation at 1139 bp leads to a stop codon at 1212 position, which results in a C-terminal truncated, unstable protein. This single base-pair mutation in TECPR2 leads to a group of disease called Hereditary Spastic Paraplegias (HSPs) characterized by spasticity in lower limbs due to axonal deformity in corticospinal motor neurons [28]. TECPR2 interacts with the autophagosomal membrane protein LC3 and regulates Endoplasmic reticulum exit sites and ER export function, thereby regulating autophagosome biogenesis [26]. Thus, autophagy was found to be impaired upon reduced expression levels of TECPR2 genes depicting its role in the autophagic pathway. A recent study has shown that C-terminal TECPR domains of TECPR2 protein associates with lysosomes via VAMP8 and interacts with HOPS complex and Atg8 family proteins via its LIR motif. TECPR2 via its C-terminal TECPR domains regulates lysosomal targeting of autophagosomes and its consequent lysosomal consumption. HSP patients show impaired autophagy flux by the accumulation of autophagosomes owing to the C-terminal mutated version of TECPR2 protein [29]. Another study reveals the accumulation of autophagosomes in the central nervous system neurons of TECPR2 knockout mouse model [30].

Significance of the project:

HOPS complex is a multisubunit tethering protein connecting late endosomes and autophagosomes with lysosomes, where it regulates vesicle fusion among these compartments. Dysfunction of HOPS complex causes cargo retention in the autophagosomes and late endosomes, resulting in defects in cargo trafficking pathways leading to Lysosomal storage disorders (LSDs) [31]. It is imperative to know the interacting partners of the HOPS complex to understand how the function of this complex is regulated during vesicular trafficking. Previous studies showed TECPR2 protein as an interacting partner of the HOPS complex and shed light on the role of TECPR2 in the formation of autophagosome intermediates and in autophagosome biogenesis by governing Endoplasmic Reticulum Exit Sites (ERES) formation and function [26].

To gain insight into the mechanism of TECPR2 interaction with HOPS complex and whether, in the case of HSPs, the mutation in TECPR2 regulates its interaction with HOPS, we aimed to answer the following questions:

a) To determine the subunits of HOPS complex that interact with TECPR2
b) To determine the domains of TECPR2 that are required for interaction with HOPS subunits.

Chapter 2: Materials and Methods

2.1 Plasmids used

Plasmids	Source	Box No.	Position No.	Resistance
hVps11-pGBKT7	Mahak Sharma Lab	3	9	Kanamycin
hVps16-pGBKT7	Mahak Sharma Lab	3	8	Kanamycin
hVps18-pGBKT7	Mahak Sharma Lab	2	75	Kanamycin
hVpsa33a-pGBKT7	Mahak Sharma Lab	4	15	Kanamycin
hVps39-pGBKT7	Steve Caplan Lab	1	70	Kanamycin
hVps41-pGBKT7	MBB lab	1	77	Kanamycin
pGBKT7	Steve Caplan Lab	1	16	Kanamycin
WT-TECPR2-pGADT7	Mahak Sharma Lab	30	5	Ampicillin
pGADT7	Steve Caplan Lab	2	3	Ampicillin
TECPR2-ΔLIR-pGADT7(1-1407aa)	Mahak Sharma Lab	30	30	Ampicillin
TECPR2-ΔTECPR-pGADT7(1-1010aa)	Mahak Sharma Lab	37	19	Ampicillin
TECPR2-ΔTECPR-pGADT7(1-944aa)	Mahak Sharma Lab	30	28	Ampicillin
TECPR2-TECPR-pGADT7(935-1411aa)	Mahak Sharma Lab	37	24	Ampicillin
N-term-HA-TECPR2-pcDNA3.1(-)	Mahak Sharma Lab	35	54	Ampicillin

2.2 Molecular Cloning

The enzymes used for cloning were from NEB Inc. WT-TECPR2-pGADT7, and WT-TECPR2-pGBKT7 were used as a template to clone domain deletion mutants of WT-TECPR2 in PGADT7 and pGBKT7 vectors. Inserts were amplified using appropriate primers. The vector and insert were digested at 37°C for 2-3 hours. Gel purified vector and insert taken in the correct ratio were kept for ligation overnight at 16°C. The ligated products were transformed in DH5-α competent cells. The positive clones were checked for their protein expression and sent for sequencing.

Sl no.	Domain deletion mutants	Forward primer	Reverse primer	Significance of the mutant
1.	TECPR2-ΔLIR(1-1407aa)	5'CTGGAGGACGAGTGAGAGGTCATCTGA3'	5'TCAGATGACCTCTCACTCGTCCTCCAG3'	LIR region deleted
2.	TECPR2-ΔTECPR (1-1010aa)	5'ATGGCATCGATATCAGAGCCTGTT 3'	5'TTAGCCAGTTCTGAACCACAGGTTC3'	Extreme C-terminal TECPR domains deleted
3.	TECPR2-ΔTECPR(1-944aa)	5'ATCACAGCCCGGAACTAAGTGGTGTGGGCGCTG3'	5'CAGCGCCCACACCACTTAGTTCCGGGCTGTGAT3'	C-terminal TECPR domains deleted
4.	TECPR2-TECPR (935-1411aa)	5'TACCCGCTGTCCCAGATCAC3'	5'TCAGATGACCTCCCACTCGTC3'	Only the C-terminal TECPR domains present

Table 1: List of domain deletion mutants created in yeast-two-hybrid vector pGADT7 by molecular cloning

2.3 Antibodies used

Two antibodies were used in the immunostaining experiment. The mouse monoclonal antibody for human LAMP1(CD107a) was purchased from BD transduction laboratories. The rabbit polyclonal antibody against HA-epitope was purchased from Sigma.

2.4 Yeast two-hybrid assay

Cloning of the Hops subunits, i.e., Vps11, Vps 16, Vps18, Vps33a, Vps39 and Vps41, was done in the GAL4-binding domain vector(pGBKT7). WT-TECPR2 and all the domain deletion mutants of TECPR2 were cloned in the GAL4-activation domain vector(pGADT7). Y2H Gold strain yeast was streaked on the YPAD media plate. After two days of incubation at 30-degree,2-3 yeast colonies were inoculated in YPAD media for 20-24 hrs. Yeast two-hybrid experiment was performed, and constructs were co-transformed in yeast strain, plated on -leu/-trp plates, and was incubated at 30 for two days. Then the yeast transformants were spotted on -leu/-trp/-his plates. For stringent selection, transformed yeast was also plated on -leu/-trp/-his+3-AT plates. The plates were incubated at 30 degree for three-four days.

2.5 Transfection and Immunostaining

HeLa cells were grown on coverslips. They were transfected with desired constructs using X-tremegene HP transfection reagent (Roche). Post 14 hours of transfection, cells were fixed with 4% PFA for 10 minutes. Then the cells were given PBS wash twice and blocked with 5% FBS made in permeabilization buffer for half an hour. Permeabilization buffer was previously made with PHEM buffer and 0.2% saponin. Cells were then incubated with primary antibodies in permeabilization buffer for 2 hours at room temperature, washed twice with PBS and incubated with secondary antibodies conjugated with Alexa-fluor conjugates (molecular probes), in permeabilization buffer for 40 minutes at room temperature. Following this, coverslips were mounted using fluoromount-G (Southern biotech) mounting medium. Images were obtained using Zeiss LSM 710 confocal microscope.

2.6 Buffer Composition

a) PHEM buffer

PIPES – 60mM

HEPES – 25mM

EGTA – 10mM

Magnesium chloride – 2mM

Adjust pH to 6.9

b) Lithium Acetate buffer

Lithium Acetate- 10g

Type 1 H2O – 100ml

Adjust the pH to 7.5

c) TE buffer

Tris- 1M

EDTA- 0.5M

Type 1 H2O – 100ml

Adjust the pH to 7.5

d) PEG

Polyethylene glycol – 50g

Type 1 H2O – 100ml

Chapter 3: Results

3.1 Interaction of HOPS subunits with WT-TECPR2 protein

In this study, we have characterized the interaction of TECPR2 protein with tethering complex HOPS. Using all six subunits of HOPS as bait in a yeast two-hybrid assay, we mapped its interaction with wild-type (WT) TECPR2 protein. Vps11, Vps16, Vps18, Vps33a, Vps39, Vps41 subunits of HOPS were cloned in fusion with the Gal4-binding domain, and WT-TECPR2 protein was cloned in fusion with Gal4-activating domain. Post transformation, yeast was plated on a non-

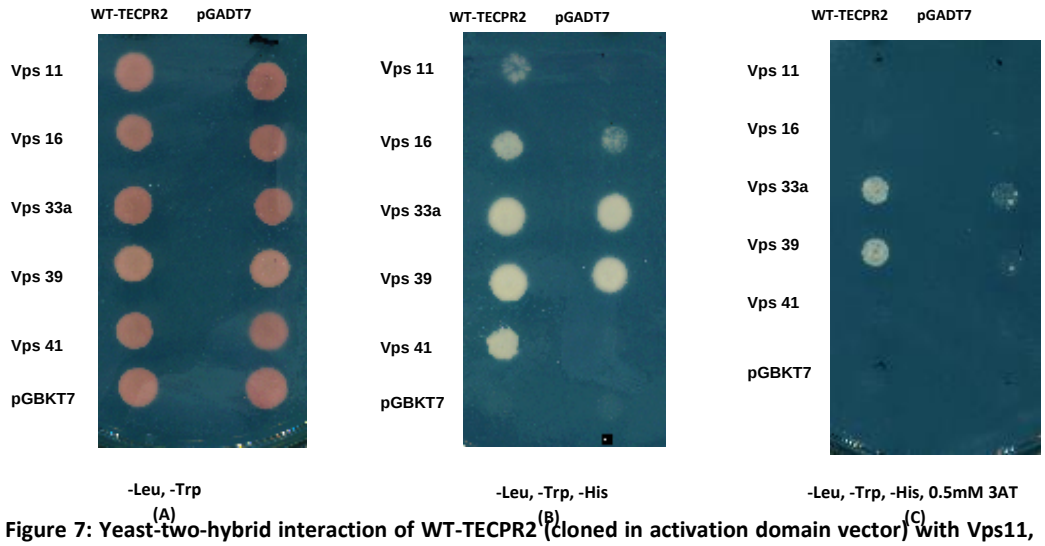

Figure 7: Yeast-two-hybrid interaction of WT-TECPR2 (cloned in activation domain vector) with Vps11, Vps16, Vps33a, Vps39 and Vps 41 subunit of the HOPS complex (Cloned in binding domain vector) were tested using pGADT7/pGBKT7 system. Transformants were plated on -leu, -trp plate to check viability(A) and on -leu,-trp,-his plate to detect interactions(B). To control self-activation of Vps33a and Vps39 HOPS subunits cloned in pGBKT7 vector, transformants were plated on 0.5mM 3AT plates(C).

selective medium to confirm viability and on a selection medium to detect interactions. After three days, it was observed that Vps11, Vps16, and Vps41 subunits of HOPS were showing interaction with WT-TECPR2 protein where Vps11 subunit was showing weak interaction, and Vps16 and Vps41 subunits were showing comparatively strong interactions in (-trp,-leu,-his). Vps33a and Vps39 subunits cloned in binding domain vectors showed self-activation on this plate. So, no conclusion could be drawn about the interaction of Vps33a and Vps39 subunits with WT-TECPR2 from this selection plate. To control self-activation effects yeasts were plated on the -trp,-leu,-his (-3)interaction plate containing 0.5 mM 3AT. 3AT is 3,4-amino-1,2,4-triazole, a competitive inhibitor of Histidine synthase (reporter

gene), so the addition of 3AT made the condition stringent. Only strong interactions manifest as the growth of yeast on the interaction plate, while low-affinity interactions were not obtained. So, on the 3AT plate, it was observed that Vps33a and Vps39 subunits showed strong interaction with WT-TECPR2. Vps18 subunit of HOPS was showing self-activation in this plate, and its self-activation could not be controlled even in a higher concentration of 3AT plates. So, this data has not been shown. Thus, the data are shown in Figure 7(A),(B), and (C) imply that Vps11, Vps16, Vps33a, Vps39, and Vps41 HOPS subunits interact with TECPR2 protein.

3.2 Molecular Cloning of domain deletion mutants of TECPR2

To determine which domain of TECPR2 is important for its interaction with the HOPS complex, we created different domain deletion mutants of TECPR2 by molecular cloning in yeast two-hybrid vector (pGADT7)

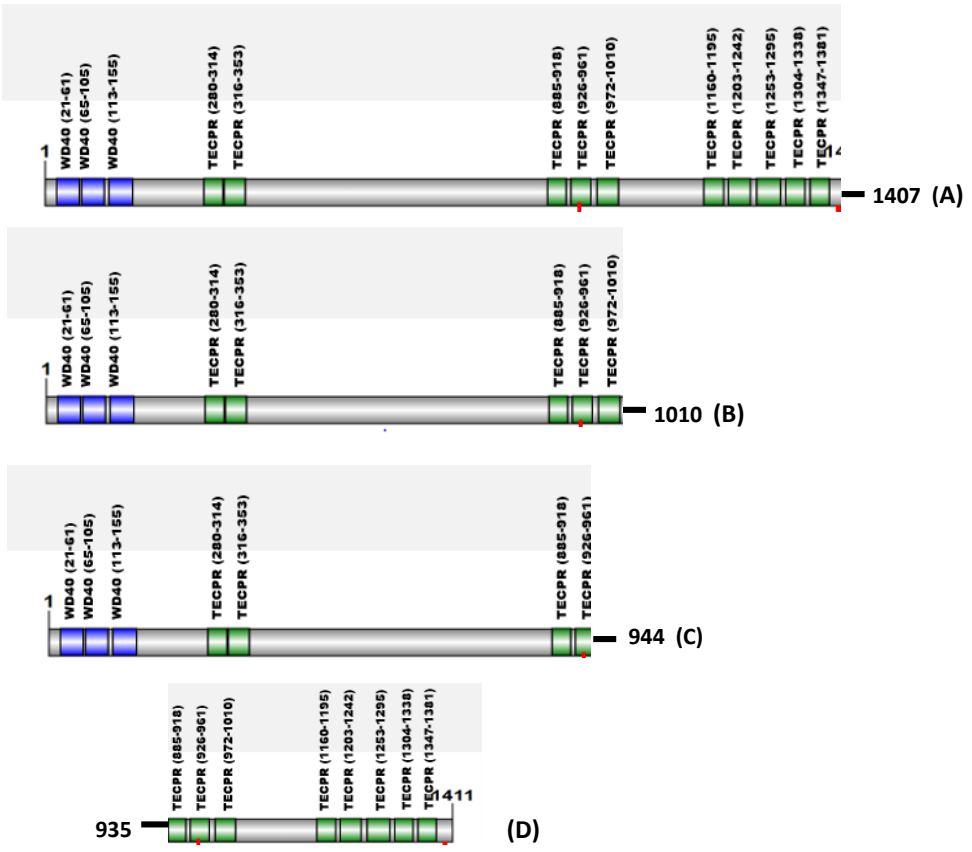

Figure 8: Schematic representation of domain deletion mutants of WT-TECPR2 cloned in Yeast-two-hybrid vector pGADT7

(A)TECPR2-ΔTECPR(1-1407a.a.)
(B) TECPR2-ΔTECPR(1-1010a.a.)
(C) TECPR2-ΔTECPR(1-944a.a.)
(D) TECPR2-TECPR(935-1411a.a.)

3.3 Vps11 and Vps 16 subunits interact in the 935-1411aa region of TECPR2, and Vps41 interacts in the C-terminal region

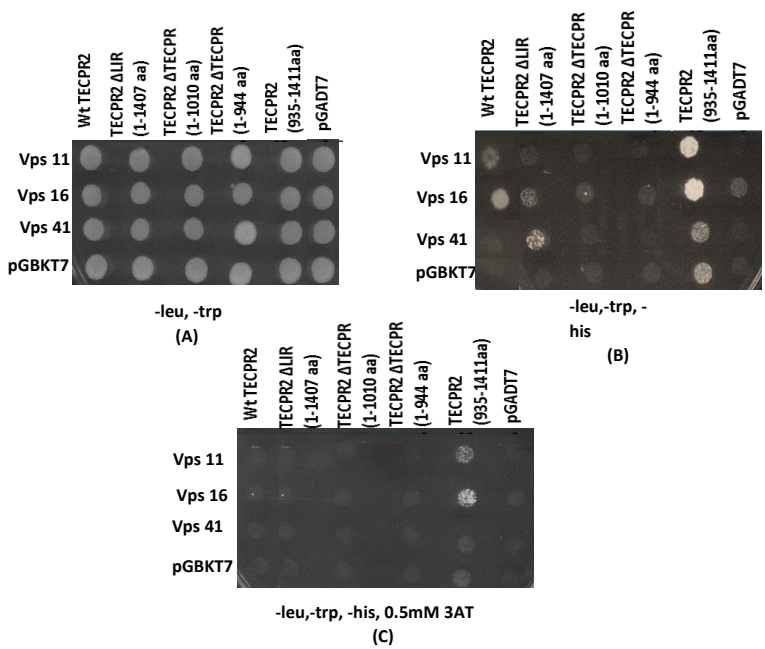

Figure 9: Yeast-two-hybrid interaction of WT-TECPR2 (cloned in activation domain vector) with Vps11, Vps16, and Vps 41 subunit of the HOPS complex (Cloned in binding domain vector) were tested using pGADT7/pGBKT7 system. Transformants were plated on -leu, -trp plate to check viability(A) and on -leu,-trp,-his plate to detect interactions(B). To control self-activation, transformants were also plated on -leu,-trp, -his, 0.5mM 3AT plate(C).

After the sequence analysis of the cloned mutants was done yeast-two-hybrid experiment was performed to check the interaction of all the HOPS subunits with the above-mentioned domain deletion mutants. HOPS subunits were present in fusion with the Gal4-binding domain, and the domain deletion mutants of TECPR2 protein were present in fusion with the Gal4-activation domain. Post transformation, yeast was plated on non-selective and on selection medium, as mentioned earlier. Three days hence, it was observed that Vps41 subunit interacts more strongly with the (1-1407aa) mutant of TECPR2 compared to its interaction with the wild type protein in -trp,-leu,-his plate(Figure 9-B). In the case of Vps11 and Vps 16 subunits, interaction with wild-type protein could be seen in this plate, but the 935-1411aa TECPR2 mutant was showing self-activation (Figure 9-B). So, we could not conclude anything about these two subunits from this interaction plate. To control self-activation, yeast transformants were also plated on the -trp,-leu,-his plate containing 0.5 mM 3AT, where we could observe that both Vps11 and Vps 16 subunits of HOPS interact strongly in the 935-1411aa region of TECPR2 protein. No interaction was observed with other domain deletion mutant constructs of the protein(Figure 9-C). Hence, it can be speculated that the presence of the LIR domain

inhibits the interaction of Vps41 with TECPR2 protein and C-terminal TECPR domains are important for its interaction with Vps11 and Vps16 subunit of the HOPS complex.

3.4 Vps33a interacts in 944-1411aa region of TECPR2 and Vps 39 in 1010 - 1411aa region

Another yeast-two-hybrid experiment was set up to check the interaction of Vps33a and Vps 39 subunits of HOPS with the domain deletion mutants of TECPR2. Three days post-transformation colonies were observed on the -trp,-leu,-his interaction plate where Vps33a and Vps39 subunits showed self-activation (Figure 10-B). So, they were plated on 0.5 mM 3AT plate. From this plate, we could observe that Vps33a and Vps39 subunit showed stronger interaction with 935-1411 aa mutant of TECPR2 compared to their interaction with WT-TECPR2. Both Vps33a and Vps39 subunits showed very weak interaction with the 1-1407 aa construct, and only Vps33a showed very weak interaction with the 1-1010 aa mutant TECPR2 construct(Figure 10-C). Hence, the data suggest that the Vps39 subunit of HOPS interacts within the 1010-1411 aa region of TECPR2 protein and Vps33a interacts within the 944-1411aa region as it did not show any interaction within the 1-944aa region of the protein.

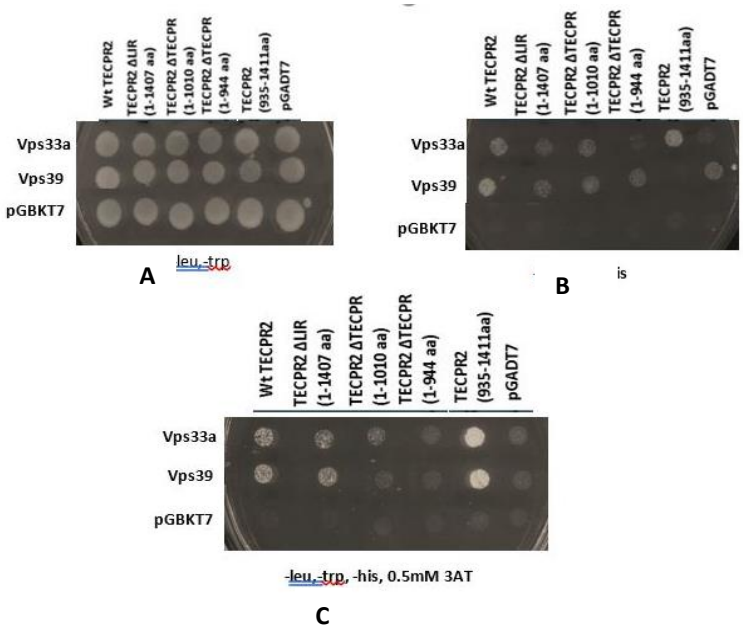

Figure 10: Yeast-two-hybrid interaction of WT-TECPR2 (cloned in activation domain vector) with Vps33a and Vps 41 subunit of the HOPS complex (Cloned in binding domain vector) were tested using pGADT7/pGBKT7 system. Transformants were plated on -leu, -trp plate to check viability(A) and on -leu,-trp,-his plate to detect interactions(B). To control self-activation, transformants were also plated on -leu,-trp, -his, 0.5mM 3AT plate(C).

3.5 Subcellular localization of WT-TECPR2 protein

A recent study has shown that TECPR2 regulates autophagosome biogenesis[26] and lysosomal targeting of autophagosomes with lysosomal SNARE, VAMP8 [29]and not much has been characterized about its subcellular localization. To determine the localization of TECPR2 protein, we overexpressed HA-tagged TECPR2 HeLa cells and costained the cells with lysosomal marker LAMP1. Analysis by confocal microscopy showed that expression of TECPR2 was mostly cytosolic, although we could observe some TECPR2 puncta colocalized with LAMP1 positive endosomes. This data indicated that the localization of TECPR2 might be on the lysosomal membranes.

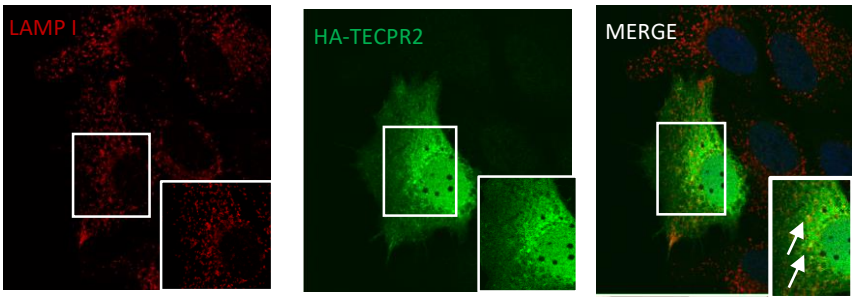

Figure 11: HeLa cells overexpressed with HA-TECPR2 localizes to cytoplasm. TECPR2 colocalize with LAMP1 positive endosomes. Hela cells were transfected with HA-TECPR2 and analyzed for lysosomal localization by confocal microscopy. Inset shows magnified view of the boxed region

Conclusion and Future outlook

In this study, we explored which subunits of the HOPS complex interact with TECPR2 and the region of TECPR2 required for interaction with HOPS complex. The yeast two-hybrid assay showed that the Vps11 subunit of the HOPS complex showed weak interaction while Vps16, Vps33a, Vps39, and Vps41 subunits showed strong interaction with WT-TECPR2 protein. To determine the potential domain of TECPR2 important for its interaction with HOPS, we created domain-deletion mutants of TECPR2 in yeast two-hybrid vector by molecular cloning. Yeast-two-hybrid data showed that the C-terminal part of the protein is important for its interaction with the HOPS complex. Vps11 and Vps 16 subunit of HOPS complex showed strong interaction in 935-1411aa region of TECPR2 protein while Vps41 subunit showed strong interaction with 1-1407aa mutant of TECPR2. Vps33a and Vps39 subunit of HOPS showed strong interaction in the 944-1411aa region and 1010-1411aa region of TECPR2, respectively. From the observed data, we could also speculate that the presence of the C-terminal LIR domain inhibits the interaction of the Vps41 subunit of HOPS with TECPR2 protein. Overexpression of HeLa cells with HA-tagged TECPR2 mostly showed cytosolic expression of the protein, and some TECPR2 punctae colocalized with LAMP1 positive endosomes. Further confirmation of TECPR2 mutants and HOPS interaction will be done by co-immunoprecipitation and GST Pulldown techniques. Apart from this, we also need to determine the localization of these TECPR2 mutants with autophagic and endocytic markers by confocal microscopy. Future experiments also include the generation of stable cell lines of HeLa cells expressing TAP-TECPR2 to perform Tandem Affinity Pulldown and analyze the potential interacting partners of TECPR2.

Bibliography

[1] P. Saftig and J. Klumperman, “Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function,” *Nat. Rev. Mol. Cell Biol.*, vol. 10, no. 9, pp. 623–635, 2009, doi: 10.1038/nrm2745.

[2] M. Schwake, B. Schröder, and P. Saftig, “Lysosomal Membrane Proteins and Their Central Role in Physiology,” *Traffic*, vol. 14, no. 7, pp. 739–748, 2013, doi: 10.1111/tra.12056.

[3] D. W. Lamming and L. Bar-Peled, “Lysosome: The metabolic signaling hub,” *Traffic*, vol. 20, no. 1, pp. 27–38, 2019, doi: 10.1111/tra.12617.

[4] F. M. Platt, B. Boland, and A. C. van der Spoel, “Lysosomal storage disorders: The cellular impact of lysosomal dysfunction,” *J. Cell Biol.*, vol. 199, no. 5, pp. 723–734, 2012, doi: 10.1083/jcb.201208152.

[5] N. Naslavsky and S. Caplan, “The enigmatic endosome - Sorting the ins and outs of endocytic trafficking,” *J. Cell Sci.*, vol. 131, no. 13, 2018, doi: 10.1242/jcs.216499.

[6] Y. Feng, D. He, Z. Yao, and D. J. Klionsky, “The machinery of macroautophagy,” *Cell Res.*, vol. 24, no. 1, pp. 24–41, 2014, doi: 10.1038/cr.2013.168.

[7] L. Yu, Y. Chen, and S. A. Tooze, “Autophagy pathway: Cellular and molecular mechanisms,” *Autophagy*, vol. 14, no. 2, pp. 207–215, 2018, doi: 10.1080/15548627.2017.1378838.

[8] I. M. Yu and F. M. Hughson, “Tethering factors as organizers of intracellular vesicular traffic,” *Annu. Rev. Cell Dev. Biol.*, vol. 26, pp. 137–156, 2010, doi: 10.1146/annurev.cellbio.042308.113327.

[9] H. J. klein. Balderhaar and C. Ungermann, “CORVET and HOPS tethering complexes - coordinators of endosome and lysosome fusion,” *J. Cell Sci.*, vol. 126, no. 6, pp. 1307–1316, 2013, doi: 10.1242/jcs.107805.

[10] F. Guerra and C. Bucci, “Multiple Roles of the Small GTPase Rab7,” *Cells*, vol. 5, no. 3, p. 34, 2016, doi: 10.3390/cells5030034.

[11] D. Khatter, V. B. Raina, D. Dwivedi, A. Sindhwani, S. Bahl, and M. Sharma, “The small GTPase Arl8b regulates assembly of the mammalian HOPS complex on lysosomes,” *J. Cell Sci.*, vol. 128, no. 9, pp. 1746–1761, 2015, doi: 10.1242/jcs.162651.

[12] P. Jiang *et al.*, “The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17,” *Mol. Biol. Cell*, vol. 25, no. 8, pp. 1327–1337, 2014, doi: 10.1091/mbc.E13-08-0447.

[13] R. Jahn and R. H. Scheller, “SNAREs - Engines for membrane fusion,” *Nat. Rev.*

Mol. Cell Biol., vol. 7, no. 9, pp. 631–643, 2006, doi: 10.1038/nrm2002.

[14] D. Fasshauer, R. B. Sutton, A. T. Brunger, and R. Jahn, "Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q-and R-SNAREs (membrane fusionneurotransmissionclostridial neurotoxins)," *Neurobiol. Commun. by Peter B. Moore*, vol. 95, no. December, pp. 15781–15786, 1998.

[15] R. W. Baker, P. D. Jeffrey, M. Zick, B. P. Phillips, W. T. Wickner, and F. M. Hughson, "A direct role for the Sec1/Munc18-family protein Vps33 as a template for SNARE assembly," *Science (80-.).*, vol. 349, no. 6252, pp. 1111–1114, 2015, doi: 10.1126/science.aac7906.

[16] C. Stroupe, K. M. Collins, R. A. Fratti, and W. Wickner, "Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p," *EMBO J.*, vol. 25, no. 8, pp. 1579–1589, 2006, doi: 10.1038/sj.emboj.7601051.

[17] E. Itakura, C. Kishi-Itakura, and N. Mizushima, "The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes," *Cell*, vol. 151, no. 6, pp. 1256–1269, 2012, doi: 10.1016/j.cell.2012.11.001.

[18] I. Dingjan, P. T. A. Linders, D. R. J. Verboogen, N. H. Revelo, M. Ter Beest, and G. Van Den Bogaart, "Endosomal and phagosomal SNAREs," *Physiol. Rev.*, vol. 98, no. 3, pp. 1465–1492, 2018, doi: 10.1152/physrev.00037.2017.

[19] A. Spang, "Membrane tethering complexes in the endosomal system," *Front. Cell Dev. Biol.*, vol. 4, no. MAY, pp. 1–7, 2016, doi: 10.3389/fcell.2016.00035.

[20] X. Lin *et al.*, "RILP interacts with HOPS complex via VPS41 subunit to regulate endocytic trafficking," *Sci. Rep.*, vol. 4, pp. 1–11, 2014, doi: 10.1038/srep07282.

[21] C. Bröcker *et al.*, "Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 6, pp. 1991–1996, 2012, doi: 10.1073/pnas.1117797109.

[22] H. Behrmann *et al.*, "Structural identification of the Vps18 β-propeller reveals a critical role in the HOPS complex stability and function," *J. Biol. Chem.*, vol. 289, no. 48, pp. 33503–33512, 2014, doi: 10.1074/jbc.M114.602714.

[23] S. C. Graham *et al.*, "Structural basis of Vps33A recruitment to the human HOPS complex by Vps16," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 33, pp. 13345–13350, 2013, doi: 10.1073/pnas.1307074110.

[24] R. Yarwood, J. Hellicar, P. G. Woodman, and M. Lowe, "Membrane trafficking in health and disease," *DMM Dis. Model. Mech.*, vol. 13, no. 4, 2020, doi: 10.1242/dmm.043448.

[25] R. E. N. van der Welle *et al.*, *VPS41 recessive mutation causes ataxia and dystonia with retinal dystrophy and mental retardation by inhibiting HOPS function and mTORC1 signaling*, no. Dc. 2019.

[26] D. Stadel *et al.*, "TECPR2 Cooperates with LC3C to Regulate COPII-Dependent ER Export," *Mol. Cell*, vol. 60, no. 1, 2015, doi: 10.1016/j.molcel.2015.09.010.

[27] R. Sommer *et al.*, "Crystal Structures of Fungal Tectonin in Complex with O-

Methylated Glycans Suggest Key Role in Innate Immune Defense," *Structure*, vol. 26, no. 3, pp. 391-402.e4, 2018, doi: 10.1016/j.str.2018.01.003.

[28] D. Oz-Levi *et al.*, "Mutation in TECPR2 reveals a role for autophagy in hereditary spastic paraparesis," *Am. J. Hum. Genet.*, vol. 91, no. 6, pp. 1065–1072, 2012, doi: 10.1016/j.ajhg.2012.09.015.

[29] M. Fraiberg *et al.*, "Lysosomal targeting of autophagosomes by the TECPR domain of TECPR2," *Autophagy*, vol. 00, no. 00, pp. 1–13, 2020, doi: 10.1080/15548627.2020.1852727.

[30] B.-C. Tamim-Yecheskel *et al.*, "A tecpr2 knockout mouse exhibits age-dependent neuroaxonal dystrophy associated with autophagosome accumulation," *Autophagy*, vol. 00, no. 00, pp. 1–14, 2020, doi: 10.1080/15548627.2020.1852724.

[31] G. Parenti, D. L. Medina, and A. Ballabio, "The rapidly evolving view of lysosomal storage diseases," *EMBO Mol. Med.*, vol. 13, no. 2, pp. 1–21, 2021, doi: 10.15252/emmm.202012836.