CHARACTERIZATION OF Vps18 SUBUNIT OF MAMMALIAN HOPS COMPLEX

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Certificate of Examination

This is to certify that the dissertation titled “Characterization of Vps18 subunit of mammalian HOPS complex” submitted by Ms. Neelam Singh (Reg. No. MS08034) for the partial fulfilment of BS-MS dual degree programme 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. Samarjit Bhattacharya Dr. Rachna Chaba Dr. Mahak Sharma

(Supervisor)

Dated: April 25, 2014

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 acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Neelam Singh

(Candidate)

Dated: April 25, 2014

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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Finally I extend my deepest gratitude and thankfulness to God who made all the things possible.

List of Figures

Notations

IPTG	Isopropyl β-D-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
FBS	Fetal Bovine Serum
PFA	Paraformaldehyde
3-AT	3-amino-1,2,4-triazole
LAMP1	Lysosomal-associated membrane protein 1
MBP	Maltose Binding Protein

Contents

Abstract

Endocytic trafficking in eukaryots is regulated by various components of vesicle fusion machinery namely, small GTPases, tethering factors, SNAREs and motor proteins. Small GTPases, in their GTP-bound/ active state, recruit downstream tethering factors which in a SNARE-dependent manner regulate vesicle fusion. Tethering factors that function in vesicular trafficking were first discovered in yeast. One such tethering factor that is well characterized in model organism *Saccharomyces cerevisiae* and that plays a role at the late endosome-lysosome junction is HOPS (HOmotypic fusion and vacuole Protein Sorting) complex. Just like its yeast counterpart, mammalian HOPS complex is a multimer comprised of six subunits namely, Vps11, Vps16, Vps18, Vps33a, Vps39 and Vps41. These proteins have stayed highly conserved across evolution. HOPS complex plays an important role in mediating membrane fusion-events at late endosome-vacuole/lysosome. In yeast, this complex has been well-studied but the molecular mechanisms involved in HOPS complex recruitment to endo-lysosomes in mammals are yet not clear. During the course of this study we observed that HOPS Complex subunits interact with each other directly, in a yeast-two-hybrid system. Upon overexpression in mammalian cells, it was seen that hVps11, hVps16 and hvps18 localize to cytoplasm of these cells, while lysosomal recruitment of one of the subunit hVps18 is promoted by its interaction with hVps41. Together these results indicate that mammalian HOPS subunits assemble as a complex and in this fashion may function to bring about late endosome-lysosome fusion.

Chapter 1

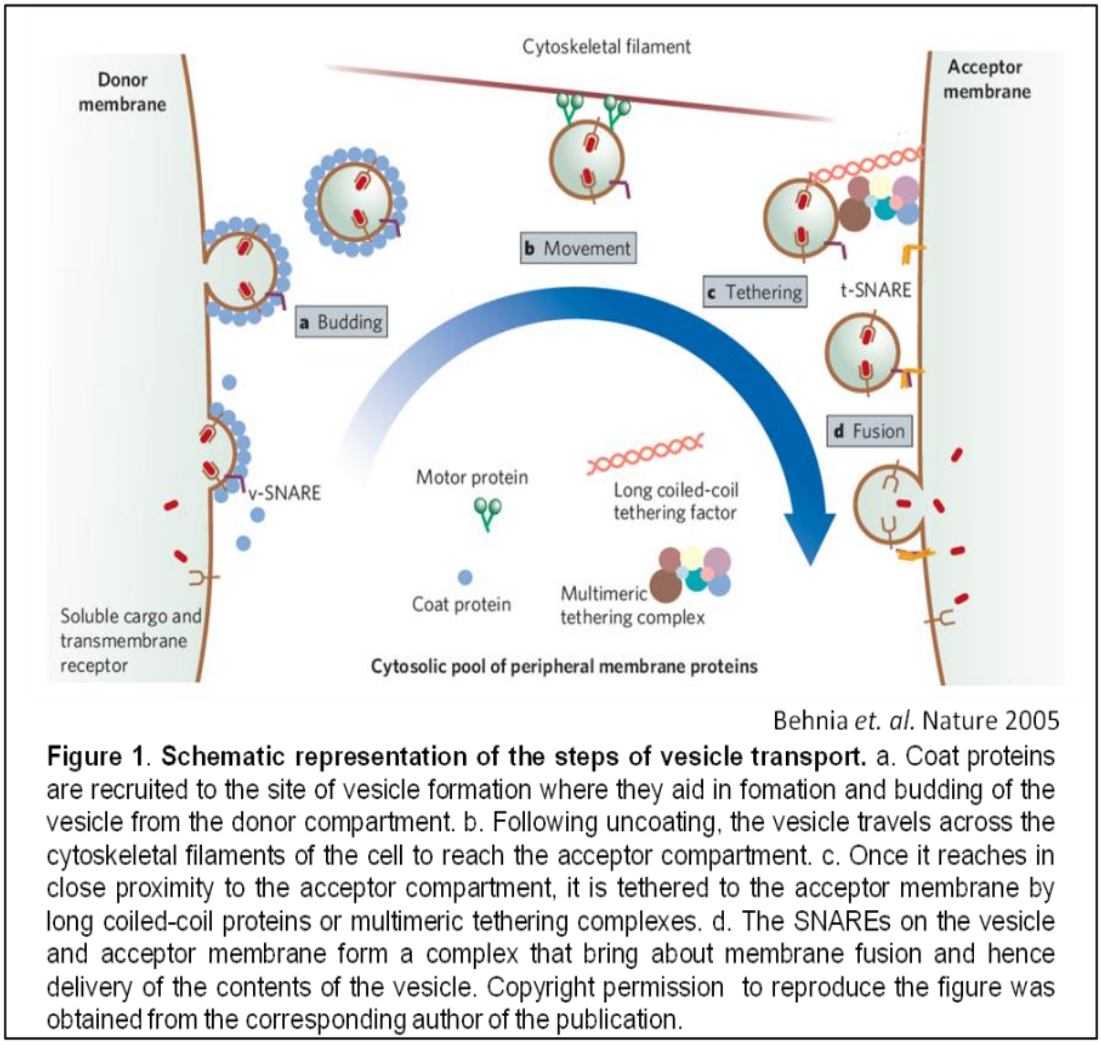
Introduction

1.1 Basic Theory
1.2 Experimental Methods

1.1 Basic Theory

Eukaryotic cells constantly communicate with their environment to perform various functions like nutrition, development, defense etc for their survival and to maintain a homeostatic state. To achieve this, cells take up matter from extracellular space in a process called endocytosis and also secrete matter outside in a process called exocytosis. Following endocytosis, the cargo then passes through an elaborate endo-membrane system and ends up either getting recycled to the plasma membrane or degraded in the lysosome. The cargo-containing vesicles fuse with early endosomes, where the cargo is sorted i.e. it is either directed to the recycling compartment (recycling pathway) or to late endosomes (degradative pathway). The late endosomes, also called multi-vesicular bodies (MVBs), fuse with the lysosomes eventually leading to cargo degradation.

Lysosomes or 'suicide bags of the cell' are single-membrane bound organelles where proteins, lipids and other molecules are degraded into simpler compounds (peptides, amino acids, carbohydrates etc.) by the action of digestive enzymes called lysosomal hydrolases. The lysosomes maintain an acidic environment (pH ~4.5 to 5), very different from other organelles in the cell with the help of ion channel pumps present in their membrane, which pump protons from the cytoplasm of the cell to the lumen of the lysosome. The acidic environment is indispensable for the activity of the enzymes.



Behnia *et. al.* Nature 2005

Figure 1. Schematic representation of the steps of vesicle transport. a. Coat proteins are recruited to the site of vesicle formation where they aid in fomation and budding of the vesicle from the donor compartment. b. Following uncoating, the vesicle travels across the cytoskeletal filaments of the cell to reach the acceptor compartment. c. Once it reaches in close proximity to the acceptor compartment, it is tethered to the acceptor membrane by long coiled-coil proteins or multimeric tethering complexes. d. The SNAREs on the vesicle and acceptor membrane form a complex that bring about membrane fusion and hence delivery of the contents of the vesicle. Copyright permission to reproduce the figure was obtained from the corresponding author of the publication.

The endocytic processes that embark the vesicular traffic in a cell can be classified into:-

1. **Pinocytosis** i.e. where the cell absorbs extracellular materials such as dissolved solutes, nutrient molecules etc. via invagination of plasma membrane and internalize them as small vesicles called pinosomes. This process is also known as 'cell drinking'.

2. **Phagocytosis** i.e. where phagocytic cells (macrophages, dendritic cells, neutrophils, etc.) engulf solid particles, for example microbes, macromolecules (e.g. proteins, lipids etc.) and internalize them as large vesicles called phagosomes.

3. **Receptor-mediated endocytosis** involves the uptake of the ligand-receptor complexes into the cell. These ligands are mostly sorted to lysosomes for degradation, while the receptors may be recycled back to the cell surface or degraded.

4. Under conditions of starvation, cells start digesting their own constituents of plasma membrane, dysfunctional or unnecessary cellular materials, etc. by internalizing them in 'autophagosomes' which directly reach lysosomes for degradation [1]. This process called **autophagocytosis,** enables maintenance of a healthy state and cellular survival during extreme conditions [2, 3].

1.1.1 Steps in a vesicle fusion pathway

The motility of an endosome is an essential component of the vesicular fusion pathway that enables a vesicle to come in close proximity to an acceptor compartment after it buds off from a donor compartment. Following this, the small GTPase localized to the vesicle membrane gets activated and it recruits a class of its downstream effectors called tethering factors. The tethering factors then bring about the tethering of two vesicles followed by their docking to each other. The SNARE molecules present on either compartments bundle up to form the trans-SNARE complexes thereby leading to fusion of the two vesicles (Figure 1).

All the fusion events in a eukaryotic cell are regulated by various components of the vesicle fusion machinery namely, coat proteins, small GTPases, tethering factors, SNAREs and molecular motors. Coat-proteins, like clathrin, form a coat on the membrane allowing the membrane to bend into a vesicle. This is followed by scission of these vesicles from the donor compartment by action of scission factors like dynamin. Small GTPases of the Rab and Arf family associate with the vesicles after uncoating and guide the vesicle to a target compartment. The tethering factors are recruited by small GTPases to specific organelles in the cell and function to tether two vesicles destined for fusion. The SNARE proteins help in providing the specificity to target vesicles and ensure that only correct vesicles fuse with the membrane. Each of the regulatory components of the fusion machinery are discussed in detail below.

1.1.2 Coat proteins and Clathrin-dependent endocytosis

The coat protein clathrin consists of three heavy chains (~190kD) and three light chains (~30kD) that form a hexameric complex containing three legged structure (called triskelion) [4, 5] arranged in pentagons and hexagons [6]. Triskelion assembles together in an organized way by linking the vertices to the polygonal patterns [7, 8] hence stabilizing the clathrin-cage assembly in the cytoplasm of the cell. Adaptor protein complexes directly interact with

clathrin subunits [9, 10] resulting in deformation of membrane and recruitment of cargo into the budding vesicle [11, 12]. The clathrin coated vesicle loaded with specific proteins and other molecules now pinches off from the donor membrane [13] and is directed towards the target organelle. Soon after the internalization of clathrin-coated vesicles in the cytoplasm of the cell, uncoating of the vesicles occurs by action of proteins such as Hsp70. Adaptor protein complexes and coat proteins disassemble back into the cytosol from the membrane of the vesicle [14].

1.1.3 Adaptor Proteins

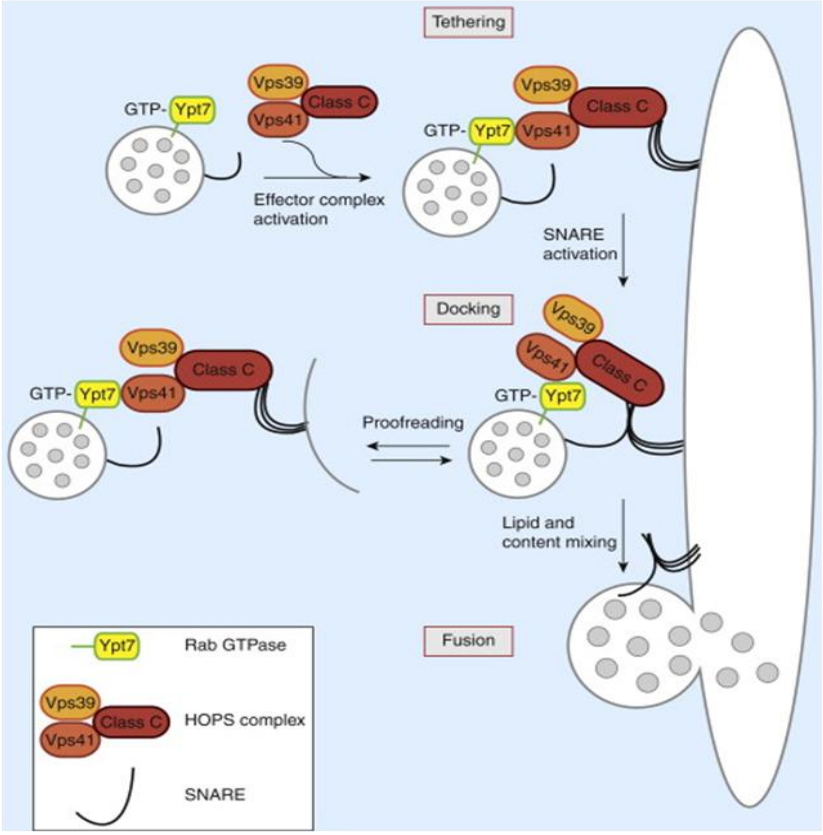
Hetero-tetrameric coat protein complexes called adaptor proteins perform their sorting function by interacting with the specific cytosolic domains of the membrane proteins [15, 16]. The adaptor protein complexes assemble together at the vesicle formation site [11, 17] and generate endocytic vesicles that are transported to target compartments [17, 18].This results in the concentrated membrane domain with adaptor and cargo proteins that are contained in the formed vesicle [11]. *The adaptor protein complexes AP-1, AP-2, AP-3 and AP-4, are recruited to the newly formed vesicles [9, 17]*. *Each adaptor protein is made of four adaptin subunits:* two large subunits (β and one of γ, α, δ, or ε), one medium subunit (μ) and one small subunit (σ) [9, 19]. The main core region of the complex interacts with the cytoplasmic tails of the cargo proteins via sorting motifs present in the cytoplasmic region of these receptors [10, 19]. Adaptor Protein complexes also interact and bind/link with the coat protein clathrin that surrounds the newly formed vesicle [5]. The specificity of adaptor proteins with the transmembrane receptors results in the formation of distinct **c**lathrin-**c**oated **v**esicles or CCVs [19]. The interaction between AP-3 and clathrin in the clathrin coated vesicles [20] is responsible for targeting vesicles to the lysosomes.

1.1.4 Small GTPases

Small GTP-binding proteins (or small GTPases) comprise various monomeric G-proteins, many of which regulate vesicular trafficking between the intracellular compartments. Now, more than 100 small G-proteins have been identified in eukaryotes from yeast to human, and they comprise a superfamily. The members of this superfamily are structurally classified into at least six families: the Ras, Rho, Rab, Sar1/Arf, Arf-like (Arl) and Ran families. These proteins undergo conformational changes between a GTP-bound form and a GDP-bound one. The GDP-bound state continues to stay inactive in the cytosol until it interacts with a guanine nucleotide exchange factor (GEF) that enables the exchange of GDP for GTP, which favors binding of the protein to the membrane [21]. In this way, GTPases act as molecular switches whose activity/function is determined by their GTP- or GDP-bound state. In the vesicular trafficking pathway, small GTPases of the Rab, Arf and Arl family play major roles as membrane organizers. Key functions of these small GTPases include vesicle motility, tethering and fusion as well as interaction with coat proteins and phospholipid generation on the membranes of the endosomes. These plethora of functions of the small GTPases are accomplished by recruitment of their diverse downstream effector proteins to the organelle membranes.

1.1.5 Tethering factors

Tethering factors are the protein complexes that are recruited to specific organelles by small GTPases. They can be broadly categorized as homodimeric coiled-coil proteins and multisubunit complexes. To date, three conserved complexes i.e. CORVET (class **C** c**or**e **v**acuole/**e**ndosome tethering), GARP/VFT (**G**olgi **A**ssociated **R**etrograde **P**rotein/ **V**ps **F**ifty **t**hree complex) and HOPS (**HO**motypic fusion and vacuole **P**rotein **S**orting) have been identified *in vivo*, that have been attributed the tethering function in endocytic trafficking [22, 23]. The role of tethering complexes in vesicle tethering was first discovered in *Saccharomyces cerevisiae* [24]. HOPS complex has been reported to function as Rab7 effector (i.e. homolog of Ypt7 in case of yeast) to bring about late endosome-vacuole fusion (Figure 2). The yeast HOPS complex regulates homotypic vacuole fusion by forming a "vertex ring" around the juxtapose vacuolar membranes before fusion events begin [25-27]. In addition, yeast HOPS complex directly interacts with SNAREs [28-30] and has been shown to recruit SNARE proteins on reconstituted membranes *in vitro.* Also, it has been shown to prevent disassembly of the SNARE complexes to mediate fusion at the vacuole [31-33].



Brocker et al., *Current Biology* 2010

Figure 2. Molecular players of vesicle fusion in yeast. The yeast HOPS complex is recruited to late endosomal-vacuolar membranes by Rab GTPase Ypt7, that then functions to tether the membranes of two compartments. Additionally, HOPS complex interacts with the SNARE proteins and allows the correctly assembled trans-SNARE pair to promote fusion with the target organelle. Copyright permission to reproduce the figure was obtained from the journal.

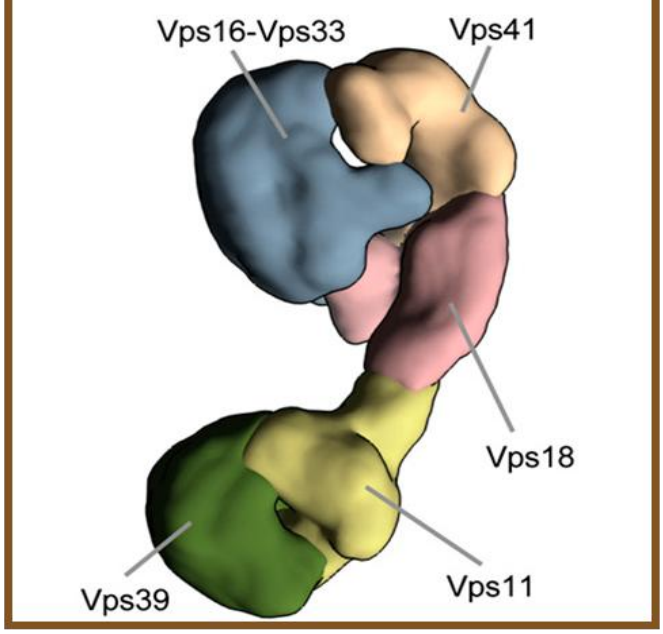
1.1.6 SNAREs

SNAREs or "SNAP (**S**oluble **N**SF **A**ttachment **P**rotein) **RE**ceptor" are membrane proteins, that catalyze fusion events by forming complexes *in trans* and bridging the opposing lipid bilayers, bringing them in proximity and inducing their fusion. These proteins are generally found as v-SNAREs or vesicular-SNAREs on the membrane of vesicle and as t-SNAREs or target SNAREs on target membranes. SNAREs usually have a transmembrane domain through which they insert into the membrane of the vesicles. Additionally, some cytosolic SNAREs localize to organelle membranes via interaction with membrane attached SNAREs leading to trans-SNARE pair/complex formation [31]. Following fusion of the two membranes, the SNARE complex gets disassembled by ATPase NSF

[31] and Sec17/Sec18 proteins [32]. In *S. cerevisiae*, HOPS subunit Vps33 is known to bind SNARE proteins Vam3p and Vam7p. In eukaryotic cells, HOPS complex subunit, Vps33, interacts with t-SNAREs on lysosomes to ensure that vesicles destined for degradation are delivered to the lysosomes.

1.1.7 HOPS complex

HOPS (**HO**motypic fusion and vacuole **P**rotein **S**orting) complex is a multi-subunit protein complex comprising of six subunits, namely, Vps11, Vps16, Vps18, Vps33 that form the core complex and Vps39, Vps41 that form the accessory part of the complex. HOPS complex subunits are conserved from yeast to mammals i.e. mammalian HOPS also assembles in a hexameric complex , similar to its yeast counterpart (Figure 3). The molecular mechanisms of late endosome- lysosome fusion in mammals are thought to be analogous to those of homotypic vacuole fusion in *S. cerevisiae*. The class B subunit Vps41p [33-35] interacts with an activated GTP-bound state of Ypt7 (homologue of mammalian Rab7), a GTPase that helps in recruitment of HOPS complex to late-endosomal and vacuolar membranes in yeast (Figure 3) [36, 37]. In addition, Vps41p has been also shown to interact with adaptor protein AP-3 [38] to mediate AP-3 dependent vesicular trafficking pathway. The class C subunit Vps33 binds to membrane SNAREs Vam3p and Vam7p in *S. cerevisiae* [32] which helps in the formation of the correct trans-SNARE complex leading to vacuolar fusion.



Bröcker C.,Kuhleeb A. *et. al.* PNAS 2012

Figure 3. Segmented model of the yeast HOPS tethering complex. Yeast HOPS complex assembles into a hexameric complex with a definite subunit arrangement. The head domain consists of Vps16-Vps33 and Vps41, while the bulky tail is composed of Vps11 and Vps39. Yeast HOPS complex has been implicated in all fusion events at the vacuole. Copyright permission to use the figure was obtained from the corresponding author of the publication.

Previous studies have shown that, in yeast, class C subunit Vps18 forms a central component of the HOPS complex and its function is required for biosynthetic, endocytic and autophagic protein transport pathways to the vacuole. Vps18 mutants are defective in vacuole biogenesis and sorting and transport of vacuolar enzymes. Deficiency of *vps18* in zebra fish larvae results in hepatomegaly and skin hypopigmentation due to defective melanosomes, a type of lysosome-related organelle [39]. *Aspergillus nidulans* homolog of Vps18 is required for nuclear migration, mitochondrial morphology and polarized growth [40]. Ablation of *vps18* in mice leads to neurodegeneration and impaired neuronal migration by blocking multiple transport pathways to the lysosome, including autophagy, endocytosis, and biosynthetic pathways [41]. The mammalian homolog of yeast Vps18p has been shown to be a member of homotypic fusion and vacuole protein sorting complex. When overexpressed,

mVps18 causes clustering of late endosomes/lysosomes indicating its role in the late endocytic pathway. hVps18 is one of the six subunits of human HOPS complex that is a key molecular player known to drive late endosome-lysosome fusion. HOPS complex subunits, including Vps18, have a secondary structure with an amino terminal beta-propeller and an alpha-solenoid towards the C-terminus. The proteins containing such motifs with repeated domains serves as a site for protein-protein interactions, thereby forming a platform for assembly of protein complexes. Bioinformatics analyses have revealed that hVps18 has a cysteine-rich zinc finger like domain or RING (i.e. **R**eally **I**nteresting **N**ew **G**ene) at its C- terminus and a CHCR (**C**lathrin **H**eavy **C**hain **R**epeat) domain at its N-terminus. Both these domains are well-conserved in other HOPS subunits as well and they are known to mediate protein-protein interactions. Studies have shown that RING-domain of hVps18 regulates monoubiquitylation of GGAs (**G**olgi-localizing, **G**amma-adaptin ear domain homology, **A**DP-ribosylation factor (ARF)-binding proteins) that plays a role in protein trafficking from trans-Golgi bodies to endosomes [42]. Mutations in the RING- domain of yeast Vps18 results in temperature-conditional **c**arboxy**p**eptidase **Y** (CPY) sorting defects [43] in *S. cerevisiae*. Vps18/PEP3 gene shares homology with *Drosophila melanogaster* dor protein, which also has RING-finger at its C-terminal [44]. Vps18p is essential for docking and fusion of autophagosomes with the vacuoles [45, 46].

1.1.8 Significance:

HOPS complex is a multisubunit tethering complex that localizes to the endo-lysosomal compartments where it regulates fusion events and delivery of lysosomal proteins [47]. Even though the yeast complex has been well characterized and understood, the molecular mechanisms governing mammalian HOPS complex recruitment and function still remain elusive. Mutations in genes encoding these regulatory proteins result in morphological defects and mislocalization of the lysosomal proteins [48]. Vacuolar trafficking defects in yeast provide the first evidence about the important role of HOPS complex in regulating SNARE-pair complex formation and membrane fusion of endo-lysosomal compartment [31, 37] . The role of the mammalian HOPS complex is just beginning to be understood and recent studies have reported critical role of different HOPS subunits in regulating endosome-lysosome, phagosome-lysosome and autophagosome-lysosome fusion [49]. The importance of this protein complex is reflected by the fact that pathogens such as *Mycobacterium.tuberculosis* evade killing in the host lysosome by targeting HOPS complex subunits [50]. Moreover, patients suffering from **a**rthrogryposis-**r**enal dysfunction-**c**holestasis (ARC) syndrome which presumably lack a functional HOPS complex suffer from recurrent bacterial infections [51]. On the other hand, recent studies have shown that Ebola and Marburg filoviruses that cause rapidly fatal haemorrhagic fever in humans use HOPS complex to undergo membrane fusion and establish infection [52]. These findings uncover HOPS as a potential target for antiviral strategies to combat these deadly diseases.

In order to gain insight into the mechanism of how mammalian HOPS complex mediates late endosome-lysosome fusion, we want to understand the assembly of HOPS subunits on the late endosomes and lysosomes.

How the interaction between different subunits guides their localization to lysosomal membranes is a key to this understanding. In view of this, this project was designed to focus on studying the localization and interaction partners of hVps18, a subunit of core HOPS complex in mammals. *We aimed to answer the following questions about human Vps18 protein to understand its role as part of the HOPS complex:*

1. Identification of the subunits of the human HOPS complex that directly interact with Vps18 using yeast-two-hybrid assay.
2. Constructing domain deletion mutants of hVps18 to identify the critical domain required for interaction with other HOPS subunits.
3. Analyzing localization of over expressed hVps18 in HeLa cells and colocalization of Vps18 with its interaction partners.
4. Purification of recombinant fragment of hVps18 to generate an antisera against Vps18 to detect its endogenous localization in cells.

1.2 Experimental Methods

1.2.1 Plasmids

Epitope tagged, full-length human HOPS subunits Vps11, Vps16, Vps18 and Vps18 cloned in mammalian expression vectors were obtained from C. Liang lab (USA) . HA-Vps33a was a generous gift from Victor Faundez lab (USA). HA-Vps41 was a gift from Wade Harper lab (USA). pGADT7 and pGBKT7 were a gift from Steve Caplan lab (USA). MBP-Vps18CT was a generous gift from Simon Richardson lab. hVps18 S619X and hVps18 A773X were cloned in pGADT7 vector using EcoRI and BamHI restriction sites. The primers used to amplify hVps18 S619X were (Forward primer 5' CGGAATTCATGGCGTCCATCCTGGATGAGTACG 3' and Reverse primer 5' CGGGATCCCTAGCCCATCTCAATCCAGGCATCTAC 3'). The primers used to amplify hVps18 S619X were (Forward primer 5' CGGAATTCATGGCGTCCATCCTGGATGAGTACG 3' and Reverse primer 5' CGGGATCCCTATGTCTGTACATCTTCCTCTTCC 3').

1.2.2 Antibodies

The mouse monoclonal antibody for human Lamp1 was purchased from BD Transduction laboratories. The mouse monoclonal antibody against Myc-epitope was procured from Invitrogen. The rabbit polyclonal antisera against HA-epitope was purchased from Sigma.

1.2.3 Transfection and Immunostaining

HeLa cells grown on coverslips were transfected with desired constructs using X-tremegene HP transfection reagent (Roche) as per manufacturer's instructions. After 20 hours of transfection, cells were fixed with 3.7% PFA for 12 minutes, washed with PBS and blocked with 5% FBS made in PHEM buffer for one hour. Cells were then incubated with primary antibodies in PHEM buffer containing 0.2% saponin for 45 minutes at room temperature, washed with PBS and incubated with secondary antibodies conjugated with Alexa-fluor conjugates (Molecular probes), in PHEM buffer containing 0.2% saponin for 30 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.

1.2.4 Yeast-two-hybrid

hVps18 WT and its domain deletion mutants were cloned in GAL4-activation domain vector while other HOPS subunits were cloned in GAL4-binding domain vector. Constructs were co-transformed in yeast strain AH109 (as indicated in the figures) and plated on -leu/-trp plates. The plates were incubated at 30°C for two days, following which yeast transformants were plated on -leu/-trp/-his plates. For stringent selection, transformed yeast was also plated on -leu/-trp/-his +3-AT plates. The plates were then incubated at 30°C for three-four days.

1.2.5 Protein-purification

MBP-Vps18CT (amino acids 766-973) cloned in pMAL was overexpressed in *E. coli* BL-21 cells and induced with 0.5mM IPTG in for five hours at 30°C. The bacterial cells were harvested and the pellet was resuspended in column buffer (20mM Tris-Cl (pH 7.4), 200 mM NaCl, 1mM EDTA, 10mM 2-mercaptoethanol) containing protease inhibitor cocktail. The bacterial cells were lysed by sonication and the lysate was cleared by centrifugation. The supernatant obtained was incubated with amylose resin for three hours. The resin was washed extensively with column buffer, and incubated with 500 µl 10mM maltose overnight to elute the protein. This eluate was labelled as fraction 1 (F1). Three successive elutions were done by incubating the beads with 500 µl elution buffer for 30 minutes at room temperature. The protein fractions were collected (F2 through F4) and stored at -80°C, until further use.

1.2.6 Buffer Compositions

1. Super Broth

   Tryptone - 1.2%

   Yeast extract - 2.4%

   Glycerol - 0.4%

2. Salt solution

   Dipotassium hydrogen phosphate - 16%

   Potassium dihydrogen phosphate - 2.3%

3. PHEM buffer

   PIPES - 60mM

   HEPES - 25mM

   EGTA - 10mM

   Magnesium chloride - 2mM

   Adjust pH to 6.9.

Chapter 2

Summary & Conclusions

2.1 Concluding Remarks
2.2 Future Outlook

2.1 Concluding Remarks

HOPS complex is a multi-subunit protein complex that regulates fusion of late endosomes with lysosomes. HOPS subunits and their function have stayed remarkably conserved from yeast to mammals. The yeast HOPS complex has been well characterized both structurally and functionally. On the contrary, little is known about the molecular mechanisms governing the assembly and functioning of mammalian HOPS complex. In this study, we have characterized the mammalian HOPS complex subunit Vps18 and how it acts as a central part of the complex to mediate recruitment of other subunits of HOPS complex to lysosomes. In summary, we have demonstrated that HOPS complex subunit hVps18 directly interacts with hVps16 and hVps41 subunits. Immunofluorescence results show that mammalian HOPS complex subunits localize to lysosomes and hVps41 promotes the recruitment of hVps18 to these compartments. Together our results indicate that mammalian HOPS complex subunits interact with each other and the assembly of HOPS complex on lysosomes is mediated via subunit-subunit interactions.

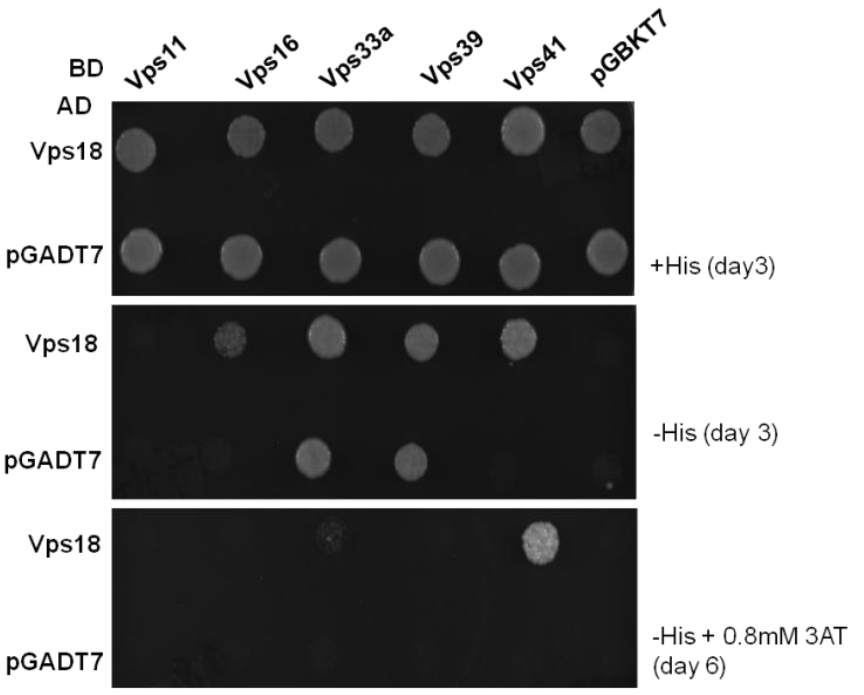


Figure4. Yeast-two-hybrid interaction of Vps18 (cloned in activation domain) with all subunits of HOPS complex (cloned in binding domain vector) was tested using pGADT7/pGBKT7 system. Yeast were plated on non-selective medium (+His) to confirm viability and on selective medium (-His) to detect interactions.

2.1.1 hVps18 interacts with Vps16 and Vps41 subunits of the HOPS complex

Using hVps18 as bait in a yeast-two-hybrid assay, we mapped its interactions with other subunits of HOPS complex. hVps18 was cloned in fusion with GAL4-activation domain while other subunits of HOPS complex were cloned in fusion with GAL4-binding domain. Post transformation, yeast were plated on non-selective medium to confirm viability and on selection medium to detect interactions. Three days hence, it was observed that hVps18 interacts with hVps16 and hVps41 subunit of HOPS complex. hVps33a and hVps39 cloned in binding domain vectors showed autoactivation, and therefore no conclusions could be drawn about their interaction with

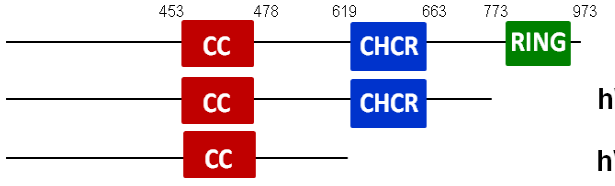


Figure5. Domain architecture of hVps18, as predicted bioinformatically, showing the functional domains present in the protein. CC: Coiled Coil, CHCR: Clathrin Heavy Chain Repeat, RING: Really Interesting New Gene.

hVps18 from the selection plate. To minimize/remove autoactivation effects, yeast were also plated on the interaction plate containing 0.8mM 3AT. 3AT is a competitive inhibitor of Histidine synthase (reporter) gene, so addition of 3AT makes the conditions stringent such that only strong interactions manifest as growth of yeast on the interaction plate while low affinity interactions are not obtained. On the 3AT plate, it was seen that hVps18 showed an interaction with hVps41 while no interaction was seen with hVps16 suggesting that it is a weak interaction. Autoactivation of hVps33a and hVps39 was controlled and hVps18 was seen to show a very weak interaction with hVps33a. This data implies that HOPS complex subunit hVps18 directly interacts with hVps41 and hVps16 subunits. These subunit-subunit interactions may play a role in assembly of entire complex on the lysosomes where it functions to mediate late endosome-lysosome fusion.

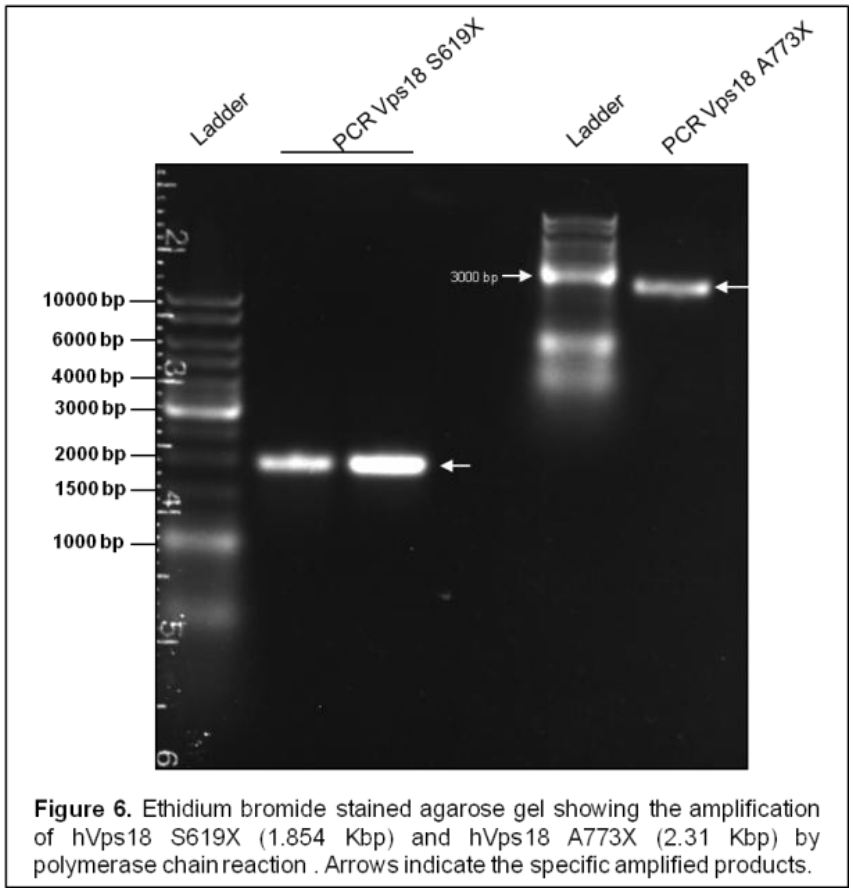


Figure 6. Ethidium bromide stained agarose gel showing the amplification of hVps18 S619X (1.854 Kbp) and hVps18 A773X (2.31 Kbp) by polymerase chain reaction . Arrows indicate the specific amplified products.

2.1.2 Cloning of hVps18 S619X and hVps18 A773X

Bioinformatics analyses predicts that hVps18 has two functional domains, namely, CHCR (Clathrin Heavy Chain Repeat) and RING (Really Interesting New Gene). Interestingly, these domains are also present in other HOPS subunits including Vps11 and Vps41.To understand the critical domains involved in interaction of hVPs18 with other subunits of HOPS complex, we constructed domain deletion mutants of Vps18 (Figure 5). hVps18 S619X and hVps18 A773X were cloned in yeast expression vector pGADT7 to study their interactions with other HOPS subunits in a yeast-two-hybrid system. PCR amplification yielded amplicons of the desired size (Figure 6). Cloning was done

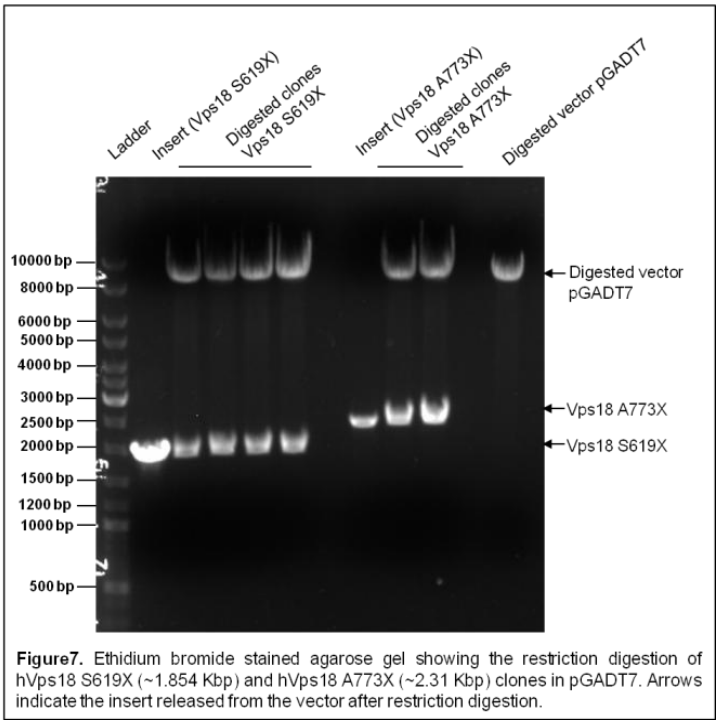


Figure7. Ethidium bromide stained agarose gel showing the restriction digestion of hVps18 S619X (~1.854 Kbp) and hVps18 A773X (~2.31 Kbp) clones in pGADT7. Arrows indicate the insert released from the vector after restriction digestion.

exploiting EcoRI and BamHI restriction sites. Four clones of Vps18S619X and two clones of Vps18A773X were screened by digesting them with the aforementioned restriction enzymes. All the digested clones were positive for the insert of the expected size (Figure 7).

2.1.3 Interaction of hVps18 WT and domain-deletion mutants with other subunits

Using hVps18 WT, hVps18 S619X (ΔCHCR+ΔRING) and hVps18 A773X (ΔRING) as bait in a yeast-two-hybrid system, we studied as to which is the critical domain required for interaction of hVps18 with other subunits of HOPS complex. hVps18 and its mutants were cloned in fusion with GAL4-activation domain while other subunits of HOPS complex were cloned in fusion with GAL4-binding domain. Post transformation, yeast were plated on non-selective medium to confirm viability and on selection medium to detect interactions. After three days, it was observed that hVps18 interacts with hVps41 subunit of HOPS complex, while hVps18 S619X (ΔCHCR+ΔRING) and hVps18 A773X (ΔRING) failed to show any interaction with Vps16 and Vps41, suggesting that RING domain is critical for this interaction. hVps33a cloned in binding domain vector showed autoactivation, and therefore no conclusions could be drawn about its interaction with hVps18 or its mutants from the selection plate (Figure 8).

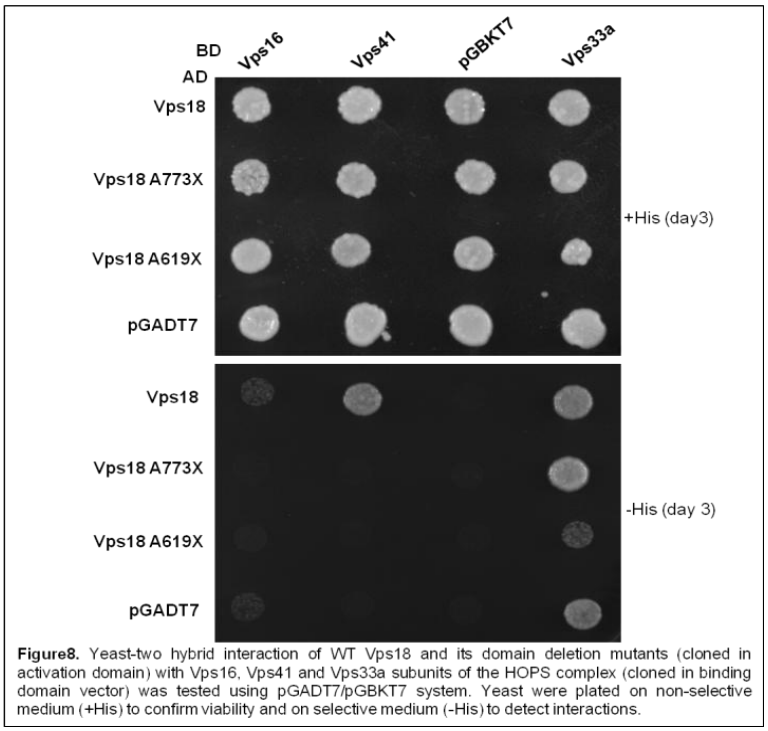


Figure8. Yeast-two hybrid interaction of WT Vps18 and its domain deletion mutants (cloned in activation domain) with Vps16, Vps41 and Vps33a subunits of the HOPS complex (cloned in binding domain vector) was tested using pGADT7/pGBKT7 system. Yeast were plated on non-selective medium (+His) to confirm viability and on selective medium (-His) to detect interactions.

2.1.4 Subcellular localization of hVps18

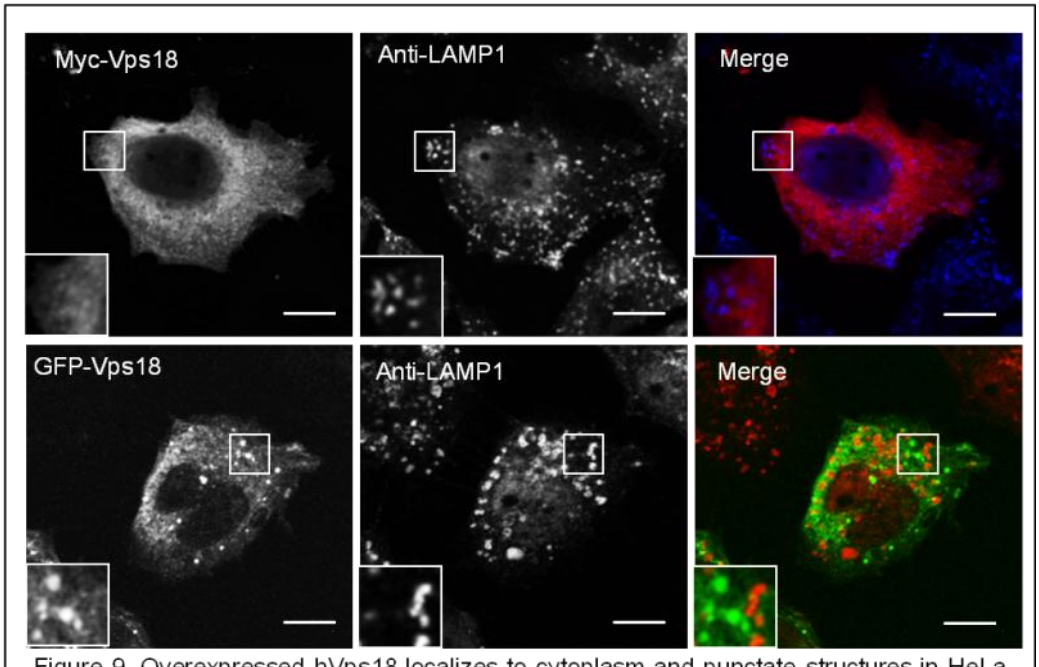


Figure 9. Overexpressed hVps18 localizes to cytoplasm and punctate structures in HeLa cells. HeLa cells were transfected with Vps18 and analyzed for lysosomal localization by confocal microscopy. Inset shows magnified view of the boxed region.

To determine the subcellular localization of HOPS subunits, hVps18 was either transfected alone or cotransfected in combination with other HOPS subunits in HeLa cells. Confocal microscopy analysis revealed that when overexpressed alone, hVps18 localizes to cytoplasm of HeLa cells and is not present on lysosomes (LAMP1 positive endosomes). In several cells we also observed the localization of Vps18 to structures which appeared as protein aggregates (Figure 9).

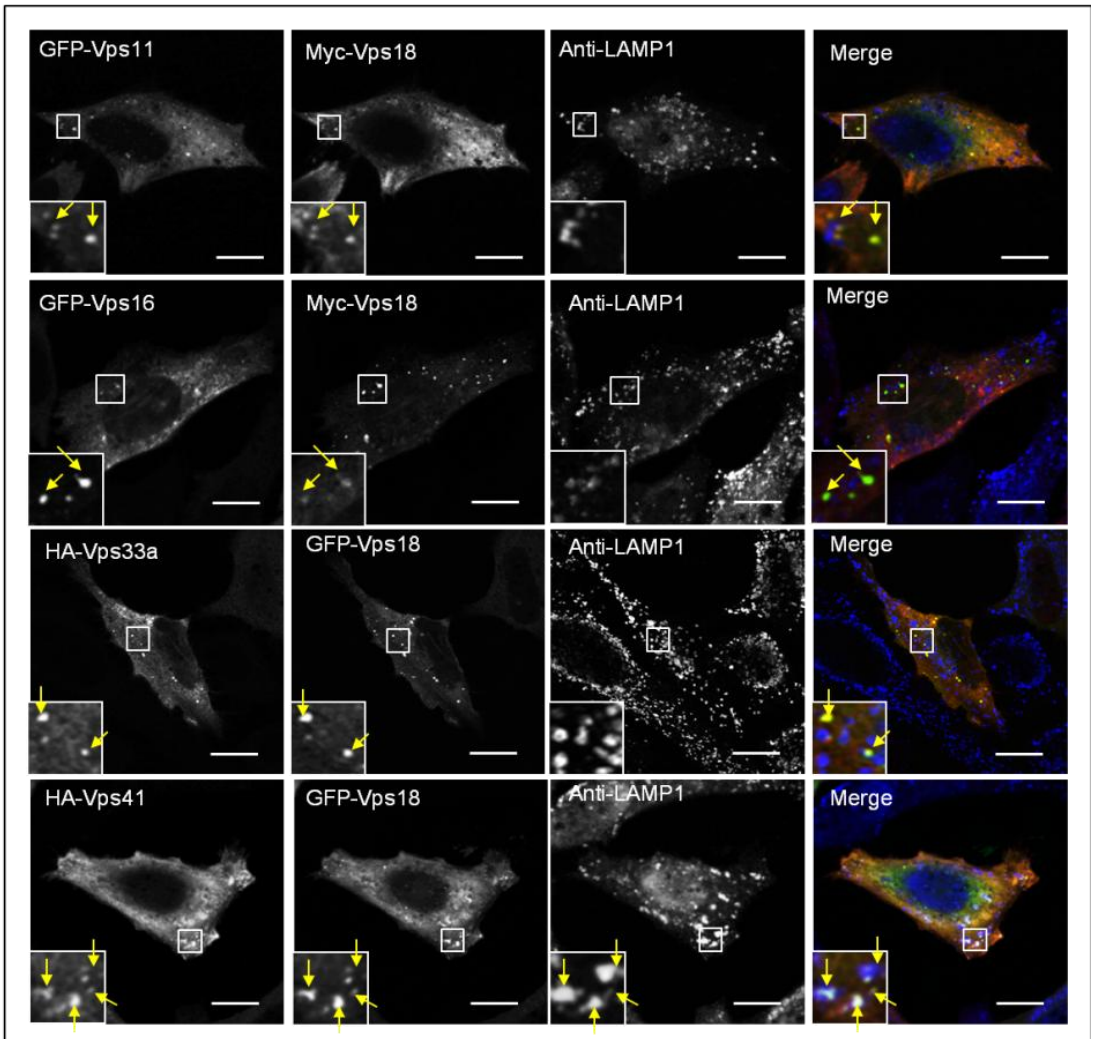


Figure 10. hVps41 recruits hVps18 to lysosomes. HeLa cells were co-transfected with hVps18 and hVps11 or hVps16 or hVps33a or hVps41, as indicated, and analyzed for lysosomal localization by confocal microscopy. Colocalized pixels are indicated in the inset.

When either hVps11, hVps16 or hVps33a were co-expressed along with hVps18 in HeLa cells, it was observed that hVps18 colocalized with these subunits on punctate structures. However, these structures were again not positive for LAMP1 and thus were not late endosomal or lysosomal compartments. Interestingly, when hVps18 was coexpressed alongwith hVps41 in HeLa cells, it was seen that hVps41 recruited hVps18 to lysosomes, suggesting that interaction of hVps41 with hVps18 is responsible for lysosomal localization of Vps18. This is expected as Vps41 subunit of HOPS complex is reported to localize to late endosomes and lysosomes (Figure 10). Thus, our data suggests that Vps41 associates with lysosomes and recruits Vps18 to

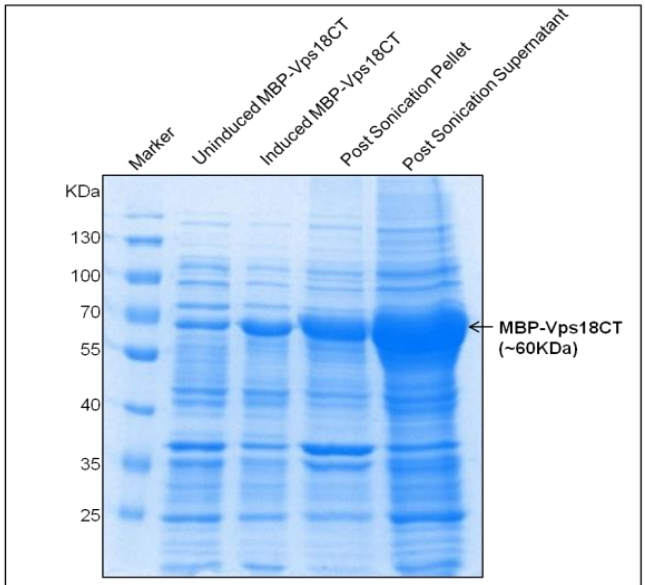


Figure11. Coomassie stained SDS-PAGE gel showing bacterial cell lysates expressing MBP-Vps18 C-terminal fragment.

lysosomal membranes via direct interaction with Vps18. Further interaction of hVps18 with hVps16 and hVps33a may be responsible for recruitment of hVps16 and hVps33a to lysosomes. Together these results indicate that HOPS subunits localize to lysosomes and their lysosomal localization is guided by subunit-subunit interactions.

2.1.5 Purification of MBP-tagged hVps18-CT (766-973aa)

hVps18 C-terminal fragment (amino acids 766-973) cloned in fusion with Maltose-binding protein in pMAL vector was obtained from the laboratory of Simon Richardson at University of Greenwich. MBP-Vps18 was transformed in *E.coli* BL-21 strain and induced using 0.5 mM IPTG at 30°C for 5 hours (Figure 11). Post-induction, MBP-Vps18 was purified from bacterial cell lysates by incubating it with amylose resin. The protein was eluted from the beads using 10 mM maltose and then subjected to SDS-PAGE. Coomassie staining of the SDS-PAGE gel revealed that the protein was eluted in all the fractions, namely, F1 through F4, however most protein was eluted in the F1 fraction. (Figure 12).

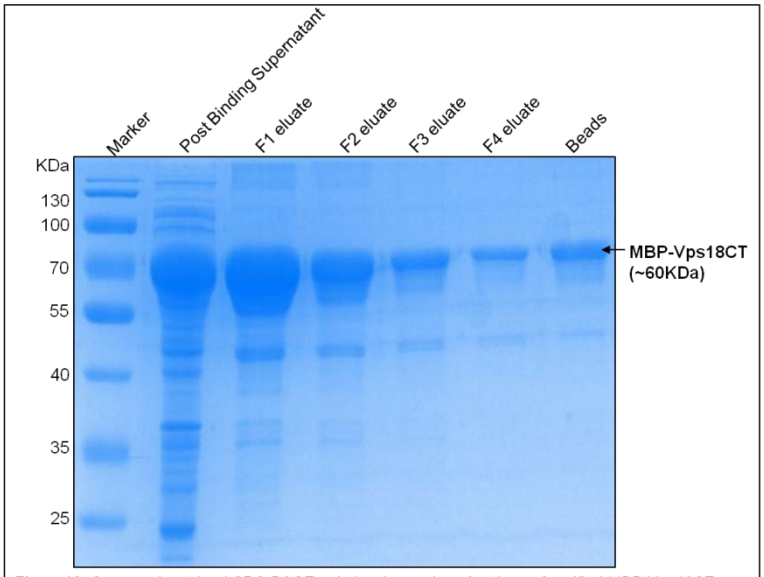


Figure12. Coomassie stained SDS-PAGE gel showing various fractions of purified MBP-Vps18CT eluted from the amylose resin (amino acids 766-973).

The eluate will be further subjected to gel filtration chromatography so as to obtain protein of higher purity that can be used for generation of antibody against hVps18. This antibody will be useful to detect endogenous hVps18 through techniques like immunofluorescence and western blotting.

Future Outlook

In this study we demonstrate for the first time the organization of human HOPS complex. HOPS complex subunit Vps18 was found to directly interact with Vps41 and Vps16 subunit but not with Vps11 and Vps39. A weak interaction was seen with Vps33a subunit of the complex. The interaction with Vps41 and Vps16 is mediated by the RING domain of Vps18 as deletion of this domain (Vps18A773X) abolished the interaction with these two subunits.

We also found that Vps18 upon overexpression localizes to cytoplasm and to punctate structures which were not positive for lysosomal marker LAMP1. Co-localization of Vps18 with Vps11, Vps16 and Vps33a was seen on these punctate structures. The identity of these structures could not be confirmed as they did not localize with most endocytic markers (images with LAMP1 and previous unpublished data from the laboratory). Interestingly, only upon co-expression with Vps41 we observed distinct endosomes containing both Vps18 and Vps41 that colocalized with LAMP1. This suggests that interaction with Vps41 drives association of Vps18 with late endosomal and lysosomal membranes. Moreover, this data is in agreement with previous reports suggesting Vps41 subunit of HOPS complex is localized to late endosomes and lysosomes [1]. Future experiments will determine if Vps18 is a central component of the human HOPS complex that interacts with multiple other subunits and regulates formation of this six subunit HOPS complex that can then function as a tethering factor to drive late endosome lysosome fusion.

In conclusion, we have defined that Vps18 directly interacts with Vps16 and Vps41 subunit of the HOPS complex. Interaction of Vps18 with Vps41 can promote recruitment of Vps18 to late endosomes and lysosomes.

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