Identification of SNAREs that interact with mammalian HOPS complex to mediate late endosome-lysosome fusion.

Irfana Saleem

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

Indian Institute of Science Education and Research Mohali
April 2015

Dedicated to

my parents, friends and teachers for their love and support

and to my nephew Yaanu

'*Happiness can be found even* in the darkest times'

- *J.K. Rowling*

Certificate of Examination

This to certify that the dissertation entitled "**Identification of SNAREs that interact with mammalian HOPS complex to mediate late endosome-lysosome fusion"** submitted by Ms.Irfana Saleem (ms10028) 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. 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 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.

Irfana Saleem
(Candidate)
Dated: April 24,2015

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

I would like to extend my deepest gratitude to my supervisor Dr. Mahak Sharma for so many things. First of all, I would like to thank her for continuous support and guidance throughout the project. Secondly for inspiring, motivating and setting up a great example for being a researcher. Thirdly for her care, affection and for spending valuable time for me in my difficult times and fourth, for the coffee maker.

Next I would like to thank my PhD mentor Divya Khatter for her guidance and time, and also lab members Aastha Sindhwani, Devashish Dwivedi, Rituraj Marwaha, Vivek B. Raina and Divya Jagga for helping and supporting for carrying out my project smoothly. And a special thanks to Aastha and Divya Jagga for the beautiful tea times.
I would like to thank all the staff and faculty of DBS for making IISER Mohali a great place to learn and conduct research.

Now its time for some great people in my life to be acknowledged. I would like to thank my Parents for making a difference from being usual and let me spread my wings and see the world. Thank you so much to both of them, for their support and unconditional love. And to my sister for being so awesome and a best friend. To my brother for his support, advice and also for the laptop which was very crucial for fulfilment of this project. I would also like to thank my uncles Mahad, Mackbool and their family and also my aunt Huzna for supporting me and my family while I'm away.

Now I would like to thank some special people in my life, my buddies at IISER, Prashant, Thoi, Rajat, Ashima, Sachikanta, Nitin and my brothers Anuj, Abhishek and Pradeep for making a great team for adventure and fun. A special thanks to my best friend Prashant for his support and care.
Last but not at all least, to God Almighty, thank you so much for giving me all the above people and making my life wonderful.

Notations

3-AT	**: 3-amino-1,2,4-triazole**
FBS	**: Foetal Bovine Serum**
LAMP1	**: Lysosomal-associated membrane protein 1**
PBS	**: Phosphate Buffer Saline**
PFA	**: Paraformaldehyde**
SDS PAGE	**: Sodium Dodecyl Sulfate- Polyacrylamide gel electrophoresis**
Vti1b	**: Vesicle transport through interacting with t-SNARE**
VAMP	**: Vesicle associated membrane protein**

List of Figures

Contents

Abstract

Eukaryotic cells dynamically communicate with their extracellular environment that involves a constant uptake and degradation of cargo, such as nutrients, in the lysosomes. The dynamic fusion and fission events that drive cargo transport to lysosomes are regulated by protein machineries such as small GTPases, tethering factors like HOPS (Homotypic fusion and vacuole Protein Sorting) complex and SNAREs (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor). Human HOPS complex is a six subunit tethering factor, conserved from yeast to mammals, that has been shown to mediate cargo trafficking to lysosomes. Previous studies suggest that Vps33 subunit of yeast HOPS complex interacts with vacuolar SNAREs such as Vam3, Vam7 and Nyv1 to mediate homotypic fusion at the vacuole/lysosome. However, little is known about the SNAREs that function in conjunction with mammalian HOPS complex to mediate late endosome-lysosome fusion. In this study, we have characterised mammalian SNARE proteins that partially localize to lysosomes, namely, VAMP7, Syntaxin7, Syntaxin8, and Vti1b. Using yeast two-hybrid assays, we have tested interaction of these SNAREs with all the six subunits of the HOPS complex. No direct binding with any of the HOPS subunits was found in this assay, suggesting that probably more than one subunit of HOPS complex is required for interaction with SNAREs. Further identification of the SNARE proteins will be carried out using tandem affinity pull down approaches using Vps41 and Vp33a subunits as bait proteins. Confocal microscopy analysis indicates that target membrane associated SNARE Vti1b co localizes with hVps41 subunit of HOPS complex. Depletion of Vps41 subunit results in severely dispersed staining of Vti1b that continues to colocalize with lysosomal marker LAMP1. Furthermore, Vti1b co localizes with other SNAREs such as Syntaxin7, Syntaxin8 and VAMP7 suggesting that they could be the potential SNAREs forming a quaternary complex that mediates membrane fusion at the lysosomes.

Chapter 1

1.1 Introduction

1.2 Materials and Methods

1.1 Introduction

Endo-lysosomal pathway is fundamental to all eukaryotes through which they mediate uptake, intracellular transport and degradation of cargo such as macromolecular nutrients, worn-out organelles and micro-organisms. The conventional endo-lysosomal pathway begins by internalization of cargo in small vesicles at the plasma membrane, followed by their fusion with the early endosomes (Huotari and Helenius 2011) The cargo from early endosomes is either directed to plasma membrane via recycling endosomes or to late endosomes, if destined for degradation.(Saftig and Klumperman 2009) Further, late endosomes undergo heterotypic fusion with lysosomes forming a hybrid organelle where the cargo is subsequently degraded. (Shown in Figure 1)

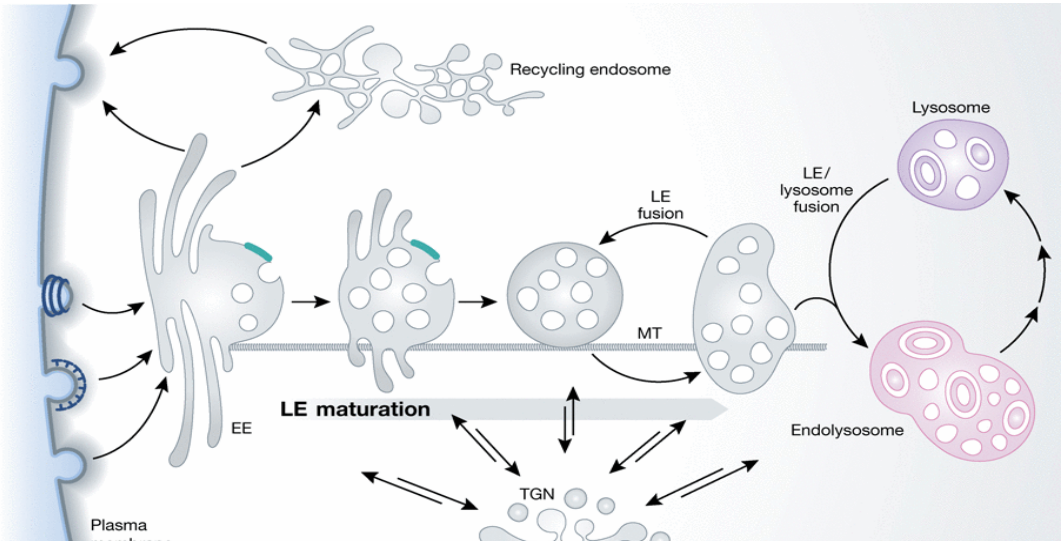


Fig.1 **Schematic representation of transport of cargo through endo-lysosomal pathway**. The cargo internalized at plasma membrane is transported to early endosomes, upon accumulation of cargo at early endosomes they are sorted to recycling pathway via recycling endosomes or degradative pathway towards late endosomes. Late endosomes fuse with lysosomes to form a hybrid organelle endo-lysosomes where the cargo is degraded. The image is reproduced from (Huotari and Helenius 2011).

The transport of cargo through the endo-lysosomal pathway is highly regulated at each step by various classes of proteins such as the coat proteins, small GTPases, tethering factors, SNAREs and motor proteins (Vazquez-Martinez and Malagon 2011) .This cargo transport is driven by dynamic fusion and fission events of vesicles carrying cargo. During the fission of a vesicle from a donor membrane, coat proteins are recruited towards the the donor membrane that aid in formation and budding of vesicles carrying the cargo (Behnia and Munro 2005).Upon uncoating of coat proteins, the vesicles travel across the cytoskeletal filaments with the help of motor proteins such as Dynein and Kinesin towards the target membrane. Once they reach their destination, the vesicles are

tethered with the target membrane with the help of tethering factors and further, they fuse with the target membrane in a SNARE-dependent manner (shown in figure2).

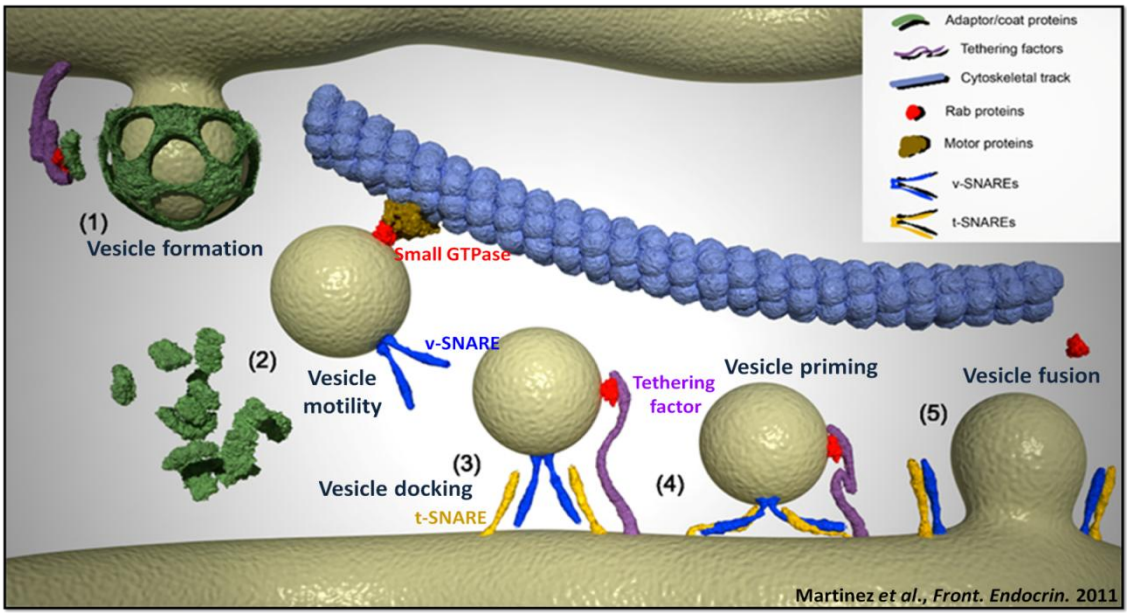


Fig2.**Steps in Vesicle fusion pathway .**Coat proteins aids in formation and budding of vesicles. Upon uncoating of coat proteins ,the vesicle travel towards the target membrane through cytoskeletal filaments. Once they reach target membrane tethering factors recruited by Rab proteins tethers and docks the vesicle to target membrane ,where they undergo vesicle fusion with help of v-SNARE and t-SNAREs. The image is reproduced from(Vazquez-Martinez and Malagon 2011)

1.1.1Coat Proteins

There are three different types of coat proteins that mediate budding and formation of vesicles from different organelles and these vesicles are specifically destined to other organelles. Clathrin-coated vesicles form from plasma membrane and trans-golgi and are directed towards late endosomes. COPII coated vesicles carry cargo from rough endoplasmic recticulum(ER) to golgi, while COP1 coated vesicle transport cargo from golgi to ER (Zanetti, Prinz et al. 2013).The coat protein Clathrin forms a triskelion structure with the help of three heavy chains and three light chains of proteins, which helps in stabilization of the clathrin coat. Once the clathrin-coated vesicle enters the cytoplasm, the coat is disassembled with help of proteins Hsp70 and auxlin .(Young, Stoilova-McPhie et al. 2013).

1.1.2 Small GTPases

Small GTP-binding monomeric proteins regulate diverse functions in eukaryotic cells which includes vesicular trafficking. They are known as molecular switches because of their existence in GTP bound active state and GDP bound inactive state .The activity of GTPases is regulated by proteins such as Guanidine exchange factors (GEF) and GTPase activating

proteins (GAP) which mediates exchange of GTP to GDP bound form and *viceversa*. The small GTPase family includes protein families such as Ras, Rho, Arf, Arl, and Rab, out of which Rab, Arf and Arl families have defined roles in vesicular trafficking. Both sequence and localization of these small GTPases are conserved from yeast to mammals. Studies of small GTPases involved in vesicle trafficking place the small GTPases such as Ypt1/Rab1 to exocytosis pathway, Rab5 to Early endosomal ,Ypt7/Rab7 and Arl8b to the late endosomal and lysosomal pathways (Segev 2001). During vesicular trafficking these small GTPases act as effectors of tethering factors involved in vesicle targeting and docking.

1.1.3 Tethering factors

Tethering of the transport vesicles to the target membranes requires the aid of proteins termed as the "tethering factors" that localize to specific organelles in the cells. For instance, GARP and COG complexes localize to Golgi, Exocyst localizes to plasma membrane and CORVET and HOPS complexes localize to the early endosomes and late endosomes, respectively(Brunet and Sacher 2014). Tethering factors can be classified into two groups, namely, long-coiled coil proteins or large multi-subunit proteins. Long coiled-coil proteins form homodimers and are mostly associated with early endosomes (e.g., EEA1) or Golgi complex (p115). Large multi-subunit proteins that are well characterized *in vivo* include the Golgi-localizing GARP complex and the CORVET and HOPS complexes of the endocytic pathway. In general, the tethering factors mediate various biochemical functions such as vesicle tethering and proof reading SNARE complex formation. HOPS (Homotypic fusion and vacuole Protein Sorting) complex is a multisubunit tethering factor with Vps11, Vps16, Vps18 as core subunits along with SNARE interacting subunit Vps33a, and Vps39 and Vps41 as accessory subunits, which interact with smallGTPases. HOPS complex, like other tethering factors is a Rab/Arl effector which interacts with small GTPase in its GTP bound form (Hong and Lev 2014). The studies in yeast vacuole fusion mutants implicates that HOPS interact with SNARE proteins to mediate membrane fusion.

1.1.4 SNAREs and SM proteins

SNARE (**S**oluble **N**-ethylmaleimide-sensitive factor **A**ttachment **RE**ceptor) proteins comprise a family of small membrane-bound proteins that mediate membrane fusion in both secretory and endocytic pathways in the cell (Rothman 2014). The SNARE hypothesis proposed by James Rothman, and later proven by his colleagues, suggests that SNAREs

associating with cargo-laden transport vesicles called v-SNAREs and with the target membrane called t-SNAREs interact with each other to form a quaternary helical complex named trans-SNARE complex which is followed by membrane fusion. There is yet another set of classification of SNAREs into R (arginine) and Q-(glutamine) SNAREs based on the presence of these amino acids in the highly conserved ionic middle layer of the four helix bundle formed by amino acid side chains of the SNAREs from both vesicle and target membrane(Hong and Lev 2014). The SNARE proteins comprise of a coiled-coil SNARE motif with a characteristic stretch of 60-70 amino acids constituting heptad repeats. The SNARE motif is implicated in formation of four helix quaternary/trans-helix complex that mediates membrane fusion of the transport vesicles and target membrane. Additionally, majority of SNAREs also possess a C-terminal transmembrane domain through which they anchor themselves into vesicle membranes and a N-terminal peptide with which they interact with SM (Sec1/Munc18) protein family members. For example, Munc18 interacting with SNARE Syntaxin1A (Toonen, de Vries et al. 2005) SM proteins either interact with SNAREs directly or by forming a part of tethering factor complex as in Sly1 binds to COG4 subunit of COG – complex. Moreover, SM proteins can interact with individual SNAREs or SNARE complexes as a whole. For example, Vps33 subunit of HOPS complex in yeast interacts with SNARE complex formed by SNAREs Vam3, Vam7 and Nyv1 .(Hong and Lev 2014)

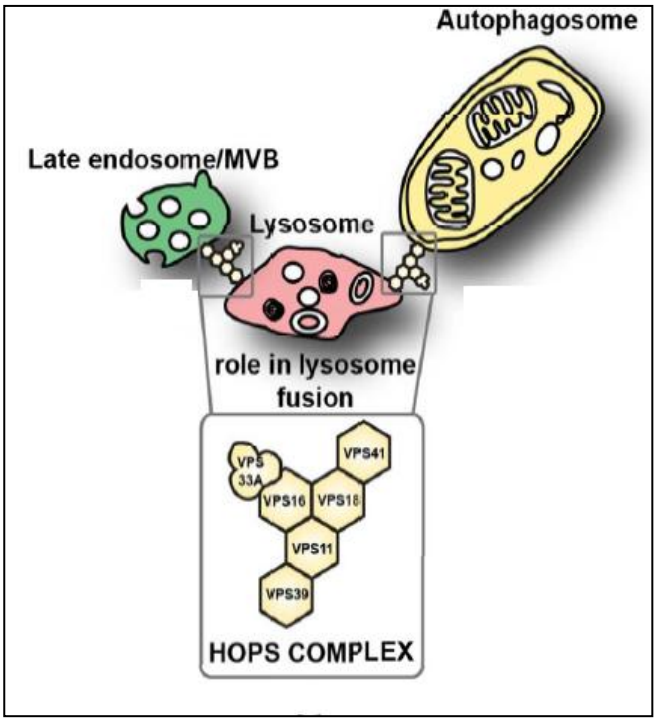


Fig3.**Role of HOPS complex** in mediating tethering and fusion of late endosome or auto-phagosome to lysosomes. The image is reproduced from (Wartosch, Gunesdogan et al. 2015)

1.1.5 HOPS complex is a multi-subunit tethering factor implicated in late-endosome lysosome fusion

HOPS complex is a multisubunit tethering factor that mediates tethering of late endosome to lysosomes and their fusion or tethering of autophagosome to lysosomes and their fusion (Wartosch, Gunesdogan et al. 2015). The Vps41 and Vps39 subunit of human HOPS complex have been shown to interact with small GTPase Rab7, which recruits them to their target organelles (Solinger and Spang 2013). Apart from this Vps41 had been shown to interact with Adaptor Protein AP-3 and act as a coat protein. Vps11 has a role in degradation of glycolipids and also retrograde transport of toxins (Kvalvaag, Pust et al. 2014). Vps18 β-Propeller plays a critical role in regulating stability and function of yeast HOPS complex (Behrmann, Lurick et al. 2014), and recruitment of Vps33a to HOPS by Vps16 subunit is crucial for fusion of lysososmes with endosomes and autophagosomes (Wartosch, Gunesdogan et al. 2015). hVps33a, similar to its counterpart in yeast Vps33p, is a known Sec1/Munc18 (SM) protein that interacts with SNAREs individually or with the quaternary complex (Hong 2005). The functions of these subunits of HOPS complex and their interaction with various proteins such as SNAREs and small GTPases are shown to be conserved in yeast, Drosophila and mammals.

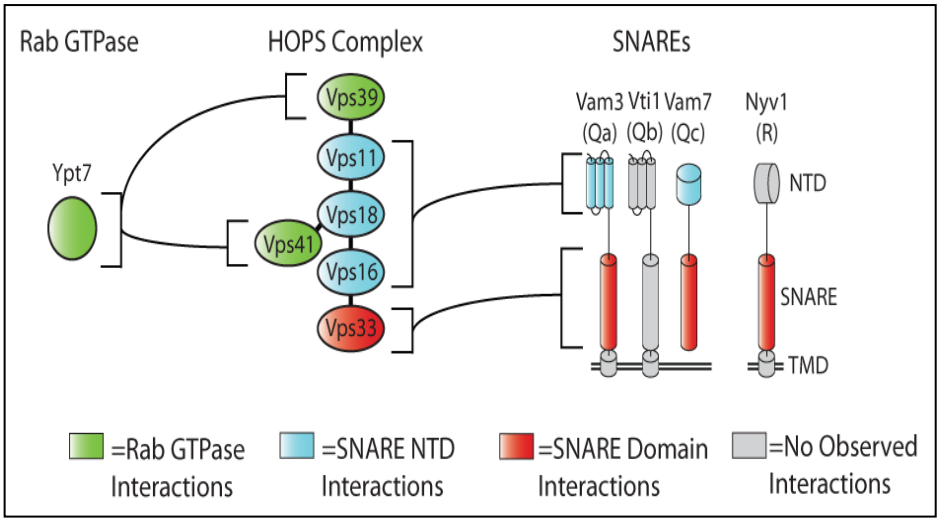


Fig4.**Yeast HOPS complex and SNARE partners.**Vps33 subunit of yeast HOPS interact with SNARE motif of t-SNAREs Vam3 , Vam7 and v-SNARE Nyv1.Along with subunits Vps11 ,Vps16 and Vps18 subunits interacting with N-terminal domain Vam3. . The image is reproduced from(Hong 2005)

1.1.6 *S.cerevisiae* HOPS complex mediates SNARE complex assembly at the vacuole

In yeast, detailed studies have shown that the HOPS complex binds to the complex of Q-SNAREs formed by SNARE motifs of Vam3, Vam7 and Vti1 and also this interaction is mediated by Vps33 subunit which is also a SM protein. Also, it is shown that Vps11, Vps16, Vps18 subunits interacts with H_{abc} domain and Px domain of t-SNAREs Vam3 and Vam7 on the vacuole or lysosomal membrane (9)

1.1.7 Mammalian HOPS complex subunits interact with SNAREs to mediate fusion at the lysosomes

The genetic studies on Drosophila and yeast HOPS complex had proven that the homologues of the different subunits of mammalian HOPS shares significant identity.

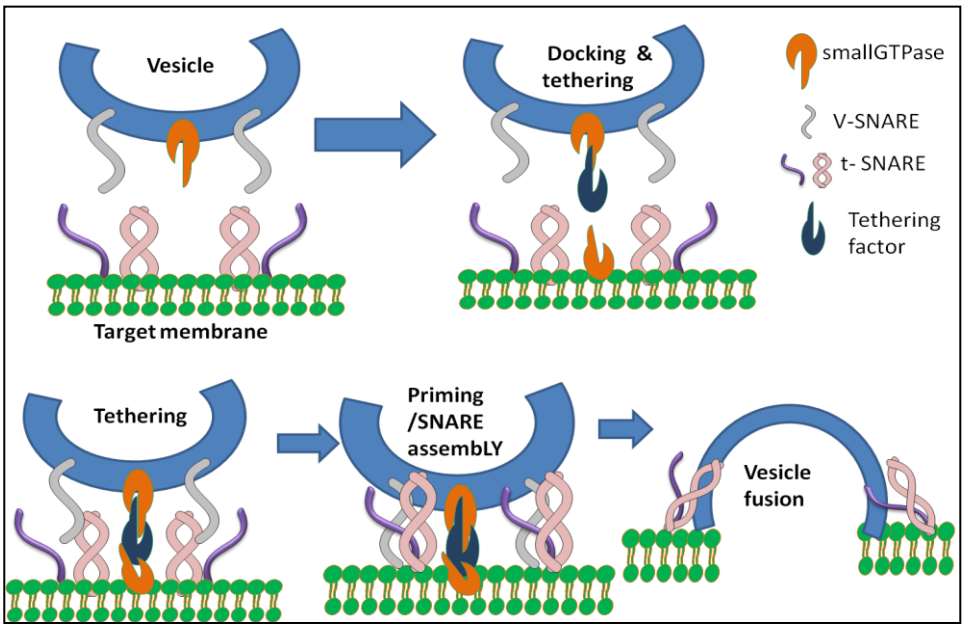


Fig5.**Tethering factor –SNARE model.** Upon recruitment by small GTPase, tethering factors act as protein bridges that brings v-SNARE of transport vesicle and t-SNARE of target membrane to interact and form 4 helical quaternary SNARE complex and mediate membrane fusion.

This implicates that the mammalian HOPS (hereby human HOPS) complex is also involved in SNARE mediated vesicle fusion and the interaction with corresponding SNARE partners is conserved along evolution. The studies in mammalian HOPS complex have shown that Syntaxin 7, a t-SNARE homologue of yeast Vam3 completely colocalizes and interacts with Vps11 and Vps18 of human HOPS (Kim, Kramer et al. 2001). Furthermore it has also been shown that Syntaxin 17, an auto-phagosomal t-SNARE interacts with Vps33a and Vps16 subunits of human HOPS complex mediating autophagosome-lysosome fusion (Jiang, Nishimura et al. 2014). Nevertheless its less known about other v-SNAREs and t-SNAREs that interact with human HOPS complex subunits, thereby mediating fusion of late endosomes with lysosomes. Hence the aim of my dissertation work was:

- To identify the candidate SNARE proteins that interact with mammalian HOPS complex and mediate fusion of late endosomes to lysosomes.
- To identify novel SNARE proteins that interact with Vps33a subunit of the HOPS complex using tandem affinity pull down approach.

Prior studies on mammalian SNAREs have shown that the SNAREs VAMP7, VAMP8, Syntaxin 7, Syntaxin8 and Syntaxin11 localize to late endosomes and lysosomes (Pryor, Mullock et al. 2004). We have, therefore, characterized these SNAREs and their interactions with HOPS complex by following experiments:

1. Identification of HOPS complex subunits that directly interact with t-SNAREs Syntaxin 11, Syntaxin 8 and Syntaxin 7 using yeast two hybrid.

2. Identification of sub cellular localization of t-SNARE Vti1b in HeLa cells and its co localization with HOPS complex subunits

3. Identification of other SNARE partners of Vti1b and their localization.

4. To perform a TAP-pull down of Vps33a subunit of HOPS complex and identify the SNAREs that interact with Vps33a

1.2 Materials and Methods.

1.2.1 Plasmids

Full length human HOPS complex subunits hVps41 and hVps39 cloned in mammalian expression vector were obtained from C.Liang lab(USA).HA- tagged Vps41 was gifted by Wade Harper lab (USA). GFP VAMP7 ,GFP VAMP8 ,GFP-Syntaxin 7 and GFP Syntaxin 8 was obtained from Kirchhausen lab,Takuma lab and Linder lab respectively. The cloning of Flag tagged Vti1b , Myc tagged Vti1b and TAP-Vps33a were done in the lab. And also the SNARE constructs for yeast two hybrid assay were cloned in the lab .The details of these clonings are shown in table (1).HOPS complex subunits in pGADT7 and tomato-Arl8b were previously cloned in the lab.

1.2.2 Antibodies.

Mouse monoclonal antibody against human LAMP1 was obtained from BD transduction laboratory and Rabbit polyclonal antibody from Abcam.Rabbit polyclonal antibody against Vps33a was obtained from Protein Tech and mouse monoclonal antibody against Vti1b was from BD transduction laboratory and Synaptic systems. Rabbit polyclonal antibody against HA epitope tag was obtained from Covans .Mouse monoclonal antibody against Myc epitope tag procured from invitrogen and Rabbit polyclonal antibody against myc from Abcam.

1.2.3 Cell culture and Transfection

Both Hela and Hek cells were grown in Dulbecco's Modified Eagle medium (DMEM,) supplemented with 10% FBS().Hela cells grown in coverslips were transfected with DNA constructs either using transfection reagent X-treme Gene HP (Roche) or Lipofectamine (Invitrogen) as per manufactures instruction.Post16 to 20 hours transfection ,the coverslips were fixed with 4% of PFA in PHEM buffer for 10 minutes and washed with PBS three times. These coverslips were blocked in PHEM buffer containing 5% FBS and 0.2% Saponin for one hour. After blocking the cells were incubated with primary bodies made in PHEM buffer for 45 minutes , after that wash with 1X PBS and incubated in secondary antibodies conjugated with Alexa flour conjugates for 30 min. After that wash by 1X PBS, was given and the cells are mounted with Fluoromount (Southern biotech) and are analysed by using Zeiss LSM 710 confocal microscope

1.2.4 Yeast two-hybrid screen of SNAREs against human HOPS complex subunits

The yeast two-hybrid system detect the proteins that interact with each other and also enables immediate availability of the clones of the genes that are interacting. Two separate plasmids of hybrids are constructed with one hybrid consisting of activation domain of the GAL4 transcriptional activator protein fused with corresponding protein of interest (bait)"and the other hybrid cloned with DNA binding domain of the GAL4 transcriptional activator protein fused with the potential interacting partner of the bait namely, (prey). If there is a interaction with prey and bait it will result in transcriptional activation of a reporter gene (His/Ade/LacZ) containing binding site for GAL4.Thus direct interaction of the protein can be detected with the two-hybrid system. However, it's necessary to mention that if two proteins failed to interact in yeast two-hybrid in does not mean that there is no direct interaction between these proteins ,further confirmation with other protein-interaction interaction test such as co-immunoprecipitation should be also performed. In the yeast two-hybrid assay of different SNAREs such as Syntaxin 11 ,Syntaxin 7 and Syntaxin 8 against human HOPS complex subunits ,SNAREs were cloned in pGBKT7 ,binding domain containing vector of Gal4 transcription factor (details of cloning shown in table 1) and HOPS complex subunits in pGADT7 ,activation domain containing vector of Gal4 transcription factor (priory cloned in the lab).The strain of yeast used is Gold strain and reporter gene used is Histidine.

1.2.5 Tandem affinity purification (TAP) of hVps33a subunit of human HOPS complex subunit.

The genetic method such as yeast two-hybrid discussed above is used in identification of interaction between two proteins. These methods limits the possibility of finding interaction of proteins in complexes such as spliceosome and ribosome. Tandem affinity purification overcome these limitation and also use of two different affinity purification tags increases the specificity of method. The two common tags used are Streptavidin Binding Peptide(SBP) and Calmodulin Binding Peptide (CBP).SBP is a synthetic sequence isolated from random peptide library which shows high affinity to streptavidin resin and can be eluted using buffer containing biotin. Whereas CBP tag is generated from C-terminal fragment of muscle myosin light chain kinase which shows affinity towards calmodulin resin in presence of calcium and can be eluted using any chelating agent such as EDTA .The major steps of the TAP pull down of TAP tagged Vps33a is listed below.

- Cloned TAP tagged Vps33a of HOPS complex subunits in pCDH lentiviral expression vector
- Create a stable cell line expressing TAP tagged Vps33a
- Lysis of cells expressing TAP-Vps33a
- Apply this cell lysate to Streptavidin resin and streptavidin binding buffer.
- Wash unbound proteins and contaminants with streptavidin buffer.
- Elute tagged protein and interacting partner with streptavidin eluate buffer.
- Apply eluate to Calmodulin and Calmodulin binding buffer.
- Wash unbound proteins and contaminants with Calmodulin buffer.
- Elute tagged protein and interacting partner with Calmodulin eluate buffer.
- Run a SDS page and analyse the proteins by mass spectrometry.

Amino acid sequence of SBP and CBP tags

SBP tag : MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG

CBP tag : KRRWKKNFIAVSAANRFKKISSSGAL

1.2.6 Imaging using confocal microscopy

Zeiss LSM 710 confocal microscope was used for imaging of the cells in immunostaining experiments. All images were captured using 60X oil immersion objectives. Optical parameters of the images were set at the beginning and kept constant throughout imaging. Excitation and Emission wavelength of Alexa 488 ,Alexa 568 and Alexa 647 fluorophores were adjusted with the help of filters attached with microscope.

Chapter 2

2.1 Results and Discussion

2.2 Future Directions

2.1 Results and discussion

Mammalian HOPS complex has been implicated in tethering and fusion of late endosome to lysosomes. Other proteins like small GTPases and SNAREs also play a crucial role in helping HOPS subunit in docking and tethering of vesicles to their target membrane. In yeast the small GTPase and the SNAREs involved in this process are completely known but in the case of mammals what SNAREs interact with HOPS are not well studied. In this study we have shown that Syntaxin 11, a t-SNARE interacts with Vps41 and Vps18 of human HOPS in yeast two hybrid whereas the other t-SNAREs Syntaxin 7 and Syntaxin 8 do not show any direct interaction in yeast two hybrid. Confocal analysis indicates that Vti1b localizes to lysosomes and also co localizes with lysosomal smallGTPase Arl8b and human HOPS complex subunit Vps41. In this study we also have shown that the SNAREs Syntaxin 7, Syntaxin 8 and VAMP7 could be the potential partners of Vti1b to form a helical quaternary SNARE complex. Also, we have confirmed the expression of TAP-tagged Vps33a cloned in pCDH vector, through Western Blotting.

2.1.1 Syntaxin 11 interacts with Vps18 and Vps41 subunits of the human HOPS complex in yeast two-hybrid assay

To test the interaction of Syntaxin 11 with HOPS complex subunits, Syntaxin11 was used as a bait in yeast two hybrid assay in which Syntaxin 11 was cloned in fusion with Gal4 binding domain containing vector and HOPS subunits were cloned in Gal4 activation domain containing vector.

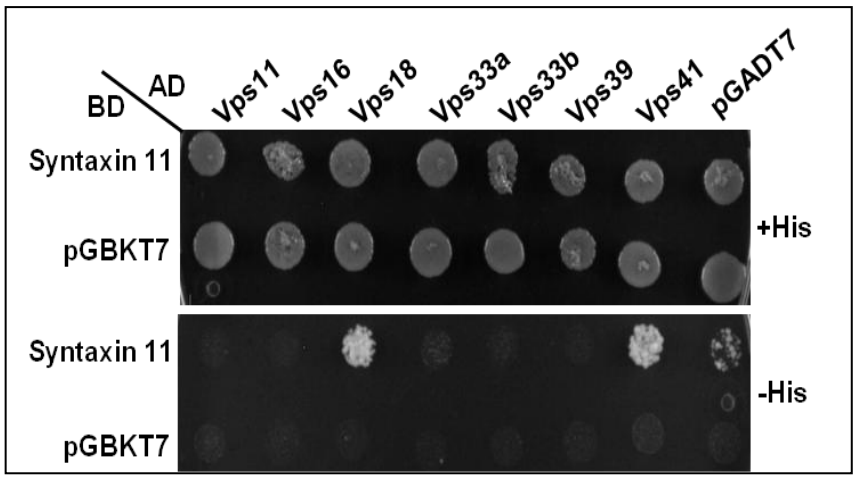


Fig6. **Yeast two hybrid interaction of syntaxin 11** (cloned in binding domain vector) with HOPS complex subunits (cloned in activation domain vector) was tested using pGBKT7/pGADT7 system. Yeast were plated on non-selective media (+His) to confirm viability and on selective media (-His) to detect interactions.

After transformation, yeast were plated on non selective medium (-Leu, -Trp, +His) for

confirmation of viability of cotransformants and on selective medium (-Leu, -Trp, -His) for detection of direct interaction. After 3 to 4 days , it was observed that Syntaxin 11 interacted with Vps18 and Vps41 subunit of human HOPS(shown in figure 6). Since Syntaxin 11 expresses more specifically in immune cells, we think that this interaction would be more meaningful to study in immune cells.

2.1.2 Syntaxin 7 and Syntaxin 8 do not show direct interaction with the human HOPS complex subunits.

Syntaxin 7 without C-terminal transmembrane domain (and Syntaxin 8 without C-terminal transmembrane domain were cloned in Gal4 binding domain containing vector (details in table 1) and human HOPS complex subunits which were cloned in Gal4 activation domain containing vector, and the interaction of Syntaxin 7 and Syntaxin 8 against human HOPS complex subunit were studied using yeast two hybrid assay. After transformation the yeast were plated on non selective media (-Leu, -Trp, +His) and on to selective media (-Leu, -Trp, -His) for detection of direct interactions. Since both Syntaxin 8 and Syntaxin 7 cloned in binding domain vector showed self activation, therefore no conclusion were drawn about their interaction with HOPS complex subunits from this selection plate. To control self activation, yeast were plated on selective media containing 0.8mM 3-AT and to provide even more stringent conditions, yeast were plated on selective media -Leu -Trp -His -Ade. Although auto-activation was controlled under stringent conditions, the yeast two hybrid assay showed no direct interaction of Syntaxin 8 and Syntaxin 7 with any HOPS complex subunit(shown in figure 7).

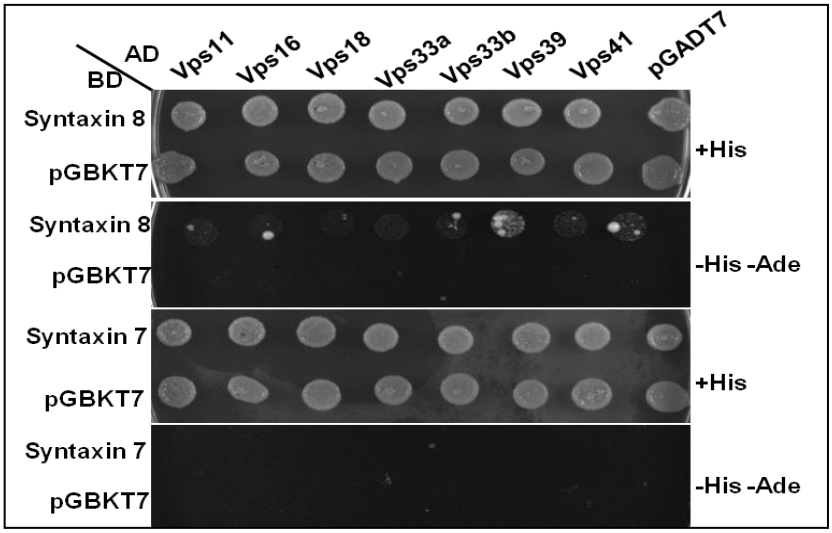


Fig.7 **Yeast two hybrid interaction of syntaxin 8 and syntaxin 7**(cloned in binding domain vector) with HOPS complex subunits (cloned in activation domain vector) was tested using pGBKT77/pGADT7 system. Yeast were plated on –Leu-Trp for viability and replated on selective media (-Leu –Trp –His –Ade) for testing interaction. No significant growth was found on selective media plates.

2.1.3 Identifying sub cellular localization of SNAREs and their interaction partners in endo-lysosomal pathway

Prior studies have shown that the SNAREs Vti1b, Syntaxin8, Syntaxin7, VAMP7 and VAMP8 localize to late endosome and lysosomes. To determine the subcellular localization of Vti1b we cloned Flag tagged Vti1b in pCDNA mammalian expression vector (details of cloning in table 1) and over expressed Flag tagged Vti1b in Hela cells and co stained the cells with LAMP1, a lysosomal marker. Confocal analysis indicated that Vti1b colocalized with LAMP1, implicating that Vti1b localizes to lysosomes (shown in figure8). Further Myc tagged Vti1b (details of cloning in table 1) was cotransfected with lysosomal small GTPase Arl8b along with HA tagged Vps41 subunit of human HOPS. Analysis by confocal microscopy revealed that Vti1b co localized with HA tagged Vps41 and with small GTPase which is

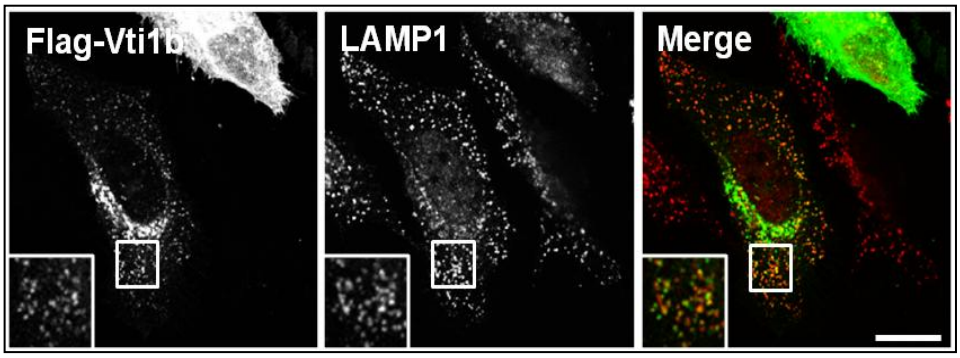


Fig8 **.Vti1b localizes to lysosomes**.Hela cells were transfected with FLAG -tagged Vtib and analyzed for lysosomal localization by confocal microscopy. Co localized puncta are indicated in the inset

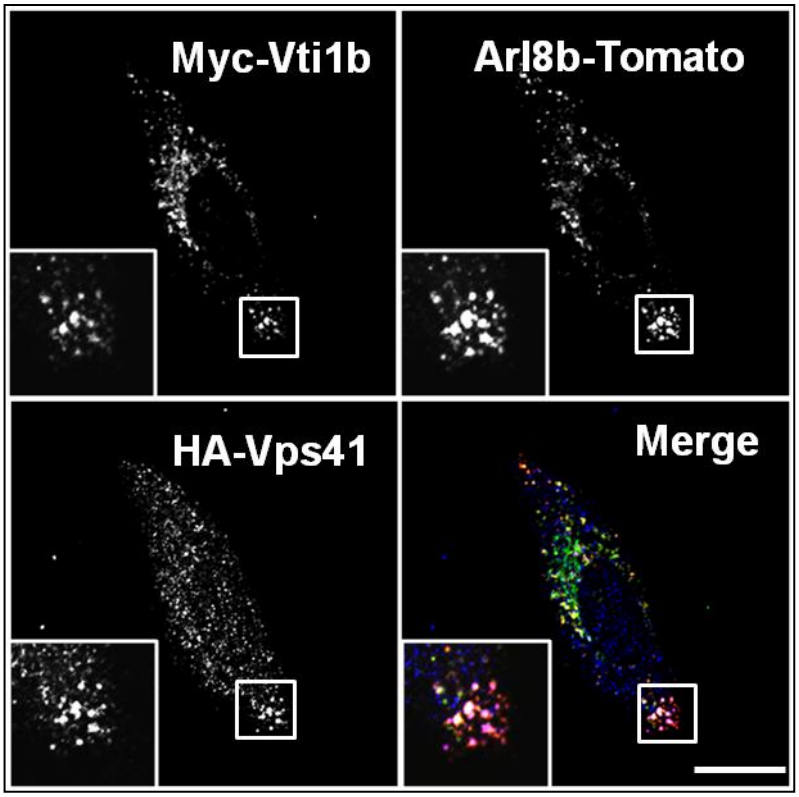


Fig.9 **Vti1b colocalizes with small GTPase Arl8b and human HOPS subunit Vps41 .** Hela cells were co-transfected with Myc-tagged Vti1b and Arl8b-Tomato and HA-tagged hVps41 and analyzed for co localization by confocal microscopy. Colocalized puncta are indicated in the inset**.**

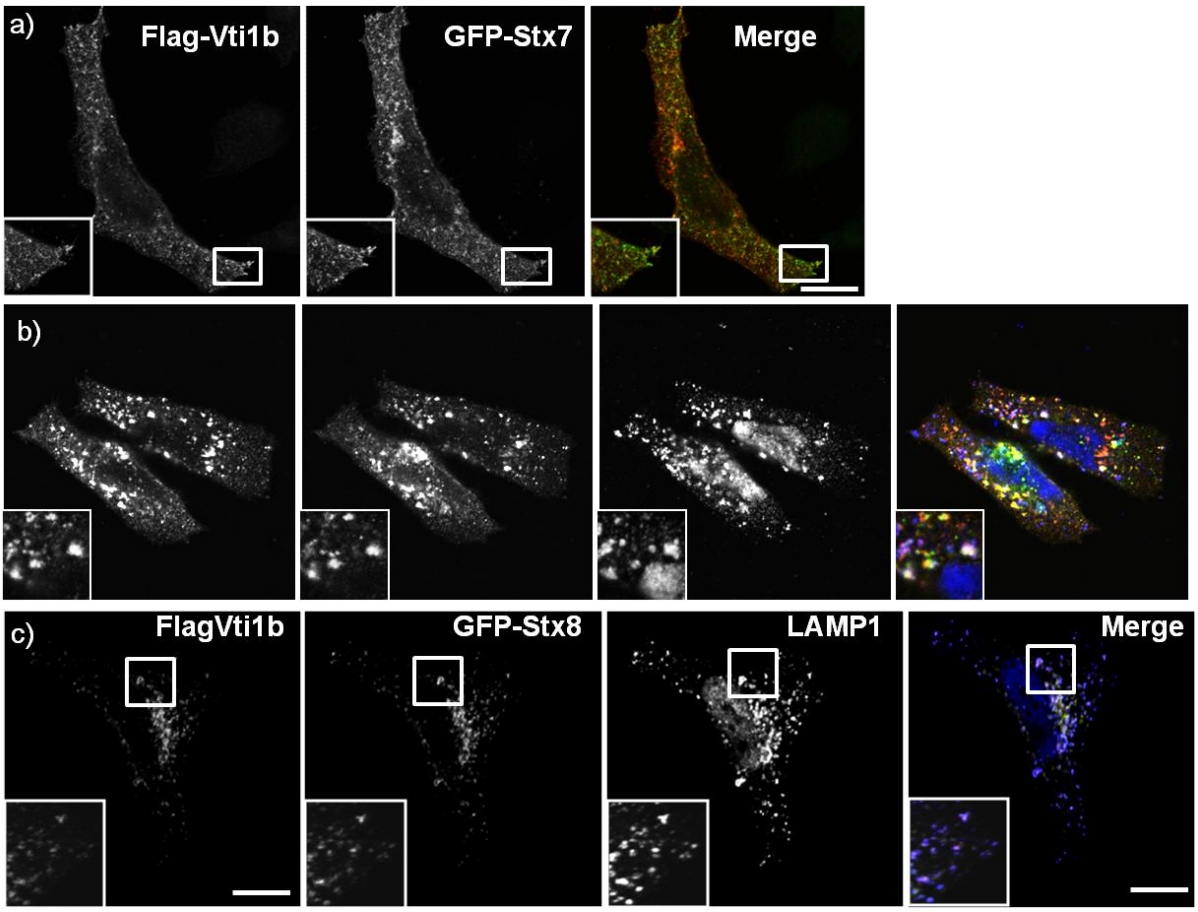


Fig.**10 Potential lysosomal trans-SNARE complex partners for Vti1b**. Hela cells were co-transfected with FLAG/Myc -tagged Vtib and SNAREs Syntaxin 7 (Fig. a) , VAMP7(Fig. b) and Syntaxin 8 (Fig. c) and analyzed for colocalization by confocal microscopy

specific for lysosomes (Shown in figure 9). This association of Vti1b with small GTPase Arl8b and Vps41 subunit of HOPS complex resembles the small GTPase, tethering factor and SNARE association shown in figure 5. In conjunction with these co localization experiments , we cotransfected Flag/Myc tagged Vti1b in combination with other SNAREs such as VAMP7, VAMP8, Syntaxin8 and Syntaxin 7 in HeLa cells and co stained for LAMP1 a lysosomal marker. The rationale behind these experiments was that, if four individual SNAREs interact with each other and form a quaternary complex to mediate membrane fusion, and if Vti1b is one of those SNAREs forming the complex, there should be other SNAREs that Vti1b interacts with to form the quaternary complex. The confocal analysis indicates that Vti1b co localizes with other t-SNAREs Syntaxin7 and Syntaxin 8 and also with v-SNARE VAMP7 and all of these structures were positive for LAMP1, a lysosomal marker .(Shown in figure 10)

2.1.4 Tandem affinity pull down using hVps33a as a bait in transiently hVps33a expressing cells

HEK cells were cotransfected with TAP tagged Vps33a subunit of human HOPS complex subunit and Myc tagged Vps16. Tandem affinity purification was done according to the manufacturer's instructions. The fractions were collected at every step of the purification and were loaded on an SDS-page gel. Later via western blot analysis the expression of TAP-Vps33a along with myc tagged Vps16 was confirmed by probing the blot against anti Vps33a and anti myc. The Vps16 dependent recruitment of Vps33a to HOPS complex to tether and mediate late endosome to lysosome fusion is already known.(Wartosch, Gunesdogan et al. 2015) We tried to test interaction of Vps33a with Vti1b by using anti-Vti1b antibody and with Vps41 by anti Vps41 antibody. However we were not able to draw any conclusion about the interaction of Vps33a to t-SNARE Vti1b and HOPS complex subunit Vps41 since no corresponding band for either endogenous Vti1b or Vps41 was present in blot.(Shown in figure 11).

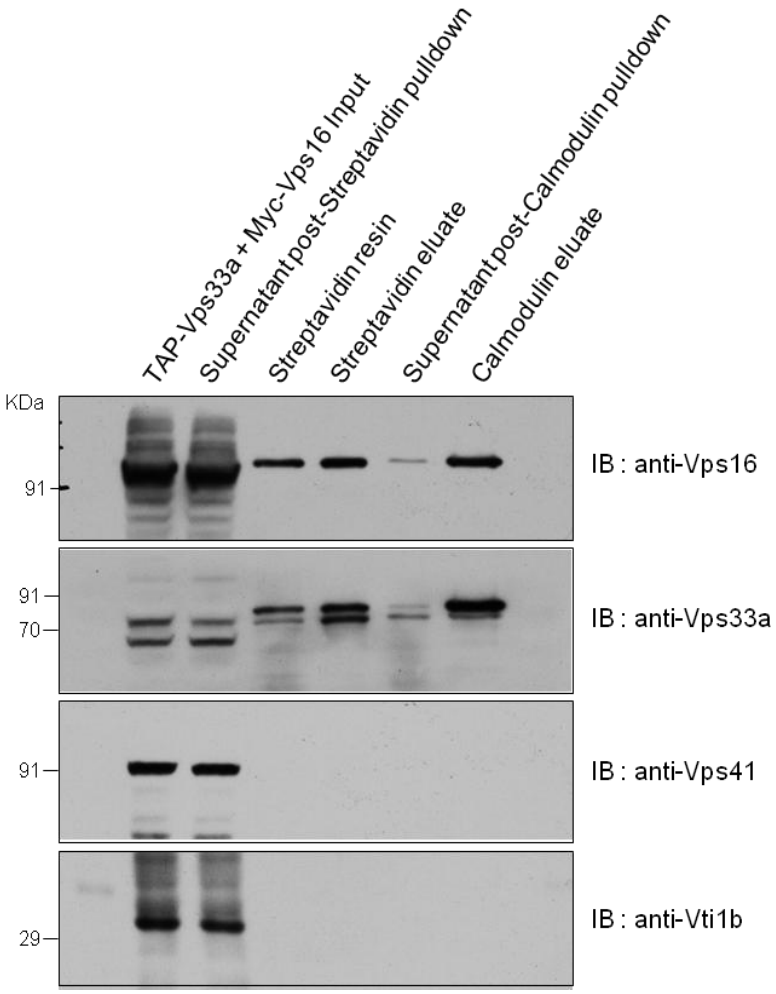
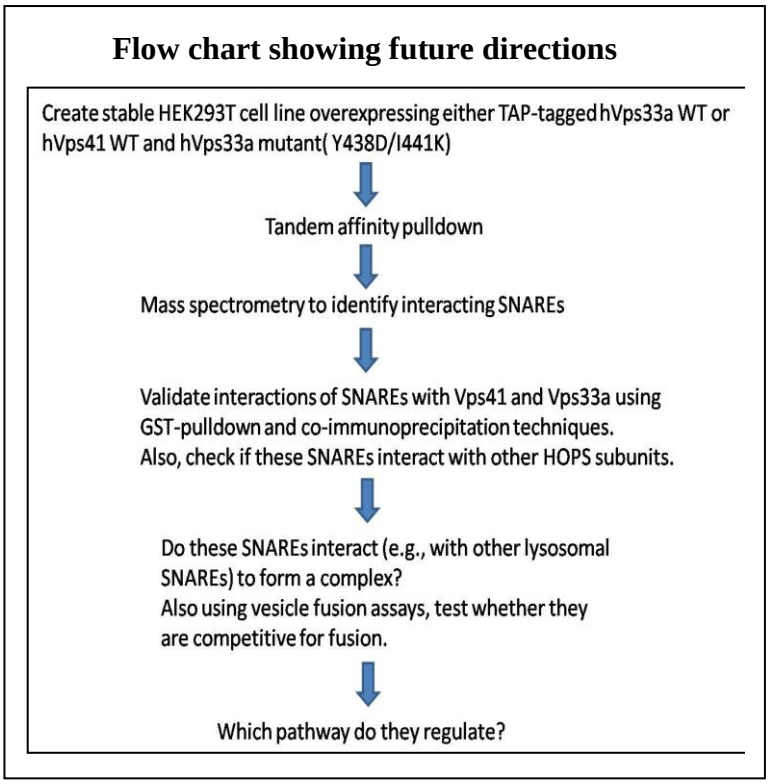


Fig11. A representative western blot depicting the tandem affinity pulldown of the indicated HOPS subunits and the SNARE Vti1b, using hVps33a as bait.

2.1.5 Table(1) containing details of Cloning done as part of this study

Name of DNA construct	**Cloned in Vector**	**Template used**	**Restriction site**	**Primer used (Forward primer F.P) and Reverse primer (R.P)**
TAP-Vps33a	PCDH(lentiViralvector)	Vps33a in PNTAPB	5'Xba1 3'Not1	F.P5'GCTCTAGAATGAAGCGACGATGGAAAAGAA3' R.P.5'ATAAGAATGCGGCCGCCTAGAAAGGTTTTTCCATCAGAG3
Syntaxin 8 without TM	pGADT7 and pGBKT7	Myc-Syntaxin 8	5'EcoR1 3'BamHI	F.P 5'CGGAATTCATGGCACCGGACCCCTGGTTCTC 3' R.P 5' CGGGATCCTCACCCACAAGAGGCTGACTTTCTG 3'
Synatxin 7 without TM	pGADT7 and pGBKT7	Myc-Syntaxin7	5'EcoR1 3'BamHI	F.P 5' CGGAATTCATGTCTTACACTCCAGGAGTTG 3' R.P 5' CGGGATCCTCAGGTTTTTCTGGATTTGCGCTG3'
VAMP7 without TM	pGADT7	Hela cDNA	5'EcoR1 3'BamHI	F.P 5' GCGAATTCATGGCGATTCTTTTTGCTGTTG 3' R.P 5' CGGGATCCTCACTTGAGGTTCTTCATACACATG 3'
Vti1b wihtout TM	pGBKT7	Flag-Vti1b	5'EcoR1 3'BamHI	F.P 5' GGAATTCATGGCCGCCTCCGCCGCCTCCTC 3' R.p5'CGGGATCCTCACTTGTTGGTTATCACTTTTCTGG3'
VAMP8 without TM	pGADT7	Hela cDNA	5'Nde1 3'EcoR1	F.P5'GGGAATTCCATATGATGGAGGAAGCCAGTGAAGGTG 3' R.P 5' GCCCATATGTCACTTCACGTTCTTCCACCAG 3'

2.2 Future Directions



In conclusion we have shown in this study that Syntaxin 11 shows direct interaction with Vps41 and Vps18 subunits of human HOPS complex in yeast two hybrid assay. Since Syntaxin 11 is expressed specifically in immune cells, this interaction would be more meaningful to study in immune cells**.** The confocal experiments indicate that Vti1b, a t-SNARE localizes to lysosomes and also colocalizes with lysosomal small GTPase Arl8b and hVps41 of human HOPS complex Furthermore, colocalization of other SNAREs such Syntaxin 8, Syntaxin 7 and VAMP7 with Vti1b indicates that they could be the potential SNARE partners of Vti1b that interact and form a four helical quaternary complex with Vti1b.

We were also able to confirm the previously reported interaction of Vps16 subunit of HOPS complex with Vps33a subunit of HOPS complex through TAP pulldown of the cells transiently expressing TAP-Vps33a by western blot. The future experiments (shown in figure 12) will be to make stable cell lines of HEK cells expressing either TAP tagged hVps33a or TAP tagged hVps41 and to perform Tandem affinity Purification and analyse the SNAREs that interact with these subunits by mass spectrometry. Further confirmation of the potential interaction of SNAREs and HOPS complex subunits will be done by co-immunoprecipitation and GST pull down techniques. Apart from this we also have planned to analyze the localization of SNAREs Vti1b, Syntaxin 8 and Syntaxin 7 in HOPS complex depleted cells and localization of different HOPS complex subunits in cells deprived of these SNAREs using siRNA.

\

2.3 Bibliography

Behnia, R. and S. Munro (2005). "Organelle identity and the signposts for membrane traffic." Nature **438**(7068): 597-604.

Behrmann, H., A. Lurick, et al. (2014). "Structural identification of the Vps18 beta-propeller reveals a critical role in the HOPS complex stability and function." J Biol Chem **289**(48): 33503-33512.

Brunet, S. and M. Sacher (2014). "Are all multisubunit tethering complexes bona fide tethers?" Traffic **15**(11): 1282-1287.

Hong, W. (2005). "SNAREs and traffic." Biochim Biophys Acta **1744**(2): 120-144.

Hong, W. and S. Lev (2014). "Tethering the assembly of SNARE complexes." Trends Cell Biol **24**(1): 35-43.

Huotari, J. and A. Helenius (2011). "Endosome maturation." EMBO J **30**(17): 3481-3500.

Jiang, P., T. Nishimura, et al. (2014). "The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17." Mol Biol Cell **25**(8): 1327-1337.

Kim, B. Y., H. Kramer, et al. (2001). "Molecular characterization of mammalian homologues of class C Vps proteins that interact with syntaxin-7." J Biol Chem **276**(31): 29393-29402.

Kvalvaag, A. S., S. Pust, et al. (2014). "Vps11, a subunit of the tethering complexes HOPS and CORVET, is involved in regulation of glycolipid degradation and retrograde toxin transport." Commun Integr Biol **7**(1): e28129.

Pryor, P. R., B. M. Mullock, et al. (2004). "Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events." EMBO Rep **5**(6): 590-595.

Rothman, J. E. (2014). "The principle of membrane fusion in the cell (Nobel lecture)." Angew Chem Int Ed Engl **53**(47): 12676-12694.

Saftig, P. and J. Klumperman (2009). "Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function." Nat Rev Mol Cell Biol **10**(9): 623-635.

Segev, N. (2001). "Ypt and Rab GTPases: insight into functions through novel interactions." Curr Opin Cell Biol **13**(4): 500-511.

Solinger, J. A. and A. Spang (2013). "Tethering complexes in the endocytic pathway: CORVET and HOPS." FEBS J **280**(12): 2743-2757.

Toonen, R. F., K. J. de Vries, et al. (2005). "Munc18-1 stabilizes syntaxin 1, but is not essential for syntaxin 1 targeting and SNARE complex formation." J Neurochem **93**(6): 1393-1400.

Vazquez-Martinez, R. and M. M. Malagon (2011). "Rab proteins and the secretory pathway: the case of rab18 in neuroendocrine cells." Front Endocrinol (Lausanne) **2**: 1.

Wartosch, L., U. Gunesdogan, et al. (2015). "Recruitment of VPS33A to HOPS by VPS16 is required for lysosome fusion with endosomes and autophagosomes." Traffic.

Young, A., S. Stoilova-McPhie, et al. (2013). "Hsc70-induced changes in clathrin-auxilin cage structure suggest a role for clathrin light chains in cage disassembly." Traffic **14**(9): 987-996.

Zanetti, G., S. Prinz, et al. (2013). "The structure of the COPII transport-vesicle coat assembled on membranes." Elife **2**: e00951.