Regulation of mammalian HOPS complex by small GTPases and their effectors

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Certificate of Examination

This is to certify that the dissertation titled *"Regulation of mammalian HOPS complex by small GTPases and their effectors"* submitted by Mr. Vivek B. Raina (Reg. No. MS10100) 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 then report be accepted.

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(Supervisor)

Dated: April 23, 2015

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.

Vivek B. Raina
(Candidate)

Dated: April 23, 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 take this opportunity to express my deep sense of gratitude to Dr. Mahak Sharma for helping me develop a keen interest and passion for science. She is a kind of mentor for whom the famous saying “A mentor empowers a person to see a possible future, and believe it can be obtained” holds true. I sincerely thank her for the constant words of encouragement and motivation.

Secondly, I would like to thank all the members of the lab especially Ms. Divya Khatter and Ms. Rituraj Marwaha (who once was my classmate!) for the continuous guidance and support throughout the project. I would also like to thank 3TL2 Lab members especially Mahima, Asha Raju, Reshme Murli, Sukriti and Poulami for the most needed tea breaks.

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-Vivek B Raina

Contents

List of Figures

Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
LiAc	Lithium Acetate
PEG	Polyethylene glycol
LB	Luria Broth
IPTG	Isopropyl β-D-1-thiogalactopyranoside
DTT	Dithiothreitol
PFA	Paraformaldeyde
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
GST	Glutathione S-transferase
LAMP1	Lysosome associated membrane protein 1
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
HOPS	Homotypic Fusion and Protein Sorting
Vps	Vacuole Protein Sorting
PLEKHM1	Pleckstrin homology domain-containing family M member 1

Abstract

Vesicular trafficking pathways in a eukaryotic cell are mediated by small GTPases of Rab, Arf and Arf-like (Arl) families and their effectors/interaction partners including tethering factors, motor proteins and SNAREs. HOmotypic fusion and Protein Sorting (HOPS) complex is a multi-subunit tethering complex conserved from yeast to mammals that regulates endocytic trafficking to vacuoles/lysosomes. The six subunits are namely Vacuole Protein Sorting (Vps) 11, Vps16, Vps18 and Vps33 subunits that form the core complex, while Vps39 and Vps41 act as the accessory subunits. Previously, a lysosomal small GTPase of the Arl family, Arl8b was shown to directly bind and recruit the human (h)Vps41 subunit to the lysosomes. Here by using GST-pull down and purified proteins, we have shown that this interaction takes place through the N-terminal WD40 domain of hVps41 and this domain is both essential and sufficient for this interaction. Further, a previously-reported single nucleotide polymorphism (T146P) within this domain disrupts the binding to Arl8b and prevents association of Vps41 with lysosomes. These results also explain how this SNP leads to loss-of-function of Vps41 in mediating delivery of cargo and their degradation in lysosomes.

Further as part of another study, I have also explored the interaction of late endosomal and lysosomal protein, Pleckstrin homology domain-containing family M member 1 (PLEKHM1), with Vps39 subunit of the HOPS complex. PLEKHM1 was previously identified as an interaction partner for late endosomal small GTPase Rab7. PLEKHM1 and Vps39 colocalize on lysosomes and this interaction is dependent upon the second Pleckstrin Homology (PH) domain of PLEKHM1. These findings will be crucial in exploring how small GTPases and their effectors collaborate to mediate vesicular trafficking towards lysosomes.

Chapter 1

Introduction

1.1 Basic Theory
1.2 Experimental methods

1.1 Basic Theory

Eukaryotic cells are highly compartmentalised into different organelles with different structure and function. For a cell to maintain homeostasis, these organelles need to remain in constant communication with each other that involves transport of materials from one compartment in the cell to the other. This transport takes place with the help of membrane-bound vesicles which bud off from a donor compartment and fuse with an acceptor compartment. There are distinct pathways through which this transport takes place namely secretory pathway, retrieval pathway and the endocytic pathway (Fig.1). Via the secretory pathway, cargo such as Lysozyme C or lysosomal hydrolases are taken from the endoplasmic reticulum through the Golgi complex to the plasma membrane or the lysosomes respectively. In retrieval pathway, molecules are retrieved back from early or late endosomes to Golgi or from Golgi to endoplasmic reticulum. Endocytic pathway is the one through which the cargo such as nutrients, ligand-bound receptors etc. are ingested at the plasma membrane in vesicles and are delivered to lysosomes for degradation. Following internalization, the cargo-containing vesicle fuses with the early endosomes which then mature into late endosomes, a process highly marked by a change in the membrane proteins. Finally, late endosomes undergo either homotypic fusion by fusing with other late endosomes or heterotypic fusion by fusing with the lysosomes to form a hybrid organelle, where the cargo is eventually degraded.

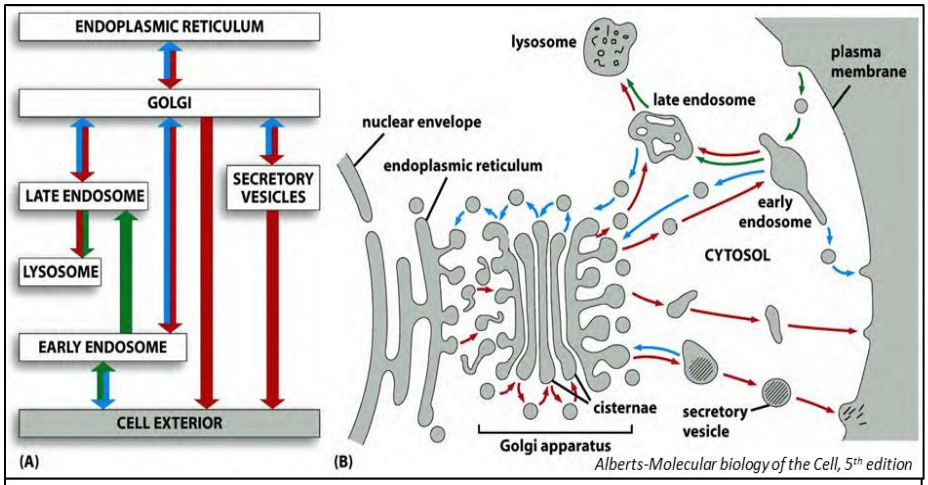

Figure 1: Different pathways of intracellular trafficking. In red is the secretory pathway, blue represents retrieval pathway, and green represents endocytic pathway. *Adapted from Molecular Biology of the Cell, by Bruce Alberts, 5th edition, Garland Science*

Based on the size of the endocytic vesicles formed, endocytosis can be categorised into two different types namely *phagocytosis* and *pinocytosis*. In phagocytosis, large particles like microbes, macromolecules etc. are ingested via the phagosomes which are generally >250nm in diameter, whereas in pinocytosis solutes are ingested via small pinocytic vesicles which are generally about 100nm in diameter.

1.1.1 MOLECULAR PLAYERS IN VESILCE FUSION:

Vesicle fusion processes, which underlie the intracellular transport in eukaryotic cells, employ various classes of regulatory proteins in the cells (Fig.2). These proteins coordinate to bring about vesicle fusion in a specific manner. Some of these regulatory proteins are described below:

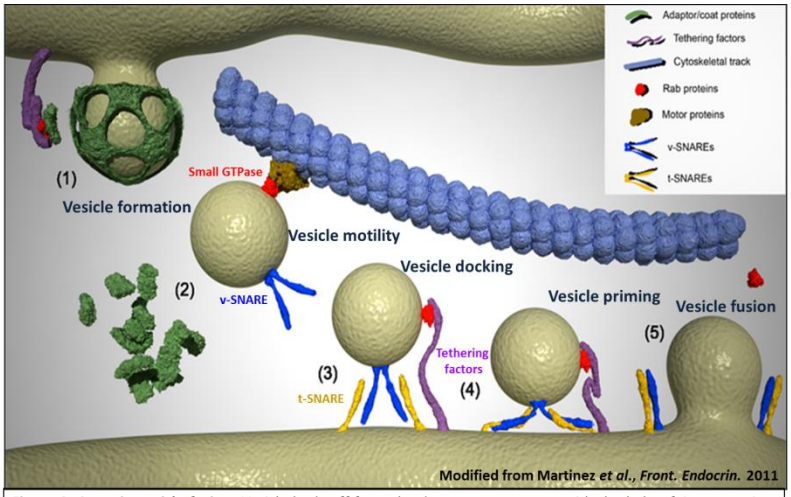

Figure 2: Steps in vesicle fusion. Vesicle buds off from the donor compartment with the help of Coat proteins. After disassembly of the coat proteins, vesicle moves on the cytoskeletal track. Tethering factors tether the vesicle near the acceptor compartment and finally the two membranes fuse with the help of SNAREs. Modified from *Martinez et. al., Frontiers in Endocrinology, 2011.*

Coat proteins:

Clathrin mediated endocytosis refers to uptake of cargo from the plasma membrane where clathrin acts as the coat protein. This protein forms a triskelion shape which is made up of three heavy chains and three light chains. Heavy chains form the structural backbone and the light chains regulate formation and disassembly of clathrin lattice. Clathrin uses adaptor proteins to bind to membranes. It is recruited to the membrane by adaptor protein AP-2 along with other accessory adaptor proteins. This clathrin-coated vesicle now buds off from the plasma membrane with the help of a mechanochemical enzyme called dynamin (Kosaka and Ikeda 1983). Following vesicle budding, this clathrin coat is disassembled by ATPase heat shock cognate (HSC70) and its cofactor auxilin (Schlossman, Schmid et al. 1984, Ungewickell, Ungewickell et al. 1995) rendering uncoated vesicle to fuse with target membrane.

Small GTPases:

Small GTPases are a family of proteins that bind and hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate (GDP). They act as molecular switches that can alternate between a GTP-bound active state and a GDP-bound inactive state (Vetter and Wittinghofer 2001). GDP-bound form is cytosolic whereas the GTP-bound form is associated with membranes. When bound to GTP, small GTPases activate their downstream effectors. Various families of small GTPases are known to regulate the endocytic trafficking like Rab family, Arf family and Arf-like (Arl) family. All Rabs, in their GTP-bound form, are associated with the membranes where they act as the target for a wide variety of proteins including tethering factors (Pfeffer 2001). For example, Rab5 recruits EEA1 which is a tethering factor on early endosomes (Simonsen, Lippe et al. 1998). Like Rabs, Arfs and Arls are also localised to specific organelles in the cell and activate a number of downstream effectors. Post-translational modifications, like myristoylation at the N-terminus, play an important role in the membrane recruitment and biological activity of Arfs and Arls. For example, Arl8b is acetylated at the N-terminus. The most identified effectors of Arf/Arl are often the coat proteins and lipid modified enzymes (Gillingham and Munro 2007)

Tethering factors:

GTP bound small-GTPase recruits tethering factors to the membranes and helps in pairing of the two membranes. Tethering factors facilitate the recognition of membranes just before fusion and helps in tethering of the vesicle with the acceptor compartment. The tethering factors are classified as coiled-coil tethering factors and large multi-subunit complexes. A characteristic of the coiled coil tethering factors is the presence of a long stretch of heptad repeats where every seventh residue has a propensity to form an alpha-helix. p115 is one such example. Multi-subunit tethering factors are made up of different proteins and form a big complex. Two multi-subunit tethering complexes known to play role in endocytic trafficking are CORVET (class C CORe Vacuole /Endosome Tethering) and HOPS (HOmotypic fusion and Protein Sorting) complex (Solinger and Spang 2013).

SNAREs:

The final step in the vesicle fusion pathway is the fusion of the membranes of the two organelles. This step is mediated by a family of Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor (SNARE) proteins. SNAREs are present on both vesicle or transport intermediate (v-SNARE) and the target (t-SNARE) compartment. Fusion of the membranes takes place because of the formation of a long, parallel four-helix bundle, in which one helix is contributed by the v-SNARE associated with the vesicle and three helices are contributed by t-SNAREs associated with the target membrane (Sutton, Fasshauer et al. 1998). This complex brings the two membranes into close proximity and facilitates fusion. After fusion has taken place, the SNARE complex gets disassembled by ATPase NSF (Jena 2008) and Sec17/18 proteins (Lobingier and Merz 2012).

Of all the key molecular players described above, tethering factor HOPS complex and small GTPases Arl8b and Rab7 are the ones which were of the main focus in my study.

1.1.2 HOPS Complex:

HOmotypic fusion and Protein Sorting (HOPS) is a multi-subunit tethering complex which comprises of six different subunits of which Vacuole Protein Sorting (Vps) 11, Vps16, Vps18 and Vps33 form the core complex and Vps39 and Vps41 form the accessory complex (Fig3). In *Saccharomyces cerevisiae,* Vps39 and Vps41 subunits interact with Rab7 homolog, Ypt7 (Brett, Plemel et al. 2008, Ostrowicz, Brocker et al. 2010)) and promote fusion of late-endosomes, AP-3 vesicles and autophagosomes with vacuoles (Nickerson, Brett et al. 2009). HOPS complex is conserved from yeast to mammals. In yeast, it has been shown that Ypt7-binding subunits Vps39 and Vps41 are located at the

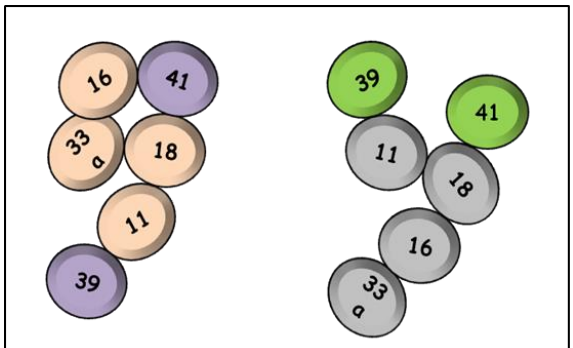

Figure3: Different subunits of HOPS complex.
A) Assembly of different subunits of HOPS complex in *S. cerevisiae.* B) Putative assembly of different HOPS subunits in humans.

opposite ends of the 30nm long seahorse-shaped particle. Vps33 subunit is assigned the function of binding with the SNAREs.

1.1.3 Small GTPases Arl8b and Rab7 and their effectors:

Rab7, a Rab family small GTPase, is known to localise on the late-endosomal membranes. It is a master regulator of endo-lysosomal trafficking regulating growth-factor receptor degradation, mannose-6-phosphate receptor retrieval to the trans-Golgi network among others (Press, Feng et al. 1998, Ceresa and Bahr 2006). In yeast, it has been shown that Rab7 homolog, Ypt7, interacts with Vps39 and Vps41 subunit of HOPS complex and mediates the fusion of late-endosomes with the vacuoles In humans, however, an ongoing study in the lab showed that Rab7 does not interact with any of the subunits of HOPS complex directly (Khatter et al, accepted in JCS 2015). Instead, it has been shown in a study that another small GTPase ADP-ribosylation factor-like 8b or Arl8b interacts with human HOPS complex (Garg, Sharma et al. 2011). Arl8b is a small GTPase of the Arf-like family that has been shown to regulate the spatial distribution of the lysosomes (Hofmann and Munro 2006). Arl8b regulates this function by microtubule-dependent motility of lysosomes (Donaldson and Jackson 2011). It interacts with human HOPS subunit Vps41 and regulates cargo trafficking to lysosomes (Garg, Sharma et al. 2011). But the exact mechanism of regulation still remained elusive.

Although, previous studies from our lab rule out the direct interaction of Rab7 with any of the human HOPS subunit (Khatter et al. accepted in JCS 2015), It has been previously shown that Rab7 associates with hVps39 and hVps41subunits of HOPS complex using approaches like co-immunoprecipitation (Rink, Ghigo et al. 2005). Keeping in mind this association of Rab7 with hVps39 and hVps41 and the role played by Rab7 in regulating the late-endosomal lysosomal trafficking, we wanted to check whether there is any Rab7 effector which is playing the intermediate role in the interaction of Rab7 with HOPS complex. One of the known effectors of Rab7 known as Pleckstrin homology domain-containing family M member 1 (PLEKHM1) did show an interaction with hVps39 in a yeast two-hybrid screen done previously in the lab. PLEKHM1 is a component of Rab7-regulated late endosomal trafficking and is shown to play a role in the bone resorption and a loss-of-function mutation of

PLEKHM1 is associated with the development of osteopetrosis with a function in vesicular transport (Van Wesenbeeck, Odgren et al. 2007). Also, PLEKHM1 has been shown to negatively regulate the endocytic pathway (Tabata, Matsunaga et al. 2010).

Keeping this information in mind, I worked on two different projects namely:

1. *Investigating the role of human HOPS complex subunit Vps41 in interaction with Arl8b, and*
2. *Characterising the interaction of Rab7 effector PLEKHM1 with human HOPS complex subunit, Vps39.*

1.2 Experimental Methods

Plasmids and reagents

hVps41 WD40 domain and WD40 domain-containing SNP T146P were made by site-directed mutagenesis using wild-type hVps41 and hVps41 WD domain in pET-45b(+) respectively as the templates. PLEKHM1wild-type was cloned in pEGFP-C1 and PLEKHM1 domain deletion constructs were created by site-directed mutagenesis using PLEKHM1 wild-type cloned in pEGFP-C1 as the template. For yeast two-hybrid assay, hVps39 was cloned in pGBKT7 whereas PLEKHM1 wild-type and different domain deletion mutants were cloned in pGADT7. For co-immunoprecipitation assay, hVps39 was cloned with Myc-tag in pcDNA3.1(-) and PLEKHM1 was cloned with N-terminal HA tag in pcDNA3.1(-).

Cell Culture

HeLa and HEK293T cells were cultured in DMEM supplemented with 10% FBS and incubated in a 5% humidified incubator at 37^0C.

Immunostaining and Confocal Microscopy

For immunostaining, DNA was transfected after seeding the cells on a coverslip. Cells were fixed using 4% PFA for 10 minutes; 16-18 hours post transfection. After fixation, cells were blocked using blocking solution (5% FBS in PHEM buffer + 0.2% saponin) followed by treatment with primary antibody dissolved in PHEM buffer containing 0.2% saponin for 45 minutes. Coverslips were then washed three times with PBS and incubated with Alexa-Flour-conjugated secondary antibody dissolved in PHEM buffer containing 0.2% saponin for 30 minutes. Coverslips were again washed three times with PBS and then mounted on a glass slide using Fluoromount-G mounting medium. Zeiss LSM 710 confocal microscope was used to image the cells.

Co-immunoprecipitation

For co-immunoprecipitation assay, HEK293T cells were harvested 20 hours post-transfection in PBS and lysed using TAP lysis buffer containing Protease inhibitor cocktail. The cell lysates were, subsequently, incubated with the anti HA-agarose beads for 4 hours at 4^0C. The beads were washed four times with TAP buffer and the proteins were eluted using 4X laemmli sample buffer. The eluates were subjected to SDS-PAGE followed by Western blotting using specific antibodies. Western blotting was performed using the ECL procedure according to the manufacturer's instructions.

Yeast two-hybrid assay

hVps39 and different domain deletion constructs of PLEKHM1 were cloned in fusion with Gal4-binding domain and Gal4-activation domain in pGBKT7 and pGADT7 plasmids, respectively. *S. cerevisiae* strain used for the experiment was "Gold strain". The yeast were co-transformed with the indicated constructs, following which they

were streaked on plates lacking leucine and tryptophan and allowed to grow at 30^0C for three days, to score for co-transformants. After this, yeast were replated on selection plates lacking Histidine and Adenine to check for interactions.

Protein purification

E.coli bacterial strain BL-21 was transformed with His-Vps41 WD40 and His-Vps41 WD40 (T146P). Bacteria, containing this gene of interest, were allowed to grow for 12-13 hours in LB at 37^0C. From this primary culture, secondary culture was inoculated using superbroth as the media and allowed to grow till O.D. reached between 0.4-0.6. At this point, culture was induced using 0.5mM IPTG and allowed to grow at 30^0C for 5 hours. Bacterial cells were harvested and resuspended in prep buffer and subjected to sonication. Pellet, obtained after sonication, was resuspended in buffer A (100mM NaH_2PO_4, 10mM Tris-HCl, 8M urea) pH-8.0 and kept at 37^0C on shaking for 3 hours. Next, it was subjected to centrifugation at 12,000 rpm for 20 minutes and the obtained supernatant was incubated with anti-His beads for 1-2 hours. Beads were washed with buffer A, pH-6.4, containing 20mM imidazole by incubating beads with this buffer for 2 minutes followed by centrifugation at 700 g. Protein was eluted using buffer A, pH-4.9 again by incubating the beads with this buffer for 2 minutes followed by a centrifugation at 700 g. Supernatant containing the eluted protein was collected. Eluted protein was subjected to step-wise dialysis against decreasing concentration of urea by first dialysing it against 4M urea for one hour, then 2M urea for one hour, followed by 1M urea for one hour (all at room temperature) and finally against 1x PBS overnight at 4^0C. Dialyzed protein was concentrated using a concentrator with a 10 KDa molecular weight cut off by centrifuging it at 2300 rpm in a swing bucket centrifuge.

Circular dichroism spectroscopy

CD spectrum was obtained on an *Applied photophysics ChiraScan* instrument. Samples were dissolved in 5mM NaH_2PO_4, 5mM NaCl keeping the concentration of sample at 5μM. Baseline was the buffer solution in which the protein was dissolved. Spectra were recorded from 260nm down to 198nm. At least three repeat scans were

obtained for each sample and its baseline. The averaged baseline spectrum was subtracted from the averaged sample spectrum.

Buffer compositions:

1. Super Broth:

    Tryptone - 1.2%

    Yeast extract - 2.4%

    Glycerol – 0.4%

2. Salt solution:

    Di-potassium hydrogen phosphate - 16%

    Potassium dihydrogen phosphate – 2.3%

3. PHEM buffer:

    PIPES – 60mM

    HEPES – 25mM

    EGTA – 10mM

    Magnesium chloride – 2mM

    Adjust final pH to 6.8

4. 1x PBS:

    NaCl - 137mM

    KCl - 2.7mM

    Na_2HPO_4 - 10mM

    KH_2PO_4 - 1.8mM

5. Prep Buffer:

Tris-Cl - 20mM

NaCl - 150mM

DTT - 0.5mM

Triton X-100 - 0.5%

Glycerol - 5%

Adjust final pH to 7.4

Chapter 2

Results and concluding remarks

2.1 Results

2.2 Summary and Future Outlook

2.1 Results

2.1.1 WD40 domain of hVps41 is sufficient for the interaction of hVps41 with Arl8b:

Previously, it has been shown that Arl8b interacts with hVps41 subunit of the HOPS complex and it recruits this subunit to the lysosomal membranes. To get more insights into the assembly of the HOPS complex onto the lysosomal membrane and its function in mediating late endosome-lysosome fusion, we were studying the interaction of hVps41 and Arl8b in more detail. Our recent results indicate that GST-tagged Arl8b can pull down wild-type hVps41 from transfected HeLa cell lysates. Moreover, different domain deletion mutants of hVps41 were employed in a similar GST pulldown assay, using GST-Arl8b as the bait, where it was revealed that the WD40 domain of hVps41 is essential for its interaction with Arl8b. To further confirm that WD40 domain of hVps41 was sufficient for interaction with Arl8b, we tested this interaction using purified proteins. Using site-directed mutagenesis, His-tagged WD40 domain of hVps41 (His-Vps41 WD40) was created in pET-45b(+) by inserting a stop codon at amino acid position 450. This construct was expressed in *E.coli* BL-21, and WD40 domain hVps41 protein was purified from inclusion bodies by first solubilising the protein using 8M urea and then refolding it by step-wise dialysis against decreasing concentrations of urea. Further, purified His-Vps41WD40 was incubated with purified GST-Arl8b bound on glutathione beads. The eluted proteins were subjected to SDS-PAGE and immunoblotting using anti-His antibody. Interestingly, it was observed that purified WD40 domain of hVps41 showed an interaction with GST-Arl8b but not GST alone. Previous studies have shown that two single nucleotide polymorphisms (SNPs) (T146P and A187T) in the WD40 domain of Vps41 abrogated the neuroprotective function of Vps41 by hindering the autophagic degradation of α-synuclein (Harrington, Yacoubian et al. 2012). We hypothesised that these SNPs might be disrupting the binding of hVps41 with Arl8b, and consequently, its function. To investigate this, we tested the interaction of hVps41 containing either of the SNPs with Arl8b in a GST-pulldown assay. We observed that, similar to the wild-type protein, hVps41 A187T showed an interaction with Arl8b (data not shown). Interestingly, hVps41 containing T146P SNP showed significantly reduced interaction with Arl8b. Again, to confirm this, His-Vps41 WD40 containing the T146P SNP (His-Vps41 WD40 (T146P)) was created and the protein was purified as

described above. Interaction of His-Vps41 WD40 (T146P) was tested with Arl8b in a purified protein interaction assay, and it was seen that, while purified wild-type WD40 domain of hVps41 was pulled down by purified GST-Arl8b, there was a significant reduction in the interaction of WD40 (T146P) with Arl8b. (Fig.4B) Finally, to prove that the substitution of threonine at position 146 by proline is not disrupting the domain secondary structure, thereby leading to random coil formation, CD spectra of both the purified proteins i.e. wild-type WD40 domain and WD40 (T146P) were obtained. (Fig.4C). Although, minor structural changes could be seen in the WD40 (T146P) when compared to wild-type WD40, this substitution did not result in the complete denaturation of this domain or formation of a random coil. Together, these results show that WD40 domain is sufficient for the interaction of hVps41 with Arl8b and the SNP T146P in the WD40 domain disrupted the binding of hVps41 with Arl8b.

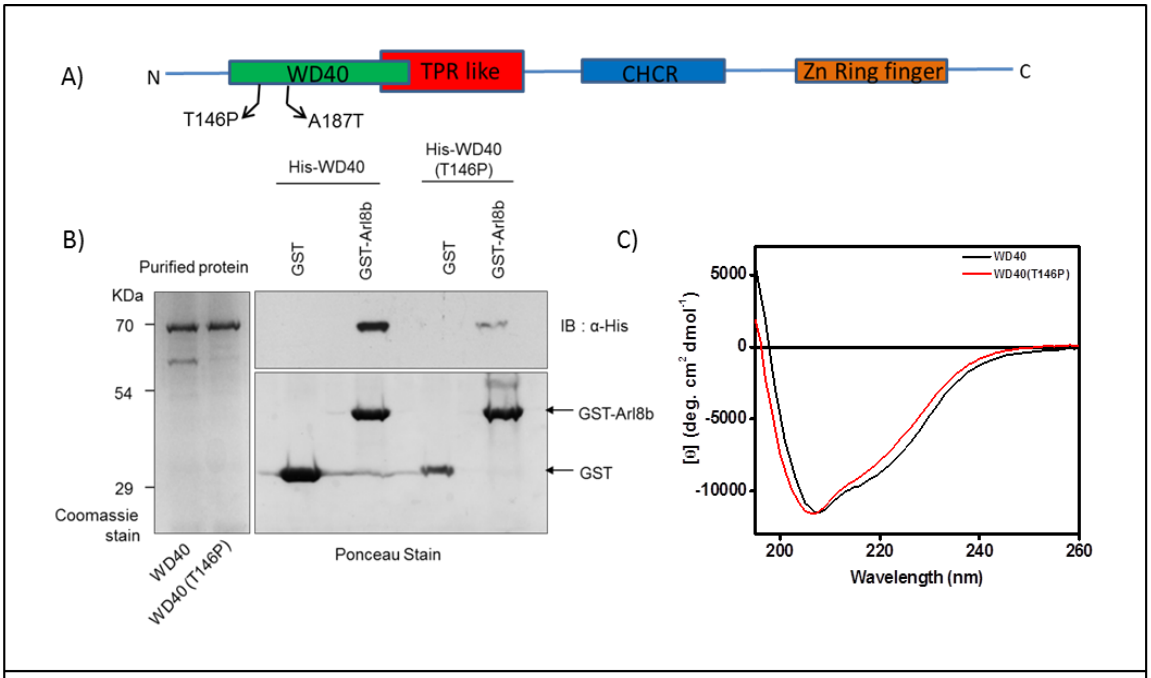

Figure 4: Interaction of WD40 domain of hVps41 with Arl8b. A) Domain architecture of hVps41 indicating the two SNPs within the WD40 domain. B) Interaction analysis of the purified proteins His-Vps41 WD40 and His-Vps41 WD40 (T146P) incubated with either purified GST or GST-Arl8b on glutathione beads C) Circular dichroism spectra of WD40 and WD40 (T146P) domain of hVps41.

2.1.2 Interaction of PLEKHM1 with hVps39

2.1.2(a) hVps39 gets co-immunoprecipitated by PLEKHM1

Previously in the lab, it was found that hVps39 interacts with PLEKHM1 subunit of HOPS complex in a yeast two-hybrid screening. In order to test this interaction in the cells, we did a co-immunoprecipitation assay in which HEK293T cells were either transfected with Myc-Vps39 alone or co-transfected with HA-PLEKHM1. Post 20 hours of transfection, the cells were lysed using TAP lysis buffer and subjected to incubation with Anti-HA agarose beads for 4 hours at 4^0C. After the incubation, the beads were then washed four times with TAP buffer and the elution was carried out in 4x Lammeli sample buffer. The samples were subjected to SDS-PAGE followed by Western blotting for analysis. It was found that HA-PLEKHM1 binds directly to Anti-HA agarose beads and Myc-Vps39 is co-immunoprecipitated by PLEKHM1 (Fig. 5). The co-immunoprecipitation was checked by using Anti-Myc antibody whereas direct immunoprecipitation was analysed by using Anti-HA antibody. We also observed that PLEKHM1 and hVps39 strongly interact with each other as the expression level of Myc-Vps39 in case of double transfection along with HA-PLEKHM1 was very low when compared to Myc-Vps39 alone but its co-immunoprecipitation was huge. These results showed that hVps39 does interact with PLEKHM1 inside the cells also and that it is a strong interaction.

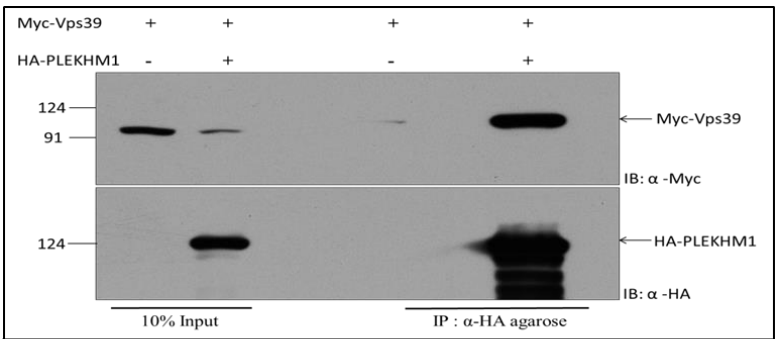

Figure 5: Co-immunoprecipitation of hVps39 by PLEKHM1. Lysates from HEK293T cells co-transfected with Myc-Vps39 and HA-PLEKHM1 (as indicated), were subjected to immunoprecipitation (IP) with anti-HA antibodies, and immunoblotted (IB) with Myc antibody.

2.1.2(b) Second PH domain of PLEKHM1 is responsible for its interaction with hVPS39:

Bioinformatic analysis has predicted that PLEKHM1 has four functional domains, namely RUN domain, two Pleckstrin homology domains (PH1 and PH2) and a

Cysteine (Cys) rich domain (Fig. 6). RUN domain, in general, is known to bind small GTPases. Pleckstrin homology domains bind to the phosphoinositides on the membrane and Cys-rich domain in case of PLEKHM1 has been shown to bind to Rab7.(20943950) To delineate the PLEKHM1 domain important for its interaction with hVps39, we created different C-terminal and N-terminal domain deletion constructs of PLEKHM1, as indicated, (Fig. 7) and tested their interaction in an yeast two-hybrid assay. Domain deletion constructs were made by introducing a stop codon at the end of respective domains using site-directed mutagenesis where wild-type PLEKHM1 cloned in pGADT7 was taken as the template for the PCR reaction. *S.cerevisiae (Gold strain)* were co-transformed with the indicated constructs and plated on selection plate (-His –Ade) to score interactions. After 3 days, it was observed that wild-type PLEKHM1 (PLEKHM1 FL) showed an interaction with hVps39, as indicated by the growth of yeast on the interaction plate (Fig. 7A). In case of C-terminal domain deletions, PLEKHM1 with deleted Cys-rich (PLEKHM1 ΔCR) also showed an interaction. However, yeast transformed with PLEKHM1 construct lacking both Cys-rich domain and second PH (PH2) domain (PLEKHM1 ΔCR+PH2) and hVps39 did not grow on the selection plate, indicating that the two proteins do not interact. Similar results were obtained for the PLEKHM1 construct containing RUN domain only. These results indicated that second PH domain plays an important role in the interaction of hVps39 with PLEKHM1. Further, to confirm this result and to show that second PH domain alone is important for the interaction, N-terminal domain deletion constructs were co-transformed with hVps39 in yeast and interaction was checked using similar approach. As expected, PLEKHM1 FL did show an interaction with hVps39 again. PLEKHM1 constructs lacking RUN domain (PLEKHM1 ΔRUN) only and both RUN domain and first PH domain (PLEKHM1 ΔRUN+PH1) also showed an interaction with hVps39 marked by the growth of yeast on selection plate. (Fig.7B) Expectedly, PLEKHM1 construct lacking second PH domain (PLEKHM1 CR only), did not show an interaction with hVps41 confirming that it is indeed the second PH domain which is important for the interaction if PLEKHM1 with hVps39.

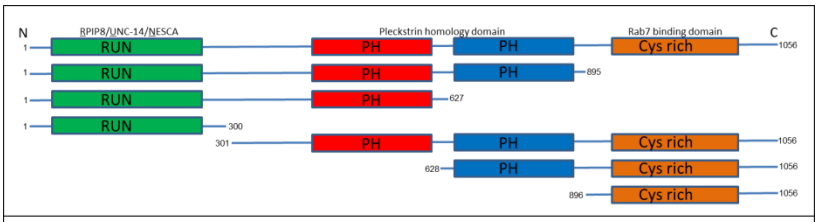

Figure 6: Domain architecture of PLEKHM1 wild-type and different domain deletion constructs

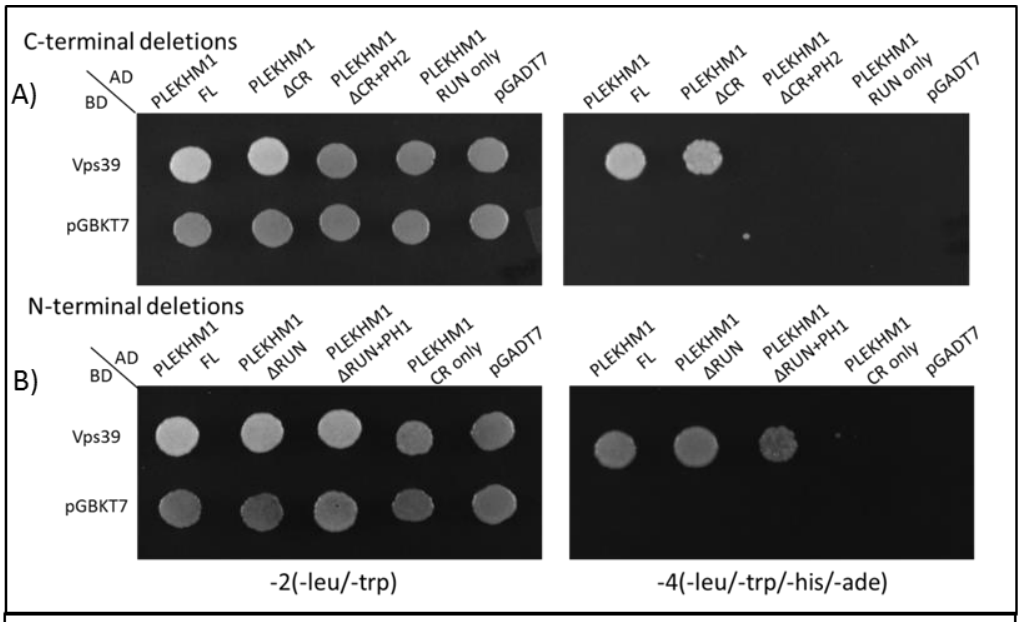

Figure 7: Yeast two-hybrid assay of hVps39 and PLEKHM1 wild-type and its different domain deletion constructs. hVps39 was cloned in fusion with GAL4 binding domain (BD) and PLEKHM1 WT and its deletion constructs were cloned in fusion with GAL4 activation domain (AD) vectors as indicated. Yeast were plated on non-selective medium (+His) to confirm viability and plated on selective medium (-His/-Ade) to detect interactions. A) Assay of hVps39 and PLEKHM1 wild-type and its C-terminal domain deletions constructs. B) Assay of hVps39 and PLEKHM1 wild-type and its N-terminal domain deletions constructs.

2.1.2(c) PLEKHM1 promotes the association of hVps39 with lysosomes:

To look at the subcellular localization of the two proteins and the effect of PLEKHM1 on the localization of hVps39, HeLa cells were either transfected with hVps39 alone or co-transfected with PLEKHM1-WT or different deletion constructs of PLEKHM1. After staining for desired proteins, it was observed that, hVps39 localizes to the cytoplasm of HeLa cells upon overexpression. (Fig.8a). However, upon co-expression of HA-Vps39 with GFP-PLEKHM1, it was observed that hVps39 was recruited to endosomes positive for GFP-PLEKHM1 and LAMP1, indicating that PLEKHM1 recruited hVPs39 to lysosomes (Fig.8b). Further, to test which domain of PLEKHM1 was responsible for this recruitment, different domain deletion constructs of PLEKHM1 cloned in pEGFP-C1 vector were co-expressed with HA-Vps39. Confocal microscopy revealed that, while PLEKHM1 ΔRUN and PLEKHM1 1-895 colocalised with hVps39 on LAMP1 positive compartments. PLEKHM1 RUN only and PLEKHM1 1-627, which lacks the second PH domain, were completely cytosolic and, therefore, had no effect on Vps39 localization (Fig.8d-e). Our immunofluorescence results are in accordance with the yeast-two-hybrid results and

further strengthen our finding that hVps39 interacts with PLEKHM1 and that this interaction takes place via the second PH domain.

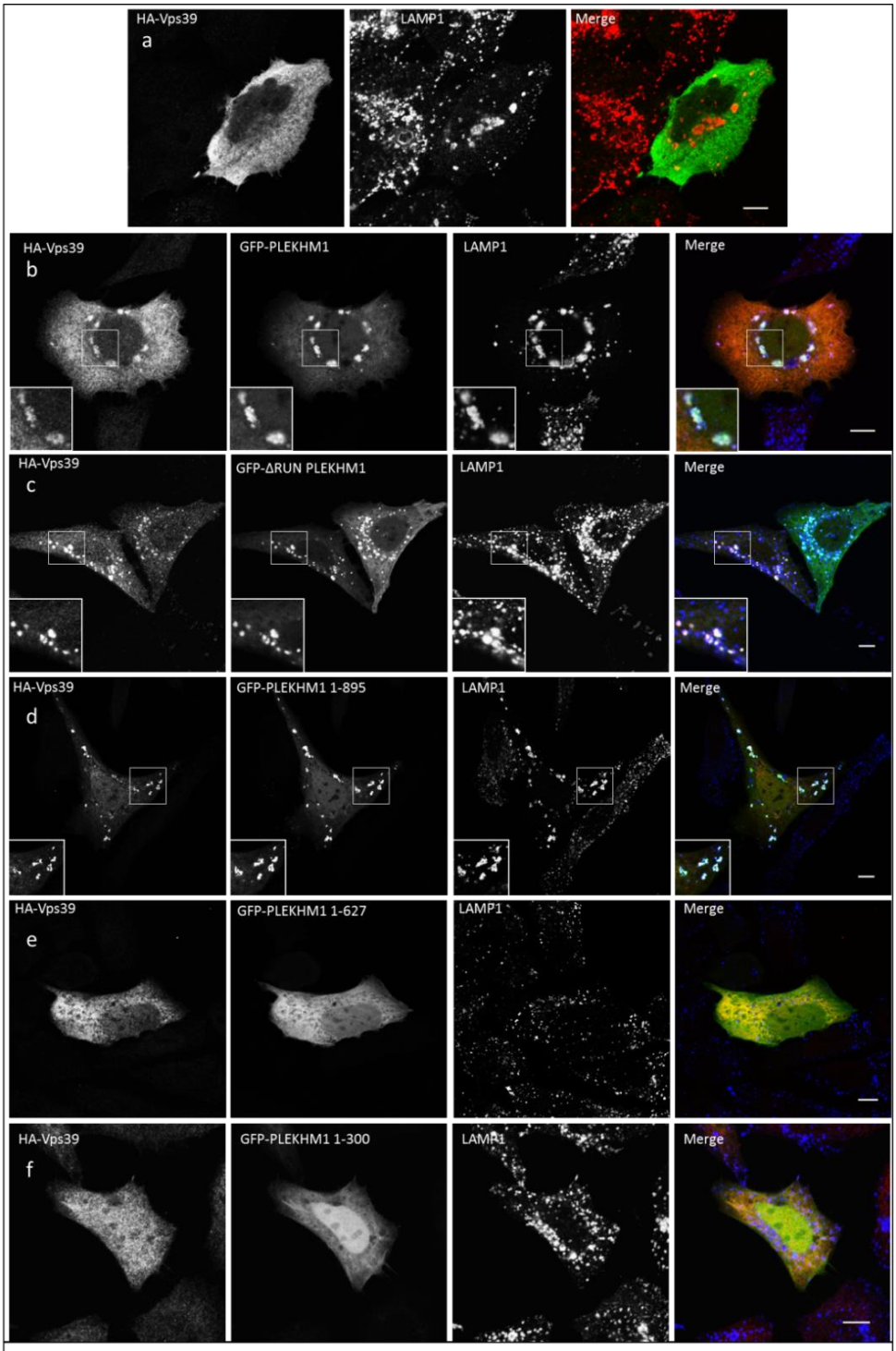

Figure 8: Confocal images of immunostained cells transfected either with hVps39 alone or with PLEKHM1 and its different domain deletion mutants. HeLa cells were either transfected with HA-Vps39 (a) or co-transfected with HA-Vps39 and GFP-PLEKHM1 WT (b) or various domain deletion mutants of PLEKHM1 (c-f) and analyzed by confocal microscopy. Co-localized pixels are represented in the inset. (Scale bar 10µm)

2.2 Summary and Future outlook

In this study, we have shown that PLEKHM1 interacts with human HOPS complex subunit Vps39 by using approaches like co-immunoprecipitation and yeast two-hybrid. Our yeast two-hybrid results indicate that the second PH domain of PLEKHM1 is responsible for its interaction with hVps39. Furthermore, confocal microscopy revealed that PLEKHM1 and hVps39 co-localise on lysosomes and again, the second PH domain of PLEKHM1 drives the localization of Vps39 on lysosomes. This study provides an insight into a novel function of PLEKHM1. Further, it would be interesting to see which domain of hVps39 is important for this interaction and whether other HOPS subunits are recruited to this complex. This study can provide insights in the role played by hVps39, function of which remains largely unknown. It would also be very interesting to see how these proteins mediate trafficking to lysosomes and whether this interaction is important for the same. The interaction between PLEKHM1 and HOPS complex subunits might also play a significant role in the clearance of pathogenic microbes like *Salmonella,* as is highlighted in a recent study by the group of Ivan Dikic (McEwan et al. 2015)

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