Identification and characterization of Rabip4' interaction partners

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

Indian Institute of Science Education and Research Mohali

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Certificate of Examination

This is to certify that the dissertation titled **"Identification and characterization of Rabip4' interaction partners"** submitted by Ms. Gitanjali (Reg. No. MS15001) for the partial fulfillment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Indranil Banerjee **Dr. Sharvan Sehrawat** **Dr. Mahak Sharma (Supervisor)**

Dated: May 4th, 2020

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.

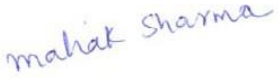
This work has not been submitted in part or in full for a degree, a diploma, or a fellowship, to any other university or Institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussion. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Gitanjali

(Candidate)

Dated: May 4th, 2020

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

My sincere gratitude first goes to Dr Mahak Sharma, who took me as her masters' student. I am much obliged to her, who, despite having two masters' student, agreed to be my masters' thesis supervisor when I was struggling academically. She has been supportive of me and continuously gave her valuable inputs throughout this project. I got to learn a lot in this lab as she would never restrict us from exploring all that was available to us.

I want to thank my PhD guide, Ms Shalini Rawat, under whom I did my masters' project, for her constant assistance and inputs.

My appreciation also extends to my lab members who were always there to cheer me and help me amid trying times and with whom I've made some cherishable moments. I thank Vidya bhaiya for his hard work for making the lab functional by maintaining the lab equipment. I also thank other labs in the department for providing me with the reagents that were not available in the lab.

I thank my family and friends for their faith and support. Lastly, I thank myself for persevering through tough phases and not give up. I acknowledge myself for being patient with my work and the unprecedented situations that I have faced during these five years.

Gitanjali

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Abstract

Lysosomes are subcellular compartments that digest intracellular and extracellular material and recycle their contents to maintain cellular homeostasis. Recent studies have shown several unconventional functions of lysosomes; such as antigen presentation, tumour invasion, transportation of RNA granules, nutrient sensing and plasma membrane repair. The positioning of lysosomes determine these functions in the mammalian cell. Our lab primarily works on Arl8b, one of the small GTPases, which relocate the lysosomes towards the periphery of the cell. Previous work suggests that Arl8b interacts with a subset of RUN domain-containing proteins. Rab4 and Rab14 effector, Rabip4' is one such protein that binds to Arl8b via its RUN domain. Here we have used tandem affinity, and GST pulldown approaches to identify interaction partners for Rabip4'. To this end, we have created a stable cell line expressing Tandem tag Rabip4', and GST full-length Rabip4'. Mass spectrometry-based identification of the eluate has revealed several interesting hits. We have also characterised Rabip4' interaction with Sorting nexin proteins that localise on the early endosomes. The newly identified interaction partners from this work will be further characterised in future studies.

Chapter 1: Introduction

1.1 Lysosome: A classical terminal degradative compartment

Lysosomes are membrane-enclosed compartment of a cell. These compartments loaded with acid hydrolases are primarily known to degrade both intracellular and extracellular cargos trafficked towards them. These hydrolytic enzymes are carried to lysosomes by mannose-6-phosphate receptors and work ideally in an acidic condition present within the lysosome lumen (Griffiths et al. 1988). The pH inside the lysosomes is ~ 4.5-5, which is maintained by V-type H^+ ATPases present in the membrane. Some of the byproducts, such as amino acids and cholesterol, is used immediately for denovo synthesis of macromolecules. Lysosomes take cargos via three routes: endocytosis, phagocytosis and autophagy. Endocytosis is a process in which cells take up extracellular materials such as nutrients, growth factors, antibodies and hormones into vesicles. These vesicles transport the internalised material to early endosomes, which are slightly acidic. Some of this material is recycled back to the plasma membrane, whereas others passed on to lysosomes for degradation. Phagocytosis is the internalisation of large particles such as microorganisms and exhibited by specialised cells like macrophages and neutrophils. Large structures known as phagosomes form after internalising the material. These structures fuse with lysosomes for further degradation (Mellman et al. 1986). The final route is autophagy, where an autophagosome sequesters damaged organelle or any other cellular component. The seized material is further delivered to lysosomes for digestion by forming autolysosomes (Mizushima 2007).

Since lysosomes play a vital role in clearing out cellular debris, any defect in lysosome biogenesis, transportation of acid hydrolases or fusion of lysosomes with other compartments can be harmful to the cell. These defects can lead to several lysosomal disorders. There are more than 60 known lysosomal storage disorders resulting from defective lysosomal function.

1.2 Atypical functions of lysosomes

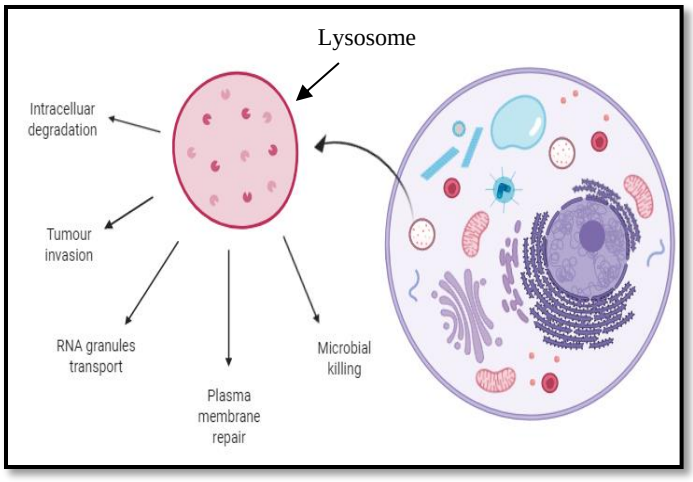


Figure 1 the diagram gives an overview of the conventional and unconventional roles of lysosomes. This schematic is adapted from Pu et al. 2016.

Recent studies have shined some light on the unconventional functions of lysosomes. These are nutrient sensing, plasma membrane repair, tumour invasion and RNA granule transport, to name a few (Ballabio 2016). For sensing nutrients, mTORC1 protein kinase gets recruited, followed by activation on the surface of lysosomes as feedback to nutrients in mammalian and yeast cell. From the surface of lysosomes, mTORC1 initiates a signalling cascade for cell growth (Saxton and Sabatini 2017). mTORC1 activation requires both growth factors and nutrients. When there is no signal, mTORC1 is present in the cytosol in its idle form. The nutrient signal activates heterodimeric GTPases which recruits mTORC1 onto the lysosome membrane, and the growth signal activates the lysosome-bound GTPase Rheb, which activates mTORC1 (Lawrence et al. 2018; Yang et al. 2017). Cells like neurons need local protein synthesis, which requires transporting RNA to greater lengths. While proteins transport in vesicles, RNA molecules interact with RNA-binding proteins (RBP) and then transported to the distal parts of the neuron (Knowles et al., 1996). Researchers have reported that RNA granules hitchhike on mobile lysosomes to reach their destination within the cell. ANXA11 facilitates this shipping by tethering RNA granules to lysosomes via its intrinsic and phase separating properties (Liao et al. 2019). Some reports infer that lysosomes also help in repairing the plasma membrane in response to Ca^{2+} influx upon plasma membrane damage (Corrotte and Castro-Gomes 2019). Lysosomes fusing with the plasma membrane is a mechanism through which *Trypanosoma cruzi* enters the mammalian cells (Tardieux et al. 1992). Some reports also indicate the role lysosomes play in tumour invasion and antigen presentation. There is a drastic change in the distribution of lysosomes during metastasis and cancer cell growth. Lysosomes are more towards the periphery in these cells (Nishimura et al. 2002). The exocytosis of lysosomes facilitates

migration of cancer cells by digesting the extracellular matrix (Mohamed and Sloane 2006). Lysosomes also present antigen peptides generated after digestion. The dendritic epithelial cells present antigen to CD4-T lymphocytes by transporting them to the plasma membrane via tubular lysosomes (Chow et al. 2002).

1.3 Motility and distribution of lysosomes within a cell

The distribution and motility of lysosomes in the cells govern their function. Although distributed throughout the cells, a distinct cluster of lysosomes appears near the nucleus. Lysosomes rapidly transverse between the nucleus and cell periphery fusing with other compartments and with each other on their way. Kinesin motor complex governs the anterograde transport of lysosomes while dynein-dynactin motor complex the retrograde transport (Perera and Zoncu 2016). BORC, a multi-subunit complex present on lysosomes recruits Arf like GTPases Arl8b on lysosomes (Pu et al. 2015), which in turn interacts with the motor protein Kinesin-1 through its effector SKIP, thus promoting the anterograde transport of lysosomes (Bagshaw et al. 2006). The retrograde movement of lysosomes is through a series of protein interactions that ultimately link lysosomes to the dynein-dynactin motor complex. Rab7 present on the lysosomes interacts with its effector RILP, which connects it to the dynactin p150-glued subunit (Cantalupo et al. 2001). Another effector, ORP1L associates via the membrane-bound βIII-spectrin to the dynein-dynactin motor complex in the presence of high cholesterol concentration and facilitate the retrograde movement of lysosomes (Johansson et al., 2007).

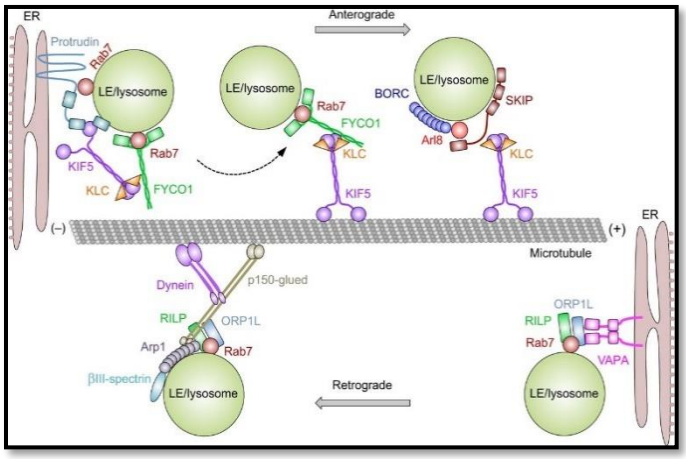


Figure 2 Anterograde motion of lysosomes is mediated by tethering them to kinesin motor complex, whereas retrograde transport is mediated through dynein-dynactin motor complex. The diagram is adapted from Pu et al. 2016

1.4 Role of small GTPases in the lysosome motility and fusion

The role of small GTPases in regulating the motility and fusion of lysosomes is indispensable as also described in the previous section. Small GTPases such as Rab7 and Arl8b determine the location of lysosomes within the cells by recruiting their effectors. Regulation of Rab7 and Arl8b is crucial for lysosome position and function. The GTPases are known as molecular switches and transit back and forth between two forms, GTP-bound 'active' form and GDP-bound 'inactive' form. GDP-bound GTPases are acted upon by GEFs and converted into the active form. The active form is then readily converted to the inactive form by the GAPs. The active GTPases are recruited onto the membrane and interact with their effectors to initiate the downstream signalling. However, in the inactive state, they remain in the cytosol. While thus far, no GEFs and GAP are known for Arl8b, the GEFs and GAPs of Rab7 are known. Mon1/CCZ1 complex acts as GEF for Rab7 and TBC1D5, TBC1D15 and TBC1D2 are known to have GAP activity towards Rab7 (Stroupe 2018). Previous reports indicate that both Rab7 and Arl8b are responsible for recruiting the multi-subunit tethering factor- HOPS complex at the lysosomes, where Arl8b directly interacts with and recruits Vps41 subunit of the complex to the lysosomal membrane (Khatter et al. 2015; Kant et al. 2013). HOPS complex tethers the lysosomes with target organelles which finally undergo fusion via the formation of SNAREpin (Luzio et al. 2007). Arl8b also interacts with PLEKHM1, a RUN domain-containing protein similar to SKIP/PLEKHM2. Interaction of PLEKHM1 and Arl8b promote autophagosome-lysosome fusion by recruiting HOPS complex to membrane contact sites (Marwaha et al. 2016). It is important to note that the RUN domain seems to be the common determinant in PLEKHM1 and PLEKHM2 required for binding to

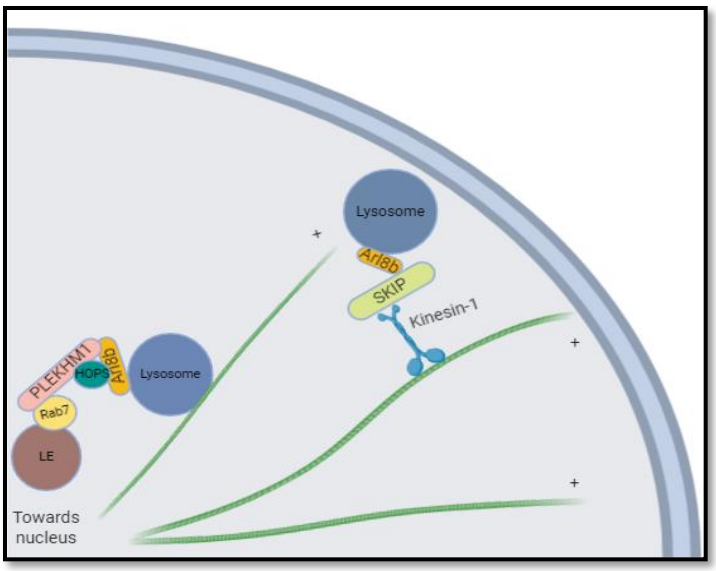


Figure 3 PLEKHM1 acts as a dual effector by binding to both Rab7 and Arl8b. Binding of Arl8b to either PLEKHM1 or SKIP determines lysosome position. Image adapted from Marwaha et al. 2016.

Arl8b. In order to gain insights into Arl8b interactome, we explored whether Arl8b binds with endosomal and lysosomal RUN domain-containing proteins. With this idea in mind, we explored whether RUFY1, a member of RUFY family of proteins present on endosomes as a potential interaction partner for Arl8b.

1.5 RUFY: A family of RUN and FYVE domain-containing proteins

RUFY proteins include an N-terminal RUN domain and a C-terminal FYVE domain (Kitagishi and Matsuda 2013). RUN domain, labelled after RPIP8, UNC-14, and NESCA proteins, is an evolutionarily conserved protein-protein interaction domain which interacts with small GTPases involved in membrane trafficking. RUN domain is organised into six conserved blocks which constitute the core of a globular structure (Callebaut et al. 2001). FYVE domain, on the other hand, allows any protein to interact with phosphatidylinositol-3-phosphate membranes (mostly early endosomes). There are four proteins which come under this family: RUFY1, RUFY2, RUFY3, and RUFY4 (Kitagishi and Matsuda 2013).

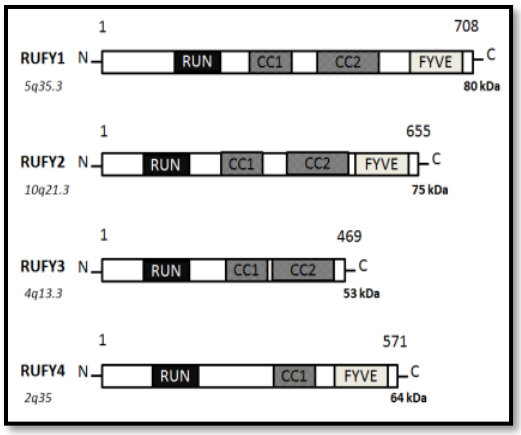


Figure 4 Domain architecture of the RUFY protein family. Adaptation from Kitagishi and Matsuda 2013.

Functions of RUFY2 and RUFY4 are not well characterised yet. Whereas, RUFY3 is reported to interact with Rab5 and plays a role in ensuring the robustness of neuronal polarity (Mori et al. 2007). RUFY1 has two isoforms, a longer one known as Rabip4' and the shorter one as Rabip4. Both the longer and shorter isoform show 95% similarity except for the N-terminal region in Rabip4' that is 108 amino acid longer than Rabip4. Previous studies have identified Rabip4' as the effector of Rab4 (Fouraux et al. 2004), a small GTPase

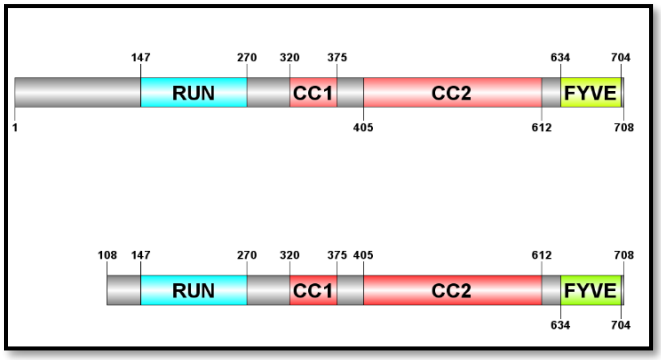


Figure 5 Domain architecture of the two isoforms of RUFY1. Upper schematic represents the longer isoform Rabip4' the lower one represents the shorter isoform Rabip4.

localised on early endosomes regulating the endosomal trafficking of cargos. Another report has shown Rabip4/RUFY1 to interact with Rab14 as well, where Rab14 determines the recruitment of RUFY1 on endosomes and RUFY1-Rab4 interaction mediates endosomal tethering and fusion (Yamamoto et al. 2010). Others have reported the role of Rabip4 in endocytic trafficking of GLUT4 and Rab4 dependent recycling of transferrin receptors from endosomes (Mari et al. 2006; Yamamoto et al. 2010). Lastly, the work done by Ivan et al. 2012 hinted Rabip4', whose function was defined so far in context to endosomes, also governs the spatial distribution of lysosomes via its interaction with adaptor protein complex AP-3. They reported dramatic relocalisation of lysosomes near the plasma membrane in HEK293T cells upon Rabip4' knockdown. This observation piqued our interest and motivated us to study for interactions between Rabip4' and lysosome-related protein, particularly Arl8b since it interacts with most of its effectors via their RUN domain and Rabip4' happen to have one.

1.6 Objective:

In this project, we are attempting to address whether Rabip4' interacts with Arl8b and function of Rabip4' in regulating cargo transport to late endosome/lysosome. So far, we have discovered that Rabip4' interacts with Arl8b through its RUN domain. Rabip4' also seems to regulate the endosome-to-Golgi transport of Cation-independent mannose-6-phosphate receptor (CI-M6PR). As a part of this thesis, we have determined the interacting partners of Rabip4' and initiated studying the interactions that help Rabip4' to regulate CI-M6PR trafficking.

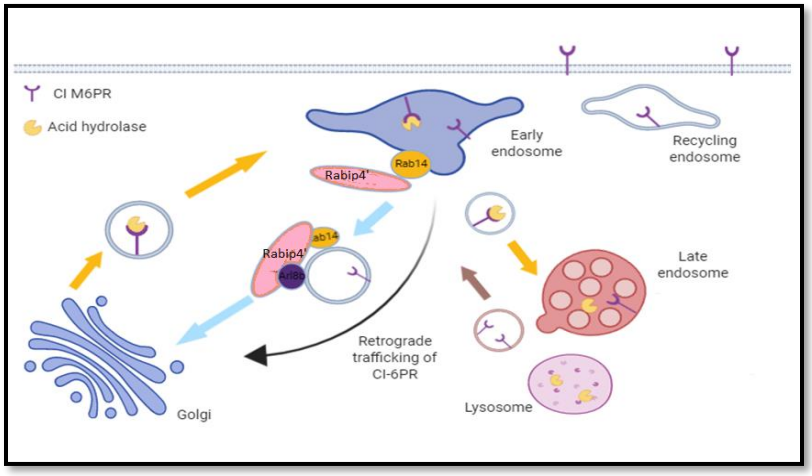


Figure 6 summarising the transportation of CI-M6PR in a cell. Our studies suggests the presence of compartments positive for both Arl8b and Rabip4', in which M6PR receptors are trafficked back to the Golgi.

Chapter 2: Materials and methods

1. Cloning:

The enzymes used for cloning were from NEB Inc. FLAG-Rabip4' was used as a template to clone Rabip4' (WT) in pGEX-4T3 and pNTAP-A vectors. TAP-Rabip4' was then cloned in pCDH-CMV-MCS-EF1-Puromycin. Inserts were amplified using appropriate primers. The vector and insert were digested at 37°C for 2-3 hours. Gel purified vector and insert taken in the right ratio were kept for ligation, overnight at 16°C. The ligated products were transformed either in DH5-α cells or 2T1R cells depending on the vectors. The positive clones were checked for their protein expression and sent for sequencing.

2. Protein expression and purification:

The expression vector with the gene of interest was transformed into E.coli Rosetta strain. From a single transformed colony, a primary culture was set up in Luria-Bertani medium (BD Difco) including vector antibiotic for 12 hours at 37°C. A secondary culture was set up using 1% inoculum in the super broth containing vector antibiotic for 3 hours at 37°C. It was then induced with 0.5mM IPTG to express the protein at 16°C for 14 hours. Bacterial cells were collected by centrifugation at 4000 rpm for 15 minutes at 4°C. The pellet was dissolved in PREP buffer (20mM Tris-Cl pH 8.0, 150mM NaCl, 1mM EDTA, 0.05mM DTT, 0.5% Triton-X, 5% glycerol) containing 1mM PMSF and protease inhibitor tablet (Roche), on ice. Cells were then lysed by sonication, followed by centrifugation at 12000 rpm for 15 minutes at 4°C. The supernatant (protein lysate) was collected and stored for further analyses.

3. GST-Pulldown:

The Protein lysates of GST and GST-tagged protein were incubated with Glutathione resins (Gbiosciences) on a hula mixer (Life technologies) for 3 hours at 4°C for binding of GST and GST-tagged protein to the resins. The resins were given ten washes with PREP buffer.

For the pulldown, HEK239T cells were lysed in TAP lysis buffer (20mM Tris-Cl pH 8.0, 0.5% NP-40, 5mM $MgCl_2$, 150mM NaCl, 1mM Na_3VO_4, 1mM NaF) containing 1mM PMSF and 1X protease inhibitor (Sigma-Aldrich), on a hula mixer for 1.5 hours at 4°C. It was followed by centrifuging the lysed cells at 12000 rpm for 15 minutes. The supernatant was pre-cleared with Glutathione resins and incubated with GST and GST-tagged protein-bound Glutathione resins for 3 hours. Samples were washed thrice with washing buffer (20mM Tris-Cl pH 8.0, 0.2% NP-40, 5mM $MgCl_2$, 150mM NaCl, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF). Samples were eluted by boiling in Laemmli buffer and loaded onto SDS-Page.

4. TAP-Pulldown:

HEK293T cells stably expressing TAP-tagged protein were lysed in lysis buffer provided in the TAP pulldown kit (InterPlay N-Terminal Mammalian TAP System Kit, Agilent) following the manufacturer's protocol. The cell-lysate was first incubated with streptavidin beads for 2 hours at 4°C. Unbound protein was removed by washing the beads thrice with streptavidin-binding buffer. Proteins bound to the beads were eluted by incubating the beads with streptavidin elution buffer, which contains 2mM biotin. The entire eluate was incubated with calmodulin beads for 2 hours at 4°C, followed by three washes with the calmodulin-binding buffer. Final elution was done by boiling the sample in Laemmli buffer. The sample was loaded onto SDS-Page.

5. Sample preparation for mass spectrometry:

GST pulldown samples were loaded on SDS-Page and stained using silver stain (Pierce Silver Staining Kit for Mass Spectrometry, ThermoFisher) following the manufacturer's protocol. The protein bands were cut and stored in DEPC water. TAP pulldown sample was loaded on SDS-Page and run till it completely crossed the stacking gel and entered about 1cm into the resolving gel. Following staining and destaining of gel, the protein band was incised and stored in DEPC water. All protein samples were sent to Taplin MS facility for mass spectrometry analysis.

6. Co-Immunoprecipitation and Immunoblotting:

HEK293T cells were transfected with the desired DNA constructs and lysed in TAP lysis buffer (20mM Tris-Cl pH 8.0, 0.5% NP-40, 5mM $MgCl_2$, 150mM NaCl, 1mM Na_3VO_4, 1mM NaF) containing 1mM PMSF and 1X protease inhibitor (Sigma-Aldrich) on a hula mixer for 1 hour at 4°C. The lysates were collected by centrifugation at 12000 rpm for 10 minutes. The lysates were incubated with indicated antibody conjugated-agarose beads for 3 hours on a hula mixer at 4°C. The beads were given three washes with washing buffer (20mM Tris-Cl pH 8.0, 0.1% NP-40, 5mM $MgCl_2$, 150mM NaCl, 1mM Na_3VO_4, 1mM NaF). Samples were eluted by boiling in Laemmli buffer and loaded on SDS-Page.

Proteins segregated on the gel were transferred onto PVDF membranes (Bio-Rad Laboratories) by wet transfer. The membranes were blocked overnight with blocking solution (10% skimmed milk in 0.05% PBS-Tween 20) at 4°C, and probed with indicated primary and secondary antibodies prepared in 0.05% PBS-Tween 20 for 3 hours and 45 minutes, respectively, at room temperature. Membranes were washed thrice with 0.05% PBS-Tween 20 for 10 minutes after probing with primary antibodies and with 0.3% PBS-Tween 20 after probing with secondary antibodies. Membranes were developed using chemiluminescent based developing solutions (Amersham ECL Select Western Blotting Detection Reagent, GE Healthcare). Membranes were washed for 30 seconds with stripping solution (3M Guanidine hydrochloride) followed by two washes of 0.05% PBS-Tween 20, 10 minutes each; and blocked overnight at 4°C before reprobing them with a different set of primary and secondary antibodies.

7. Stable cell-line preparation:

HEK293T cells were transfected with lentiviral plasmids and plasmid with the gene of interest. The virus produced by the cells was obtained by collecting the cell media and concentrating it with lenti X concentrator (Clonetech). The virus pellet obtained after centrifuging the media at 1500xg for 45 minutes at 4°C was resuspended in plain media. The virus suspension was added to the media of HEK293T cells growing in a 35mm dish, containing polybrene (Sigma), for transduction. Another 35mm dish of HEK293T cells growing in polybrene containing media was labelled as mock since no virus was

added to it. Post-transduction, the media of both dishes was replaced with complete media containing a selection drug. Transduced cells were cultured in selection drug-containing media until all cells in the mock dish died; post-selection cells were checked for the expression of the desired protein and stocked in -80°C freezer until further use.

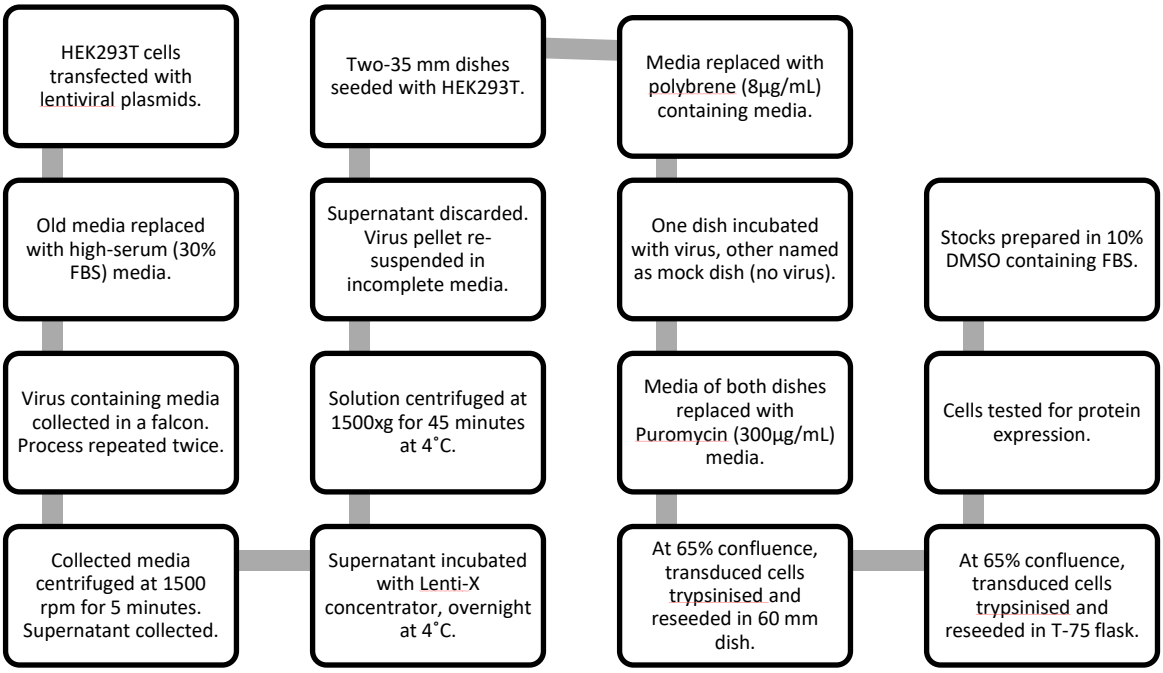


Figure 7 A flowchart describing the steps of stable cell line preparation. Note: Reseeded transduced cells were cultured in selection media.

8. Cell Culture and RNAi:

All plasticware used in cell culture were from BD. HeLa and HEK293T cells (from ATCC) were cultured in DMEM (Lonza) supplemented with 10% FBS (Gibco) in a humified incubator supplied with 5% CO_2 at 37°C. Each cell line was checked for mycoplasma contamination. For gene silencing, siRNAs were ordered from GE Healthcare and prepared according to the manufacturer's instructions. The sequence of control siRNA is TGGTTTACATGTCGACTAA, and h-RUFY1 560 oligo is CATCAGATATAGCGACTAG.

9. Plasmids used:

Plasmid	Source
pNTAP-A	InterPlay N-terminal Mammalian TAP vectors, Agilent
pGEX-4T3	Aimee L. Edinger Lab
pCDH-CMV-MCS-EF1-Puro	Mahak Sharma Lab
pCAGGS-EGFP-SNX5	Michiyuki Matsuda Lab
pCAGGS-FLAG-SNX5	Michiyuki Matsuda Lab
pCAGGS-FLAG-SNX2	Michiyuki Matsuda Lab
pCAGGS-FLAG-SNX4	Michiyuki Matsuda Lab
pCXN2-mRFP-SNX5	Michiyuki Matsuda Lab
pCAGGS-FLAG-SNX1	Michiyuki Matsuda Lab
pEGFPC1-SNX8	Kristen S. Lab
pCAGGS-FLAG-SNX6B	Michiyuki Matsuda Lab
SNX17-GFP	Lawrence Banks Lab
SNX17-GFP	Steve Caplan Lab
pCDNA 3.1(-)-SNX17(WT) FLAG	Lawrence Banks Lab

pCDNA 3.1(+)-Rabip4'-FLAG	GenScript
pEGFPN1-Rabip4'	Mahak Sharma Lab
Human SNX29-C-term Flag-pcDNA3.1(+)	GenScript
pCDNA-3.1(-)-Arl8b-HA	Mahak Sharma Lab
pEGFPN3-PLD3	Matthew Seaman Lab
pCMV-dR8.91	Coen Kuijl Lab
pCMV-VSV-G	Coen Kuijl Lab

10. Antibodies used:

Following are the antibodies used in this project: rabbit anti-CBP (Millipore), rabbit anti-FLAG (Invitrogen), mouse anti-GFP (SCBT), mouse anti-CD107A (BD), mouse anti-CD107B (BD), rabbit anti-EEA1 (CST), rabbit anti-Rab14 (Abcam), mouse anti-Rab7 (SCBT), rabbit anti-HA (Sigma), mouse anti-FLAG (Sigma), mouse anti-RUFY1 (SCBT). All Alexa fluorophore-conjugated secondary antibodies were from Thermo Fisher Scientific. HRP-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson Immunoresearch Laboratories.

11. Transfections, Immunofluorescence, and Cell imaging:

For DNA transfection, 0.3M HeLa cells; and for RNAi, 0.06M HeLa cells were grown on glass coverslips for 24 hours. Cells were transfected with the indicated DNA construct using X-tremeGENE-HP (Roche) transfection agent for 8-12 hours. Cells for

knockdown experiments were transfected with the desired siRNA using DharmaFECT transfection agent for 60 hours.

After transfection, cells were fixed with 4% PFA in PHEM (60 mM PIPES, 10mM EGTA, 25mM HEPES, and 2mM $MgCl_2$ in autoclaved water and the final pH set to 6.8) for 10 minutes at room temperature. Cells were incubated with the primary antibody in the blocking solution (0.2% Saponin + 5% FBS in PHEM buffer) overnight at 4°C. Coverslips were washed thrice using 1X PBS (pH 7.4) and incubated with Alexa Fluorophore-Conjugated secondary antibody in staining solution (0.2% Saponin + PHEM buffer) for 30 minutes at room temperature. The coverslips were mounted on Fluoromount G (Southern Biotech) after three washes with 1X PBS.

Coverslips were observed under the 710 Confocal Laser Scan Microscope (ZEISS) using a 63X/1.4 NA oil immersion objective. Single-plane images were acquired with the ZEN Pro 2011 (ZEISS) software. All settings were adjusted accordingly and maintained for a given set of experiments.

12. Image Processing and Quantifications:

Images were processed using ImageJ and Adobe Photoshop software. For co-localisation analysis, 10-15 cells from one experiment for each treatment were taken. Pearson coefficient and Manders overlap coefficients were calculated using the JACoP plugin provided in ImageJ. All graphical representations were prepared in MS Excel.

Chapter 3: Results, Conclusion and Future direction

3.1 Results

Our previous studies hint that Rabip4' interacts with Arl8b via its RUN domain. HeLa cells depleted with Rabip4' show enlarged lysosomes as well as a significant defect in the retrograde trafficking of CI-M6PR from endosomes to Golgi (Rawat et al., unpublished work). Piqued by this observation, we aim to understand the function of Rabip4' in the retrograde trafficking of CI-M6PR.

To begin with, we wanted to know the interacting partners of Rabip4'. For this, we used two different pulldown approaches; GST pulldown and Tandem affinity (TA) pulldown (TAP). We cloned Rabip4' in pGEX-4T3 and pNTAP-A vectors, and from pNTAP-A vector, TAP-Rabip4' was subcloned into a lentiviral vector (pCDH-Puromycin) to make a stable cell line (Fig 8). We checked all positive clones for their protein expression. Some clones had better expression than the rest (Fig 9).

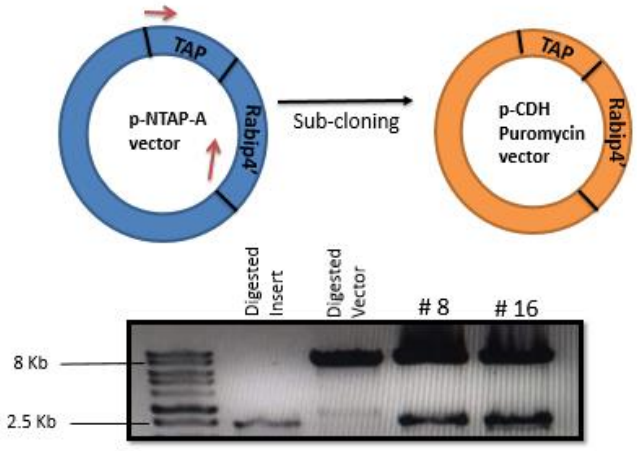


Figure 8 Upper schematic represents sub-cloning of TAP-Rabip4' from pNTAP-A to pCDH-Puromycin lentivector. The gel image below shows confirmed clones after the sub-cloning.

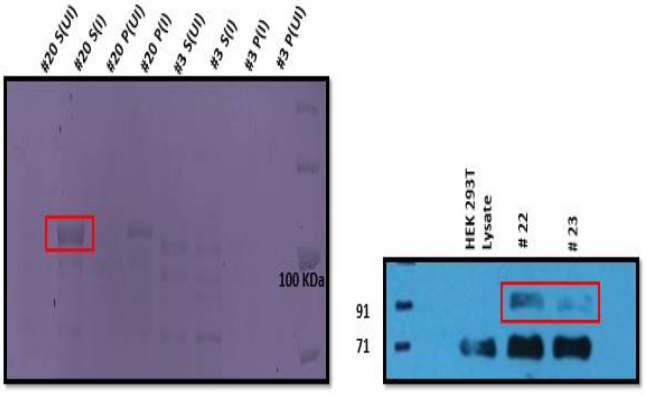


Figure 9 Image on the left shows the expression of GST-Rabip4' of positive clones. Size of GST-Rabip4' is ~ 105 kDa. Image on the right shows the expression of TAP-Rabip4' of positive clones. Size of TAP-Rabip4' is ~ 87 kDa. The blot is probed with rabbit anti-CBP (Millipore) in 1:5000 dilution.

Additionally, to check if the introduction of a tag has not interfered with the protein's function, we transfected HeLa cells with TAP-Rabip4' construct and co-stained the cells with EEA1 or Rab14, which are known to co-localise with Rabip4' (Fouraux et al. 2004). TAP-Rabip4' showing co-localisation with the

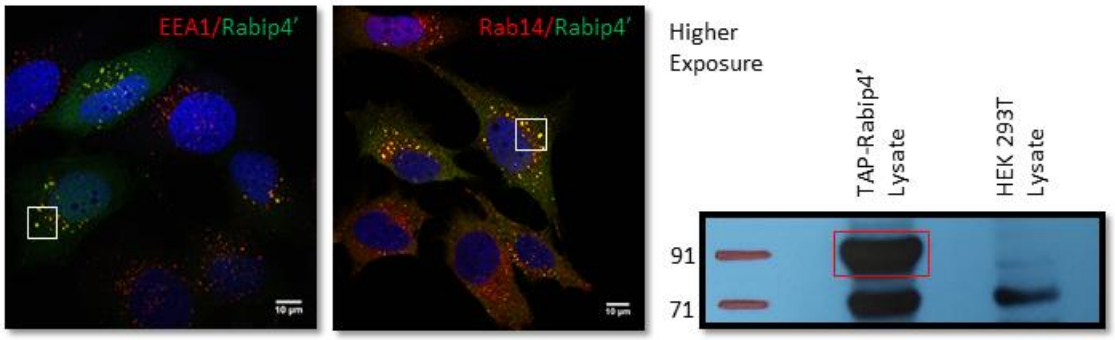


Figure 10 (Left) HeLa cells were transfected with TAP-Rabip4' construct and immunostained for EEA1 and Rab14. TAP-Rabip4' punctae showing co-localisation with EEA1 and Rab14. Antibody used: rabbit anti-EEA1 (CST) in 1:200 dilution, rabbit anti-Rab14 (Abcam) in 1:100 dilution. (Right) Expression of TAP-Rabip4' stable cell line in HEK293T cells after selection. The blot is probed with rabbit anti-CBP (Millipore) in 1:5000 dilution.

markers (Fig 10, left) further drove us to proceed with the TAP approach. We then proceeded with preparing a stable cell line of TAP-Rabip4' in HEK293T cells. The expression analysis showed that the transduced cells were expressing TAP-Rabip4' in fair amounts (Fig 10, right). The eluates from the pulldown experiments were sent for mass spectrometry analysis to identify the interacting partners. Below, we have reported some relevant hits along with their known function and localisation (Table 1 and Table 2). We are planning to study whether some of these hits, such as dynein and the adaptor protein complexes, are relevant binding partners for Rabip4' and significance of their binding on Rabip4' function.

Table 1: List of important hits obtained in the mass spectrometry analysis of TAP pulldown samples.

Unique Peptides identified	Gene	Protein	Localisation	Known function
25	DYNC1H1	Cytoplasmic dynein-1 heavy chain 1	Cytoskeleton	It is a motor protein responsible for the retrograde transport of vesicles.
10	RUFY2	RUN and FYVE domain-containing protein 2,	Nucleus	It is involved in the trafficking of vesicles via its interaction with Etk.
6	RUFY3	Protein RUFY 3	Cytoplasm	It plays a role in the generation of neuronal polarity formation and axon growth.
6	CLTC	Clathrin heavy chain 1	Cytoskeleton	Clathrin is the major protein of coated pits, vesicles and has a role in clathrin-dependent endocytosis and autophagosome formation.
3	DYNC1LI1	Cytoplasmic dynein-1 light intermediate chain 1	Cytoskeleton	Acts as one of several non-catalytic accessory components of the cytoplasmic dynein-1 complex that are thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein function.
1	AP3B1	AP-3 complex subunit beta-1	Golgi apparatus	It is a subunit of non-clathrin- and clathrin-associated adaptor protein complex 3 (AP-3) that plays a role in protein sorting in the trans-Golgi network (TGN) and endosomes.

1	KIF5A	Kinesin heavy chain isoform 5A	Cytoskeleton	It is required for anterograde axonal transportation of MAPK8IP3/JIP3. The ZFYVE27-KIF5A complex contributes to the vesicular transport of VAPA, VAPB, SURF4, RAB11A, RAB11B and RTN3 proteins in neurons.
1	ARL1	ADP-ribosylation factor-like protein 1	Golgi apparatus	GTP-binding protein that recruits several effectors, and Arf-GEFs to the trans-Golgi network, and modulates their functions at the Golgi complex
1	ARF4	ADP-ribosylation factor 4	Golgi apparatus	Involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus.
1	GAPVD1	GTPase-activating protein and VPS9 domain-containing protein 1	Endosomes	Acts both as a GAP and GEF, and participates in various processes such as endocytosis, insulin receptor internalization or LC2A4/GLUT4 trafficking. It shows GEF activity for Rab5 and GAP activity for Ras.
1	HTT	Huntingtin	Endosomes and Nucleus	May play a role in microtubule-mediated transport or vesicle function.
1	SNAPIN	SNARE-associated protein Snapin	Golgi apparatus, Cytosol and Lysosomes	It is a component of the BLOC-1 complex and plays a role in intracellular vesicle trafficking and synaptic vesicle recycling.
1	AP1M2	AP-1 complex subunit mu-2	Golgi apparatus	It is a subunit of clathrin-associated adaptor protein complex 1and plays a role in protein sorting in the trans-Golgi network (TGN) and endosomes. The AP complexes mediate the recruitment of clathrin to membranes and the recognition of sorting signals within the cytosolic tails of transmembrane cargo molecules.
2	AP1M1	AP-1 complex subunit mu-1	Golgi apparatus	Similar to AP1M2
2	DCTN1	Dynactin subunit 1	Nucleus and Cytoskeleton	Plays a crucial role in dynein-mediated retrograde transport of vesicles and organelles along microtubules by recruiting and tethering dynein to microtubules. It binds to both dynein and microtubules providing a link between specific cargos, microtubules and dynein.
2	PLD3	5'-3' exonuclease PLD3	Endosomes, Lysosomes, Golgi apparatus and ER	Involved in the regulation of endosomal protein sorting
5	COPB1	Coatomer subunit beta	Plasma membrane and Golgi apparatus	A cytosolic protein complex that associates with Golgi non-clathrin-coated vesicles mediates biosynthetic protein transport from the ER, via the Golgi up to the trans-Golgi network—required for budding from Golgi membranes retrograde, and Golgi-to-ER transport of dilysine-tagged proteins.
5	COPA	Coatomer subunit alpha	Golgi apparatus and Extracellular region	Similar to COPB1
2	COPG1	Coatomer subunit gamma-1	Golgi apparatus	Similar to COPB1
1	COPE	Coatomer subunit epsilon	Golgi apparatus	Similar to COPB1
1	COPG2	Coatomer subunit gamma-2	Cytosol and Golgi apparatus	Similar to COPB1

1	GABARAPL2	Gamma-aminobutyric acid receptor-associated protein-like 2	Golgi apparatus	It modulates intra-Golgi transport through coupling between NSF activity and SNAREs activation. It is also involved in autophagy.

Table 2: List of important hits obtained in the mass spectrometry analysis of GST pulldown samples.

Unique peptides identified	**Gene**	**Protein**	**Location**	**Function**
4	Rab14	Ras-related protein Rab-14	Endosomes and Golgi apparatus	Plays a role in membrane trafficking between the Golgi complex and endosomes, and regulates the transport of FGFR-containing vesicles during early embryonic development.
3	Rab5B	Ras-related protein Rab-5B	Endosomes	Probably involved in vesicular traffic
2	Rab5C	Ras-related protein Rab-5C	Endosomes	Probably involved in vesicular traffic
2	Rab34	Ras-related protein Rab-34	Golgi apparatus	It is involved in protein transport and redistribution of lysosomes to the peri-Golgi region and plays a role in phagosome maturation and their fusion with lysosomes.
1	SCAMP3	Secretory carrier-associated membrane protein 3	Early and late endosomes	Essential for the biogenesis of multivesicular endosomes. Also, it plays a role in the recycling of EGFR receptor and prevents its degradation.
1	DENND6A	Protein DENND6A	Endosomes	It acts as GEF for RAB14 and necessary for RAB14 recruitment to recycling endosomes.
1	VPS35	Vacuolar protein sorting-associated protein 35	Endosomes	Acts as component of the retromer cargo-selective complex (CSC), a complex to prevent missorting of selected transmembrane cargo proteins into the lysosomal degradation pathway.
1	SNX29	Sorting Nexin 29	--	--

The defect in retrograde trafficking of CI-M6PR further prompted us to check for any interactions between the components of retromer complex and Rabip4'. Retromer complex is known to play a vital role in endosome-to-Golgi retrieval pathway, known as retrograde trafficking. It has two subcomplexes: a trimeric complex of Vps35, Vps 26 and Vps 29; and a dimer of SNX1 or SNX 2 and SNX5 or SNX6 (Seaman 2012). Our primary focus was on sorting nexin proteins, and we wanted to identify which sorting nexins co-localise with Rabip4'. Mainly, SNX8, since its role in the retrograde trafficking has been reported previously (Dyve et al. 2009) and it appeared as

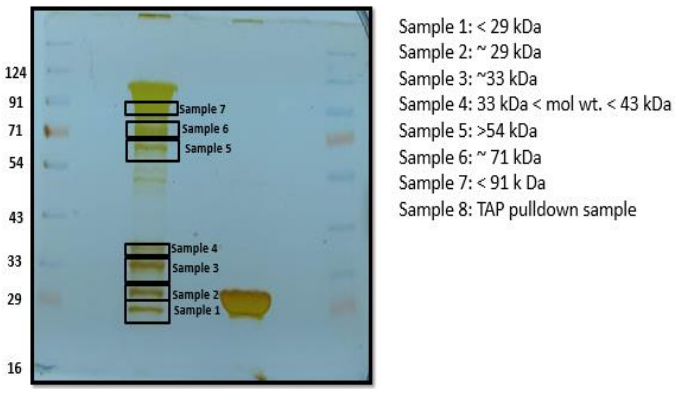


Figure 11 Pull down samples were loaded on polyacrylamide gel and stained using silver staining. The highlighted bands were cut and sent for mass spectrometry analysis.

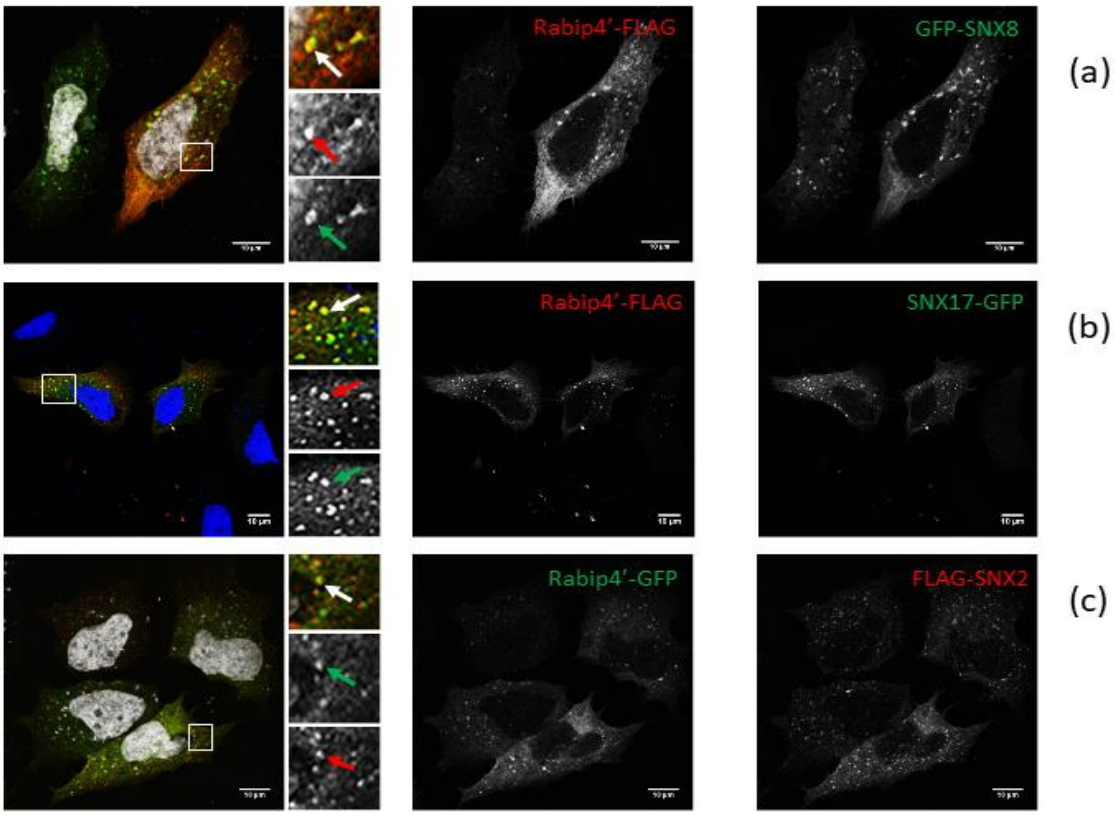


Figure 12 Double transfections in HeLa cells were done using a Rabip4' construct and an SNX construct. Cells were stained with mouse anti-FLAG (Sigma) in 1:500 dilution. (a) HeLa cells expressing Rabip4-FLAG and GFP-SNX8. (b) HeLa cells expressing Rabip4'-FLAG and SNX17-GFP. (c) HeLa cells expressing Rabip4'-GFP and FLAG-SNX2.

one of the interacting partners of Rabip4' in a bioinformatics software called BioPLEX2.0. All SNX constructs available in the lab were screened for their expression by co-transfecting them with either Rabip4'-FLAG or Rabip4'-GFP in HeLa cells. We also observed SNX29- a potential hit in our mass spectrometry data, to co-localise with Rabip4'. However, this was our preliminary observation with only one experiment. Of all the remaining SNX proteins screened, SNX5 and SNX6B did not exhibit any co-localisation with Rabip4' whereas SNX17, SNX2, and SNX8 were co-localised with Rabip4' (Fig 12). To test these interactions between SNX proteins and Rabip4', we performed a co-immunoprecipitation assay. The following experiments were done once, and thus, these are all preliminary observations that need to be repeated. In the case of SNX17-GFP, anti-FLAG beads precipitated a notable amount of the protein, whereas very less amount of SNX17-GFP precipitated with Rabip4'-FLAG (Fig 13). The anti-GFP beads precipitated a significant amount of FLAG-SNX2, although it was immunoprecipitated with Rabip4'-FLAG as well (Fig 14). GFP-SNX8 was immunoprecipitated with Rabip4'-FLAG and displayed a low affinity for the anti-FLAG beads (Fig 15). It suggests that SNX8-Rabip4' interaction is more real.

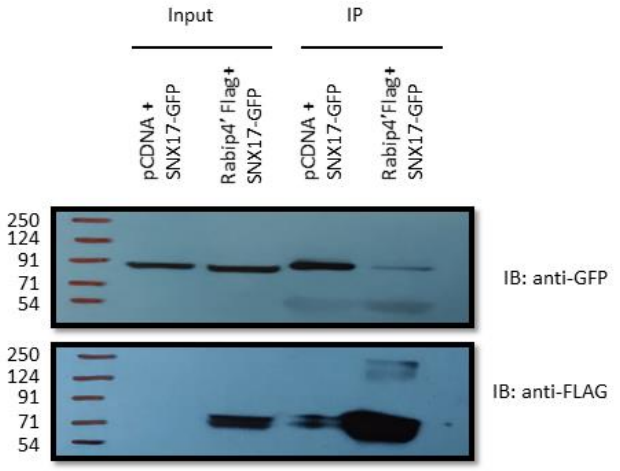


Figure 13 (Upper panel) Co-IP of SNX17-GFP, size of SNX17-GFP 80 kDa. The blot is probed with mouse anti-GFP (SCBT) in 1:3000 dilution. SNX17-GFP has non-specifically precipitated with anti-FLAG beads. It shows very less interaction with Rabip4'-FLAG. (Lower panel) IP of Rabip4'-FLAG, size of Rabip4'-FLAG is 81 kDa. The blot is probed with rabbit anti-FLAG (Invitrogen) in 1:3000 dilution.

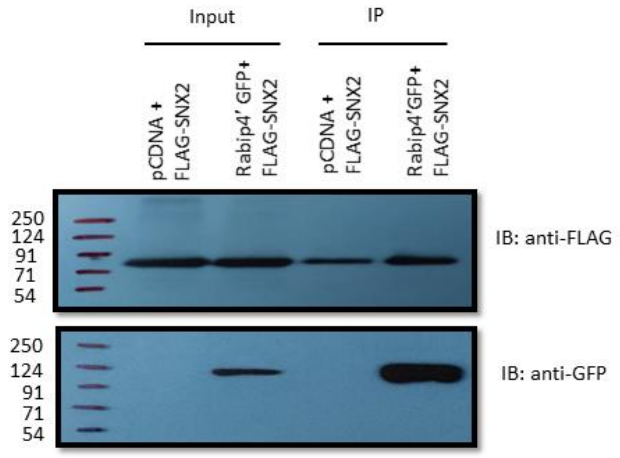


Figure 14 (Upper panel) Co-IP of FLAG-SNX2, size of FLAG-SNX2 59 kDa; however, the band size is running higher than the expected value. The blot is probed with rabbit anti-FLAG (Invitrogen) in 1:3000 dilution. Some amount of FLAG-SNX2 is immunoprecipitated by anti-FLAG beads. (Lower panel) IP of Rabip4'-GFP, size of Rabip4'-GFP is 107 kDa. The blot is probed with rabbit anti-GFP (SCBT) in 1:3000 dilution.

To further know the function of these SNX proteins, we suppressed the expression of Rabip4' in HeLa cells via siRNA knockdown and transfected the knock down cells with either SNX17-GFP, GFP-SNX8 or FLAG-SNX2. Cells were then co-stained with Rab14. In control knockdown, many Rab14 compartments were positive for SNX8, whereas, in Rabip4' knockdown cells, this co-localisation was reduced as observed in data from one experiment (Fig 16). No notable changes were

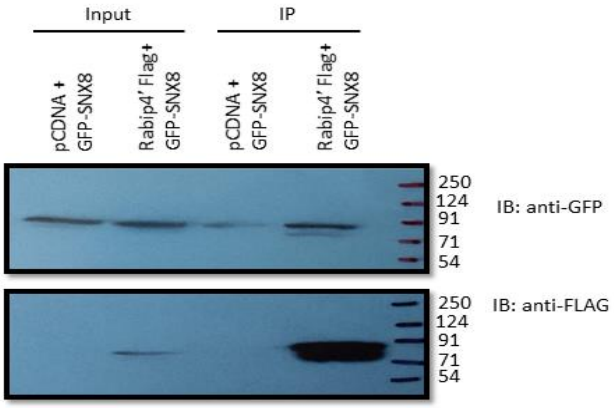
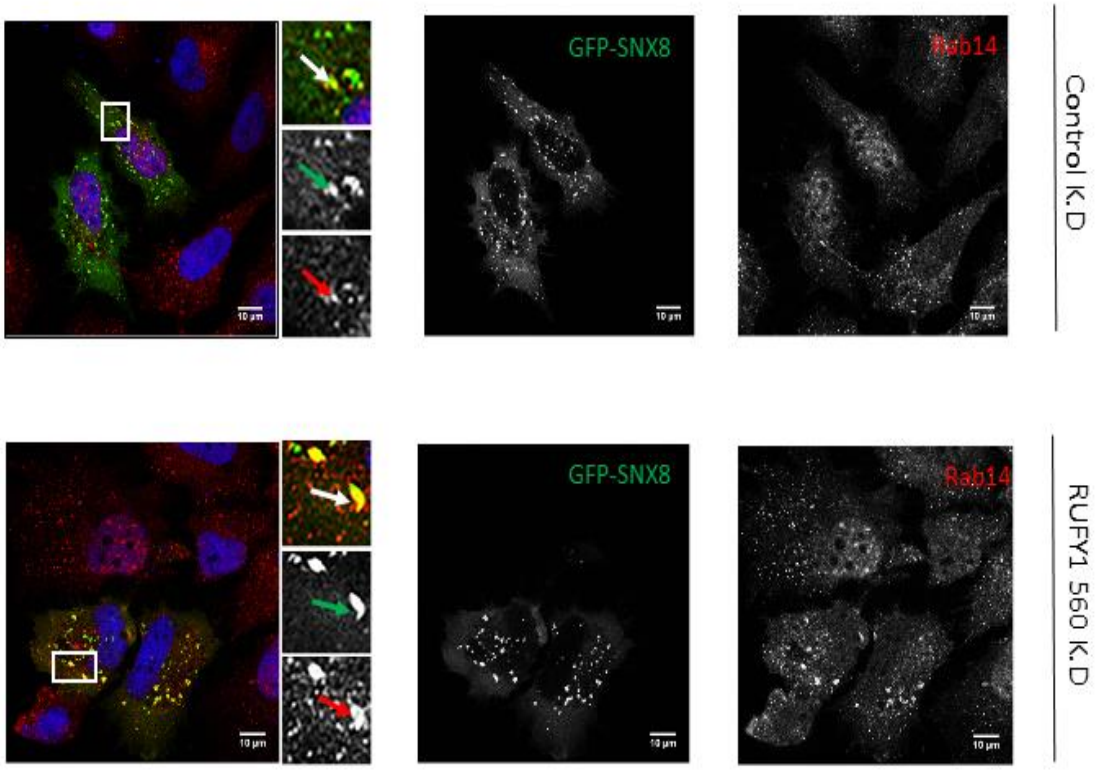


Figure 15 (Upper panel) Co-IP of GFP-SNX8, size of GFP-SNX8 80 kDa. The blot is probed with mouse anti-GFP (SCBT) in 1:3000 dilution. GFP-SNX8 is precipitated along with Rabip4'-FLAG showing minimum non-specific binding with anti-FLAG beads. (Lower panel) IP of Rabip4'-FLAG, size of Rabip4'-FLAG is 81 kDa. The blot is probed with rabbit anti-FLAG (Invitrogen) in 1:3000 dilution.



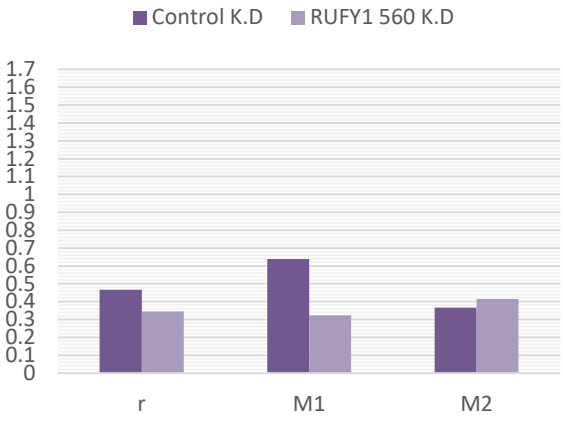
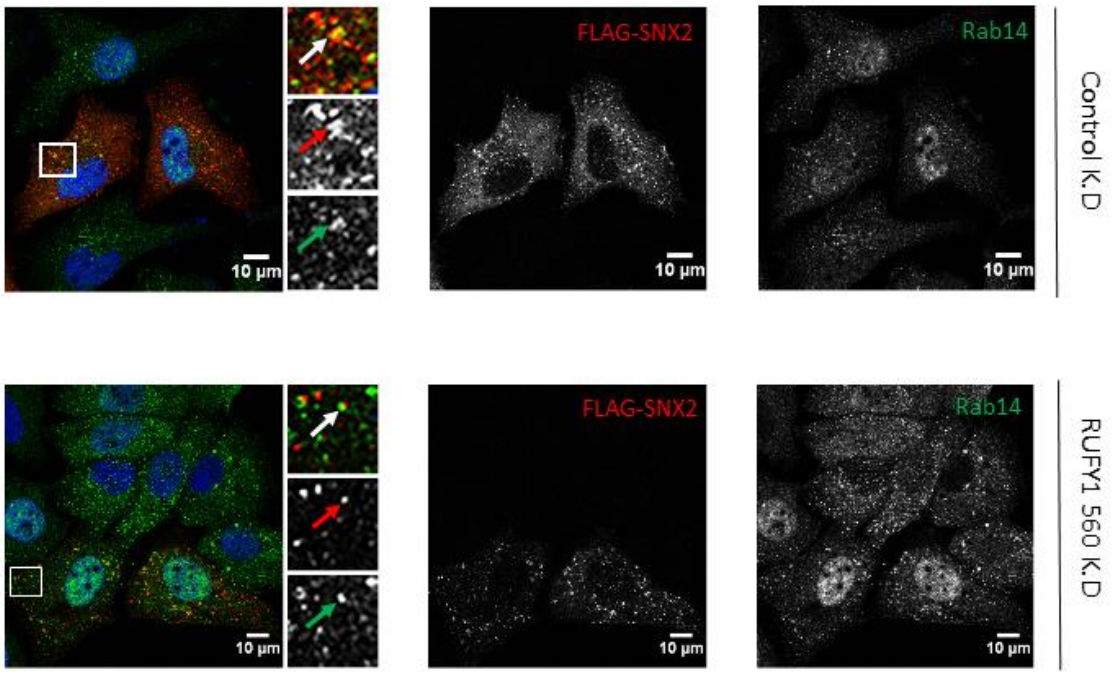


Figure 16 Hela cells were transfected with indicated siRNA oligos and transfected again with GFP-SNX8 post 48 hours of knockdown. Cells were co-stained for Rab14 with rabbit anti-Rab14 (Abcam) in 1:100 dilution. Most Rab14 punctae co-localise with GFP-SNX8. No significant change in the co-localisation between SNX8 and Rab14 is observed upon Rabip4' knockdown. 10-15 cells were taken from one experiment for quantification of Pearson correlation coefficient (r) and Manders overlap coefficient.



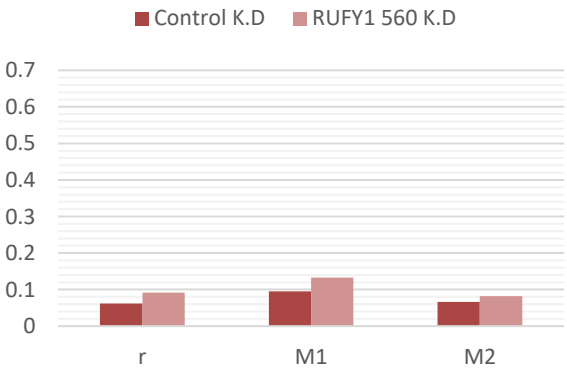
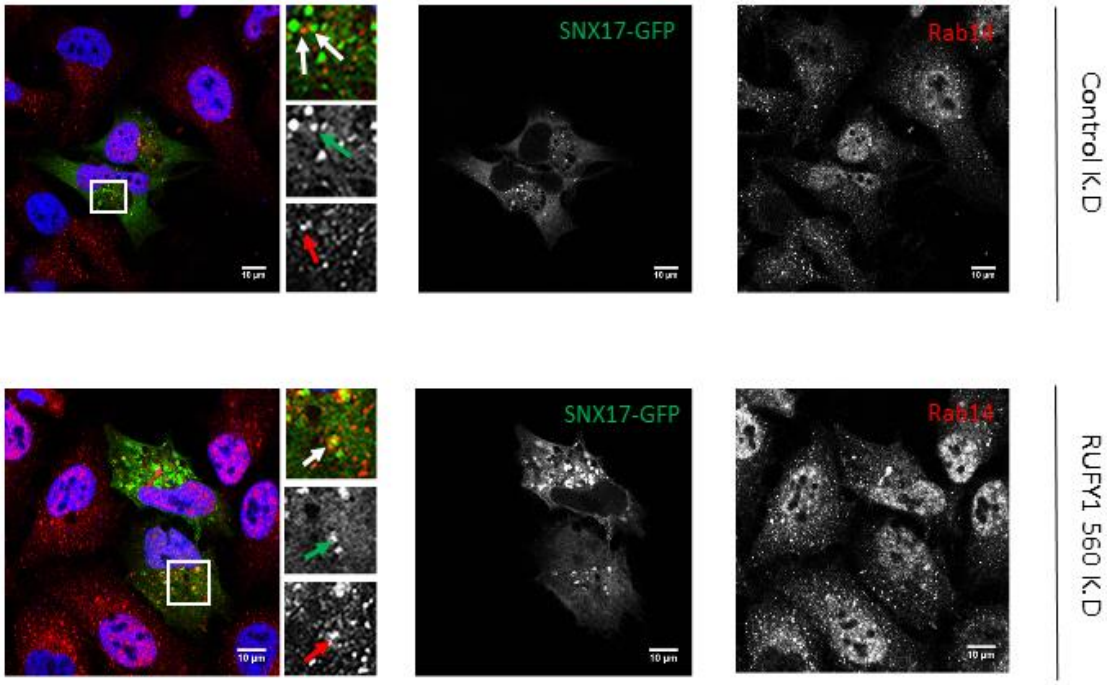


Figure 17 Hela cells were transfected with indicated siRNA oligos and transfected again with FLAG-SNX2 post 48 hours of knockdown. Cells were co-stained for Rab14 with rabbit anti-Rab14 (Abcam), used in 1:100 dilution. No punctae of Rab14 co-localise with SNX2. No significant change in the co-localisation between SNX2 and Rab14 is observed upon Rabip4' knockdown. 10-15 cells were taken from one experiment for quantification of Pearson correlation coefficient (r) and Manders overlap coefficient.

observed in the co-localisation between SNX2 and Rab14 upon Rabip4' knockdown; also, they

did not co-localise in control cells (Fig 17). Likewise, with SNX17, no punctae of Rab14 were positive for SNX17; also, no significant change in their distribution seen upon Rabip4' knockdown (Fig 18). Co-localisation of all SNX proteins with Rab14 in control and Rabip4' knockdown cells were quantified from one experiment (10-15 transfected cells from each treatment).



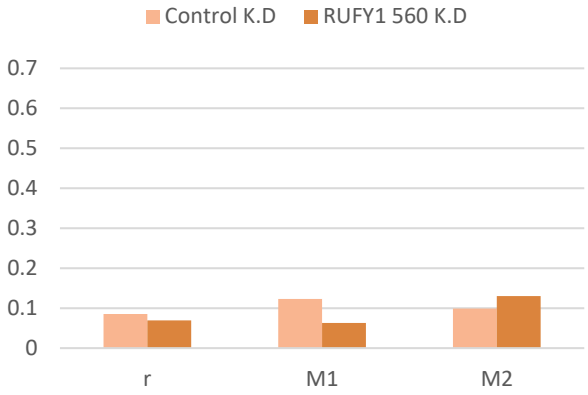


Figure 18 HeLa cells were transfected with indicated siRNA oligos and transfected again with SNX17-GFP post 48 hours of knockdown. Cells were co-stained for Rab14 with rabbit anti-Rab14 (Abcam), used in 1:100 dilution. No punctae of Rab14 co-localise with SNX17. No significant change in the co-localisation between SNX17 and Rab14 is observed upon Rabip4' knockdown. 10-15 cells were taken from one experiment for quantification of Pearson correlation coefficient (r) and Manders overlap coefficient.

We also investigated Phospholipase D3 (PLD3), one of the proteins identified as Rabip4' interaction partner in the TAP experiment, for its interaction with Rabip4'. PLD3 is a type II transmembrane protein belonging to the superfamily of phospholipase D enzymes, whose catalytic function has not been well-characterised. However, its role in regulating endosomal

protein sorting is previously reported (Mukadam et al. 2018). We first wanted to determine the localisation of PLD3. HeLa cells were transfected with PLD3-GFP and co-stained with

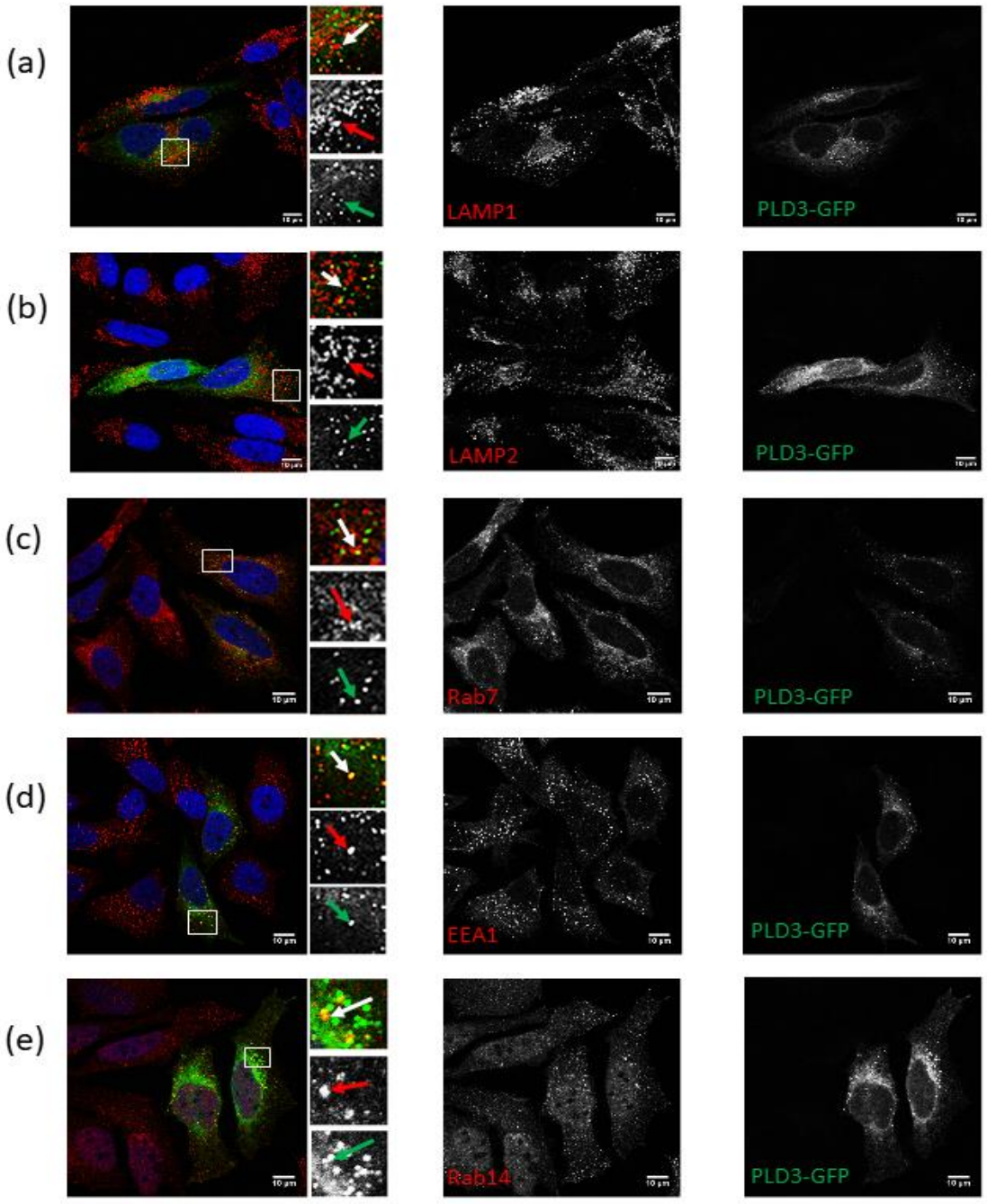


Figure 19 HeLa cells transfected with PLD3-GFP were stained for (a) LAMP1, using mouse-anti-LAMP1 (BD) in 1:1000 dilution, (b) LAMP2, using mouse anti-LAMP2 (BD) in 1:1000 dilution, (c) Rab7, using mouse anti-Rab7 (SCBT) in 1:70 dilution, (d) EEA1, using rabbit anti-EEA1 (CST) in 1:200 dilution, and (e) Rab14, using rabbit anti-Rab14 (Abcam) in 1:100 dilution. Most PLD3-GFP punctae co-localised with endosomal marker.

markers such as Rab7, LAMP1, LAMP2 representing lysosomes, and Rab14 and EEA1 were chosen for Endosomes (Fig 19). We observed that PLD3 co-localise better with endosomal markers than lysosomal markers. Thus, we conclude that PLD3 is mostly on endosomes. After confirming its localisation, we co-transfected HeLa cells with PLD3-GFP and Rabip 4'-FLAG or Arl8b-HA and observed several PLD3 punctae co-localising with Arl8b as well as Rabip4' (Fig 20). Furthermore, we triple-transfected HeLa cells with PLD3-GFP, Rabip4'-FLAG and Arl8b-HA. There were numerous punctae where all three proteins were co-localising with each other (Fig 21).

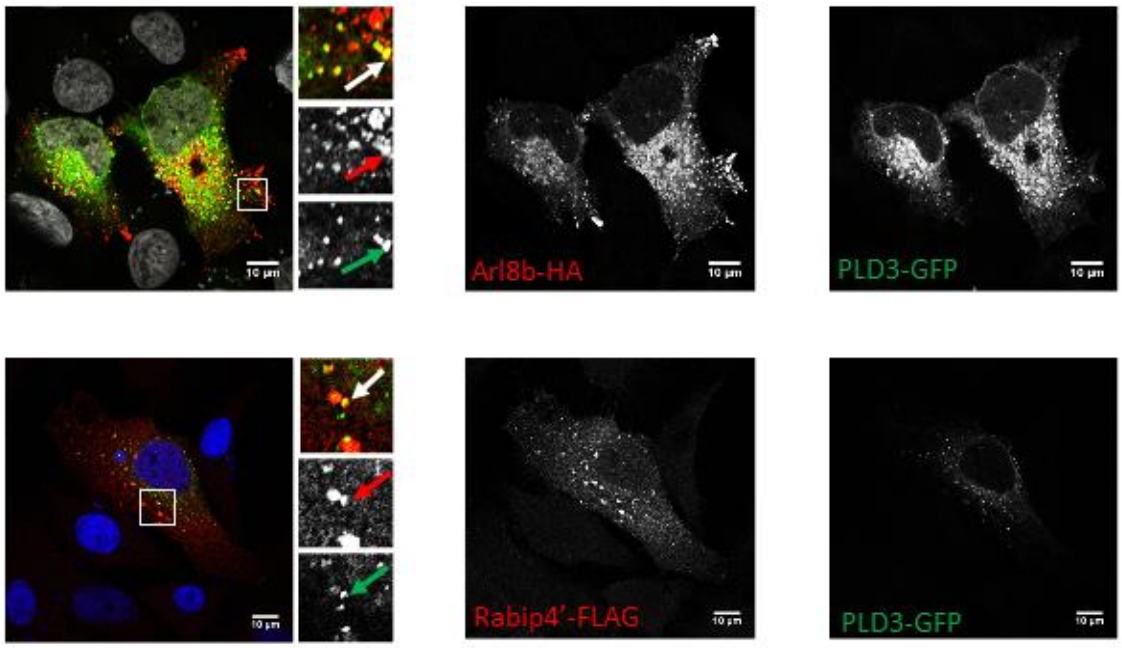


Figure 20 (Upper panel) HeLa transfected with PLD3-GFP and Arl8b-HA were stained with rabbit anti-HA (Sigma) in 1:250 dilution. Many punctae of Arl8b co-localise well with PLD3. (Lower panel) HeLa cells transfected with PLD3-GFP and Rabip4'-FLAG were stained with mouse anti-FLAG (Sigma) in 1:500 dilution. Several PLD3 compartments are also positive for Rabip4'.

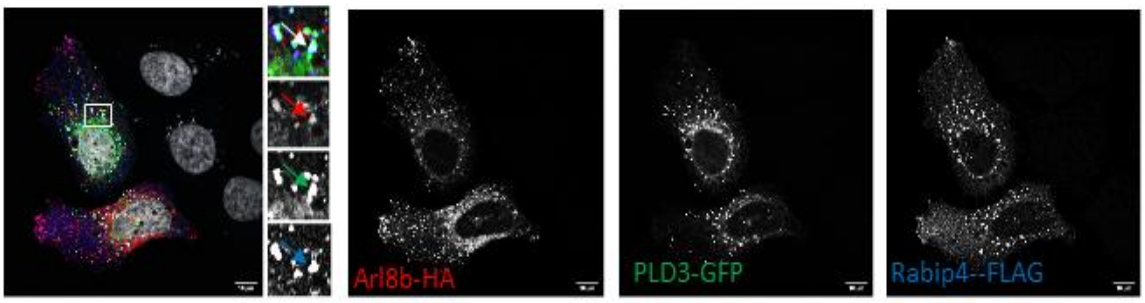


Figure 21 HeLa cells triple transfected with PLD3-GFP, Ar8b-HA, and Rabip4'-FLAG were stained with rabbit anti-HA (Sigma) and mouse anti-FLAG (Sigma) in 1:250 and 1:500 dilution, respectively.

3.2 Conclusion and Future Direction:

From our findings so far, Rabip4' interacts with Arl8b through RUN domain and appears to play a crucial role in the retrieval of CI-M6PR receptor from endosomes-to-Golgi. We identified several interacting partners of Rabip4' through mass spectrometry analysis of the pulldowns performed using Rabip4' as bait. We also discovered sorting nexin proteins; specifically, SNX8 (potential hit from the server BioPLEX2.0) and PLD3 interact with Rabip4' and the significance of these interactions should be explored in future studies.

Based on our observations in this study, we plan to study PLD3 further and find whether its interaction with Rabip4' is vital for our investigation. We further want to validate their interaction by performing pulldown assays. We are keen to know whether Rabip4' knockdown has any effect on the distribution and function of PLD3 and vice-versa. We are also interested in working on other relevant hits and come up with a mechanism which provides an insight into the retrograde trafficking of CI-M6PR.

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