Role of novel Arl8b effector in retrograde trafficking of cation-independent mannose 6-phosphate receptor from early endosomes to Golgi

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Certificate of Examination

This is to certify that the dissertation entitled "**Role of novel Arl8b effector in retrograde trafficking of cation- independent mannose 6-phosphate receptor from early endosomes to Golgi**" submitted by Miss Shrestha Shaw (MS16135) for the partial fulfillment of the 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 the report to be accepted.

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

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 (IISER-Mohali).

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university orInstitute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment 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.

Shrestha Shaw
(Candidate)
Dated: 17th April2021

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)

Acknowledgment

Firstly I would like to sincerely thank Dr.Mahak Sharma for giving me the opportunity to work in her lab since 2018 and never restricting the curiosity I had towards the field of science. She has been highly supportive throughout these years and every time motivated me to give my best. She showed her immense enthusiasm and shared her valuable inputs, which constantly urged me to excel in my conduct.

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Shrestha Shaw

Contents

List of figures

ABSTRACT

Endocytic trafficking is a multistep process where cargo initially gets internalized at the plasma membrane and eventually enters early endosomes. The cargo from here either moves to late endosomes/lysosomes for degradation or gets recycled back to the plasma membrane. Cargo packaging in vesicles, budding of vesicles from different endocytic compartments, and fusion of these vesicles with early or late endocytic compartments take place in a highly dynamic and regulated manner. The various steps of endocytic trafficking regulated by small G proteins of Ras superfamily, which includes Rabs, Arfs, and Arf-like proteins (Arls). Our lab is mostly exploring the role of Arf like small G protein Arl8b and its effector in regulating cargo trafficking to lysosomes. A subset of Arl8b effectors contain RUN-domain via which it interacts with Arl8b. We have recently identified a novel RUN domain-containing Arl8b effector, which regulates lysosome composition and in turn, its function. Upon knockdown of this gene, we could see enlarged lysosomes along with the change in lysosomal hydrolases expression and levels implicating a lysosomal stress condition. Cation independent mannose-6-phosphate receptor (CI-M6PR) positioning also seems to be changed upon depletion of the effector, which depicts that the recycling of the receptor is being hampered. Therefore, our study signifies the importance of this effector in the sorting of CI-M6PR.Our data also suggests that the recycling of CI-M6PR mediated by this effector is dynein-dynactin complex dependent

Chapter 1: INTRODUCTION

I.1 Overview of different routes of Cargo trafficking to Lysosomes

Lysosomes are membrane-bound sub-cellular organelles found in eukaryotes that are primarily known to participate in cargo degradation and nutrient recycling. Since its discovery in the 1950s by Christian de Duve, the role of lysosomes has been extensively studied in cargo degradation[1]. Cargo (proteins, pathogens, other macromolecules) destined to be degraded reach lysosomes via endocytic, phagocytic, or autophagic pathway (Figure-1)[2].

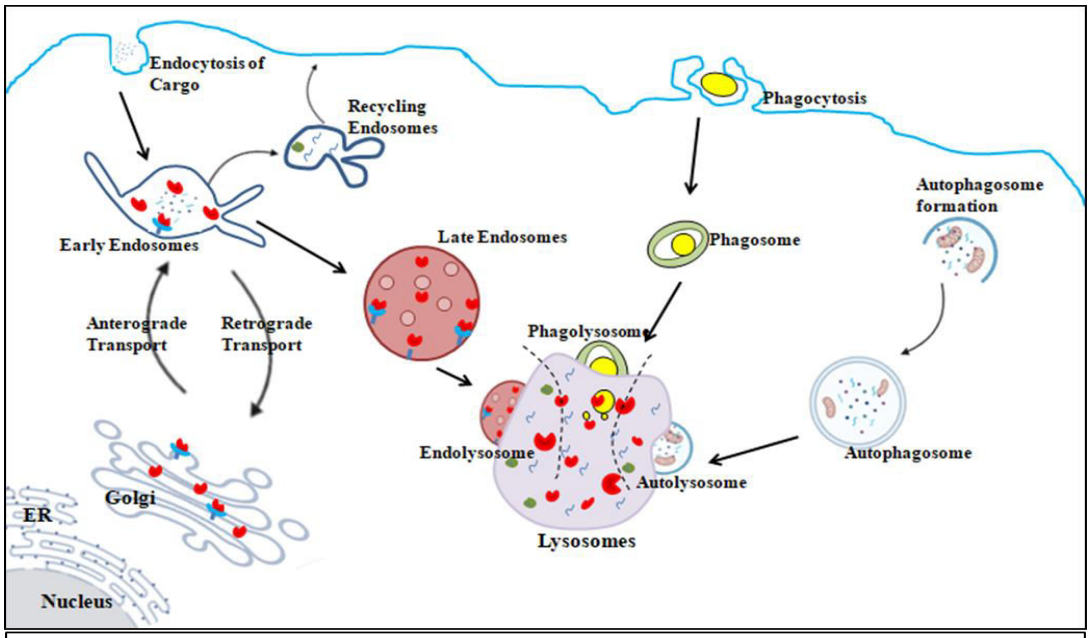

Fig.1 Different routes of cargo trafficking to lysosomes: Endocytic (left), Phagocytic (middle) and Autophagic (right) pathway. Adapted from Char et al, Frontiers, 2020.

The macromolecule endocytosed from the plasma membrane enters the early endosomal compartments from where it is recycled back to the plasma membrane through recycling endosomes or else the cargo is sorted to late endosomes and finally degrades in lysosomes[3]. Cargos like pathogens enter the cell via the formation of phagosomes and eventually fuse with lysosomes forming a phagolysosome. During autophagy, misfolded proteins and damaged old organelles are degraded, and building blocks are recycled for new macromolecule synthesis. The cytoplasmic contents of the cell are packed into a double membrane vesicle called autophagosomes. This autophagosome finally fuses with lysosomes, becoming an autolysosome, where the cargo is degraded[4]. Lysosomes being

crucial regulator of degradative processes help in termination of signaling pathway, nutrient provision, autophagic clearance of protein aggregates and microbial killing. Thus lysosomal storage disorders, neurodegenerative diseases and different variety of cancers can be caused due to damage in lysosomal functions[1].

I.2 Diverse roles played by Lysosomes

Lysosomes not only play a key role as a degradative hub of the cell, but also facilitate various other cellular functions like cell migration, metabolic signaling, tumor invasion, metastasis, plasma membrane repair and gene regulation (Figure-2). And, in a span of the past ten years, various non-canonical roles of lysosomes have been explored.

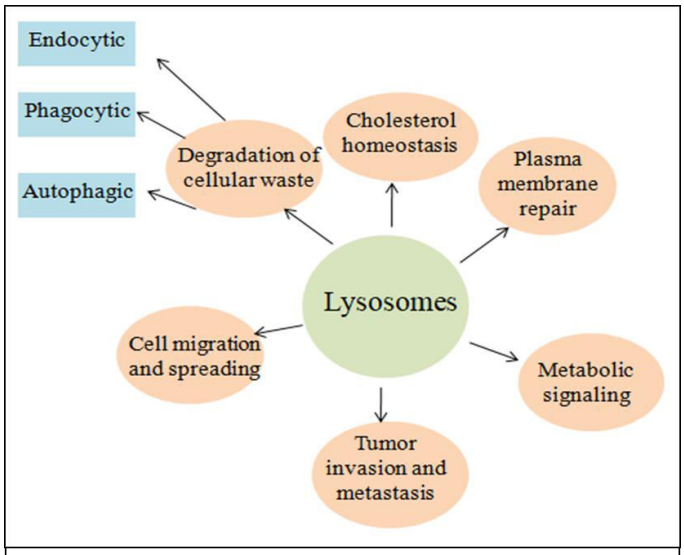

Fig. 2- Lysosomes are hub of degradation and signaling. Image depicts conventional (in blue) and unconventional (in red) functions of lysosomes in mammalian cells. Adapted from Jing Pu et al, JCS,2016

The mechanical stress or pathogenic invasion leads to rupture of the plasma membrane. The lysosome eventually fuses to the ruptured membrane sites in a calcium-dependent pathway and internalizes the damaged membrane[5]. Also, lysosomes play an essential role in cell migration via the remodeling of the actin cytoskeleton found beneath the plasma membrane[6].Another proliferating field of research is nutrient sensing by lysosomes, where signaling from lysosome-localized mTORC1facilitate growth and anabolism. The activation of mTORC1 is mediated by lysosomal cholesterol via binding to Niemann-Pick type protein C1 (NPC1)[7][1]. Additionally, permanent damage to lysosomal membranes leads to leakage of hydrolases that result in programmed cell death, known as lysosome-dependent cell death which is essential for various endolysoosmal damage-response mechanism that mediate removal of compromised lysosomes [8][1].Reports also indicate the role of lysosomes in tumor invasion and antigen presentation, where a drastic change in the distribution of lysosomes during metastasis and cancer cell growth has been observed[9]–[11].Cargo degradation and above mentioned non-canonical roles by

lysosomes are governed by the diverse distribution of lysosomes and their ability to undergo multiple fusion events with other compartments.

I.3 Role of Small G proteins Rabs, Arfs, and Arls in membrane trafficking

The small G proteins of Ras superfamily play a pivotal role in the regulation of membrane trafficking. These include different Rabs, Arfs, and Arf-like (Arl) proteins. All these small G proteins switch between GTP-bound active form from GDP-bound inactive form via GEFs (Guanine nucleotide exchange factor) and vice versa via GAPs (small G activating protein)[12]. In its activated GTP-bound state, the small G protein interacts with its effector proteins and facilitates vesicular transport by regulating multiple events of vesicle budding ,motility and fusion. At the very beginning, small G proteins were thought to play a role in signal transduction only, but later its role in vesicular trafficking came to prominence when Rab1 homolog in yeast was found to mediate membrane trafficking, and it was localized to Golgi[12]. Further studies showed the role of Arfs and its GAPs in regulating coat assembly and disassembly, cytokinesis, cholesterol homeostasis. Roles of Rabs have also been shown in vesicular targeting and tethering, along with vesicular coat assembly[13]. For instance, Rab7 localizes to late endosomes and lysosomes and regulates the maturation of early endosomes to late endosomes, along with clustering and fusion of late endosomes and lysosomes in the perinuclear region. Another Rab protein, Rab11, helps in the recycling of cargo back to the plasma membrane from the recycling endosome[14]. Our lab is primarily interested in studying the role of Arf-like small G protein, Arl8b that localizes to lysosomes, and regulates lysosome positioning and function.

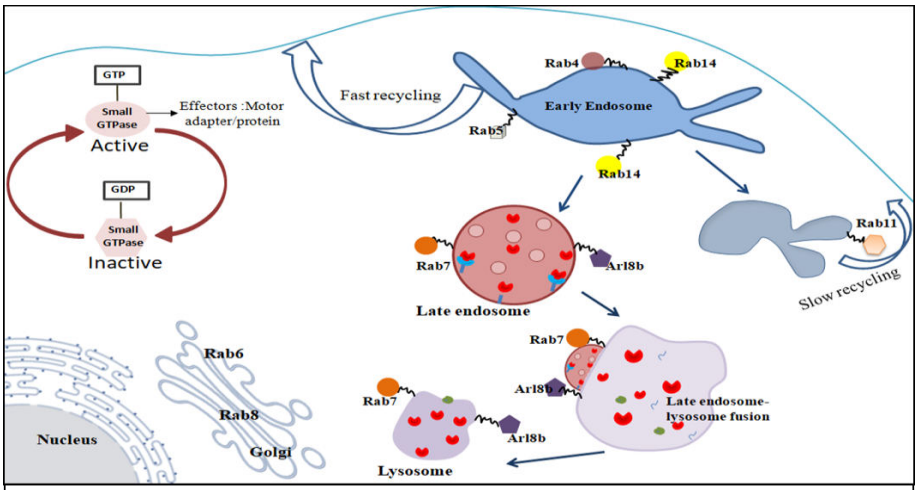

Fig.3 Schematic showing localization of different small G proteins on different intercellular compartments It also shows the activation of G proteins via GTP-GDP switch

I.4 Arl G protein, Arl8b, role in the Fusion and Motility of Lysosome

The small G protein Arl8b is a member of Arl family of protein which primarily decorates late endosomal and lysosomal compartments. They play an essential role in the regulation of lysosome positioning and membrane trafficking toward lysosomes. Arl8b is evolutionarily conserved throughout all species starting from protozoans except in yeast which has only Rab7 homolog. Arl8b being a small G protein, switches from GTP to GDP and vice versa via action of its GAP and GEF, respectively. A previous study from Pu J et al., and Falcon-Perez JM et al., suggests BORC (BLOC-one related complex) multi-subunit protein complex regulates membrane localization of Arl8b[2].Additional reports also suggest that Arl8b in its active form interacts with its effector protein PLEKHM2/SKIP and mediates plus end-directed movement of lysosomes in a kinesin-dependent manner[15]. Also, our lab has previously shown that PLEKHM1, known effector of Rab7, interacts with Arl8b and mediate late endosome-lysosome fusion and also mediate autolysosome formation[16].Arl8b interacts with PLEKHM1 and PLEKHM2 via their N terminal RUN domain. This excited us to search for other RUN domain-containing proteins that localize to endocytic compartments and test whether they interact with Arl8b.

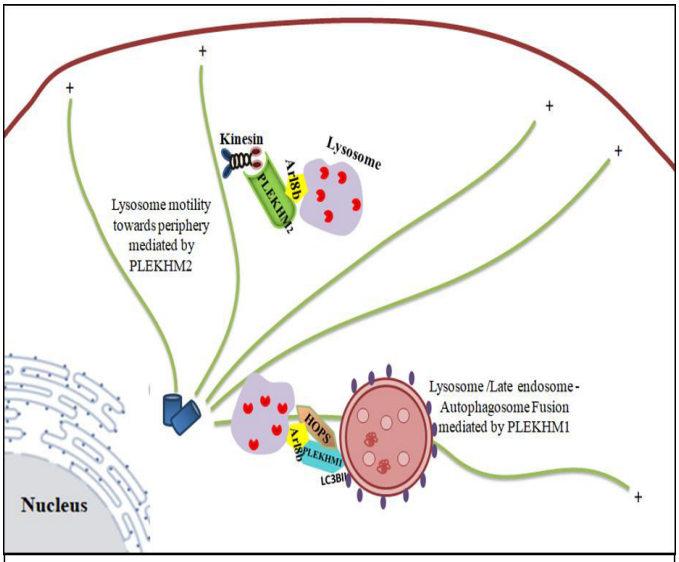

Fig.4- Role of Arf like G protein Arl8b and its effectors. PLEKHM2 regulates plus end directed motility of lysosomes and PLEKHM mediates late endosome and lysosome fusion. Image adapted from Marwaha et al, JCB, 2016

I.5 Rabip4/4': Novel Arl8b interaction partner

Rabip4/4' belongs to the family of N-terminal RUN domain and C-terminal FYVE domain-containing protein; hence it is also known as RUFY1. The RUN domain is named after RPIP8,UNC-14, and NESCA proteins, and it is organized into six conserved blocks which constitute the core of a globular structure [17]. The RUN domain is an

evolutionarily conserved domain known for protein-protein interaction. This region mediates the binding with small G proteins. The FYVE domain is required for interaction with phospholipid phosphatidylinositol-3-phosphate (PI3P) present mostly on early endosomes. RUFY1 gene encodes one shorter isoform Rabip4 (600 amino acid long; 69kDa) and a longer isoform Rabip4' (708 amino acid long; 80kDa). Rabip4',the longer isoform, has 108 amino acid long extension towards its N-terminal, which is absent from the shorter isoform Rabip4[4].

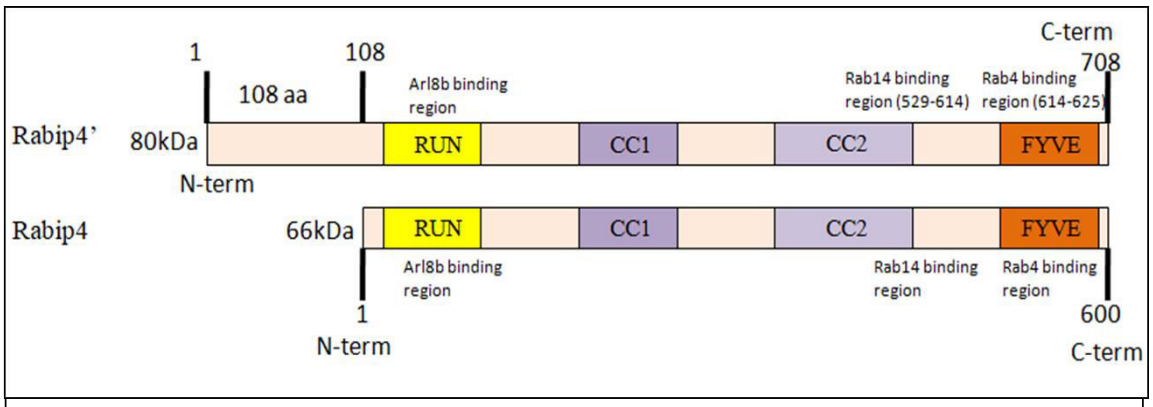

Fig. 5- Domain architecture of Rabip4'(upper) and Rabip4 (lower)

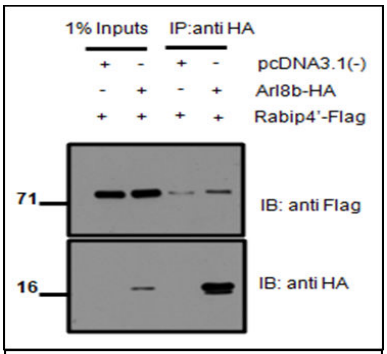

Fig. 6– Anti-HA beads used to co-immunoprecipitateRabip4' Flag from the HEK293T lysatescotransfected with Arl8b-HA and Rabip4'-Flag

Rabip4 initially identified as an effector of small G protein Rab4(hence was named Rab4 binding protein, Rabip4) and was known to play a role in endosomal trafficking by integrin trafficking and migration of NIH 3T3 fibroblast [18], [19].Rabip4/4' as acts a downstream effector of Rab14 by which it gets recruited to endosomes and later interacts with Rab4 [20], [21]. Further studies revealed that it acts as a dual effector of Rab4 andRab14, and recycles transferrin receptors from early endosomes via interaction with Rab14[22]. But from a recent study, the role of Rabip4/4', which was thought to be in the early endosomal compartment, also shown to regulate spatial distribution of lysosomes via its interaction with AP-3(adaptor protein complex) where they observed significant relocalization of lysosomes towards plasma membrane upon Rabip4/4' depletion [23].This observation drew our interest and motivated us to study for interactions between Rabip4' and lysosome-related protein, particularly Arl8b.The studies from our lab (Rawat et al.) have shown that Rabip4/4' could interact with Arl8b as it is evident from the co-

immunoprecipitation assay (figure 6). This suggests that Rabip4/4'area potential effector of Arl8b and further, the role of Rabip4/4 in regulating lysosome function has been explored in this study.

I.6 Objective

Our previous studies by Rawat et al., shows enlarged endo-lysosomal compartments upon Rabip4/4' depletion. Enlargement of lysosomes is indicative of lysosome dysfunction and suggests that Rabip4' regulate lysosome function. Further, it has been observed that Rabip4/4' and Arl8b positive compartments are also positive for CI-M6PR containing compartments which suggested its role in the CI-M6PR delivery. Also, in order to know other potential interacting partners of Rabip4/4', tandem affinity pull-down of Rabip4/4'was performed followed by a Mass spectrometry. The results of Mass spec were analyzed and we got dynein heavy chain and other dynein subunits as potential interacting partners of Rabip4/4'. Therefore the main objective of my study is:-

a. To investigate changes in lysosomes upon Rabip4/4' depletion.

b. To characterize the role of Rabip4/4' as dynein adaptor.

Chapter 2: MATERIALS and METHODS

1. Cell culture and RNAi

HeLa cells (from ATCC) were cultured in DMEM (Gibco) and were supplemented with 10% FBS (Gibco) in a humified incubator supplied with 5% CO2 at 37°C. Each cell was thoroughly examined for mycoplasma contamination. siRNAs were ordered from GE Healthcare for gene silencing and prepared according to the manufacturer's instructions. The sequence of control siRNA is TGGTTTACATGTCGACTAA, human-RUFY1 560 oligo is CATCAGATATAGCGACTAG, human-RUFY1 Smartpool, and Dynein HC, 5′-GAGAGGAGGTTATGTTTAATT-3′.

2. Cell lysate preparation, protein estimation, and Immunoblotting

The HeLa cells were collected in 1X PBS and pelleted down. The PBS was aspirated out and RIPA buffer containing EDTA (1mM), EGTA(0.5mM), Tris HCL of pH 8.0 (10mM), NaCl(140mM), Triton 100X (1%) Sodium dodecylsulphate(SDS)(0.1%) and Sodium deoxycholate(0.1%) was added. They were then vortexed every 5mins for a period of 30min. They should be kept on ice as soon as vortexing gets over. They were then centrifuged at 14000 rpm for 10mins at 4°C. The supernatant was then collected, which contained all the desired protein. This lysate was then estimated for the amount of protein present by doing a BCA protein estimation using Solution A (Bicinchoninic acid) and Solution B (copper(II) sulfate) (from Sigma) in 49:1 ratio along with 3µL of sample and 22µL of 1XPBS. This mixture was made in 96 well plates and incubated at 37°C for 30mins followed by taking the absorbance at 562 nm range. Samples (35-50µl) were eluted by boiling in Laemmli buffer and loaded on SDS-Page gel.

The proteins on the gel were transferred onto PVDF membranes (Bio-Rad Laboratories) by wet transfer at 100 volts for 1 hour 30 min. The membranes were blocked overnight with blocking solution (10% skim milk in 0.05% PBS-Tween 20) at 4°C or for 2hours at room temperature in blocking solution and examined with indicated primary and secondary antibodies prepared in 0.05% PBS-Tween20 for 3 hours and 45 minutes,

respectively, at room temperature. Membranes were washed thrice with 0.05% PBS-Tween 20 for 10 minutes each after examining 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 stripped for 30 seconds with stripping solution (3M Guanidine hydrochloride) and blocked overnight at 4°C before re-probing them with a set of different primary and secondary antibodies.

3. Plasmids used

Plasmid	**Source**
Str-KDEL-IRES-SBP-mCherry-CTSZ	Dr.Junjie Hu lab (China)
Chicken GFP-Lamp1	Dr. Steve Caplan Lab(USA)
FKBP-GFP-Rabip4'	Our Study
FRB-Tom70p	Our Study
FKBP-GFP only vector	Our Study
GFP-Tubulin	Dr.Steve Caplan Lab(USA)
mCherry-Human-Rufy1(shorter isoform Rabip4)	Dr. Shin lab

4. Antibodies used

The following are the antibodies used in this study:Rabbit anti-CathepsinD (Abcam), Rabbit anti-M6PR(Abcam), Mouse anti-Tubulin (Sigma), Mouse anti-Lamp1(BD),Rabbit anti-Tom20p (SCBT),Mouse anti-M6PR (Abcam),Mouse anti-Giantin (Abcam). All Alexafluorophore-conjugated secondary antibodies were from Thermo Fisher Scientific. HRP-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson Immunoresearch Laboratories.

5. Transfections, Immunofluorescence, and Cell imaging

For RNAi transfection, 0.07M to 0.8M HeLa cells in 35mm dish; for DNA transfection, 0.3M HeLa cells in a 35mm dish were grown on a glass coverslip for 24 hours. Cells were transfected with the indicated DNA construct using X-tremeGENE-HP (Roche) transfection agent for 8-12 hours. For knockdown experiments, the cells were then transfected with the desired siRNA using the DharmaFECT transfection agent for 60 hours.

After the transfection process, 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 is 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) for overnight at 4°C or for 3hours at room temperature. Coverslips were then washed three times with 1X PBS (pH 7.4) and incubated with AlexaFluor-Conjugated secondary antibody in staining solution (0.2% Saponin + PHEM buffer) for 30 to 45 minutes at room temperature. After washing thrice with 1X PBS, the coverslips were then mounted on glass slides using fluoromount G (Southern Biotech).

Coverslips were kept on observation 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.

6. RUSH Transport Assay

HeLa cells were cultured as described above and transfected using KDEL-RUSH-IRES-SBP-CTSZ-mCherry1 DNA (Box-35, Position-9; -80°C refrigerator) and Xtreme GeneXP (in 1ng:1µl ratio, respectively). Following 16 hours after transfection, cells were incubated with 40 µM biotin (Sigma) for different time points starting from 0 hours to 3 hours. Images were acquired by Zeiss LSM700 confocal microscopy for each time point. For live-cell imaging of the same, cells were seeded on a 35 mm glass-bottom live

imaging dish (Eppendorf) followed by transfection with the indicated plasmids. Live-cell imaging was performed using a Zeiss LSM 710 confocal microscope equipped with an environmental chamber set at 37°C and 5% CO2.

7. Image processing and quantification

Images were analyzed with the help of Fiji and Adobe Photoshop. We calculated colocalization coefficients of 50 cells for a given treatment from 3 independent experiments. Pearson's colocalization coefficient and Manders overlap were calculated using the JACoP plug-in provided in the ImageJ software. All graphical representations were made in either MS-Excel or Graphpad prism.

8. Mitochondrial distribution

Mitochondrial distribution was also quantified using ImageJ software. Tom20p signal intensity was quantified for individual cells by drawing circles at an increment of every 5µm (till the cell periphery) from the nuclear rim. First, the Tom20p intensity was measured at 5 µm (from the nuclear rim) and 5-10 µm (from the nuclear rim) then 10-15µm and lastly 15-20 µm.30 cells were quantified per experiment and are averaged over 3 independent experiments. The graphical representations were made in MS-Excel.

CHAPTER 3: OBJECTIVES and RESULTS

The work shown in this section is being prepared for a manuscript entitled "*Rabip4' binds to Arl8b and mediates endosome to TGN retrieval of CI-M6PR*" by Shalini Rawat[1], Rituraj Marwaha[1,2],**Shrestha Shaw**, Gitanjali and Mahak Sharma.

3.1Rabip4/4' plays role in lysosome composition and function

Co-immunoprecipitation and GST pull-down data (Rawat et al.) shows that Rabip4/4' binds with Arl8b via the N-terminal RUN domain of Rabip4/4'. Also, HeLa cells treated with Rabip4/4' siRNA shows an enlargement in the endo-lysosomal compartment when compared to control siRNA treated cells, which was quantified by measuring average size of Lamp1 positive compartments (Rawat et al.).

We also stained for another lysosomal marker, Cathepsin D, a lysosomal aspartic protease in control- and Rabip4/4'-siRNA treated cells. Surprisingly, we observed an increase in the intensity of cathepsin D levels upon Rabip4' depletion. To quantitate the change in cathepsin D intensity, we calculated the corrected total cell fluorescence. (CTCF) value of CathepsinD from confocal images in control- vs. Rabip4'-siRNA. We found an approximately two-fold increase in levels of Cathepsin D levels in of Rabip4/4'-siRNA treated cells when compared to control cells (Figure-7). Similar results were found from western blot analysis of whole-cell lysate of control or Rabip4/4'-siRNA. There was an almost two-fold increase in pro-Cathepsin D levels in Rabip4/4'- siRNA-treated cells compared to control-siRNA treated cells. Also, we observed a slight increase in the levels of mature Cathepsin D in Rabip4/4'-siRNA treated cells. We also examined the levels of lysosomal membrane protein Lamp1, and we found a slight rise in Rabip4/4' siRNA treated cells (Figure-8). Similar upregulation in lysosomal hydrolases and lysosomal membrane proteins like Lamp1 have been reported in previous studies where there is lysosomal stress or dysfunction [24][25][26] . The study is consistent with our data suggesting Rabip4/4' plays a role in maintaining lysosomal composition and function.

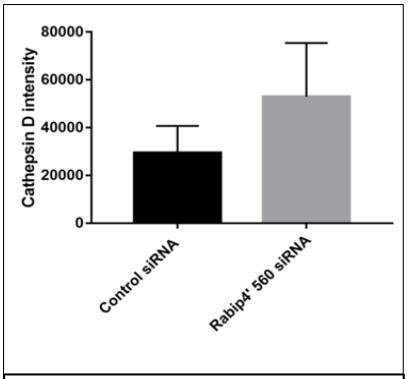

Fig.7- Quantification of Confocal Micrographs of HeLa cells treated with control or Rabip4' siRNA and immunostained for CathepsinD (n=3 experiments; 50 cells/experiment).

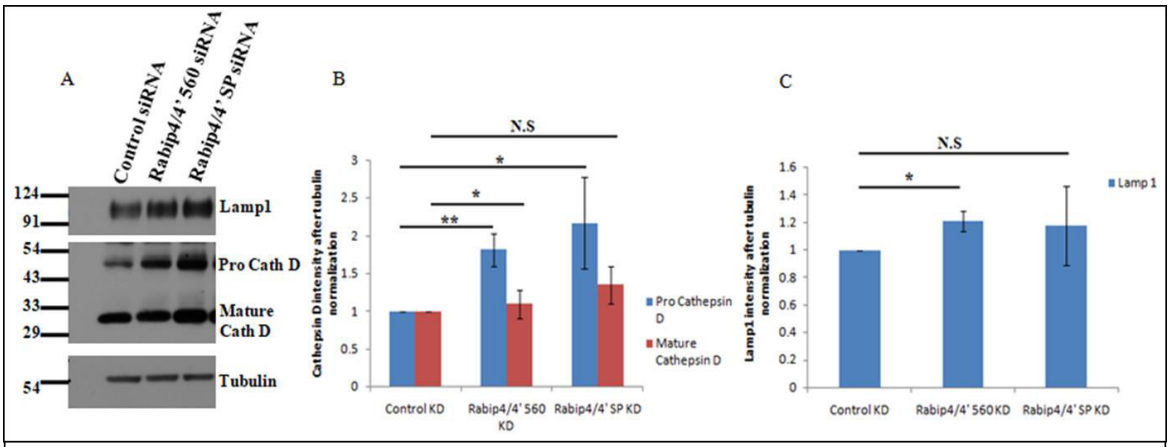

Figure 8.(A) Immunoblotting of HeLa lysate treated with either control or Rabip4' siRNA(560 oligo and Smartpool) using anti Cath D and anti Lamp1.(B) Densitometric quantification of pro-cathepsin D and mature cathepsin D normalized to α-tubulin.(*n= 3 experiments)*(C) Densitometric analysis of Lamp1 normalized to α-tubulin.(*n= 3 experiments).*Data represent mean±SEM. Unpaired t-test performed. '*' and '**' reflects significance. N.S-Non-significant

3.2 Rabip4/4' mediates retrieval of CI-M6PR receptor from endosomes to Trans Golgi network

We were next interested in finding the mechanism by which Rabip4' might be regulating lysosome composition. Therefore, we looked back at our colocalization studies of Rabip4' with various endocytic markers like EEA1, Rab14, and Lamp1 (Rawat et al.).We found that Rabip4' colocalizes with cation independent mannose-6 phosphate-receptor (CI-M6PR).CI-M6PR is a well-known receptor for various lysosomal hydrolases, and defects in its recycling from early endosome to trans-Golgi leads to impaired delivery of hydrolases in the lysosome, resulting in lysosome dysfunction. Therefore, we next assessed the localization of the CI-M6PR receptor in control vs. Rabip4/4' siRNA. As compared to control siRNA treated cells where CI-M6PR punctae (in red channel) were colocalizing with Golgi (in green channel) in the perinuclear area, we observed CI-M6PR were dispersed and enlarged uponRabip4/4'knockdown (Figure-9).

Also, we looked for CI-M6PR receptor colocalization with sorting nexin SNX1, a retromer compartment protein, in control Vs. Rabip4/4' siRNA. We observed an increased colocalization of CI-M6PR punctae with SNX1 in Rabip4/4' siRNA cells as compared to control siRNA (data not shown here). These observations suggest that CI-M6PR cannot reach Golgi upon Rabip4/4' depletion and retain in the SNX1 positive retromer compartments, a phenotype usually observed in defect of the CI-M6PR recycling from endosome to Golgi.

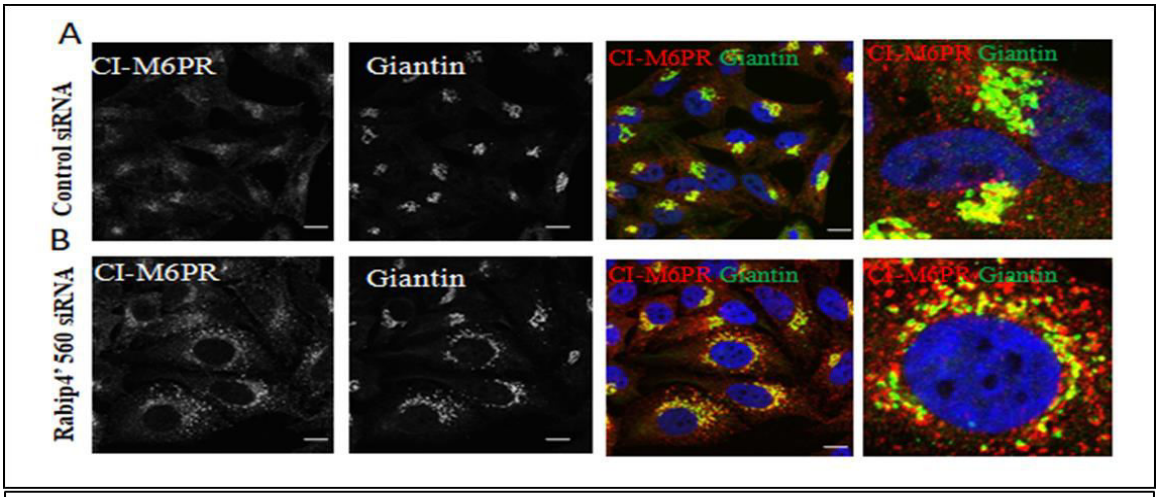

Fig. 9.(A) and (B) Representative confocal Micrographs of HeLa cells treated with control or Rabip4' siRNA and immunostained for CI-M6PR and Giantin.

Further to confirm the change in CI-M6PR distribution is due to delay in trafficking of CI-M6PR from early endosome to Golgi, we employed CD8 trafficking assay. We used a CD8-CI-M6PR chimera which had a CI-M6PR tail region attached to CD8 acto-domain thereby only permitting the movement of the chimera from early endosome to Golgi. The construct was transfected on the control and Rabip4/4' siRNA treated HeLa cells. These cells were then labeled with antibodies against CD8 and it was chased for different time-points. In control treated cells we observed chimera colocalizes with Golgi by 30mins whereas in Rabip4/4, treated cell only a fraction of CD8 reaches Golgi but majorly remains in early endosome (Rawat et al.) (data not shown here). So our data suggests defect in the trafficking of CI-M6PR from early endosomes to Golgi upon Rabip4' knockdown.

3.3 Rabip4/4' depletion leads to delayed delivery of lysosomal hydrolases cathepsin Z from Golgi

We next monitored CI-M6PR cargo (lysosomal hydrolases) delivery to the lysosome in Rabip4' siRNA treated cells. To study the delivery of lysosomal hydrolases from Golgi, we used Retention Using Selective Hook (RUSH) assay. In this assay, mCherry tagged lysosomal hydrolases Cathepsin Z(cargo) was fused to streptavidin binding protein (SBP) and was initially trapped in Endoplasmic reticulum (ER) by streptavidin (Str) fused with ER retention signal KDEL (Hook protein). Upon addition of biotin (20µM), the biotin competes with SBP and leads to a synchronized release of mCherry Cathepsin Z from ER

and then follows its general trafficking route to Golgi and then finally from Golgi to lysosomes (Figure-10).

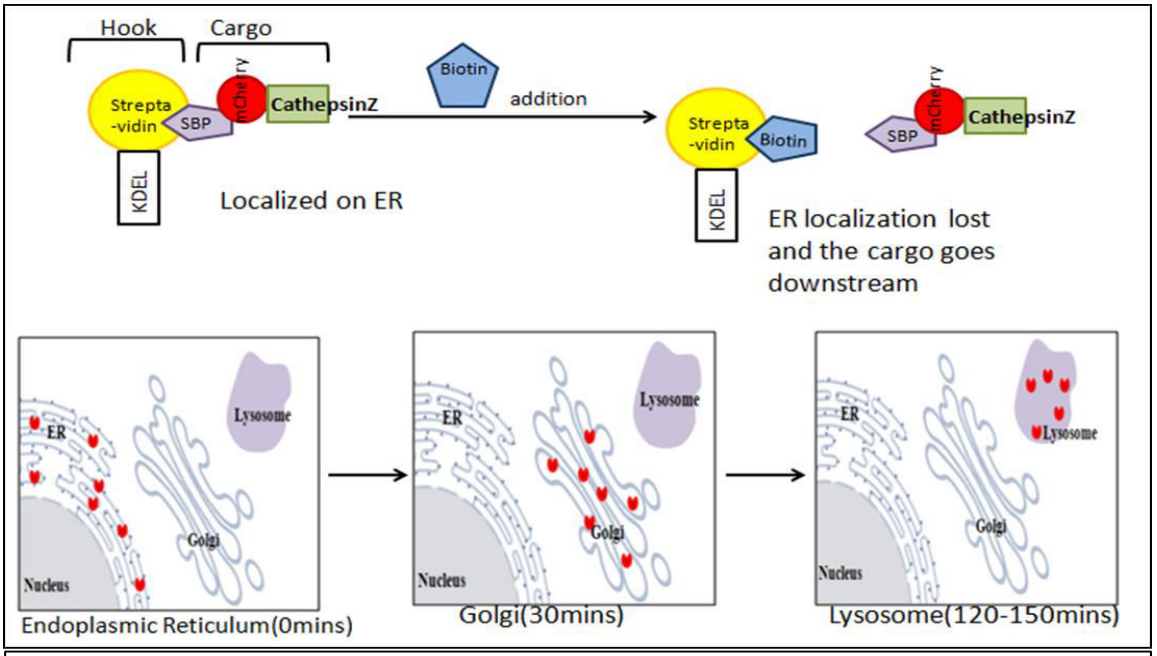

Fig.10- (Upper)Schematic of RUSH assay to study the delivery of hydrolases from ER to Lysosomes. (Lower) Also represents different time points of localizing in the specified compartment.

The transfection efficiency of KDEL-IRES-Str-SBP-mCherry-CathepsinZ was very low, so we used a higher amount of DNA and were kept for 18hours. This helped us obtain a higher number of transfected cells. We firstly checked whether, upon addition of biotin, it was losing the ER association. We observed cathepsinZ successfully localized to Golgi after 30mins of biotin addition (Figure-11)

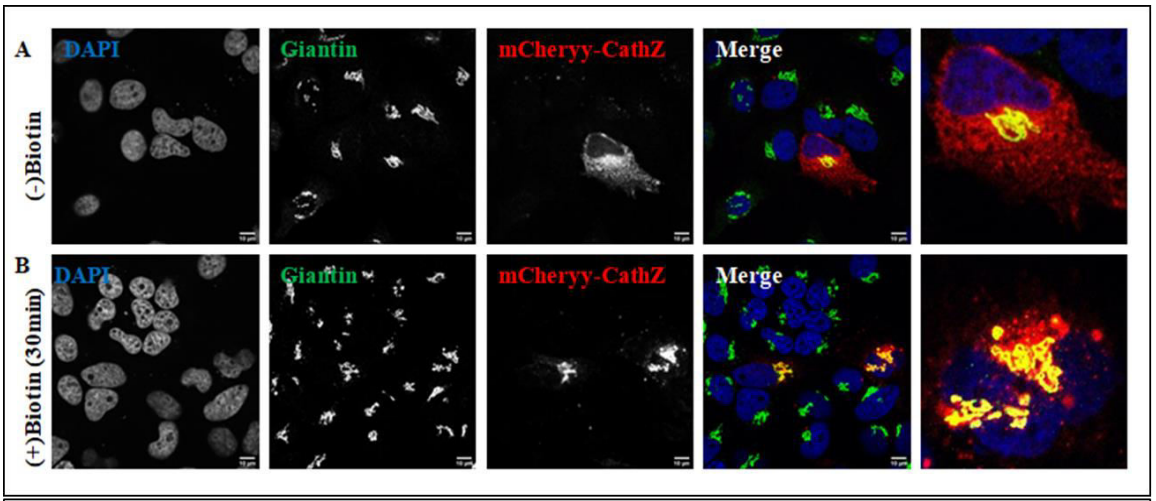

Fig.11 (A-B) Representative confocal micrographs of HeLa cells transfected with mCherry-CathepsinZ. After 18 hours of transfection, cells were treated with or with out rapamycin and immunostained for Giantin.

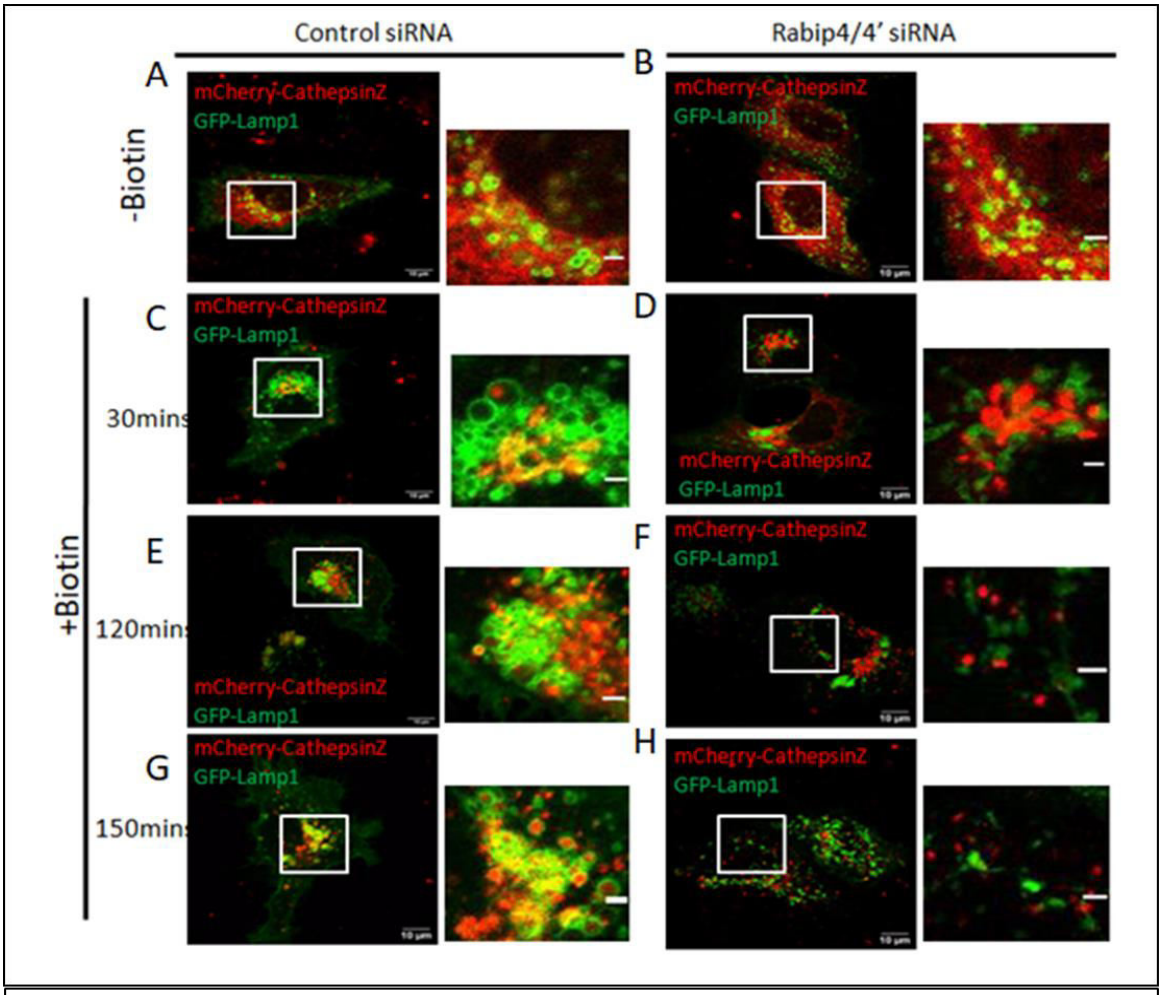

Fig.12 (A-H) Representative confocal micrographs of Hela cells treated indicated siRNA followed by co-transfection with mCherry-CTSZ and Lamp1 GFP plasmids. After 18 hours of transfection, live imaging of cells was done with images captured without biotin or after addition for different time.

We performed a RUSH assay in HeLa cells treated with control siRNA or Rabip4/4' siRNA. KDEL-IRES-Str-SBP-mCherry Cathepsin Z (red channel) and Lamp1-GFP (green channel) were co-transfected in HeLa cells, followed by live-cell imaging of the cells. Compared to control siRNA treated cells where Cathepsin Z starts colocalizing with lysosomes by 120-180 minutes, reduced colocalization of Cathepsin Z with lysosomes was observed in Rabip4/4'siRNA. Slight to negligible colocalization between GFP-Lamp1 and Cathepsin Z was observed upon knockdown of Rabip4/4' (Figure-12). RUSH data suggests there is delay/impairment in the delivery of lysosomal hydrolases from Golgi to lysosomes.

3.4FKBP-FRB heterodimerization assay to check the interaction of Rabip4/4'with dynein

To determine the mechanism by which Rabip4/4' is regulating CI-M6PR trafficking from early endosome to Golgi, we did a TAP pull-down of TAP-Rabip4/4' followed by a Mass spectrometry analysis. We obtained a higher unique number of peptides of dynein heavy chain, dynein light chain and dynactin subunits. Additionally, the co-immunoprecipitation experiment shows the interaction of Rabip4/4' with dynein and dynactin subunits (data not shown here). Dynein being a retrograde motor, Rabip4/4' interaction with dynein can be one of the possible mechanisms by which it is regulating CI-M6PR trafficking from early endosome to Golgi.

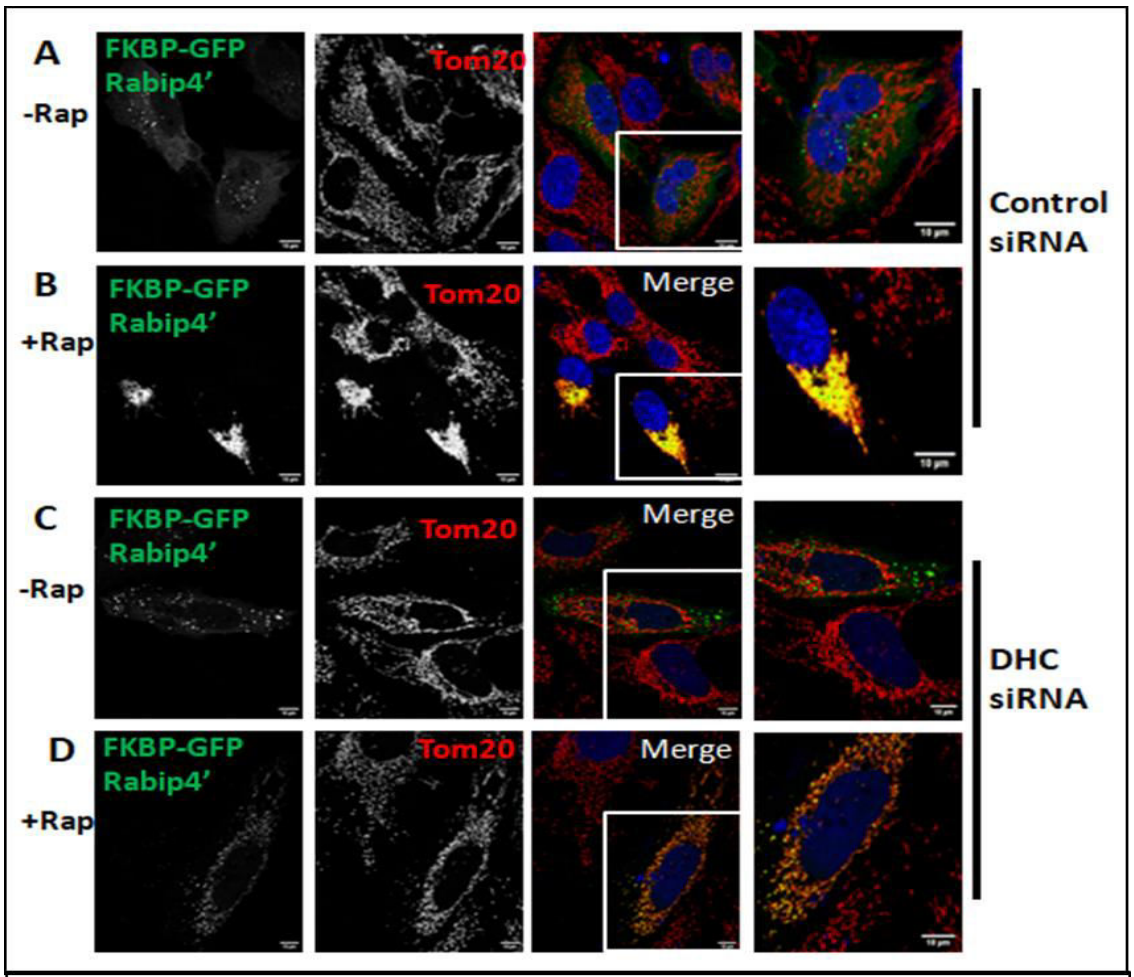

Fig. 13- (A-D) Representative confocal Micrographs of HeLa cells treated with control or DHC siRNA followed by co-transfection with FKBP-GFP Rabip4' plasmids and FRB-Tom70p. After 12 hours of transfection, cells were treated with or with out rapamycin and immunostained for Tom20.

To further check that Rabip4' can recruit dynein onto CI-M6PR compartments, we mislocalized the Rabip4' to entirely different compartments like mitochondria where it is usually not present, using a Rapamycin induced FKBP (FK506 binding protein)-FRB

(FKBP-Rapamycin binding) heterodimerization system. In this assay, HeLa cells were transfected with GFP-Rabip4' fused with FKBP tag (green) and FRB tag fused Tom70p (mitochondrial outer membrane localization). Upon addition of rapamycin (200nM), after 45mins it was observed that the FKBP-GFP-Rabip4' was translocated to the mitochondrial compartment containing FRB-Tom70p (which was stained using Tom20p antibody). A strong perinuclear clustering of mitochondria observed in cells expressing FKBP-GFP Rabip4' upon treatment with rapamycin. To further check whether the mitochondrial clustering by Rabip4' is dynein dependent, we performed an FKBP-FRB experiment in Dynein knockdown. We observed that FKBP-GFP-Rabip4/4' expressing cells upon DHC siRNA treatment did not show perinuclear clustering as compared to control siRNA treated cells (Figure-13). This observation suggests that Rabip4/4' indeed interacts with dynein and causes the perinuclear clustering of mitochondria.

3.5Rabip4/4' endosomes associates with tubulin

The previously performed data analysis of mass spectrometry results showed a high number of peptides of tubulin subunits also. (Rawat et al., not shown here) which prompted us to check the interaction of tubulin with Rabip4/4'. So, we performed live-cell imaging of Hela cells upon transfection with GFP-tubulin and mCherry-Rabip4.We observed that many Rabip4 endosomes (red channel) associates with microtubules (green channel). However, this is our preliminary observation, and further experiments need to be done to confirm this association.

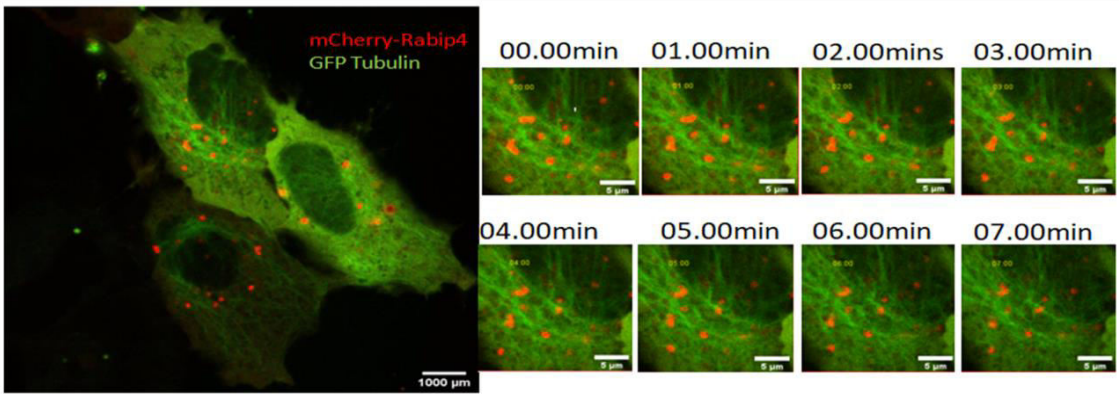

Fig. 14- Representative confocal micrographs of HeLa cells co-transfected with mCherry-Rabip4 and GFP-Tubulin plasmids. After 12 hours of transfection, live imaging of cells done. Time Lapse snapshots are shown alongside.

Chapter 4: Conclusion and future directions

The data from our studies propose that Rabip4/4' binds with Arl8b via its N-terminal RUN domain and helps in sorting of CI-M6PR from early endosome to Golgi. CI-M6PR binds to lysosomal hydrolases Golgi and traffics them to lysosomes. If this receptor does not reach Golgi upon Rabip4/4' depletion, then cargo sorting of cathepsins at Golgi gets impaired, leading to lysosomal stress conditions like enlargement of the endo-lysosomal compartment, upregulation of lysosomal hydrolases, and lysosomal membrane proteins. Our current findings also suggest that Rabip4/4' via interaction with dynein-dynactin complex mediates retrograde recycling of CI-M6PR to Golgi from early endosomes.

Recent studies show that a single nucleotide polymorphism (SNP) in Rabip4/4' plays can lead to Anorexia Nervosa [27] and high blood sugar levels [28]. These findings correlate with the role of Rabip4/4' in the sorting of GLUT4 [20].In these studies, it has been shown that Rabip4/4' has a role in sorting of GLUT4 toward storage compartments or TGN, and in the absence of Rabip4/4', the cargo (GLUT4) gets recycled back to the plasma membrane, thereby increasing the overall number of transporters on the plasma membrane. This would lead to increased uptake of glucose, a possible explanation of Rabip4/4' SNP leading to diabetic condition or lean body mass. Recently from our study, we show that there is a decrease in the amount of another cargo, CI-M6PR, being recycled to Golgi upon Rabip4/4' depletion. We, for the first time in our study elucidate the role of Rabip4/4' in sorting of cargo towards Golgi. Arl8b might also be important for the process of sorting and recycling of cargo along with Rabip4/4'. The literature studies and our results intrigued us to look into the fate of CI-M6PR, which are unable to reach Golgi upon Rabip4/4' depletion. We predict that the cargo also might move towards the plasma membrane upon depletion of Rabip4/4'. Further experiments need to be done in order to confirm our predictions. Also, studying Rabip4/4' in different cell types could reveal its role in sorting various other cargos.

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