Characterization of Rabip4' as an effector of the lysosomal small GTPase Arl8b

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Certificate of Examination

This is to certify that the dissertation titled “Characterization of Rabip4’ as an effector of the lysosomal small GTPase Arl8b” submitted by Ms.Vaishnavi Sridhar (Reg. No. MS12083) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Mahak Sharma	Dr. Rajesh Ramachandran	Dr. Sharvan Sehrawat
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Dated: April 21, 2017

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.

Vaishnavi Sridhar
(Candidate)
Dated: April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Mahak Sharma
(Supervisor)

Acknowledgement

I would like to thank my thesis supervisor Dr. Mahak Sharma for her constant guidance and supervision. I would also like to express my gratitude to my lab members Aastha, Ritu, Divya Khatter, Devashish, Siddhi, Ashwin and other 3TL2 members for helping and teaching me. All my faculty members for being a source of encouragement. I am also thankful to IISER Mohali, all the resources and staff of the institute. I deeply grateful to my friends for being my support system in times of need. I am grateful to my parents and brother for being there for me always. Last but not the least, I would like to thank god for the beautiful life he has given me.

Vaishnavi

List of Figures

List of Tables

List of Abbreviations

BSA : Bovine Serum Albumin

DMEM: Dulbecco's Modified Eagle's Medium

DTT: Dithiothreitol

EDTA : Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

EEA1: Early endosome antigen 1

GST : Glutathione S-transferase

GTP : Guanine Triphosphate

HA : Hemagglutinin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP : Horseradish Peroxidase

IPTG : Isopropyl β-D-1-thiogalactopyranoside

LAMP1: Lysosomal-associated membrane protein 1

PBS: Phosphate buffer saline

PBST: PBS-Tween

PFA: Paraformaldehyde

PIPES: Piperazine-N,N′-bis(2-ethanesulfonic acid)

PMSF: Phenylmethane Sulfonyl Fluoride or Phenylmethylsulfonyl Fluoride

SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TAP: Tandem affinity purification

Table of Contents

Abstract

Small GTPases recruit effectors to carry out downstream functions. Arl8b, a small GTPase localizes to lysosomes. Some of the known effectors of Arl8b like PLEKHM2 and PLEKHM1 contain a N terminal RUN domain. Another family of RUN domain containing proteins is the RUFY (RUN and FYVE domain containing) family of proteins. It has four members -RUFY1, RUFY2, RUFY3 and RUFY4. RUFY1 has two isoforms-Rabip4' and Rabip4. These are early endosomal and are the effectors of Rab4 and Rab14. Previous studies had shown that Rabip4' silencing leads to lysosomal clustering towards the cell periphery. We find that Rabip4' colocalizes with Arl8b and also pulls down HA-Arl8b in a GST pull down assay. We propose to show that Arl8b and Rabip4' interact directly via the latter's RUN domain. We make use of the purified protein-protein interaction assay in trying to determine the interaction.

Chapter 1
Introduction

1.1 Basic Knowledge
1.2 Materials and Methods

1.1 Basic Knowledge

1.1.1 Small GTPases

Small GTPases are monomeric GTPases, whose size is between 20-25kDa. They alternate between an active GTP bound state and an inactive GDP bound state. The GEF (guanine nucleotide exchange factor) proteins help activate these and GAP (GTPase activating protein) proteins help inactivate them by providing a catalytic group for GTP hydrolysis (Fig 1) (Donaldson and Jackson (2011)).

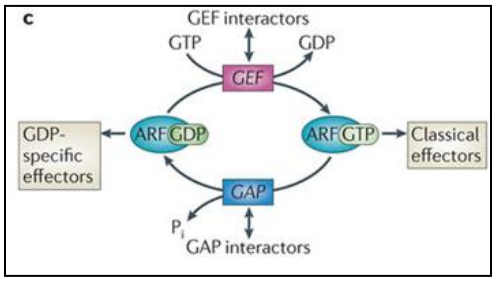

Fig 1: The small GTPase cycle. Small GTPases alternate between a GTP bound and a GDP bound form. Adapted from (Donaldson and Jackson (2011)).

In their GTP bound form, these proteins recruit effectors to carry out downstream functions and localize to specific compartments (Fig 3). These effectors could be adaptor proteins, motor proteins, tethering factors etc. (Fig 2) (especially in case of Rab, Arf and Arl proteins). Arl (Arf like proteins) are small GTPases that belong to the Arf family of small GTPases. This family has about 22 members. They are involved in endocytic trafficking pathways (Donaldson and Jackson (2011)).

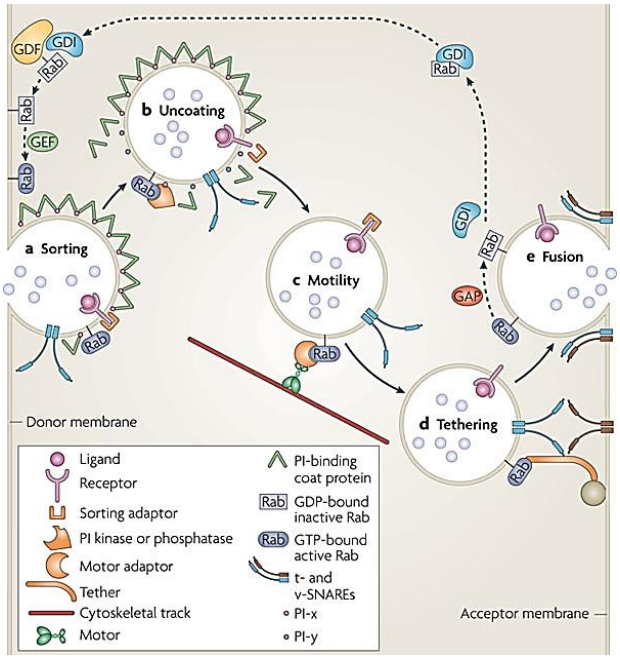

Fig 2: The different effector functions of small GTPases in membrane trafficking. Small GTPases effectors play roles in cargo sorting, vesicle motility and tethering. Adapted from (Stenmark 2009)

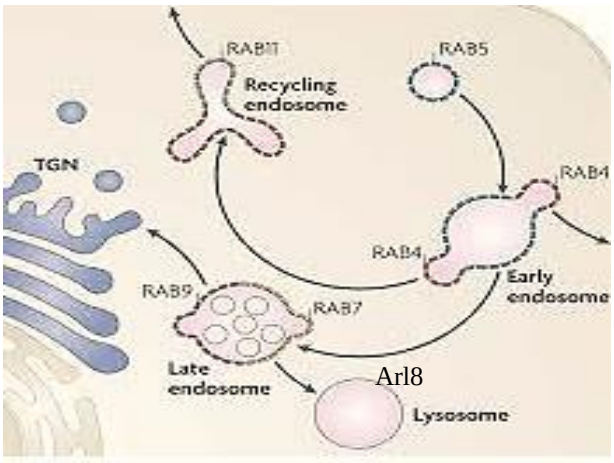

Fig 3: Small GTPases of the Rab, Arf and Arl families localize to specific compartments in the endocytic pathway. Adapted from (Stenmark 2009)

Like other Arf family small GTPases, these localize to membranes through an amphipathic N terminal helix. Some of these are myristoylated however others are not. ARFRP1, ARL8a,

ARL8b are acetylated at their N terminus and a methionine residue has been observed as the site of acetylation in case of Arl8b. Arl8b is conserved from humans to plants and protozoa, although it is absent from some yeasts and fungi, and there are two closely related paralogs in vertebrates, Arl8a and Arl8b (Donaldson and Jackson (2011)).

Arl8b is cytosolic when GDP bound and inserts into the organelle (lysosomal/late endosomal) membrane when GTP bound. Arl8b localizes to late endosomes and lysosomes in both humans and C. elegans, and mediates the transport of endocytic cargo between these two compartments. The localization of Arl8b to lysosomes is highly conserved across species.

Lysosomes are usually present in the perinuclear region and some are scattered in the cytoplasm. However, they are peripherally localized on Arl8b overexpression in a microtubule dependent manner (Fig 4) (Khatter, Sindhwani et al. (2015)).

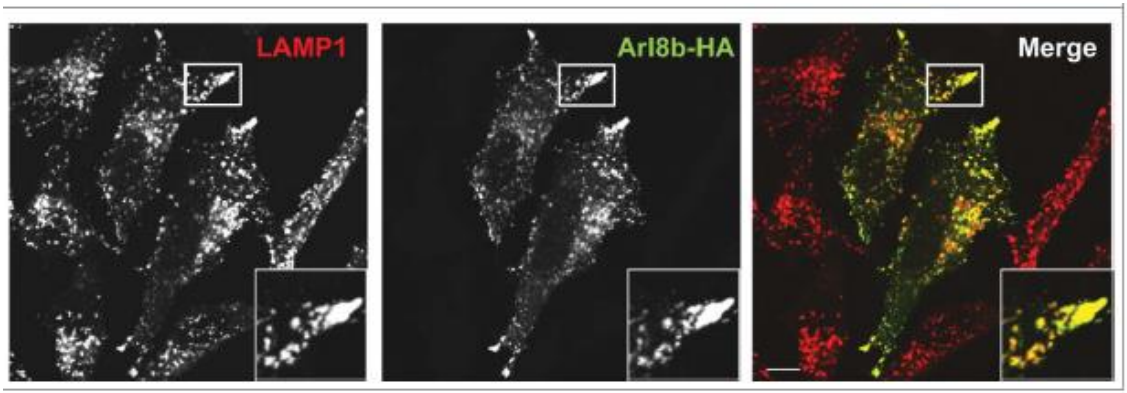

Fig 4: Arl8b localizes to lysosomes. LAMP1 is a lysosomal marker and indicated in red**,** Arl8b in green and yellow indicates that Arl8b colocalizes with lysosomes. (Khatter, Sindhwani et al. (2015)).

Like all other small GTPases, in its GTP bound form Arl8b recruits effectors that carry out downstream functions. Few known effectors of Arl8b are SKIP (PLEKHM2), PLEKHM1 and HOPS Complex- a multi subunit tethering factor (Fig 5). PLEKHM2 interacts with Kinesin to aid the anterograde motility of lysosomes. PLEKHM1 along with HOPS Complex aids in the late endosomal to lysosomal fusion (Marwaha, Arya et al. 2017).

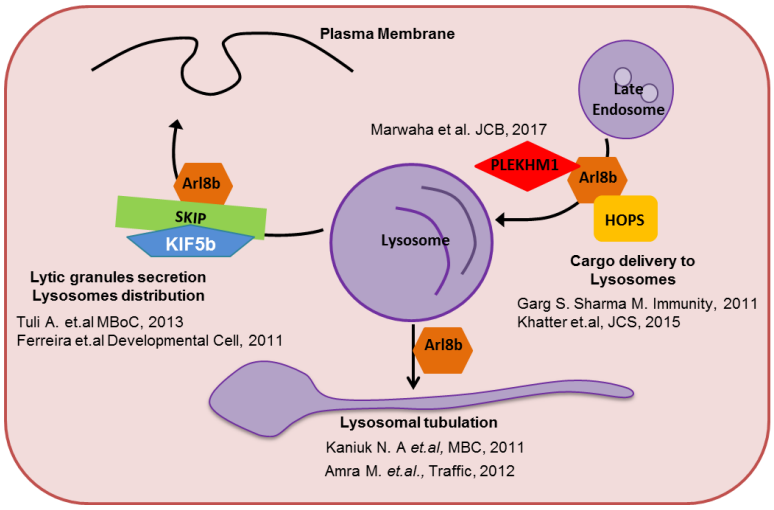

Fig 5: Arl8b and its effectors. Using information from(Khatter, Raina et al. 2015), (Marwaha, Arya et al. 2017), (Rosa-Ferreira and Munro 2011)

1.1.2 RUN domain-containing proteins

Two of the known effectors of Arl8b- PLEKHM1 and PLEKHM2 contain a RUN domain. RUN domain containing proteins tend to be small GTPase effectors. The name RUN comes from the proteins RPIP8, UNC-14, and NESCA (Fig 6) (Yoshida, Kitagishi et al. 2011). It has been shown that PLEKHM1 and PLEKHM2 are recruited by Arl8b via their RUN domain. Studies by Claudia Rose and Sean Munro show that Arl8b recruits the motor protein kinesin-1 to lysosomes via SKIP. The N terminus 300 residues form the RUN domain and are necessary for SKIP being recruited by Arl8b to lysosomes. Arl8b along with its effector SKIP is responsible for the anterograde movement of lysosomes. A recent paper from our lab (Marwaha, Arya et al. 2017) shows that the N terminal RUN domain of PLEKHM1 is necessary and sufficient for binding to Arl8b and PLEKHM1 recruitment to lysosomes. The authors identify conserved basic residues (arginines and histidines) required for binding to Arl8b. Our search for other RUN domain containing proteins involved in the endocytic pathway led us to RUFY family of proteins.

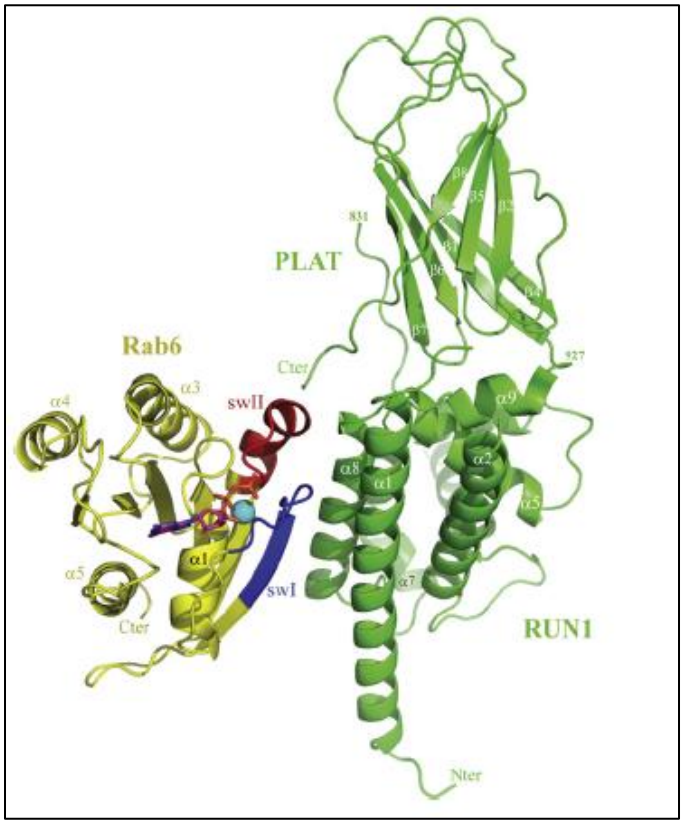

Fig 6: Ribbon model of Rab6- Rab6IP1 complex. There are eight alpha helices in the RUN domain of Rab6IP1 (Green). Rab6 is shown in yellow. Adapted from (Recacha, Boulet et al. 2009)

1.1.3 RUFY family of proteins

The RUFY (RUN and FYVE domain containing proteins) family of proteins have a RUN domain at their amino terminus and FYVE domain at the carboxyl terminus. The FYVE domain associates with PI3P on early endosomal membranes. This family has four members. Domain architecture of each of these has been indicated in Fig. 7.

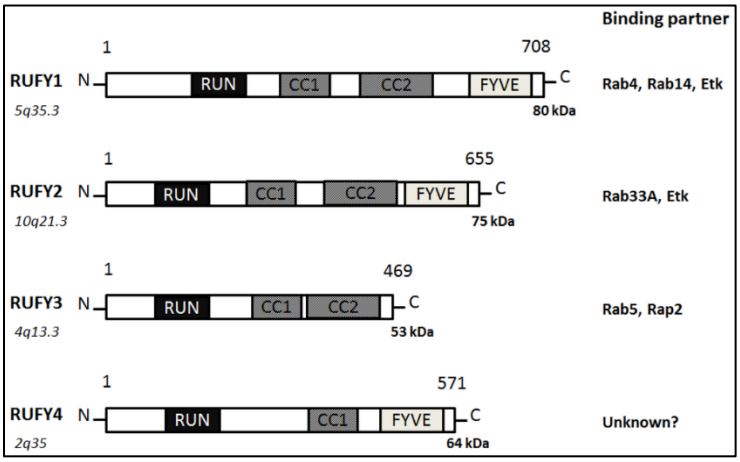

Fig 7: Domain architecture of RUFY proteins. The various domains and their positions have been indicated. Adapted from (Kitagishi, Matsuda et al. 2013)

RUFY1 has two isoforms- the longer Rabip4' and shorter Rabip4, the former being longer by 108aa at the N terminus. It localizes primarily to early endosomes and participates in early endosomal trafficking. Rabip4' is a known effector of Rab4 binding via its C terminus.

It was observed that the silencing of Rabip4s (referred to Rabip4 and Rabip4') leads to the peripheral clustering of lysosomes and outgrowth of plasma membrane protrusions (Ivan, Martinez-Sanchez et al. 2012). Rabip4' silencing leads to lysosomal protein accumulation in periphery of cell. A new complex between AP-3 and Rabip4' was found that regulates the spatial distribution of lysosomes (Fig 8).

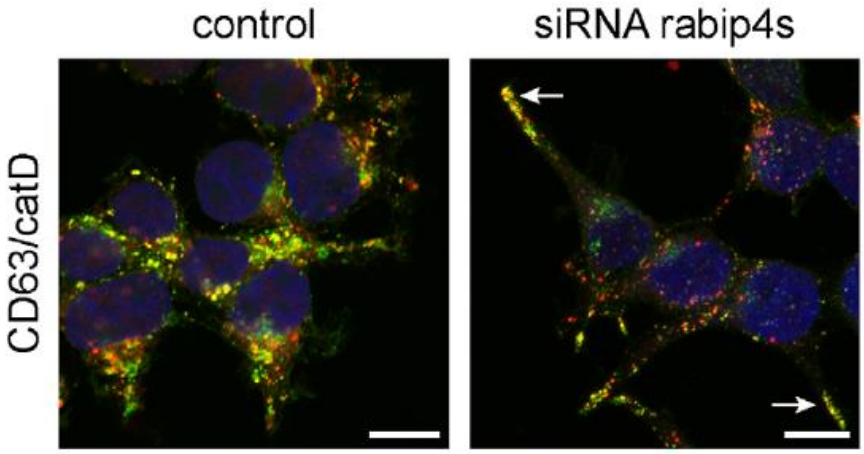

Fig 8: Rabip4s knockdown redistributes lysosomal markers to cell periphery. CD63 is stained in green, cathepsin D in red and nucleus in DAPI. (Ivan, Martinez-Sanchez et al. 2012)

On aligning the RUN domains of PLEKHM2, PLEKHM1, RUFY 1 mouse and human proteins, we find in RUFY 1 as well the conserved residues required for Arl8b binding (Fig 9). Preliminary colocalization experiments (images included in results section, Fig 10) indicate that Rabip4' colocalizes with Arl8b.

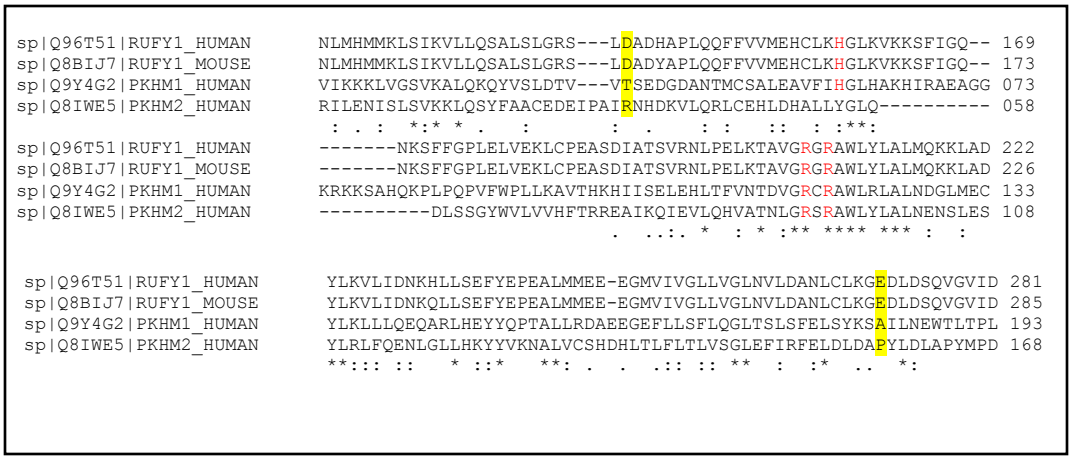

Fig 9: Alignment of the RUN domains of known Arl8b effectors with RUFY1. The starting and ending of RUN domains are highlighted in yellow. The conserved arginine and histidine residues are colored red .

Thus, the objective of my study was to investigate if Rabip4' is a novel Arl8b effector. Further, I wanted to study if this interaction is direct and mediated via the RUN domain of Rabip4'.

1.2 Materials and methods

1.2.1 Plasmids

His- Arl8b WT in pET15b+, GST-PLEKHM1 1-300 and 1-198 in pGEX6p2, GST-mouse Rabip4'(prime) 1-302 in pGEX6p2, GST-human Rabip4'(prime) 1-296 in pGEX4T3, pGEX4T3, Arl8b WT- HA in pCDNA3.1(-), GST-human Rabip4 1-194 in pGEX4T3, Rab4Q76L in pEGFPC1, Rab14 WT in pEGFPC1 (Coppens lab) were obtained from lab stock boxes. Human Rabip4'(prime) C terminal Flag was obtained from GENSCRIPT.

Details of rest of the clonings have been indicate table 1.

1.2.2 Antibodies

Mouse anti-poly His was procured from Sigma, Goat anti-mouse IgG HRP, Mouse anti-human LAMP1 from BD Transduction Laboratory, Rabbit anti- human EEA1 was procured from ABCAM, Mouse anti-HA epitope tag was obtained from Covance. Alexa-Fluor-conjugated secondary goat anti-mouse-IgG and goat anti-rabbit-IgG antibodies 488, 568, 647 were purchased from Invitrogen. Mouse anti-FLAG epitope tag antibody was procured from Sigma.

1.2.3 Transfections for colocalization experiments

HeLa Cells were allowed to grow on coverslips for 18-24 hours before being transfected with the desired plasmid constructs. It was checked whether the cells had spread well on the coverslips. 750µl Complete DMEM media was added to the required number of wells of a 24 well plate. The plate is placed in the CO_2 incubator

to warm up the media to physiological temperature. Meanwhile the transfection mixture is prepared in Tissue culture hood (contents added in the order mentioned below) :

0.5µg of each plasmid construct required

50µl OptiMEM

2.5µl Poly ethyleneamine transfection reagent/ plasmid construct.

This mixture is allowed to stay for 20-30 minutes and added on the coverslips (placed in the media containing wells)

1.2.4 Immunostaining

HeLa cells were plated on coverslips and transfected with required combinations of plasmid DNA. Post 12 to 16 hours of transfection, cells were fixed with 4% of PFA in PHEM (60mM PIPES, 10mM EGTA, 25mM HEPES, 2mM $MgCl_2$) buffer for 10 minutes and washed with 1X PBS thrice. Further, cells were incubated with primary antibody in PHEM buffer + 0.2% Saponin for 1 hour followed by three times wash with 1X PBS. Cells were then incubated with secondary antibodies conjugated with Alexa fluorophores in PHEM buffer + 0.2% Saponin for 30 minutes. After three washes with 1X PBS, the cells were mounted onto glass slides using Fluoromount G (Southern Biotech) and were analyzed using confocal microscopy.

1.2.5 Imaging using Confocal Microscopy

Zeiss Laser Scanning Microscope 710 confocal microscope was used for imaging immunostained coverslips. Images were captured using 60X objective lenses. Optical parameters were set at the beginning of imaging a single coverslip and kept constant throughout. Excitation and emission wavelength of the various fluorophores were adjusted with the help of filters. The images were edited using Photoshop, ImageJ Launcher and Zeiss Program. (Referred from Arsila Ashraf (2016) Thesis)

1.2.6 Protein purification of GST tagged proteins

Composition of solutions:

Superbroth (Tryptone, Yeast Extract and Glycerol) and salt solution (KH_2PO_4 and K_2HPO_4)

Prep Buffer for GST proteins (Tris-HCl pH 7.4, 20mM NaCl, 150mM EDTA, 1mM DTT, 0.5mM Triton X 100, Glycerol 5 %)

The gene of interest was transformed into *Eschericia coli* BL21 competent cells. The primary culture was set up using a single colony from a freshly transformed plate in 3ml LB with antibiotic resistance (1:1000) at 37 °C with shaking of 220 rpm for 12 h. The secondary culture was set up by adding 1% of primary culture to media comprising of Superbroth , 10% salt solution and antibiotic (1µl/1ml) . The flask was kept at 37 °C incubation with shaking of 220 rpm. The culture was induced at OD_{600} 0.5-0.6 with 0.5mM IPTG (usually takes 1.5-2 hrs). The culture was incubated at 16 °C for 12 hours or at 30°C for 5 hours with shaking at 220 rpm. The culture pellet after centrifugation was resuspended in Prep Buffer with protease inhibitor cocktail tablet and 1 mM PMSF. The pellet was sonicated at 20 amplitude with pulse-on-time 20 sec and pulse-off-time 1 minute. The sonicated suspension was centrifuged at 10,000 rpm for 25 min at 4 °C. Meanwhile Glutathione beads were washed twice using PBS and once using Prep Buffer in swinging bucket centrifuge at 4 °C for 1500 rpm, 2 min. These beads were added to the supernatant in a hula-mixer at 4 °C for 2 h to allow for binding of the protein to beads. The protein bound to beads was washed 10 times with 4 slurry volumes of Prep Buffer and 1mM PMSF.

1.2.7 Elution of proteins

The bead bound proteins were eluted using Glutathione elution buffer (composition mentioned below). This involved addition of 300µl elution buffer and overnight elution at 4°C using Hula mixer. This mixture is subjected to centrifugation at 1500 rpm, 4°C for 7 min. The supernatant is collected as F1 fraction. Further, a second elution step at room temperature is conducted for 30 min. The supernatant from this step is collected as F2 fraction. Both F1 and F2 are pooled and Absorbance at 280nm is noted.

This is subjected to a buffer exchange using cold 1X PBS and 1mM protease inhibitor. Amicon Ultra-0.5 Centrifugal Filter Devices were used for the purpose.

Glutathione elution buffer:

50mM TrisCl pH 8, 150mM NaCl , 10mM reduced glutathione; pH adjusted to 8.

1.2.8 Western Blotting

After running SDS PAGE, the gel was transferred onto a PVDF membrane by creating a sandwich of the gel and membrane between filter papers for two hours. The blot was blocked using 10% skim milk in 0.05% PBST (PBS, 0.05% Tween 20). After blocking, the blot was washed with 0.3% PBST and incubated with the primary antibody in 0.05% PBST for 2 hours. The blot was washed with 0.05% PBST for three times and incubated with secondary antibody in 0.05% for 1 hour. After three washes with 0.3% PBST, enhanced chemiluminescence substrate reaction was performed using Thermo Scientific ECL and the signal was obtained on the X- ray film.

1.2.9 Purified protein-protein interaction assay

Method I

Appropriate amount of bead bound GST protein was blocked using 5% BSA for appropriate time (mentioned with each of the results). After 2hrs, the beads were washed twice with ice cold 1X PBS and once with TAP lysis buffer (20mM TrisHCl pH 7.4, 150mM NaCl, 1mM Sodium orthovandate, 1mM NaF, 1mm MgCl2 1 % NP-40) with 1X Protease Inhibitor and 1X PMSF for 2 minutes at 1500 rpm and 4^0C. The beads were then incubated with appropriate amount of prey protein in TAP lysis buffer for appropriate amount of time. The unbound proteins were washed off with TAP wash buffer five times. The bound proteins were eluted from the beads by boiling with 4X sample buffer at 99 ^{0}C for 10min. The input samples and the GST pull down samples were run on SDS PAGE gel. The gel was subjected to Western Blotting analysis.

Method II

A slight modification of the above method involved elution of the bait proteins using the Glutathione elution buffer (composition as stated above) and rebinding them onto GSH beads.

Method III

10µl of glutathione slurry was blocked using 5% BSA for 2 hrs. Meanwhile, 3µg of eluted bait and 1µg prey protein were allowed to bind in 300µl TAP lysis buffer (composition as mentioned above) for 1 hour. The blocked beads were washed twice with ice cold 1X PBS and once with TAP lysis buffer. The solution of proteins was now allowed to bind to the blocked beads for 15min. The unbound protein was washed off using TAP lysis buffer five times. Rest of the steps were followed as stated in Method I.

1.2.10 **Silver staining**

It is a sensitive method to look at proteins of the order 0.5µg. The gel of purified protein-protein interaction was subjected to silver staining to look at which condition prevents non specific interaction .

The Pierce Silver Stain for Mass Spectrometry 24600 kit was used

The manual given along with the kit was followed for the solutions and the procedure.

1.2.11 **Dot Blot assay**

For dot-blot assay, 5µg purified GST and GST-fusion proteins were spotted on

nitrocellulose membrane, blocked with 10% skim milk in 0.05% PBS-Tween 20 and washed.

The blots were then incubated overnight with purified His-Arl8b (in 2% skimmed milk in 0.05% PBS-Tween 20) at 4°C. The blot was further probed for analysis as described in the western blotting protocol.

Chapter 2

Results and Conclusions

2.1 Results and Discussion

2.2 Future Prospects

2.1 Results and Discussions

All proteins are human and wild type unless mentioned otherwise. IP4' refers to Rabip4'.

Transfection and immunostaining was performed as indicated in the materials and methods section. HeLa cells were transfected with HA-Arl8b and Rabip4'(prime) FLAG in mammalian expression vector. Colocalization studies performed in the lab by me and others indicate that Rabip4'(prime) colocalizes with Arl8b. Moreover, Rabip4' colocalizes to EEA1 positive structures (already known), but not to LAMP1 positive structures (indicated in Fig 10).

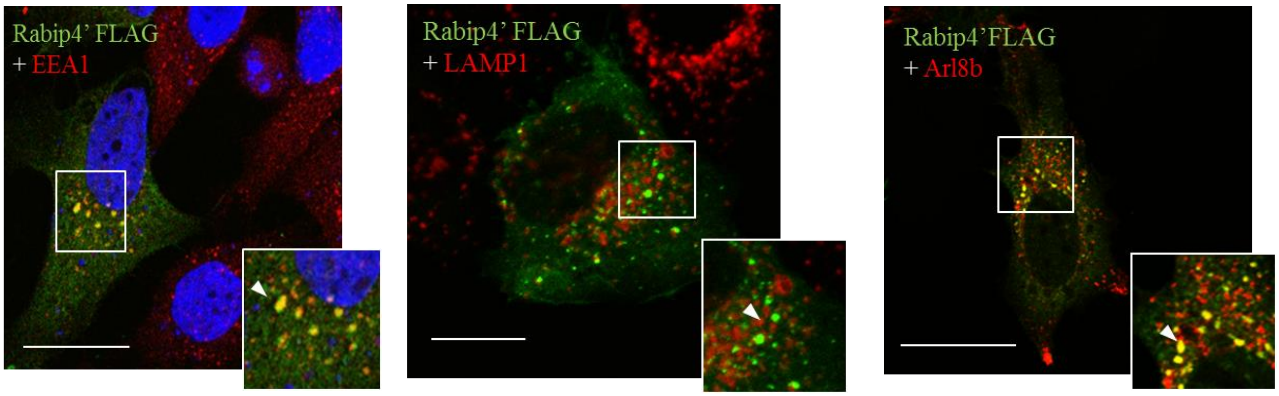

Fig 10: Colocalization images of Rabip4' FLAG with EEA1, LAMP1 and HA Arl8b. This figure indicates that Rabip4' (green) colocalizes with EEA1 positive structures (early endosomes), Arl8b and not with LAMP1 structures. EEA1, LAMP1 and Arl8b are in red.

GST Pull down experiment in our lab suggested that GST tagged human Rabip4' pulls down HA- Arl8b. Purified protein-protein interaction assay is done to investigate direct binding of two proteins. This had been previously performed in the lab to observe the interaction of Arl8b with PLEKHM1 RUN domain containing region (1-300).

Hence, we decided to proceed with the purified protein-protein interaction assay to investigate if Rabip4' interacts directly with Arl8b. For this, GST tagged proteins were taken as bait and His tagged Arl8b as prey. Previous experiments in the lab have shown that the experiment does not work if performed the other way round i.e. using His Arl8b bound to cobalt resin and eluted GST tagged proteins. Proteins purified for the assay are indicated in Fig 11.

GST was taken as the negative control, as it is known that it doesn't interact with Arl8b and GST PLEKHM1 (1-300) was used as the positive control as it is known to interact with Arl8b. For PLEKHM1, 1-300 is the RUN domain containing region. We took 1-300 regions of our test proteins as well- Human and mouse Rabip4', as they are their RUN domain containing regions.

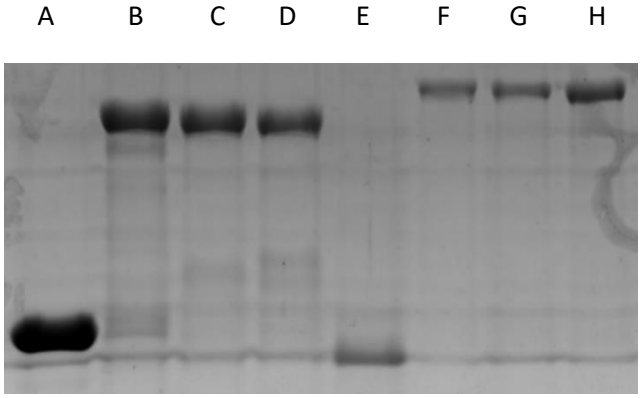

Fig 11: Proteins that were used for purified protein-protein interaction assay. Lane A indicates GST, lane B indicates GST PLEKHM1 (1-300), lane C indicates GST Mouse Rabip4' (1-300), lane D indicates Human Rabip4'(1-298). The prey protein His Arl8b was run in lane E. BSA standards have been run in lanes F-H.

The initial experiment involved the use of method I. 6µg of GST tagged proteins that were bound to Glutathione beads were taken. They were blocked for either 2 hours or overnight with 5% BSA. Excess BSA was washed off as described in the methods section. The bait proteins were then incubated with 4µg prey protein for 45 minutes in TAP lysis buffer. Three washes were given as described earlier. The rest of the procedure was followed as in the methods section. The blots were probed using Mouse anti-His tag antibody in 1:5000 dilution.

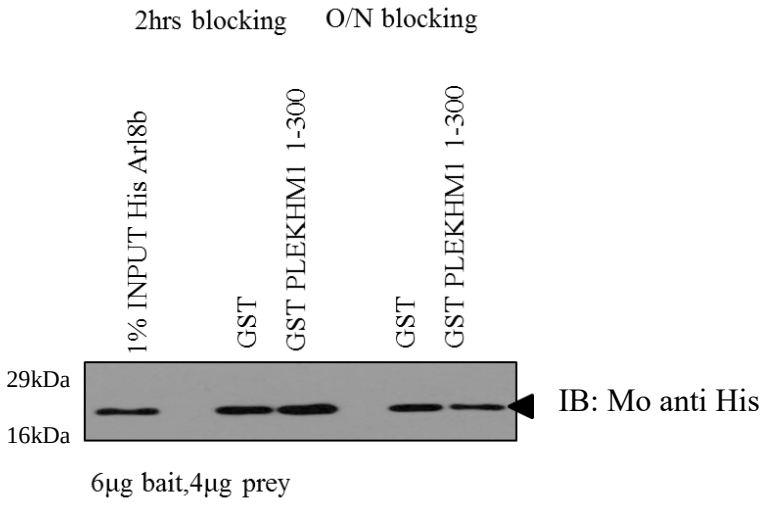

Fig 12: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. 6µg of GST tagged bait and 4µg prey proteins were used and a great non specificity was observed.

A non-specific interaction with GST was observed in case of using the above concentrations and blocking times (Fig 12). We then decided to reduce the concentrations of proteins used and observe what happens in that case.

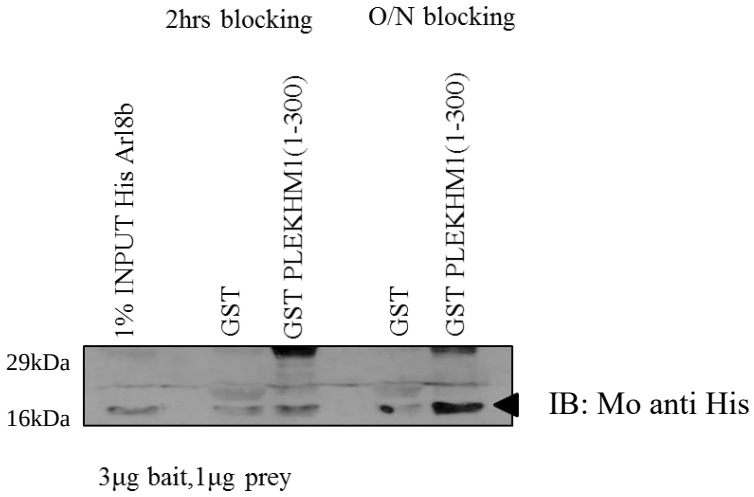

Fig 13: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. With reduced amounts of proteins i.e. 3µg of GST tagged bait and 1µg prey protein we observed lowered non specificity.

On reducing the amount of proteins used and blocking the bait proteins overnight, considerably less non -specific binding was observed (Fig 13). We decided to repeat the experiment to be sure about the results.

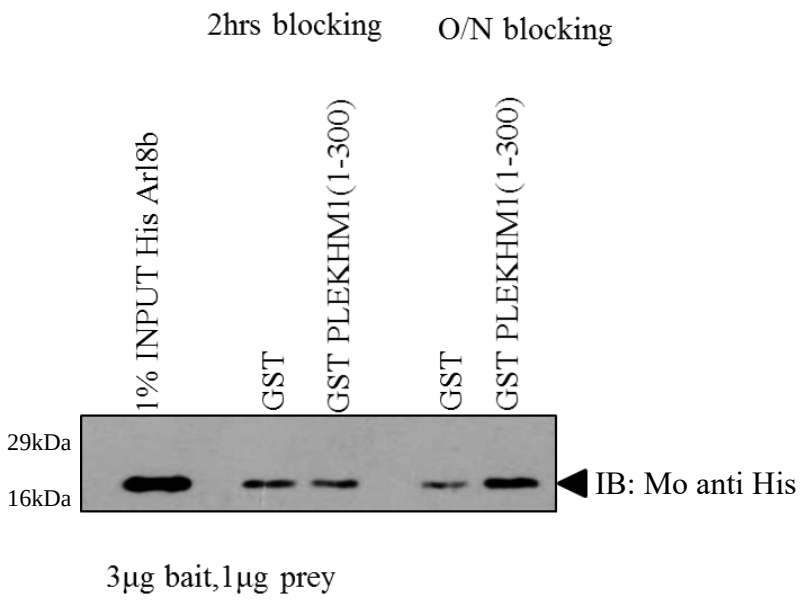

Fig 14: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Repeat of the experiment in fig 13, indicates reduced non specific binding with GST.

The repeat of the experiment also showed a reduced non-specific binding with GST, when the bait proteins were blocked overnight (Fig 14). Thus, we decided to proceed with 3µg GST tagged bait, 1µg prey protein and overnight blocking conditions. Moreover, the binding time was reduced to 15 minutes and maintained at that for the rest of the experiments. Next, we used GST Mouse Rabip4' (1-300) and GST Human Rabip4' (1-298) along with GST and GST PLEKHM1 (1-300).

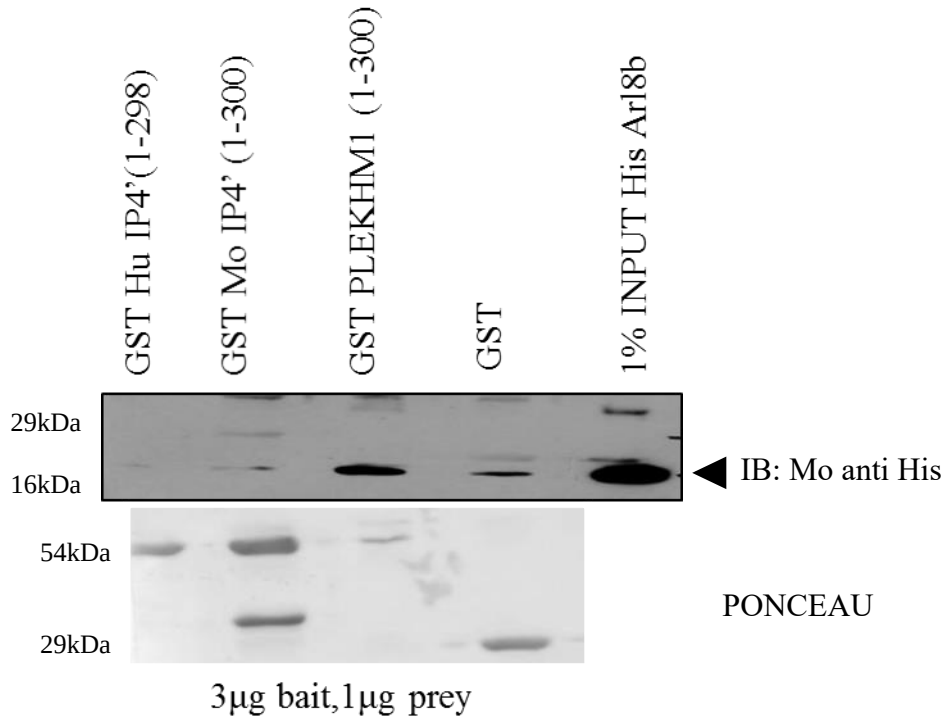

Fig 15: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Test proteins and both the positive and negative controls were used.

A considerably lowered non- specific binding with GST was observed. The bait proteins were bound to beads and the amount of beads taken were of the order of 2µl. This amount would vary with each assay. To ensure the reproducibility of experiments, we decided to elute the GST tagged bait proteins using Glutathione elution buffer and then rebound the appropriate amount to Glutathione slurry (method II).

The rest of the protocol was followed as mentioned for method I, with slight changes.

5µg of the bait proteins were bound to 15µl of slurry, the beads were blocked for either 2 hours or overnight. The bait proteins were allowed to bind to 5µg of prey protein for 15 minutes. The concentration of Mg2+ divalent cations were increased in the TAP lysis buffer. These divalent cations are required for the binding of small GTPases to GTP. The number of washes was increased to 5.

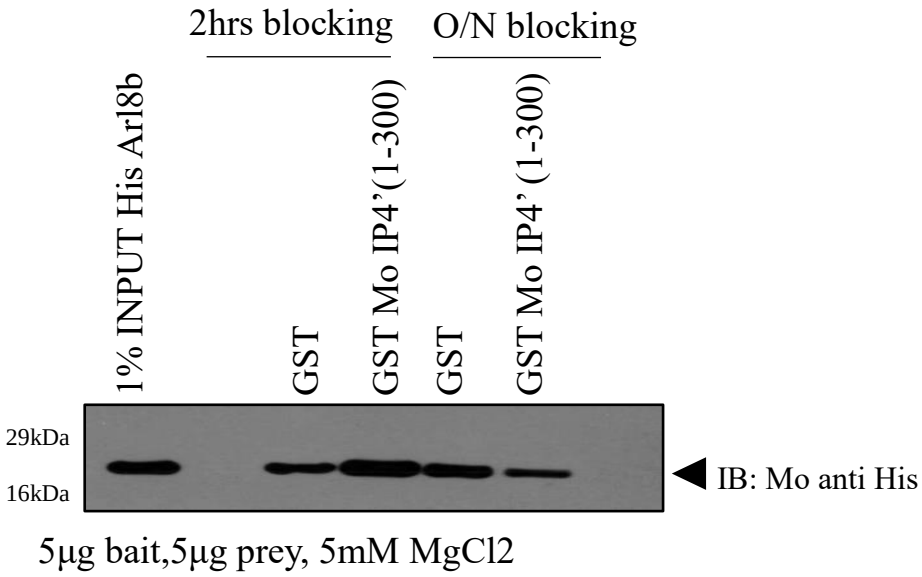

Fig 16: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Eluted proteins were taken and rebound to beads, moreover the MgCl2 concentration was increased.

A considerable non-specific binding with GST was observed with the above mentioned conditions (Fig 16), thus we decided to check what happens in case the amounts of proteins used are reduced. The amount of prey protein was reduced to 2.5µg, rest of the conditions were kept the same.

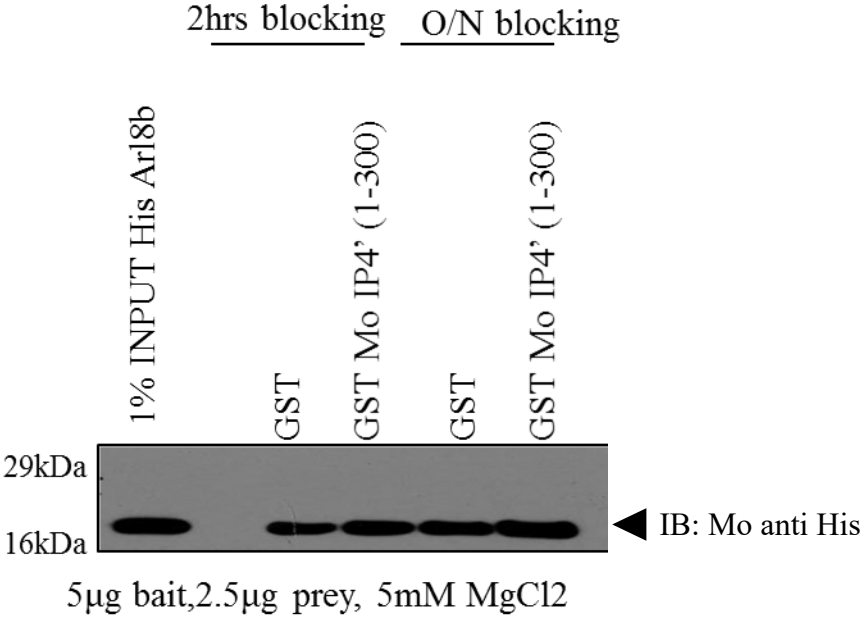

Fig 17: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Reduced amounts of protein as compared to Fig: 16 were considered. However, Non-specific interactions were observed.

A considerable non- specific binding with the negative control was observed (Fig 17). We decided to revert back to using 1mM MgCl2, with rest of the conditions staying the same.

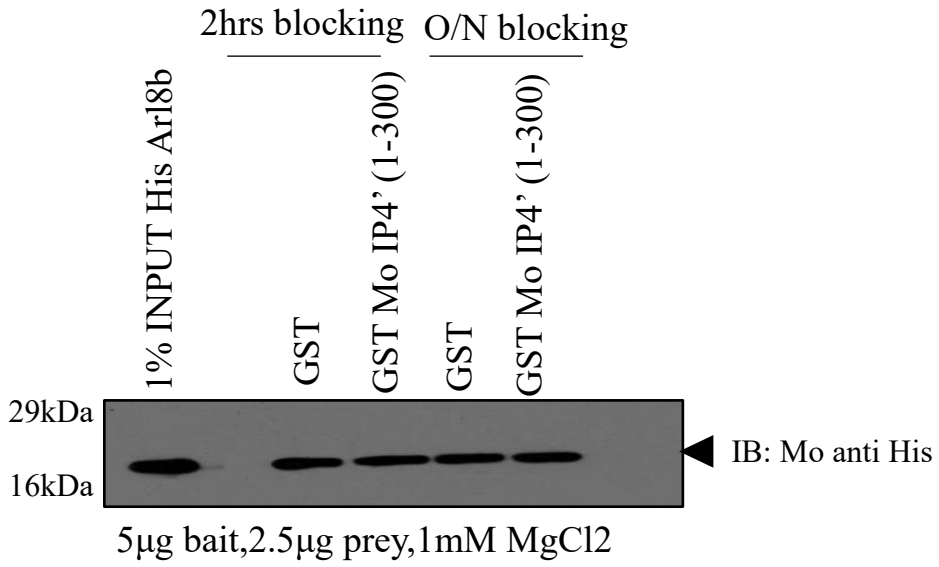

Fig 18: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Non specific interaction continued to be observed.

Even on reducing the MgCl2 concentration to the original, non specific interaction with GST continued to be observed (Fig 18). Certain protocols suggested the use of glycerol to get about the problem of non specific interactions. We decided to go ahead with the same. In the next set of experiments, we decided to use glycerol in the lysis buffer. For this, either 5% glycerol was added to the lysis buffer or not added and blocking was done for 2 hrs.

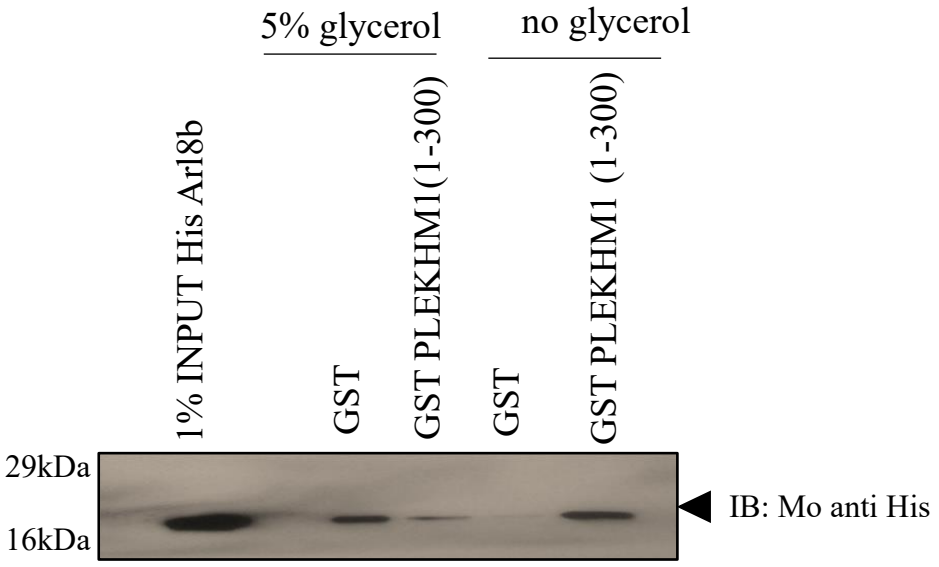

Fig 19: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Reduced non-specificity was observed in case of excluding glycerol from the lysis buffer.

We observed a very reduced, close to nil non-specific binding when glycerol was not added in the lysis buffer (Fig 19). We decided to repeat the experiment and observed a similar trend (Fig 20). Hence, it was decided to not include glycerol in the lysis buffer.

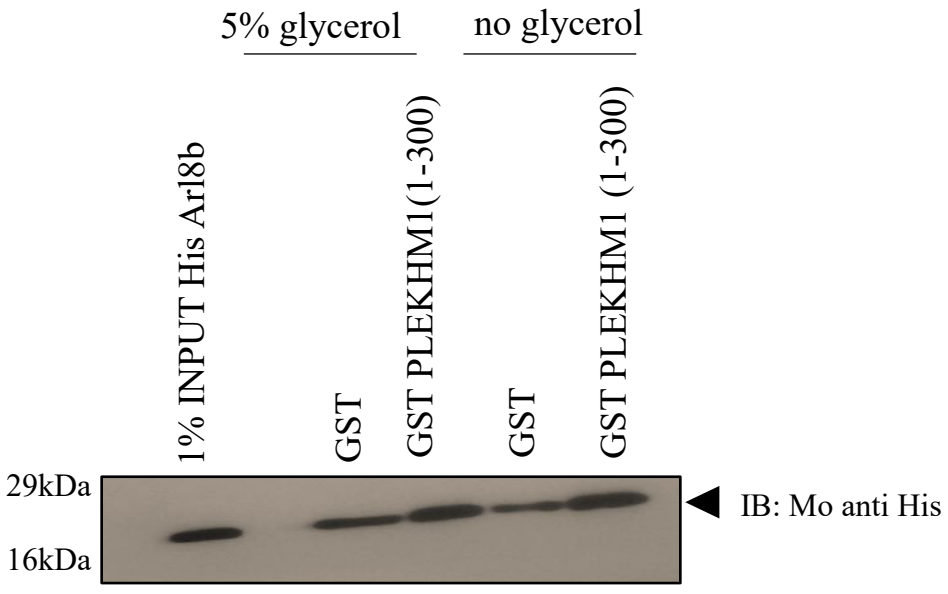

Fig 20: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Repeat of experiment in Fig: 19.

For the next experiment, the detergent concentration in the wash buffer was increased to 1%.

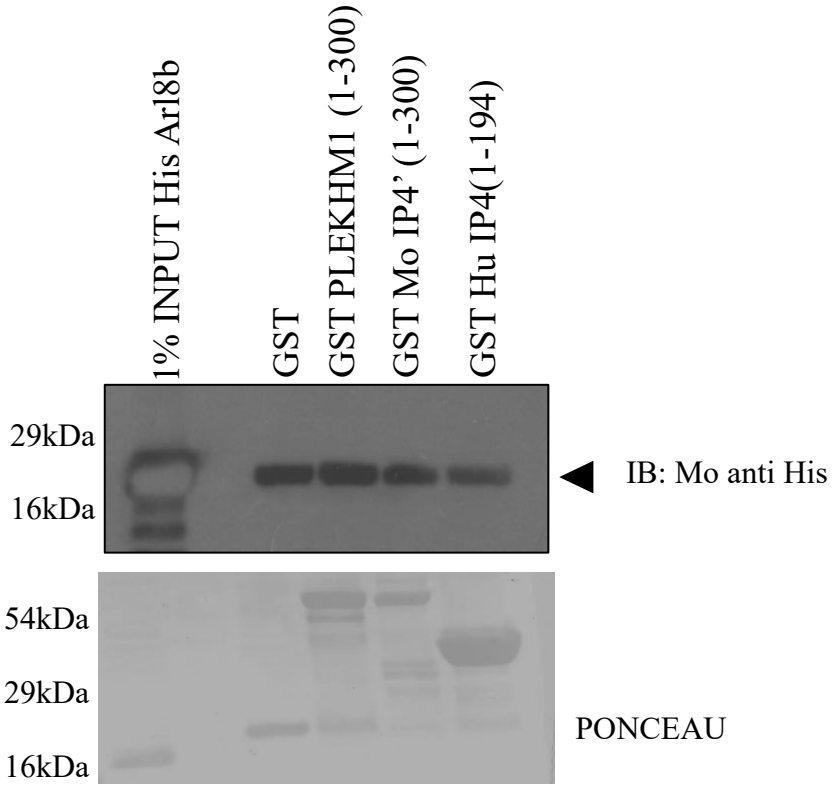

Fig 21: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. A continued non specific interaction with GST is observed even on increasing detergent concentration.

We observed non-specific binding with GST, which was comparable to binding with the other proteins (Fig 21).

For the further experiments, we decided to elute both the GST tagged bait proteins and His Arl8b and bind them in solution (as described in detail in Method III).

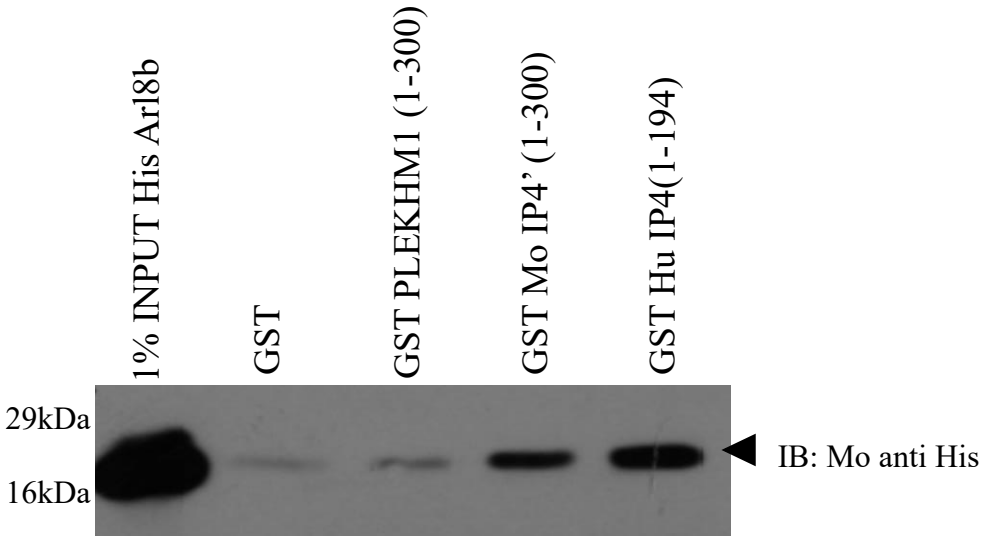

Fig 22: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. The bait and prey proteins were allowed to bind in solution. A reduced non-specificity was observed.

Almost negligible non specific binding was observed, however an issue with this experiment was that a very less binding with the positive control was observed (Fig 22). It was thus decided to repeat the experiment.

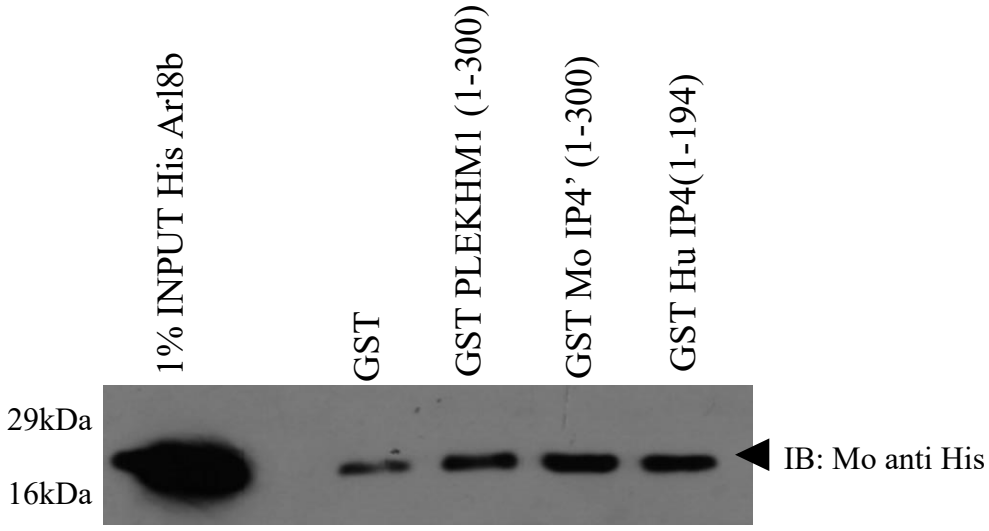

Fig 23: Purified protein-protein interaction assay to investigate interaction between His Arl8b and GST tagged bait proteins. Repeat of experiment indicated in Fig: 22

The repeat experiment indicated a lowered nonspecific binding with GST (Fig 23). However, it was not as low as in the previous experiment.

Table 1: List of clonings

This table indicates the clonings I have done as part of my thesis project. The expression of these clones was tested by me and Divya Khatter (Graduate student in the lab). The sequencing results were analyzed and verified. Cloning 1 was done to investigate the colocalization of Arl8b and Rabip4' ΔRUN mutant. Cloning 2 was done to investigate the colocalization of Rab14, Rab4 with clone 2. Cloning 3 was performed to observe the colocalization of Rabip4 with Arl8b.

S.No.	Insert	Vector	Primer # (MSI-)	Restriction Enzymes
1	Human Rabip4' ΔRUN FLAG	pCDNA 3.1(-)	598,599	EcoRI, BamHI
2	Human Rabip4' Δ615-625 FLAG	pCDNA 3.1(-)	601,602	EcoRI, BamHI
3	Human Rabip4 FLAG	pCDNA 3.1(-)	599,600	EcoRI,BamHI

2.2 Future Prospects

In summary, it is very difficult to conclude whether indeed there is a direct interaction between Rabip4' and Arl8b or not. In most of the assays performed, there appears to be a non-specific binding with GST. The assay would need to be further standardized to eliminate this non-specificity.

By far, binding the eluted bait and prey proteins in solution seems to be a good method to assay the interaction. However, the experiment would have to be repeated few more times to determine if we should indeed invest on this method.

We could incorporate better controls such as His Rab7 in future experiments. It would also be interesting to investigate what happens to Rabip4'-Arl8b binding when the conserved arginine residues of Rabip4' RUN domain are mutated.

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