Comparison of RUN Domain-containing Proteins for binding to the Small GTPase Arl8b

Partha Sankar R P S

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Certificate of Examination

This is to certify that the dissertation titled **"Comparison of RUN Domain-containing Proteins for binding to the Small GTPase Arl8b"** submitted by Mr. Partha Sankar R P S (MS11049) for the partial fulfilment 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. Mahak Sharma (Supervisor)	Dr. Samarjit Bhattacharya (Committee Member)	Dr. Kausik Chattopadhyay (Committee Member)

Dated: April 20, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Mahak Sharma at the 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.

Partha Sankar R P S

(Candidate)

Dated : April 20, 2016

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)

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List of Abbreviations

BSA : Bovine Serum Albumin

EDTA : Ethylenediaminetetraacetic acid

FBS : Foetal Bovine Serum

GST : Glutathione S-transferase

GTP : Guanine Triphosphate

NP-40 : Nonyl Phenoxypolyethoxylethanol-40

PFA : Paraformaldehyde

HA : Hemagglutinin

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

HRP : Horseradish Peroxidase

IgG : Immunoglobulin G

IPTG : Isopropyl β-D-1-thiogalactopyranoside

LAMP1 : Lysosomal-associated membrane protein 1

LB : Luria Broth

PMSF : Phenylmethane Sulfonyl Fluoride or Phenylmethylsulfonyl Fluoride

SDS PAGE : Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

M1: Pleckstrin homology domain-containing family M member 1 (PLEKHM1)

Contents

Abstract

Cells have a dynamic environment which varies with space and time. In such a heterogeneous environment, homeostasis can only be maintained by the regulated trafficking of molecules in and out of the cell, a generalized process termed as endocytic and membrane trafficking. During endocytosis, vesicles that pinch off from the membrane are transported by motor proteins to different target organelles, and finally fuse with the destination to deliver the cargo molecules. The entire mechanism of vesicular fission and fusion is rather complex wherein a number of molecular players are involved. Small GTPases are active regulators in this process. They recruit different effector molecules that promote vesicular budding, transport, tethering and fusion. RUN domain containing proteins are well-known effectors of small GTPases, and are known to play a key role in mediating membrane trafficking, organelle motility and endosomal fusion. Arf-like (Arl) GTPase, Arl8b, is a lysosomal small GTPase that facilitates late endosome-lysosome fusion. SifA and kinesin-interacting protein (SKIP) is a well known effector of Arl8b that regulate the anterograde motility of lysosomes by binding to Arl8b via its RUN domain. Recently our lab has identified a new effector of Arl8b, PLEKHM1 (Pleckstrin homology domain-containing family M member 1), which also binds to Arl8b via its RUN-domain. So as a part of this thesis work, I have analyzed the interaction between Arl8b and the other RUN-domain containing proteins that specifically localizes to late endosomes/lysosomes. Our preliminary results indicate that the RUN-domain of SKIP recruits Arl8b much more strongly as compared to PLEKHM1 or RUBICON (Run domain protein as Beclin-1 interacting and cysteine-rich containing).

Chapter I

Introduction

1.1 Small GTPases

Small GTPases form a class of GTP binding proteins known to regulate various cell signaling and membrane trafficking processes inside the cell. They act like molecular switches that could bind to GTP or GDP that differ in its confirmation. In their GDP bound form, they are considered as inactive whereas the GTP bound form is the active state and recruit downstream effector proteins that bring about a cellular response. GTPase activating proteins (GAPs) stimulate GTP hydrolysis and Guanine nucleotide exchange factors (GEFs) cause the GDP dissociation. Small GTPases are known to alternate in cytosol or membrane with the help of a farnesyl or any such moiety (shown in Figure 1).

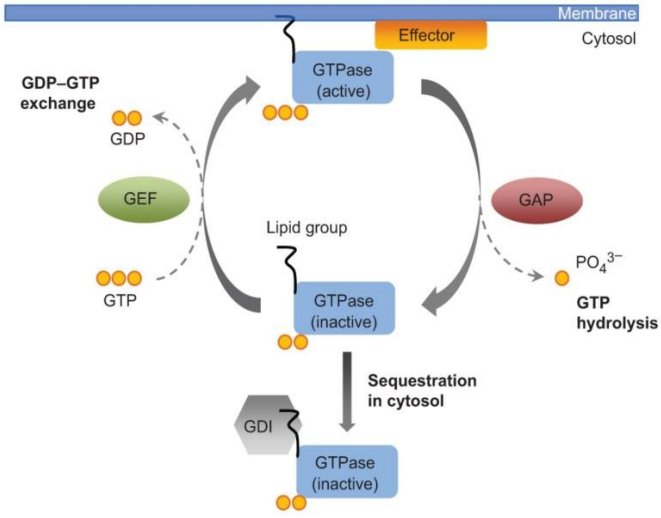

Figure 1: **GTP-GDP cycle of a GTPase.** The GTPase (Rab, Arf or Arl) is loaded with GTP by the guanine nucleotide-exchange factor (GEF). RanGTP adopts a distinct conformation that allows it to interact with an effector protein. Hydrolysis of the GTPase GTP to GDP by a GTPase-activating protein, GAP renders it inactive (Adapted from Bento *et al* 2013).

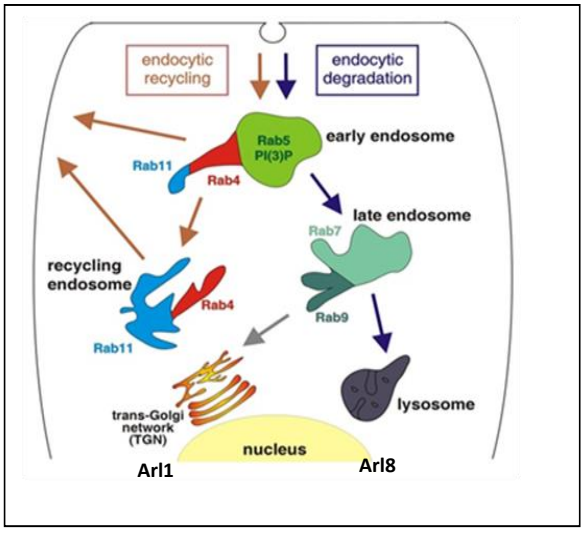

Figure 2: **GTPases involved in the endocytic pathway.** Different GTPases localize to different compartments within the cell. Rab5 localize to early endosome. Rab7 localize to late endosome. Arl8 is found to localize lysosome. Arl1 localize to trans Golgi network (From http://zerial.mpi-cbg.de/page/project-rab-gtpases)

Among the GTPases, the Rabs and the Arfs are well-known regulators of intracellular traffic and localize to distinct compartments within the cell (shown in Figure 2). Very recently, Arf-like (Arl) family of GTP-binding proteins has been shown to play very important role in controlling membrane trafficking events inside the cells. Currently, Arl family includes 20 members, and they localizes to distinct compartments inside the cell. For example, Arl1 and Arl15 localize to the trans-Golgi network, Arl13b localizes to recycling endosomes, Arl4 is present in the nucleus etc. Arl8b, which is the main focus of my thesis work, is a small GTPase of the Arl family that specifically localizes to lysosomes and regulates late endosome-lysosome fusion, and lysosome motility (shown in Figure 3).[1] Previously, it has been shown that Arl8b regulates lysosome motility by binding to its effector, SKIP.[13] A recent study from our lab has shown that Arl8b also regulates late endosome-lysosome fusion by binding to Vps41 subunit of the HOPS (Homotypic Fusion and Protein Sorting) complex, a multi-subunit complex that mediates vesicle tethering and fusion. [2, 10]

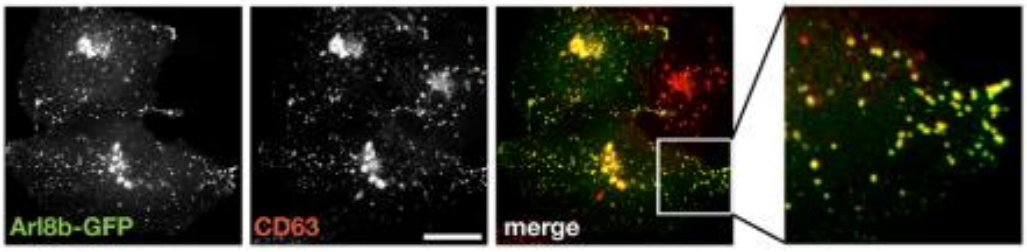

Figure 3: Arl8b localize to lysosomes. Confocal micrographs of COS cells transfected with plasmids expressing Arl8b-GFP (green channel) from a CMV promoter. CD63 localize to lysosomes (red channel) (Adapted from Hofmann and Munro 2006).

1.2 RUN domains

RUN-domain (an acronym for RPIP8, UNC-14 and NESCA) containing proteins are well-known effectors of small GTPases, and have been shown to regulate intracellular trafficking pathways inside the cell. The crystal structure of several RUN-domain proteins along with their cognate GTPases has been solved. Based on these studies, RUN-domain is shown to adopt an alpha helical structure with hydrophobic amino acids at conserved positions (shown in Figure 4). Moreover, basic amino acids present in the RUN domain are critical for mediating interaction with GTPases.[3,4]

Besides binding to GTPases, RUN-domain containing proteins have been shown to interact directly with motor protein and cytoskeletal elements, and thereby promote endosomal fusion and vesicular transport. A hypothesized model depicting how vesicular traffic is regulated by a complex of a small GTPase and the RUN-domain containing protein is shown in Figure 5. Vesicles move on the microtubules or actin when bound to different motor proteins which are recruited on to the vesicular membrane by small GTPases and RUN domain-containing proteins.

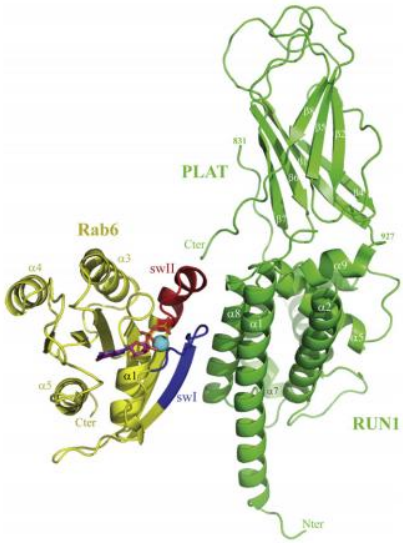

Figure 4. Ribbon Model of Rab6-Rab6IP1 complex. There are eight alpha helices in the RUN domain of Rab6 interacting protein 1(Green). Rab6, GTPase is shown in yellow (Adapted from Recacha *et al* 2009)

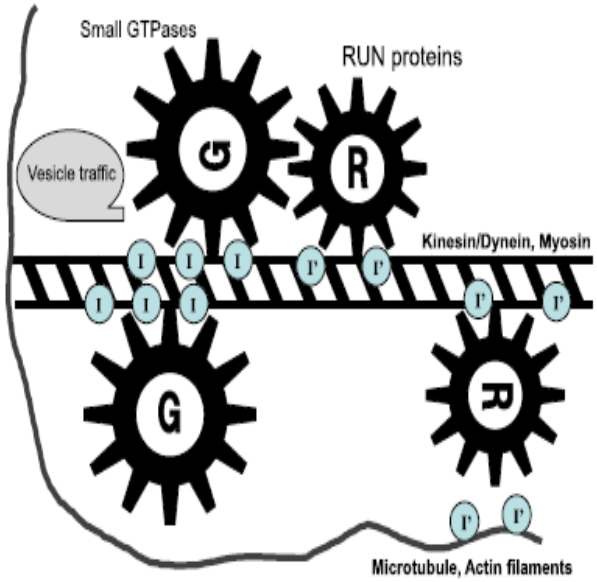

Figure 5. Hypothesized model of vesicular traffic by RUN domain containing proteins. RUN domain-containing protein associate with small GTPases (G), RUN domain-containing proteins (R), motor protein (I') and cytoskeleton (Adapted from Yoshida *et al* 2011).

1.3 Organelle Positioning

In eukaryotic cells, there is a distinct division of cellular functions. This is brought about by compartmentalized structures – the organelles – within the cell, each possessing a unique set of macromolecules. Further the relative spatial organization of organelles is of prime importance in a cell. Several cellular processes such as polarity, signaling, and growth are

intricately connected to the subcellular localization of various organelles.[5] As of now, very little is known about the mechanisms that control organelle positioning, and the effect of organelle positioning on different cellular processes (shown in Figure 6).

In recent years the role of motor proteins in controlling organelle positioning and motility is well characterized. For example, kinesin and dynein motor proteins regulate anterograde and retrograde movement of organelles, respectively, on microtubule network. Similarly, myosin motor proteins mediate organelle movements on actin cytoskeleton inside the cell.

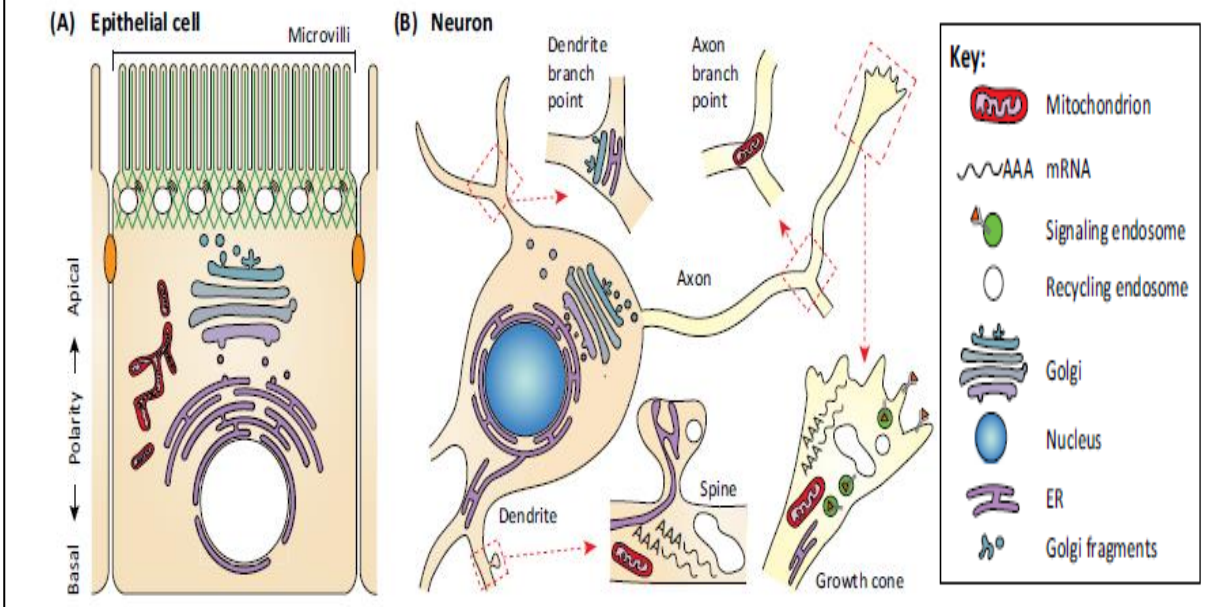

Figure 6. Organelle Positioning in Different Types of Cell (A) Epithelial cells showing nucleus placed basally and ER and Golgi placed apically. Actin polymerization is induced by the microvilli, recycling endosomes and their kinases are positioned underneath the apex. Actin is represented by green lines in the figure. (B) Neurons also shows polarity in terms of organelle distribution. ER is found in the cell body, near dendritic shafts and at growth cones. Mitochondria is found near dendritic spines, axonal branch points, and growth cones. Recycling endosomes are found at the tip of growth cone and at the base of dendritic spines to facilitate delivery of membranes and receptors to the surface. (Adapted from Bergeijk *et al*, 2016).

Very recently, it has been shown that positioning of an organelle inside the cell can also control the cellular signaling pathways. The subcellular localization of lysosomes can dictate cell motility, cell migration and mTORC1 (mammalian target of rapamycin complex 1) activation and the induction of autophagy. In a nutrient rich environment, lysosomes relocate

to the periphery of the cell and recruit mTORC1. Activated mTORC1 phosphorylates effector proteins to cause changes in cell growth and inhibit autophagy. In sharp contrast, starvation causes a preferential clustering of lysosomes at the perinuclear region by the acidification of cytoplasm. mTORC1 is not activated but autophagy is induced. (shown in Figure 7). Overexpression of kinesins or Arl8 enhanced mTORC1 activity by the movement of lysosomes to the cell periphery.[6] This clearly depicts the importance of lysosome positioning - by the activation of mTORC1 - in cell growth and proliferation.

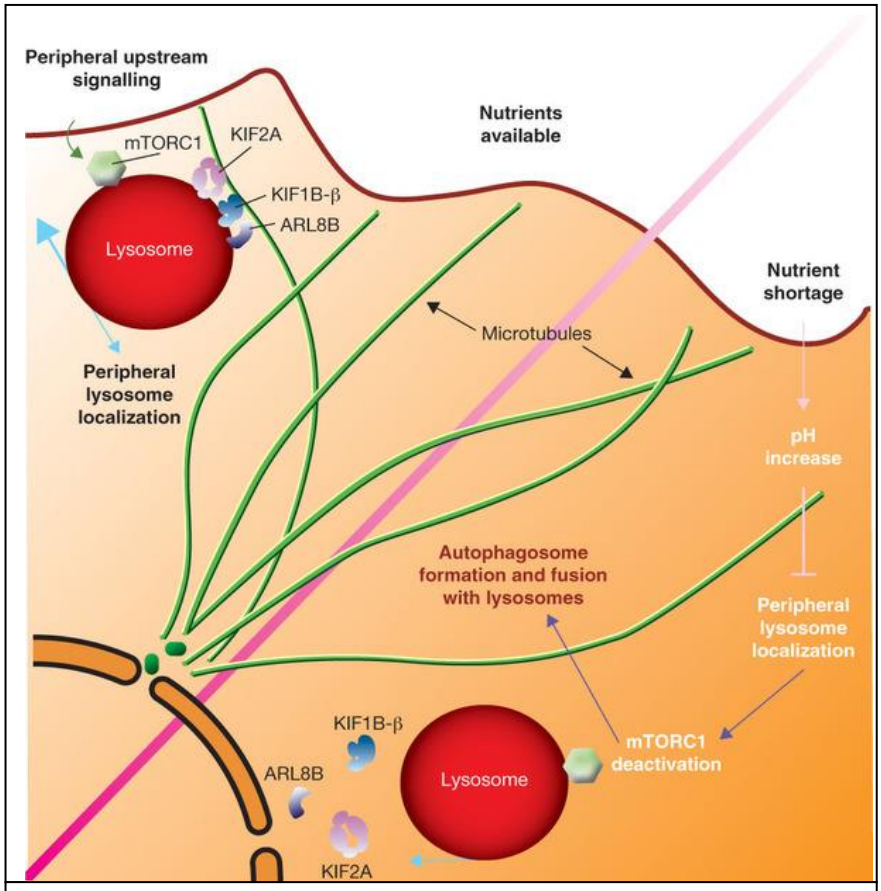

Figure 7. The positioning of lysosomes regulates mTORC1 activity and autophagy. When nutrients are available, lysosomes are maintained at the periphery of the cell through a microtubule-dependent mechanism involving kinesins (KIF1B-β and KIF2A) and the GTPase Arl8b. mTORC1, which is bound to the cytoplasmic surface of lysosomes, is activated by upstream signalling when at the cell periphery. In the absence of nutrients, kinesins and Arl8b are released from microtubules, resulting in the accumulation of lysosomes in the perinuclear area and inactivation of mTORC1 (Adapted from Pous *et al* 2011).

In a cellular model of Huntington's disease (HD), perinuclear accumulation of lysosomes was increased in HD knock in mice and primary fibroblast from an HD patient indicating a functional importance of lysosomal positioning in HD.[7] In hepatocytes, high copper

concentration causes the repositioning of the ATPase, ATP7B from Golgi to lysosome. Later copper is recruited from the cytosol to the lumen of lysosome which is later exocytosed as bile (shown in Figure 8). [8]

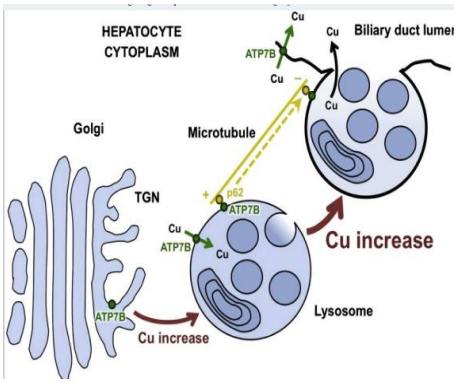

Figure 8. Wilson Disease Protein ATP7B Utilizes Lysosomal Exocytosis to Maintain Copper Homeostasis. Under conditions of high copper concentrations, ATP7B translocates from Golgi to lysosomes. Further, lysosomes exocytose to release Copper (Adapted from Polishchuk *et al* 2014).

A number of proteins are known to regulate the position of lysosomes. It would be interesting to identify novel factors involved in organelle positioning and the mechanism involved therein. Aberrant lysosomal positioning is implicated in cellular model of HD. It would be interesting to see if the position of lysosome could be regulated *in vivo* to help treat such neurodegenerative diseases.

1.4 RUN-domain containing proteins as Arl8b effectors

SKIP, a RUN domain-containing protein binds to Arl8b, a lysosomal small GTPase via its RUN domain (Figure 9). SKIP further binds to kinesin light chain to recruit kinesin motor to regulate the anterograde movement of lysosomes.[13] The model which comes up here is in strict compliance with the hypothesized one (shown in Figure 10). There is a GTPase, Arl8b, a RUN domain-containing protein, SKIP, and a motor protein kinesin that interact with microtubules that bring about lysosomal motility.

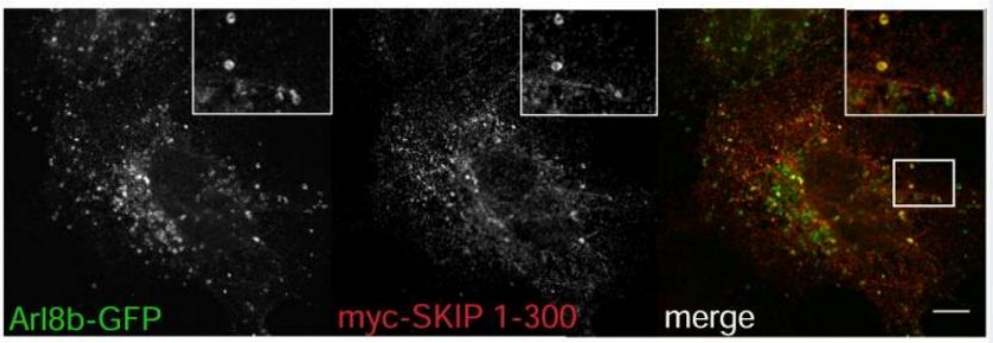

Figure 9. Arl8 Binds to the RUN Domain of SKIP and the two Proteins co-localize on lysosomes. Confocal micrographs of COS cells co-transfected with plasmids expressing Arl8b-GFP and the indicated versions of SKIP. For SKIP (1-300) a region is enlarged in the inset panel to show the co-localization and the tubes that are particularly prominent with this truncation (Adapted from Munro *et al*, 2011).

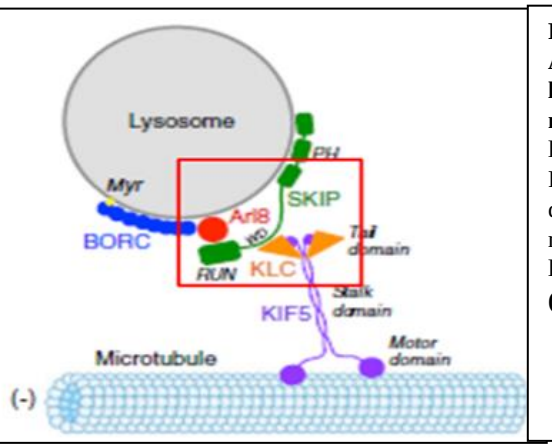

Figure 10. Schematic representation of the Arl8b-SKIP-Kinesin-1 pathway of lysosome movement toward the plus end of microtubules. This model shows that Arl8b localizes to lysosomes. Arl8b recruits SKIP a RUN domain-containing protein via its RUN domain. Further SKIP via its RUN domain, regulates the anterograde motility of lysosome via interaction with kinesin. **(**Adapted from Pu *et al* 2015).

Since Arl8b is involved in the late endosomal lysosomal pathway we looked at literature for other RUN domain containing proteins that localizes to late endosome or lysosome. We identified at least three such proteins besides (SKIP/PLEKHM2): PLEKHM1, RUBICON and RABIP4'. The domain architecture of these proteins is shown below for comparison (shown in Figure 11).

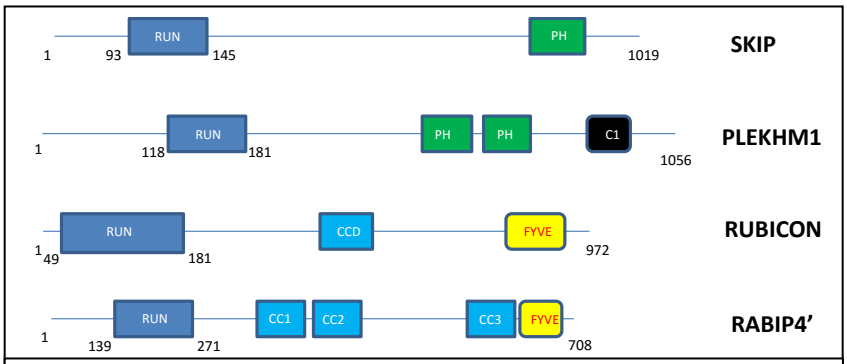

Figure 11. Domain architecture of RUN domain-containing proteins. SKIP, PLEKHM1, RUBICON, RABIP4' localize in the late endosomal lysosomal pathway.

Previous work in our laboratory showed that RUBICON and PLEKHM1 interact with constitutively active form of Arl8b via yeast two-hybrid (shown in Figure 12). Another Yeast two hybrid showed that the deletion of RUN domain of PLEKHM1 abrogated its interaction with small GTPase Arl8b.

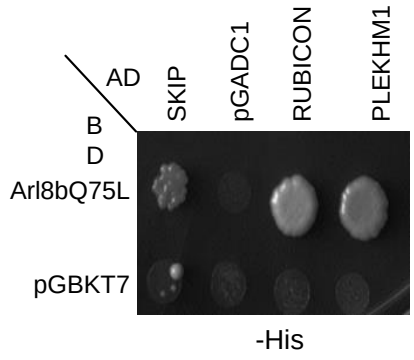

Figure 12. Yeast two hybrid showing that SKIP, RUBICON and PLEKHM1 interact with Arl8b. The constitutive active form of Arl8b interacts with SKIP, RUBICON, PLEKHM1 (Unpublished work from Tuli *et al*).

HeLa cells transfected with SKIP and Arl8b showed the predicted peripheral clustering of lysosomes (Figure. 13). Overexpression of PLEKHM1 in HeLa cells produced a perinuclear clustering of lysosomes (Figure. 14). Both SKIP and PLEKHM1 – with a similar domain architecture - interact by its respective RUN domains to the small GTPase Arl8b resulting in antagonistic phenotypes. PLEKHM1 interact with Lis1, a component of dynein complex for retrograde motility.[14] SKIP interact with kinesins and enable anterograde motility. Hence, it is evident that there exists a competition between these proteins for Arl8b to regulate the lysosomal positioning in functionally relevant scenarios within the cell.

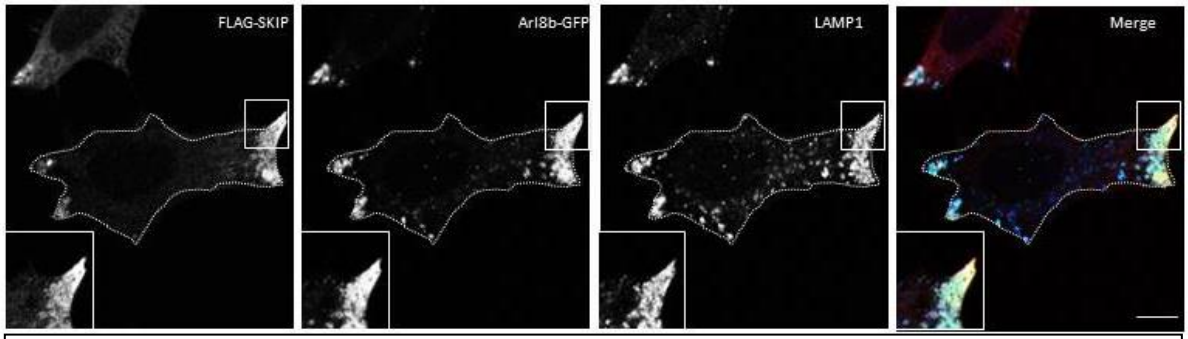

Figure 13. Overexpression of PLEKHM1 and Arl8b result in the perinuclear distribution of lysosome. FLAG-PLEKHM1 and Arl8b-GFP is overexpressed in HeLa cells. Both of them co-localize to LAMP1, a lysosomal marker, indicating that PLEKHM1 is responsible for the retrograde movement of lysosomes. (Unpublished work from R. Marwaha et al)

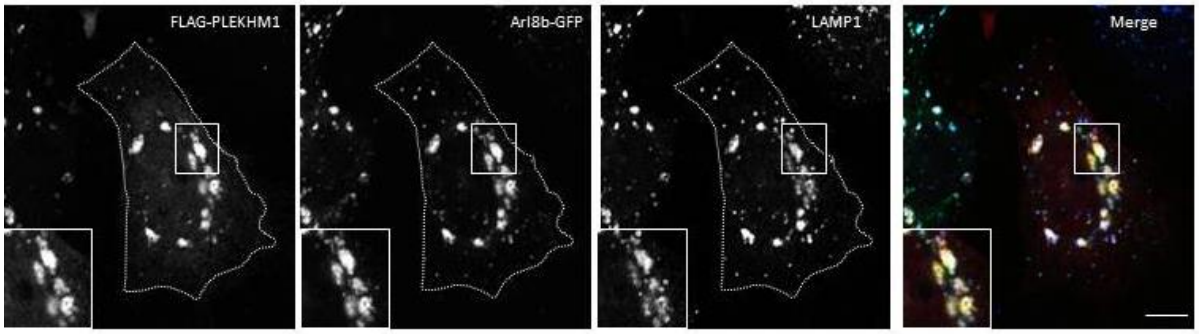

Figure 14. Overexpression of SKIP and Arl8b result in the peripheral distribution of lysosomes. FLAG-SKIP and Arl8b-GFP is overexpressed in HeLa cells. Both of them co-localize to LAMP1, a lysosomal marker indicating that SKIP is responsible for anterograde recruitment of lysosomes. (Unpublished work from R. Marwaha et al)

We wanted to quantify the binding affinity of SKIP and PLEKHM1 for the small GTPase by Surface Plasmon Resonance. The binding affinities together with the expression levels and localization of the proteins could give us a better picture of lysosomal positioning and its regulation.

Moreover, recent unpublished observations in our laboratory indicate a set of crucial basic residues in the RUN domain of PLEKHM1 that when mutated disrupt binding to

Arl8b but not to another interaction partner of PLEKHM1, Rab7.[9] These mutants were H60A, R117119A and R123A (shown in Figure 15).

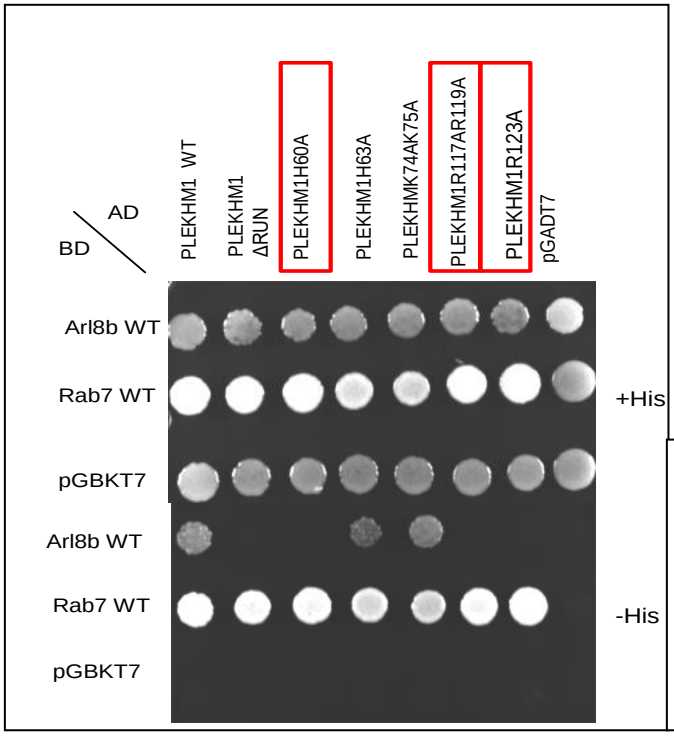

Figure 15. Yeast-two-hybrid assay which shows the interaction of PLEKHM1 and its RUN domain point mutants with Arl8b WT and Rab7 WT. This shows that Arl8b specifically interacts to PLEKHM1 via its RUN domain. The mutants H60A, R117119A and R123A specifically do not interact with Arl8b but interact with Rab7. (Unpublished work by R. Marwaha *et al*).

A sequence alignment of the RUN domain indicated us the probable basic residues in other RUN domain proteins that might be important for the interaction with Arl8b (shown in Figure 16).

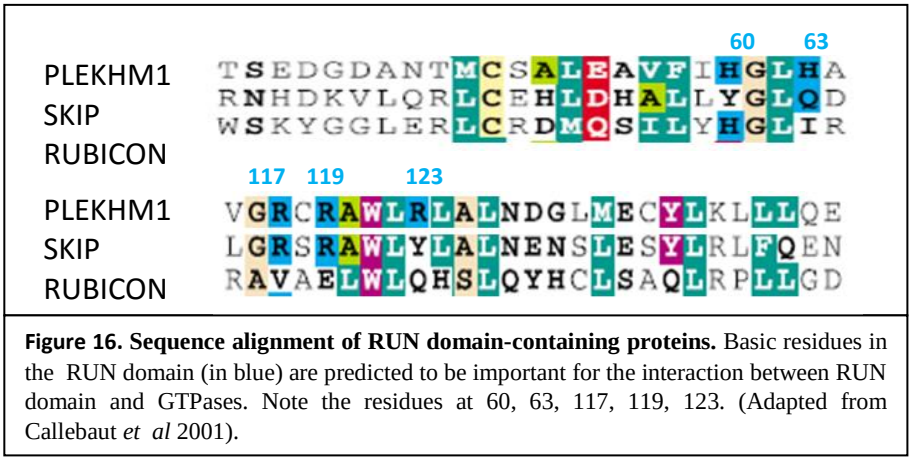

Figure 16. Sequence alignment of RUN domain-containing proteins. Basic residues in the RUN domain (in blue) are predicted to be important for the interaction between RUN domain and GTPases. Note the residues at 60, 63, 117, 119, 123. (Adapted from Callebaut *et al* 2001).

With this background we wanted to study the interaction of RUN domain-containing proteins for Arl8b by biochemical approaches such as a GST or Histidine pull down assay and quantify the affinity of RUN domains of these proteins for the small GTPase Arl8b by techniques such as Surface Plasmon Resonance. Further if the mutants abrogate the

interaction with Arl8b it would be interesting to see if the charge on the conserved basic residues plays an important role for this interaction.

Chapter 2

Material and Methods

1. Plasmids

Arl8b, Arl8b T34N cloned in pET15b^{+}, SKIP 1-300 in pGEX6P2 was available in the lab stocks. GST-PLEKHM1 RUN (1-300 amino acid) was cloned in pGEX6P2. PLEKHM1 1-198 amino acid was cloned in pGEX6P2. Rubicon 1-300 amino acid was cloned in pGEX5X1and Rabip4' RUN domain was cloned in pGEX5X1.

2. Antibodies

Rabbit polyclonal antibody against HA epitope tag was obtained from Covans. Mouse anti-FLAG was obtained from Sigma. Anti GST and anti GFP were obtained from Millipore and Abcam respectively

3. Cell Culture and Transfection

HEK293T cell lines were grown and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 % FBS at 37 ^{0}C in CO_2 incubator. For pulldown experiments like GST or His pulldown, HEK cells were seeded at 1.5 million/60mm dish which was transfected with the DNA constructs after 18-20hours of seeding using transfection reagents like Lipofectamine or Fugene as per the manufacturer's instruction.

4. Site-directed Mutagenesis

Stratagene's QuikChange™ site-directed mutagenesis kit was used for incorporating site-specific mutation in the double-stranded plasmids.

5. GST pull down assay

The major steps of GST pulldown are as below: GST protein was incubated with GSH beads for 2hr. Blocking was done by adding 5 % BSA to the bound beads for 2hrs. HEK 293T cells that were transfected with the respective DNA construct using the transfection reagent Lipofectamine. After 12-16 hours of transfection, the cells were collected and lysed in TAP lysis buffer (20mM TrisHCl pH 7.4, 150mM NaCl, 1mM Sodium orthovandate, 0.5 % or 1

% NP-40) with 1X PI and 1X PMSF for 20 minutes at 4 ^{0}C under tumbling. After lysis, the cell debris was separated from the supernatant by high speed centrifugation (13,000 rpm at 4 ^{0}C for 15mins). The supernatant was collected and 5% input was saved. Pre-clearing: 20 µl of slurry was added to the beads and tumbled in a Hula mixer at 4 ^{0}C to remove non specific interaction if any. Centrifuge at 2000 rpm, 2 min and collect the supernatant. After blocking wash the beads with TAP lysis buffer 3 times and add the above supernatant under tumbling at 4 ^{0}C for 2 hours. The unbound proteins were washed off with TAP lysis buffer four times. The bound proteins were eluted from the beads by boiling with 4X sample buffer at 99 ^{0}C. The input samples and the GST pull down samples were run in SDS PAGE gel.

6. His pull down assay

Protein was bound to the cobalt resin. Blocking was done by 5 % BSA for 1hr. The remaining procedure was identical to that of GST pull down assay.

7. Purified protein- protein pull down

The blocking for His tagged proteins was 1hr and for the GST tagged 2 h. The interaction time incubation was for 1 h. The remaining procedure was identical to that of GST pull down assay.

8. Western blotting

After running SDS Page, the gel was transferred onto a nitrocellulose membrane in transfer buffer for one and half hour. The blot was blocked in 10% skim milk for an hour. After blocking, the blot was washed with PBS + 0.3% Tween20 and incubated with the primary antibody in PBS + 0.3% Tween 20 for 1 hour. The blot was washed with PBS+ 0.3% Tween20 for three times and incubated with secondary antibody for 30 min. After three washes with PBS + 0.3% Tween20, the blot was developed after treating with ECL mix (Solution A: Solution B: Water in 1:1:8 ratio) for 2 min using developing machine.

10. Protein purification

The solutions used were:

HBS – HEPES Buffer Saline (20mM HEPES 150mM NaCl)

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, 20 mM NaCl, 150 mM EDTA, 1 mM DTT, 0.5 mM Tritox X 100, Glycerol 5 %)

Prep Buffer for His tagged proteins (20mM, 150mM NaCL, 0.5% triton X-100, Glycerol 5%) The gene of interest is transformed into *E.coli* BL21 competent cells. The primary culture was set up using a single colony in 5ml LB with antibiotic resistance (1:1000) at 37 °C incubator with shaking of 220 rpm for 12 h. Then secondary culture was set up after adding 10 % of salt solution and antibiotic (1 µl /ml) and 1% of primary culture was added to it. 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 1 mM IPTG. The culture was incubated at 16 °C with shaking of 220 rpm for adequate amount of time. The culture pellet after centrifugation was resuspended in Prep Buffer with 1 mM Protease Inhibitor and 1 mM PMSF. The pellet sonicated at 20 amplitude with pulse-on-time 15 sec and pulse-off-time 20sec. The sonicated suspension was centrifuged at 12,000 rpm for 15 min at 4 °C. The GSH beads were added to the supernatant in a hula-mixer at 4 °C for 2 h. The protein bound to beads was washed 10 times by 4 slurry volumes of Prep Buffer. Samples were collected after incubation with the glutathione elution buffer and an aliquot was run on SDS-PAGE to observe purification.

Chapter 3

Results

I Protein-protein interaction to test His-PLEKHM1 RUN domain binding to GST-Arl8b.

We cloned the RUN domain of PLEKHM1 – the first 300 residues – into a His tagged vector pET15b+ and expressed it. The protein purification protocol was standardised. Arl8b was cloned into a GST vector and expressed. GST-Arl8b was purified and eluted in Glutathione Elution Buffer. Buffer exchange was done using Hepes Buffer Saline (HBS). We looked at the secondary structure of His PLEKHM1 1-300 by CD spectroscopy. Using CDNN software we found that the RUN domain is largely alpha helical (54.5%) as expected of RUN domain-containing proteins (see Figure 17).

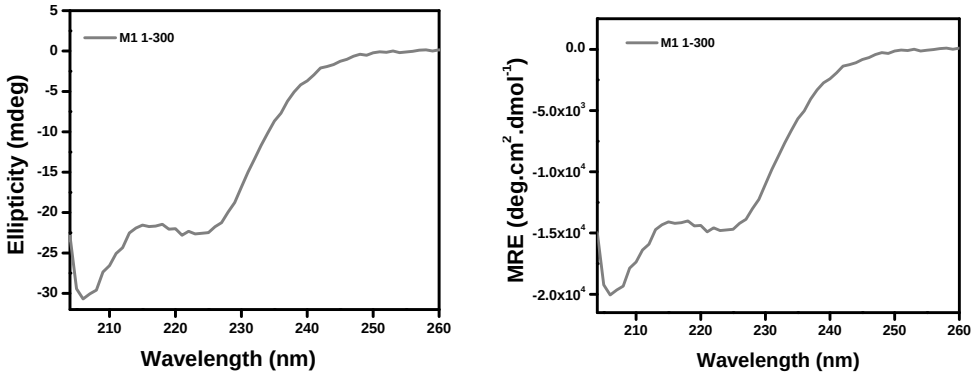

Figure 17. CD spectra of His PLEKHM1 1-300 in HBS. The CD spectra indicated a primarily alpha helical structure - Minima at 208 nm and 222 nm - as predicted for other RUN domain-containing proteins. Using CDNN software the secondary structure was found to be 54.5 % alpha helix 9.2 % beta sheet 13.8 % beta turn 20.8 % random coil.

Now we performed a purified protein-protein interaction assay. We tried to pull down GST-Arl8b using His PLEKHM1 1-300 on Cobalt resin. GST and GST-Arl3 was taken as negative control for this experiment. The pull down assay showed us that the RUN domain of PLEKHM1 is not able to pull down the small GTPase, Arl8b (see Figure 18).

In an improved version of the same experiment we transfected HEK 293T cells with Arl8b-HA and tried to pull down Arl8b-HA by His PLEKHM1 1-300 from the HEK293T lysates. This experiment failed to show any interaction (see Figure 19). We repeated this experiment with different buffer conditions (0.5% and 1% NP-40, 150mM NaCl) but result was the same.

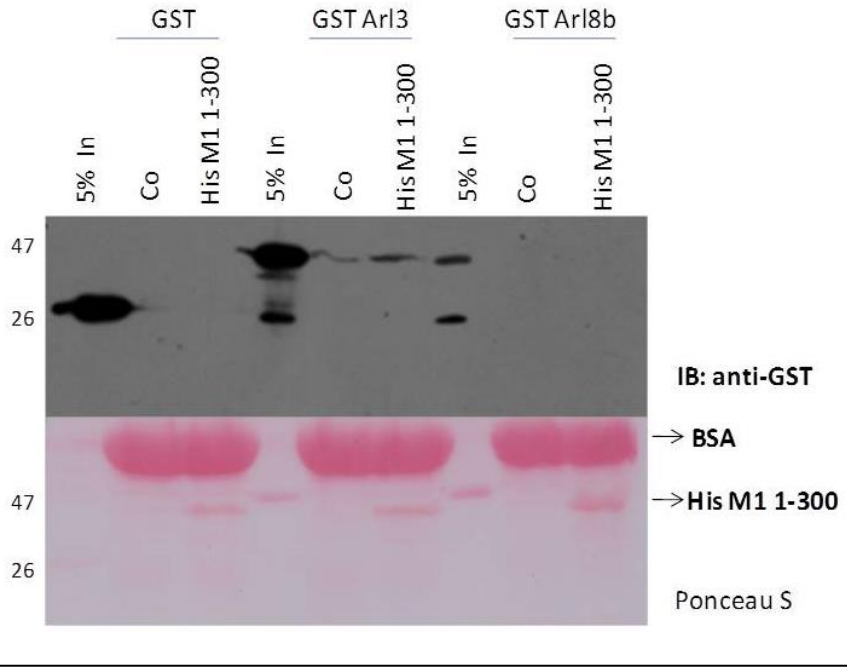

Figure 18. A purified protein-protein interaction assay was performed with GST and GST- Arl3 as control and GST- Arl8b as experiment where the GST protein were to be pulled down by His M1 1-300 that was on Cobalt beads. GST- Arl8b showed no interaction with His M1 RUN.

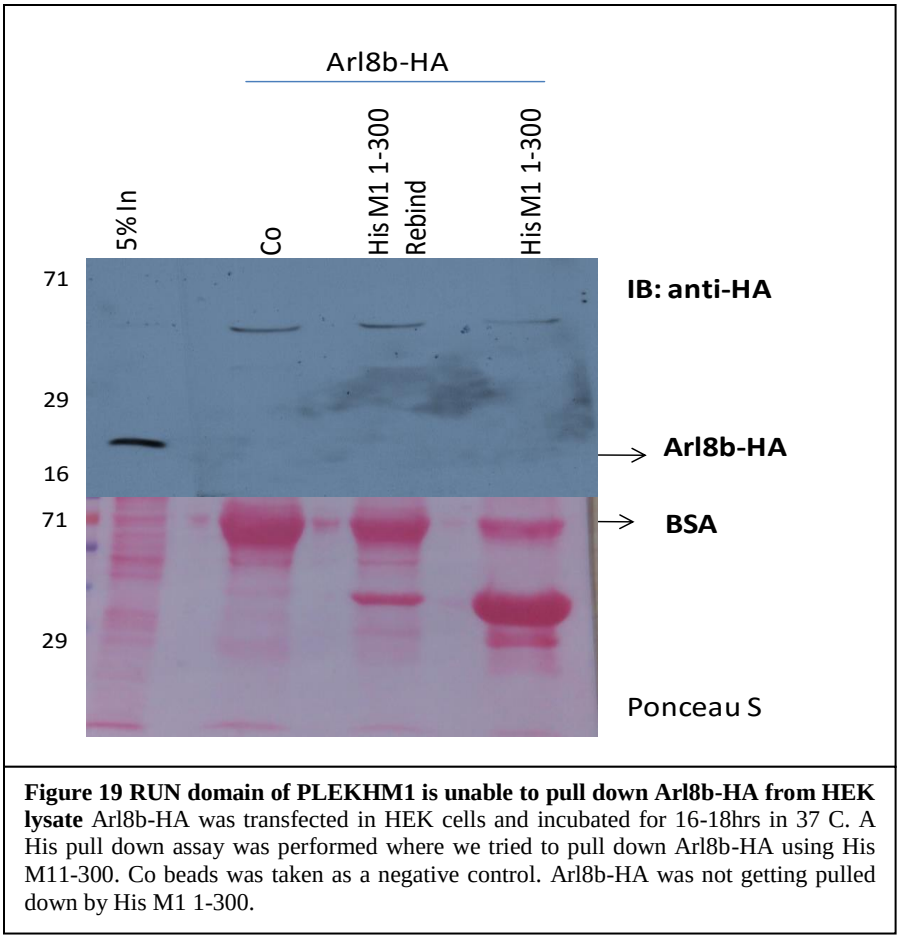

Figure 19 RUN domain of PLEKHM1 is unable to pull down Arl8b-HA from HEK lysate Arl8b-HA was transfected in HEK cells and incubated for 16-18hrs in 37 C. A His pull down assay was performed where we tried to pull down Arl8b-HA using His M11-300. Co beads was taken as a negative control. Arl8b-HA was not getting pulled down by His M1 1-300.

We have earlier observed specific binding of the GST-RUN domain of PLEKHM1 to purified His-tagged Arl8b as well as Arl8b expressed in cell lysates, however my experiments showed that the His PLEKHM1 1-300 did not bind to GST-tagged Arl8b. One possible reason for this could be the misfolding of the RUN domain with the His tag. Therefore, next we tried to employ His-tagged Arl8b to pull down PLEKHM1. We had Arl8b and its dominant negative mutant Arl8bT34N cloned in pET15b+ cloned in our lab. We expressed these proteins and standardised the purification protocols. We repeated the assay where we tried to pull down PLEKHM1-GFP by His Arl8b from HEK lysate. The dominant negative form of Arl8b T34N was the negative control. We observed a non-specific pull down in this case (Figure 20).

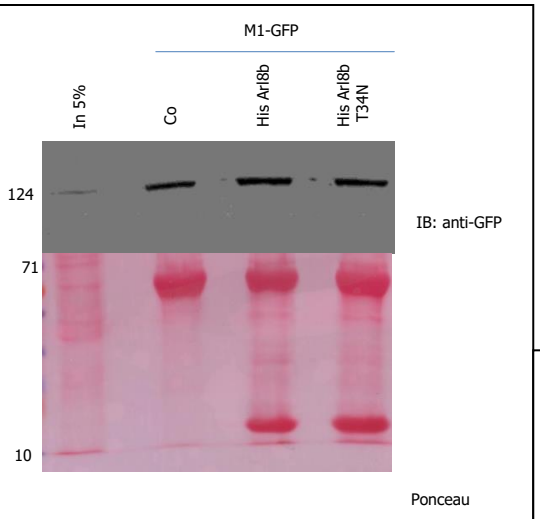

Figure 20. Pull down of M1-GFP by His Arl8b. M1-GFP was transfected in HEK cells and incubated for 16-18hrs in 37 C. A His pull down assay was performed where we tried to pull down M1-GFP using His Arl8b. Co beads and dominant negative form of Arl8b were taken as a negative control. There was a non specific pull down of M1 which again yielded inconclusive results.

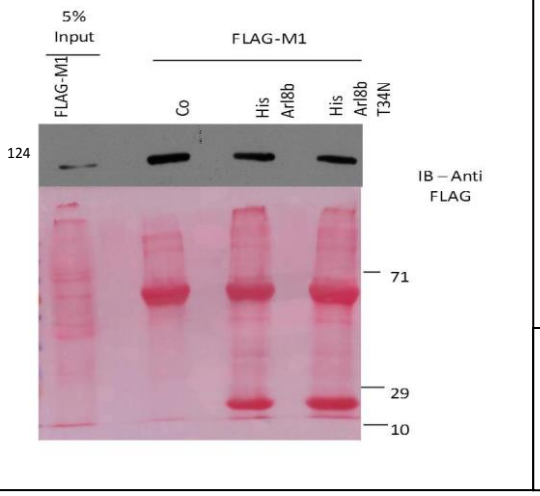

Figure 21. Pull down of FLAG M1 by His Arl8b FLAG-PLEKHM1 was transfected in HEK-293T cells. FLAG-M1 showed a non-specific pull down with His Arl8b. Co beads alone and dominant negative form of Arl8b are also nonspecifically pull down FLAG-M1

We tried the pull down of PLEKHM1 and its RUN-domain using a different tag. We used His-tagged Arl8b to pull down FLAG PLEKHM1. The experiment showed no interaction (see Figure 21). We repeated the experiment, with a stringent buffer condition for washing (1% NP-40, 300mM NaCl) and tried to pull down FLAG tagged PLEKHM1 and RUN domain of PLEKHM1. This time there was specific pull down for PLEKHM1 compared to negative control. But we got a non-specific pull down for the RUN domain alone (Figure 22). With these experiments we concluded that this approach would be rather difficult to achieve the specific interaction required for performing Surface Plasmon Resonance (SPR).

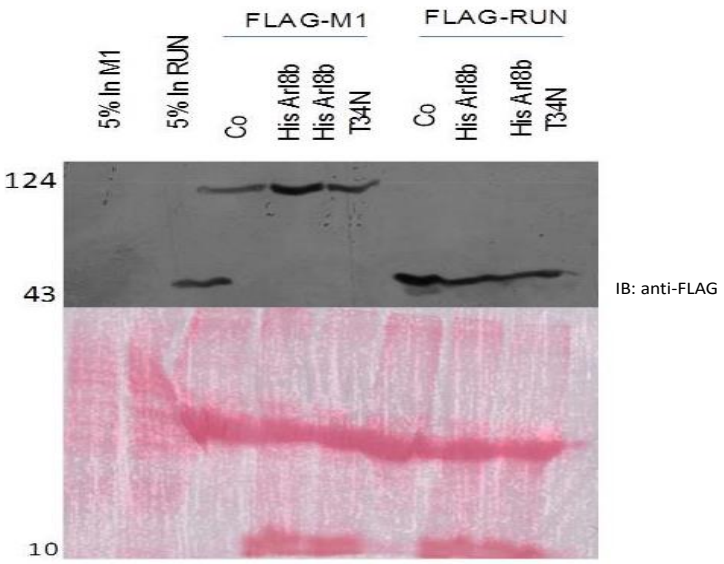

Figure 22. Pull down of FLAG-M1 or FLAG-M1 RUN by His Arl8b FLAG-M1 and FLAG-RUN was transfected in HEK cells and incubated for 16-18hrs in 37 C. A His pull down assay was performed where we tried to pull down FLAG-M1 and FLAG-M1 RUN using His Arl8b. Co beads and dominant negative form of Arl8b were taken as a negative control. There was a non-specific pull down of PLEKHM1 and PLEKHM1 RUN.

II Compare the binding of different RUN domain-containing proteins for Arl8b

Our second objective was to compare the binding of different RUN domain-containing proteins for Arl8b. To this end, RUN domains of SKIP, PLEKHM1, RUBICON and RABIP4' was cloned in GST vector. The protein production protocol was standardised. Arl8b-HA expressing HEK293T lysates was divided in five equal parts and incubated with GST or the GST tagged RUN domain proteins. GST was used as the negative control. Here, we obtained a specific pull down for SKIP, PLEKHM1 and RUBICON RUN domains. The experiment was repeated and a densitometric analysis was performed to quantify the affinity of the RUN domain-containing proteins for the small GTPase Arl8b. We found that the RUN domain of SKIP interacts with Arl8b more robustly than RUN domains of PLEKHM1 or RUBICON. We also found that the first 198 amino acid of PLEKHM1 was sufficient for its interaction with Arl8b. The RUN domain of RUBICON bound to Arl8b weakly.

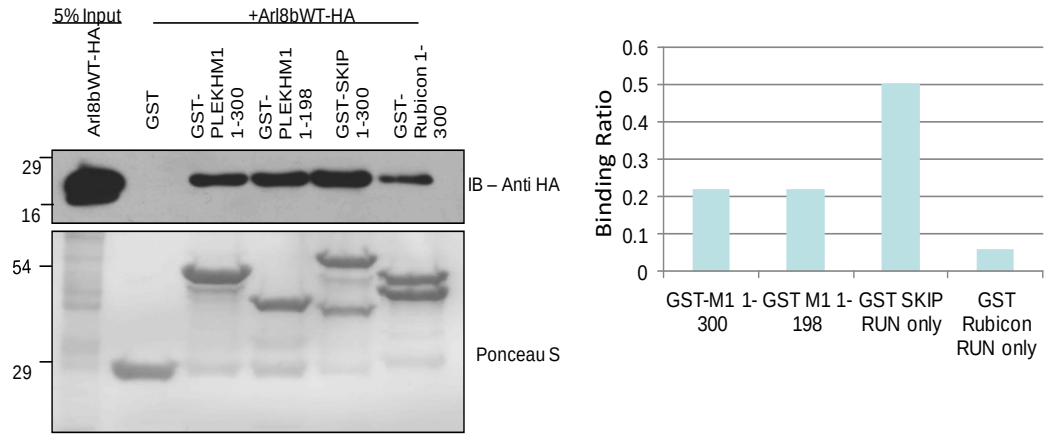

Figure 18. . RUN domain proteins have different affinities for Arl8b Arl8b-HA was transfected in HEK-293T cells. Arl8b-HA was pulled down from lysate using GST tagged RUN domain proteins. GST was a negative control. This experiment was repeated and the results of two such experiments were quantitated by densitometry analysis. The Y-axis represents the binding ratio which is defined as the amount of GST protein pulled down for unit of input Arl8b-HA. Densitometry showed that SKIP RUN interacts strongly with Arl8b as compared to RUN domain of PLEKHM1 or RUBICON. This also indicates that the first 198 amino acids of PLEKHM1 is sufficient for its interaction with Arl8b.

3.2 Conclusion

The RUN domain of SKIP has the highest affinity for Arl8b in the GST pull down assay. This could imply that under steady state SKIP recruits Arl8b much more strongly than PLEKHM1 or Rubicon. The RUN domain of PLEKHM1 only requires the first 198 amino acid for its interaction with Arl8b. It is interesting to note that Rubicon, which has been implicated in autophagy, interacts weakly with Arl8b under steady state nutrient rich conditions.

3.3 Future Directions

Arl8b can regulate lysosomal positioning within the cell. Different effector proteins are recruited on the lysosomes via Arl8b. At least two such proteins – SKIP and PLEKHM1 – regulate anterograde and retrograde motility respectively. Though the complete mechanism is unclear, there is a tug of war between SKIP and PLEKHM1 for Arl8b. In a cell, the position of lysosomes depends on the strength of interaction for Arl8b. A stronger interaction of Arl8b with SKIP indicates that under normal conditions, Arl8b is bound to SKIP more prominently than PLEKHM1. Surface Plasmon Resonance could be another good approach to test their binding affinities but we were unable to standardize this approach for our experiments. The pull down using His tag RUN domain proteins did not work probably because PLEKHM1 RUN domain was not properly folded. As part of future studies, it would be interesting to see how the strength of this interaction of Arl8b with different RUN domain effectors is modulated in conditions such as autophagy where autophagy related proteins could be playing a much more functional role in regulating the retrograde motility and perinuclear clustering of lysosomes. We are trying to see whether other RUN domain-containing proteins like Rabip4' (RUN and FYVE domain containing 1) are also involved in interaction with Arl8b as well as in regulation of lysosomal motility.

A. List of Cloning Completed

S.No	Insert	Vector	Template	Primers	Restriction Enzymes
1	FLAG PLEKHM1 Rescue 1273	pcDNA3.1(-)	GFP-PLEKHM1 Rescue 1273 C9	MSI-191,192	XhoI, KpnI
2	sec22b	pcDNA3.1(-)	HeLa cDNA	MSI-467, 468	EcoRI, BamHI
3	PH-SKIP	pET15b+	FLAG-SKIP WT	MSI-501, 502	NdeI, XhoI
4	ΔRUN PLEKHM1 Rescue	pEGFPC1	GFP-PLEKHM1 Rescue 1273 C9		XhoI, KpnI
5	mRabip4' 1-302 in pGEX6P2	pGEX-6P2	mRabip4' in pGEX6P2		
6	FLAG Δ198 PLEKHM1 1273 Rescue	pcDNA3.1(-)	FLAG M1 1273 Rescue	MSI-352, 192	XhoI, KpnI
7	GFP Δ198 PLEKHM1 1273 Rescue	pEGFP-C1	FLAG M1 1273 Rescue	MSI-527, 192	Hind III, KpnI
8	GST 1-198 PLEKHM1 pGEX6P2	pGEX-6P2	GST PLEKHM1 RUN pGEX6P2	MSI-374, 375	
9	Rubicon 1-300 pGEX-5X1	pGEX-5X1	Rubicon pGEX-5X1	AT lab	
10	GST M1 1-300 H60E pGEX-6P2	pGEX-6P2	GST-PLEKHM1 1-300 WT pGEX6P2		
11	FLAG FLAG PLEKHM1 H60A	pcDNA3.1(-)	FLAG-PLEKHM1 WT 1273 Rescue pcDNA3.1-		

	1273 Rescue pcDNA3.1(-)				
12	FLAG ΔRUN 1-895 pcDNA3.1(-)	pcDNA3.1(-)	FLAG-PLEKHM1 WT 1273 Rescue pcDNA3.1-		
13	GFP-PLEKHM1 triple mutant Rescue 1273	pEGFP-C1	GFP-PLEKHM1 H60A 1273 Rescue pEGFP-C1		
14	GST PLEKHM1 1-198 Triple mutant pGEX-6P2	pGEX-6P2	GST-PLEKHM1 1-198 Triple Mutant pGEX-6P2		

B. Protein Standardization

Protein Standardized Sl. No	**Name of Protein Construct**	**IPTG(in mM)**	**Temperature(^{0}C)**	**Time (hrs)**
1	Plekhm1 1-198	0.5	16	12
2	Rubicon 1-300	0.5	16 or 30	12 or 3
3	mRabip4’	0.5 or 0.2	30 or 16	3 or 12
4	PH-SKIP	0.5	30	3

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