To elucidate the role of Arl8b interaction partner RUFY3 in regulating lysosome positioning

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Certificate of Examination

This is to certify that the dissertation entitled "**To elucidate the role of Arl8b interaction partner RUFY3 in regulating lysosome positioning**" submitted by Mr. Prateek Chawla (MP16018) for the partial fulfillment of Masters in Biological Sciences 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.

Mahak Sharma

Dr. Mahak Sharma (Supervisor)

Dr. Shravan Kumar Mishra (Committee Member)

Dr. Shashi Bhushan Pandit (Committee Member)

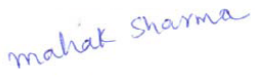
Declaration

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 of 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.

Prateek Chawla
(Candidate)
Dated: 19th April'2019

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

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Mahak Sharma for her constant support and guidance throughout the project. The scientific discussions that we have had over my stay in the lab have definitely enhanced my capabilities at the professional level. I would also like to thank my committee members Dr. Shravan Kumar Mishra and Dr. Shashi Bhushan Pandit for their valuable suggestions on my work. In addition, I would like to thank Dr. Amit Tuli for his extended help and support in the work carried out during my project.

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Abstract

Recent advancements in the field of lysosome biology have uncovered that in addition to its degradative function, lysosomes play an essential role in regulating several key biological processes such as plasma membrane repair, antigen presentation, cell migration/invasion and metabolic signalling. It has become increasingly evident that cellular distribution of lysosomes plays an essential role in regulating most of these unconventional functions. Researchers have identified several molecular players which efficiently regulate lysosome positioning in the cell. Interestingly, small GTPases have emerged as one of the key players in the process. Our group has a keen interest in understanding the role of lysosomal small GTP binding protein Arl8b and its effectors in regulating lysosome positioning and cargo trafficking. Recently, we identified RUFY3 (RUN and FYVE domain protein) as an interaction partner of Arl8b. Our findings suggest that Rufy3, via its N-terminal RUN domain, interacts with active GTP bound Arl8b and also regulates cellular distribution of lysosomes, which is dependent on dynein-dynactin machinery. Currently, we are trying to elucidate the mechanism of RUFY3 mediated retrograde transport and also decipher the significance of RUFY3 mediated lysosome positioning.

Chapter 1

Introduction

1.1 Lysosomes: a dynamic hub of degradation and cellular signalling

Lysosomes are eukaryotic, membrane-enclosed sub-cellular compartments known to degrade cellular cargo. Discovered in the 1950s by Christian de Duve, lysosomes are known to be enriched with more than 60 different hydrolases which require an optimum acidic pH (pH=4.6) to ensure efficient degradation of cellular cargo (Novikoff et al, 1956). The limiting membrane of lysosomes comprises of nearly 200 integral membrane proteins including the proton-importing V-ATPases which regulate and maintain the acidic pH of the lumen (Mellman I. 1989). Because of its degradative ability, lysosomes are termed as the "waste disposal system" or the "suicide bag" of the cell (de Duve, 2005). The cargo destined for degradation is delivered to the lysosomes via three pathways: Endocytosis, Phagocytosis and Autophagy (Kolter T, Sandhoff K, 2005). Endocytosis, coined by Christian de Duve in 1963, is a cellular process by which extracellular material is internalized either in a clathrin-dependent or independent manner and delivered to early endosomes, where the cargo is sorted. This cargo is either recycled back to the plasma membrane or directed towards the late endosomes, which eventually fuses with the lysosomes (Luzio et al., 2007). Phagocytosis is a cellular process by which relatively large particles (>0.5 μm) are internalized and delivered to lysosomes for degradation (Botelho et al, 2011). Lastly, autophagy (selective or non-selective) is a self-degradative process which plays a key role in degrading protein aggregates, damaged organelles and intracellular pathogens, thereby maintaining cellular homeostasis (Glick et al, 2010). Perturbations in lysosomal homeostasis due to dysfunctional lysosomal hydrolases, impaired lysosome traffic and biogenesis lead to accumulation of unprocessed substrate in these organelles. This becomes a leading cause of nearly 50 different lysosome storage disorders, neurodegenerative diseases and cancer (Samie MA et al, 2014; Schultz et al, 2011).

1.2 Unconventional roles of lysosomes

Apart from its intracellular degradative ability, lysosomes play an essential role in regulating plethora of physiological processes such as plasma membrane repair, gene regulation, metabolic signalling, antigen presentation, cell migration, tumor invasion and metastasis (Figure 1). In this section, we would be discussing these non-canonical roles of lysosomes in detail.

Plasma membrane is the primary barrier to the exchange of components between extracellular matrix (ECM) and the cytoplasm. When the plasma membrane is ruptured due to mechanical stress or pathogenic microorganisms, lysosomes are recruited to these damaged sites in a calcium dependent manner. These lysosomes ultimately fuse with the plasma membrane, release their enzymatic content and mediate internalization of damaged membrane surfaces (Tam C et al., 2010, JCB). This pathway is widely exploited by several pathogens to gain entry into the host cell and develop their cellular niche. For example, Chagas disease causing pathogen *Trypanosoma cruzi* injures the plasma membrane and evokes exocytosis of lysosomes, followed by compensatory endocytosis and internalization of these pathogens into the host. Additionally, lysosomes also play an essential role in regulating cell migration via remodeling the actin cytoskeleton present beneath the plasma membrane. According to Natalia et al., 2014, p14-MP1 positive late endosomes/lysosomes, which move to the cell periphery, mediate dissociation of IQGAP1 thereby, altering the focal adhesion dynamics. Another independent study has noted that activated dendritic cell (DC) promote release of lysosomal calcium by ionic TRPML1 channel. This activates actin based motor protein myosin II and mediates their migration (Bretou M et al., Sci. Immunol, 2017). Lysosomes also play an essential role in clearance of pathogenic microorganisms. Pathogenic bacteria invading the cell (via phagocytosis) are degraded by the acidic luminal hydrolases. This releases various antigenic peptides

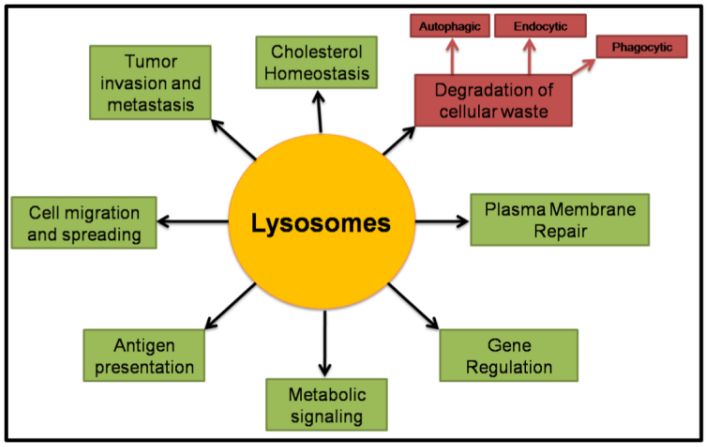

Figure 1: Lysosomes are dynamic hub of degradation and cellular signalling Representatitive image depicting conventional (in red) and unconventional (in green) functions of lysosomes in mammalian cells. Adapted from Jing Pu et al., Journal of Cell Sciences (2016)

which bind to major histocompatibility complex II (MHC II) and are presented at the cell surface to CD4+ T lymphocytes (Saric et al., 2015, MBOC; Chow et al., 2002; Vyas et al., 2007). Over the years, it has become increasingly evident that these unconventional functions of lysosomes are majorly governed by their cellular distribution.

1.3 Cellular distribution of lysosome in mammalian cells

Lysosomes are dynamic organelles that are well-dispersed throughout the cytoplasm. In non-polarized cells, lysosomes have a typical distribution, with majority of lysosomes localized near the MTOC known as the "perinuclear cloud" and some dispersed near cells periphery, reaching out to the plasma membrane and cell protrusions (Jongsma et al.,2016). However, in polarized cells, lysosomes are widely distributed in the axon, the dendrites and the cell body (soma) and tend to "mature" as they transit from the axon to cell body. Studies suggest that a small population of lysosomes is relatively static and localized to defined location in the cytoplasm, however, others traverse bidirectionally along microtubule tracks; transiting from MTOC to cell periphery and vice versa (Matteoni and Kreis, 1987). Lysosomes move on dynamic and polarized microtubule tracks. The growing plus end of these tracks are directed towards the cell periphery and minus end projected near the MTOC. Previous studies suggest that this transport is primarily mediated by molecular motor proteins, kinesin and cytoplasmic dynein (hereafter referred to as dynein) (Figure 2). While nearly 45 members in kinesin super-family of proteins mediate transport towards the cell periphery (anterograde transport), a single dynein motor protein regulates the bulk of the transport towards the MTOC (retrograde transport).

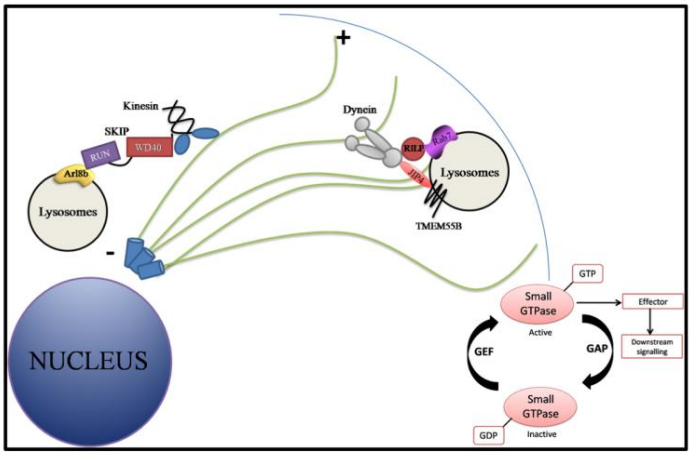

Figure 2: Lysosomes move bidirectionally along microtubule tracks Representatitive image depicting lysosome positioning in mammalian cells mediated by small GTPases and their effectors. Lysosomes move along microtubule tracks. MTOC to cell periphery (anterograde) and cell periphery to MTOC (retrograde)

1.4 Factors regulating distribution of lysosomes

Lysosome positioning is under the control of several intracellular and extracellular factors. For example, cytosolic acidification disrupts the perinuclear pool of lysosomes, mediating movement to cell periphery. However, cytosolic alkalization revives the perinuclear pool (Heuser et al, 1989; Parton et al., 1991). Furthermore, nutrient deprivation, drug-induced apoptosis and antigen presentation also mediate perinuclear clustering of lysosomes (Korolchuk et al., 2011, Erie et al., 2015,Yu et al., 2016), promoting autophagosome-lysosome fusion and clearance of cellular debris. Nutrient dependent positioning of lysosomes is primarily regulated by mTORC1 (mechanistic target of rapamycin complex 1), a serine/threonine kinase which (under nutrient rich conditions) is activated and recruited to the lysosome membrane in the cell periphery. However, during starvation mTORC1 is inactivated and promotes dephosphorylation of autophagy stimulating protein targets: ULK1 (promotes formation of phagophore) and TFEB (upregulates autophagy associated genes). Recent studies suggest that even sub-cellular compartments like endoplasmic reticulum (ER), trans-Golgi network (TGN) and peroxisome associate with limiting membrane of lysosomes and spatiotemporally regulate their distribution. For example, ER-lysosome contact sites regulate endosomal maturation and Golgi associated small GTP binding protein Rab34 mediates perinuclear redistribution of lysosomes. Additionally, a novel mechanism of lysosome positioning has been discovered where lysosome transmembrane protein TMEM55B recruits dynein adaptor JIP4 to the lysosome membrane, thereby mediating retrograde transport along microtubule tracks. Currently, our research group has a keen interest to decipher the role of small GTP binding proteins and their effectors in regulating membrane trafficking and cellular distribution of lysosomes.

1.5 Role of small GTP binding proteins in regulating lysosome positioning

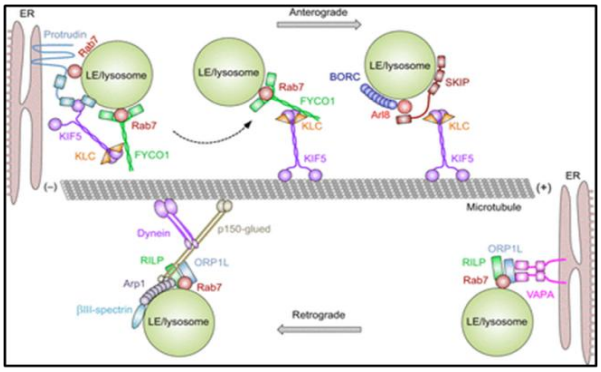

Figure 3: Mechanism of small GTPase mediated transport of late endosomes/lysosomes Representative schematic showing the role of small GTPases in regulating bidirectional transport of lysosomes along microtubule tracks (Adapted from Jing Pu et al., 2016)

Members of Rab, Arf and Arl (Arf like) GTP binding proteins associate with distinct sub-cellular compartments and play an essential role in regulating membrane trafficking and cellular distribution

of the lysosomes (Figure 3). GTP binding protein alternate between 2 conformations: active GTP bound (membrane localized) and inactive GDP bound (cytosolic). These states are spatiotemporally regulated by actions of respective guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). These small GTP binding proteins in GTP bound state recruit effector proteins which mediate downstream signalling processes. A wide range of studies suggest that they play an essential role in mediating the positioning of lysosomes, regulating transient exchange between the peripheral and perinuclear pool. Previous studies suggest that anterograde transport of lysosomes is mediated by BORC, Arl8b-SKIP and kinesin-1 complex. BORC (BLOC-1-related complex) associates with the cytosolic face of lysosomes and recruits small GTP binding protein Arl8b on the surface of lysosomes. Membrane localized Arl8b interacts with its effector PLEKHM2/SKIP via its N-terminal RUN domain and SKIP via its WD motif interacts with motor protein kinesin-1 (Pu et al., 2015; Rosa-Ferreira and Munro, 2011; Bagshaw et al, 2006). Additionally, an alternative pathway of anterograde transport of lysosomes is mediated by small GTP binding protein Rab7. Endoplasmic reticulum (ER) anchored protein 'protrudin' bridges ER and lysosome membrane by simultaneously binding Rab7 and phosphotidylinositol-3-phosphate (PI3P). Protrudin transfers these lysosomes to the Rab7 effector FYCO1 (FYVE and coiled-coil containing protein), which associates with kinesin-1 and mediates transport to the cell periphery (Raiborg et al., 2016; Matsuzaki et al., 2011). On the other hand, retrograde transport involves the recruitment of dynein-dynactin machinery on the peripheral lysosomes, majorly mediated by quissential small GTP binding protein Rab7. Various downstream effector proteins couple lysosomes to dynein and accelerate transport to the MTOC. Studies have noted that RILP (Rab7-interacting lysosomal protein) associates with the p150 subunit of the dynein-dynactin machinery (Cantulopo et al., 2001; Jordens et al., 2001; Progida et al., 2007). In addition, ORP1L (OSBP-related protein 1L) complexes with Rab7-RILP, followed by βIII spectrin attachment to Arp1 subunit of dynactin (Johansson et al., 2007). Other members of Rab family of proteins have also been implicated in regulating lysosome positioning, with RNAi mediated depletion of Rab9A and over-expression of Rab34 and Rab36 (via association with RILP) have been known to mediate juxtanuclear clustering of lysosomes. These small GTPases efficiently regulates the distribution of late endosomes/lysosomes. Our group has a

keen interest to understand the role of small GTP binding protein Arl8b and its effectors in regulating lysosome positioning in the cell.

1.6 Role of Arl8b in mediating lysosome positioning

Arl8b, a primitive small GTP binding protein, predominantly localizes on mature lysosomes, and plays a crucial role in regulating membrane trafficking and lysosome positioning in mammalian cells (Figure 4). In its active GTP bound state, it recruits effector on the surface of late endosomes/lysosomes which regulate downstream signalling. One such class of effectors is the RUN domain containing proteins. RUN (after RPIP8, UNC14 and NESCA) domain is an evolutionarily conserved protein binding domain. These domains predominantly adopt an alpha-helical fold and are divided into 6 sub-domains. Studies suggest that basic amino acid residues in A and D sub-domain play essential role in mediating interaction with small GTP binding protein (Callebaut et al., 2001). Various research groups have elucidated the role of RUN-domain containing proteins as Arl8b effectors. For example, PLEKHM2/SKIP, an Arl8b effector, mediates peripheral distribution of lysosomes via motor protein kinesin-1 (Munro and Rosa-Ferreira, 2011). In addition, a study conducted by our research group has deciphered the role of PLEKHM1 as a dual effector of small GTP binding protein Rab7 and Arl8b, which mediates endosome-lysosome fusion (Marwaha R et al., 2016). Currently, our research group has a keen interest to study the role of another class of RUN domain containing proteins: RUFY family of proteins (RUN and FYVE domain containing proteins). RUFY proteins have 4 members: RUFY1, RUFY2, RUFY3 and RUFY4. All these members have conserved domain architecture: an N-terminal RUN domain, central coiled coil domains and C-terminal FYVE domain.

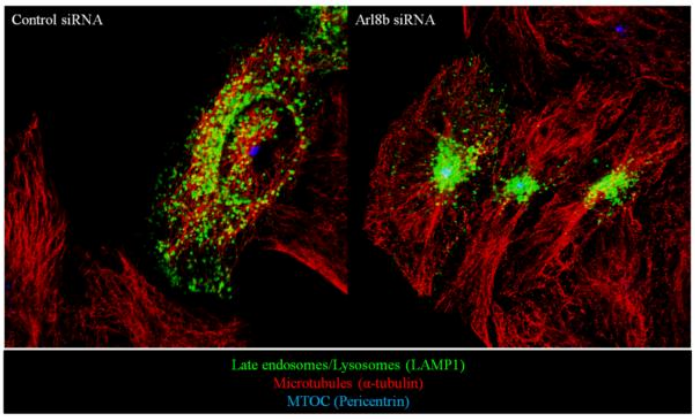

Figure 4: Arl8b is a crucial regulator of lysosome positioning Representatitive confocal image of HeLa cells treated with control and Arl8b siRNA. Stained with LAMP1 (Green), Microtubules (Red) and MTOC (Blue). Courtesy: Dr. Mahak Sharma, IISER-Mohali

This project focuses towards deciphering the role of Arl8b interaction partner RUFY3 in regulating cellular distribution of lysosomes. Human RUFY3 has 6 transcript variants and they exhibit conserved domain architecture (Figure 5). Previous studies, conducted with respect to transcript variant 2, have implicated the role of RUFY3 for maintenance of neuronal polarity, suppressing formation of multiple axons and mediating gastric cancer cell migration and invasion via interaction with P-21 activated kinase-1 (PAK-1) (Honda et al., 2017; Wei et al., 2014; Xie et al., 2017). However, other transcript variants have not been explored much. In a yeast-two hybrid screen (Conducted by Dr. Mahak Sharma and Dr. Amit Tuli), we found that RUFY3 transcript variant 1 strongly interacts with Arl8b. Subsequently, results from GST pull-down and purified protein interaction assays, strongly suggest that RUFY3 variant 1 interacts with Arl8b, via its N-terminal RUN domain. However, this project aimed at determining its significance in mammalian cells.

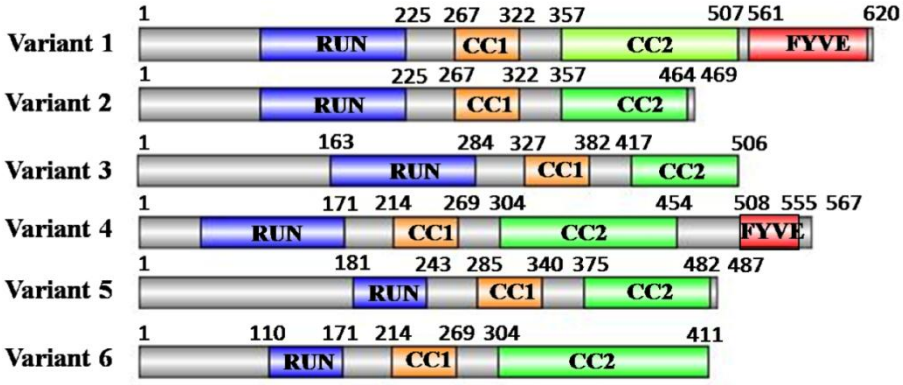

Figure 5: Arl8b interaction partner RUFY3 has 6 transcript variants Image depicting domain architecture of human RUFY3 transcript variants (generated by alternative splicing). It has 4 conserved domains: N-terminal RUN domain, central coiled coil domains (CC1 and CC2) and C-terminal FYVE domain

Chapter 2

Materials and Methods

2.1 Cell Culture and RNAi:

HeLa and HEK293T (from ATCC) were cultured in DMEM (Lonza) supplemented with 10% FBS (Gibco, Life Technologies) at 37°C with 5% CO_2 in a humidified cell culture chamber. Each cell line was regularly screened for absence of mycoplasma contamination by using MycoAlert™ Mycoplasma Detection Kit (Lonza) and was cultured for no more than 15 passages.

For gene silencing, siRNA oligos were purchased from Dharmacon and prepared according to the manufacturer's instructions. Sequences of siRNA oligos used in this study are as follows: control siRNA (TGGTTTACATGTCGACTAA); human RUFY3 siRNA, GAUGCCUGUUCAACAAAUGAAUU; human JIP4 siRNA, GCAUCACAGUGGUUGGUUGUU

2.2 Constructs used:

Table I: List of molecular constructs used in this study

Plasmid Name	Description	Source
Human RUFY3 (variant1) Flag in pcDNA3.1 (-)	Full-length human RUFY3 variant 1 (1-620 aa)with C-terminal FLAG tag cloned in pcDNA 3.1(-) vector	This study
Human RUFY3 (variant 1) in pEGFPC1	Full-length human RUFY3 (1-620 aa) with N-terminal GFP tag cloned into pEGFPC1 vector	This study
Human RUFY3 (variant 2) Flag in pcDNA3.1 (-)	Full-length human RUFY3 variant 2 (1-469 aa) with C-terminal FLAG tag cloned in pcDNA3.1(-) vector	This study
Human RUFY3 (variant 1) (233-620)Flag in pcDNA3.1(-)	Human RUFY3 variant 1 (233-620 aa) with C-terminal FLAG tag cloned in pcDNA 3.1(-) vector	This study
Human RUFY3 (variant 1) R162A/R164A Flag in pcDNA 3.1 (-)	Full-length Human RUFY3 variant 1 with point mutation at amino acid 162 and 164 changing R to A, with C-terminal FLAG tag cloned in pcDNA	This study

	3.1 (-) vector	
Human RUFY3 (variant 1) HA in pcDNA 3.1(-)	Full-length human RUFY3 (variant 1) with C-terminal HA tag; cloned in pcDNA 3.1(-) vector	This study
Human RUFY3 (variant 1) (Δ1-232) HA in pcDNA 3.1(-)	Human RUFY3 variant 1 (233-620 aa) with C-terminal HA tag; cloned in pcDNA 3.1(-) vector	This study
Human RUFY3 (variant 1) (Δ 1-232 aa) in pcDNA 3.1 (-)	Human RUFY3 variant 1 (233-620 aa) cloned in pcDNA 3.1(-) vector	This study
Human RUFY3 (variant 1) untagged in pcDNA 3.1(-)	Full-length human RUFY3 variant 1 cloned in pcDNA 3.1(-) vector	This study
Human RUFY 3 (variant1) H114A FLAG in pcDNA 3.1 (-)	Full-length human RUFY3 variant 1 with point mutation at amino acid 114 changing H to A, with C-terminal FLAG tag; cloned in pcDNA 3.1 (-)	This study
Human RUFY3 (variant 1) (H114A)-HA in pcDNA 3.1 (-)	Full length human RUFY3 variant 1 with point mutation at amino acid 114 changing H to A, with C-terminal HA tag, cloned in pcDNA 3.1 (-) vector	This study
Human RUFY3 (variant 1) HA in pCDH-Puro	Full-length human RUFY3 variant 1 with C-terminal HA tag; cloned in pCDH-Puro vector	This study
Human RUFY3 (Variant 1) FLAG (Rescue against RUFY3 1753 siRNA)	Full-length human RUFY3 (Rescue construct) against human RUFY3 1753 siRNA (with C-terminal Flag tag); cloned in pcDNA 3.1 (-) vector	This study
2X FKBP GFP RUFY3 (variant1) Flag in pcDNA 3.1(-)	Full length human RUFY3 variant 1 with N-terminal 2X FKBP and GFP tag and C-terminal FLAG tag, cloned in pcDNA 3.1 (-)	This study
2X FKBP GFP RUFY3 (variant1) in pcDNA 3.1(-)	Full length human RUFY3 variant 1 with N-terminal 2X FKBP and GFP tag, cloned in pcDNA 3.1 (-)	This study
Human GST-RUFY3 Variant 1 (1-232 aa) in pGEX6P2	Human RUFY3 (1-232 aa) with N-terminal GST tag; cloned in pGEX6P2 vector	This study
Human GST-RUFY3 Variant 1 (233-620 aa) in pGEX6P2	Human RUFY3 (232-620 aa) with N-terminal GST tag; cloned in pGEX6P2 vector	This study
Human RUFY3 (variant 1) in pGADT7	Full-length human RUFY3 (variant 1); cloned in pGADT7 vector	This study
Human RUFY3 Variant 1	Human RUFY3 Variant 1 (233-620	This study

(Δ 1-232 aa) in pGADT7	aa); cloned in pGADT7 vector	
Human RUFY3 (variant 1) R162A/R164A in pGADT7	Full-length Human RUFY3 variant 1 with point mutation at amino acid 162 and 164 changing R to A, cloned in pGADT7 vector	This study
Human RUFY3 (variant 1) R162A/R164A/R168A in pGADT7	Full-length Human RUFY3 variant 1 with point mutation at amino acid 162, 164 and 168 changing R to A, cloned in pGADT7 vector	This study
Human RUFY3 (variant 1) Δ446-561 in pGADT7	Human RUFY3 (variant 1) with 446-561 amino acids deleted by SOE PCR, cloned in pGADT7 vector	This study
Human RUFY3 (variant 1) Δ446-561 in pGADT7-HA	Human RUFY3 (variant 1) with 446-561 amino acids deleted by SOE PCR, cloned in pcDNA 3.1 (-) with C-term HA tag	This study
Human Delta RUN RUFY3 (Δ446-561)HA in pcDNA3.1 (-)	Human Delta RUN RUFY3 (Δ446-561) with C-terminal HA tag cloned by PCR in pcDNA 3.1 (-)	This study

2.3 Transfection, Immunofluorescence and Live-Cell Imaging

Cells grown on glass coverslips were transfected with desired constructs using X-tremeGENE-HP DNA transfection reagent (Roche) for 16-18h. Cells were fixed in 4% PFA in PHEM buffer (60mM PIPES, 10mM EGTA, 25mM HEPES and 2mM $MgCl_2$ and final pH 6.8) for 10min at room temperature. Post-fixation, cells were incubated with blocking solution (0.2% saponin + 5% FBS in PHEM buffer) at room temperature for 30min, followed by three washes with 1X PBS. Following this blocking step, cells were incubated with primary antibodies in staining solution (PHEM buffer + 0.2% saponin) for 45min to 1h at room temperature, washed thrice with 1X PBS and further incubated for 30min with alexa fluorophore-conjugated secondary antibodies made in staining solution. Cells were mounted in Fluoromount G (Southern Biotech) and confocal images were acquired using Carl Zeiss 710 Confocal Laser Scanning Microscope.

For live-cell imaging, cells were seeded on glass bottom tissue culture treated cell imaging dish (Eppendorf) and transfected 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% CO_2.

2.4 Antibodies and Chemicals

The following antibodies were used in this study: mouse anti-FLAG M2 clone (F1804, Sigma), rabbit anti-HA (sc-805, Santa Cruz), mouse anti-HA (MMS-101P, Covance), mouse anti-LAMP1 (555798, BD Biosciences, rabbit anti-LAMP1 (ab24170, abcam). All the Alexa fluorophore-conjugated secondary antibodies were purchased from Life Technologies. HRP-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jacksons ImmunoResearch Laboratories.

2.5 Lysosome distribution quantification:

Lysosome distribution was quantified using ImageJ software. LAMP1 signal intensity was quantified for individual cells by drawing circles at an increment of 5um (till the cell periphery) from the nuclear rim. LAMP1 intensity was measured at 5um (from the nuclear rim) and 5-15um (from the nuclear rim). No cell was measured more than once. 25-30 cells were quantified per experiment and averaged over 3 independent experiments.

2.6 Statistical Analysis

Data were presented as means ± standard error of the mean unless otherwise specified. *p* values were calculated using Student's *t-test* from three independent biological replicates.

Chapter 3

Results

3.1. RUFY3 V.1 colocalizes with Arl8b and promotes perinuclear clustering of lysosomes:

Using a GST pull-down assay followed by purified protein-protein interaction study, we could conclusively show that RUFY3 V.1, via its N-terminal RUN domain, interacts with active GTP bound Arl8b. Further, we were intrigued to determine its sub-cellular localization; however, we observed its majorly cytosolic in HeLa cells expressing Flag tagged RUFY3 V.1. Surprisingly, on staining with LAMP1, we observed dramatic perinuclear clustering of lysosomes, compared to well-dispersed lysosome pool in untransfected cells (Figure 6C). LAMP1 distribution over distance (from the nuclear rime to the cell periphery) was quantified over three independent experiments (25-30 cells/experiment) and an enhanced perinuclear distribution of lysosomes was observed in RUFY3 V.1 expressing cells (Figure 6D). In addition, this effect was restricted only to the late endocytic compartments, as the sub-cellular distribution of other organelles was not altered.

In accordance with our observations that RUFY3 V.1 interacts with Arl8b, we speculated if these proteins are localized on the same compartment. We observed that cotransfection of RUFY3 V.1 and Arl8b led to a dramatic perinuclear clustering of Arl8b positive compartments, whereas transfection of Arl8b alone promoted lysosome positioning towards the cell periphery (Figure 6A and 6B). Additionally, we also observed that RUFY3 V.1 (initially cytosolic) was recruited on these Arl8b positive compartments, which colocalized near the MTOC. We thereby speculated the role of RUFY3 V.1 in regulating cellular distribution of lysosomes.

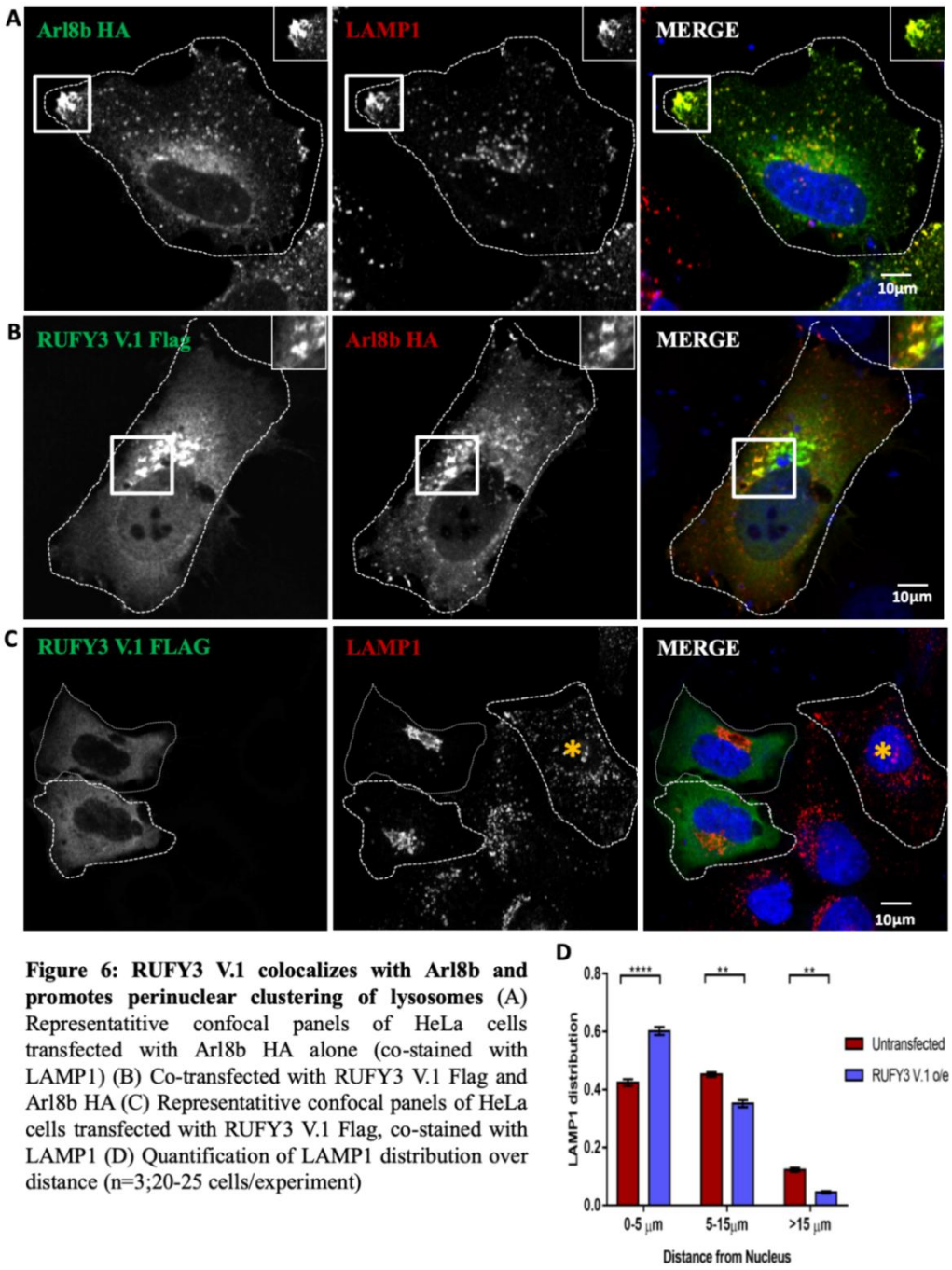

Figure 6: RUFY3 V.1 colocalizes with Arl8b and promotes perinuclear clustering of lysosomes (A) Representatitive confocal panels of HeLa cells transfected with Arl8b HA alone (co-stained with LAMP1) (B) Co-transfected with RUFY3 V.1 Flag and Arl8b HA (C) Representatitive confocal panels of HeLa cells transfected with RUFY3 V.1 Flag, co-stained with LAMP1 (D) Quantification of LAMP1 distribution over distance (n=3;20-25 cells/experiment)

3.2 RUFY3 depletion mediates peripheral repositioning of lysosomes

Next, we depleted RUFY3 from HeLa cells to assess its role in regulating cellular distribution of lysosomes and in RUFY3-mediated perinuclear clustering of lysosomes. The efficiency of RUFY3 depletion in HeLa cells, using siRNA which specifically targets RUFY3 transcript variant 1 and 4 was ~70% (confirmed by quantitative RT PCR) (Figure 7A). Interestingly, we observed that RUFY3 depletion led to a dramatic peripheral repositioning of lysosomes, compared to a well-dispersed pool of lysosomes in control cells (Figure 7A and 7B). LAMP1 distribution over

distance (from the nuclear rim to the cell periphery) was quantified over three independent experiments (30-40 cells/experiment) and we observed that RUFY3 depletion led to an enhanced LAMP1 distribution near the cell periphery. This effect was rescued by expressing siRNA resistant RUFY3 V.1 in RUFY3 depletion background (Figure 7D). This construct was made by incorporating point mutations in siRNA binding region of RUFY3. LAMP1 distribution was quantified over three independent experiments (15-20 cells/ experiment) (Figure 7E). These findings strongly suggest the role of RUFY3 V.1 in regulating lysosome positioning in cells.

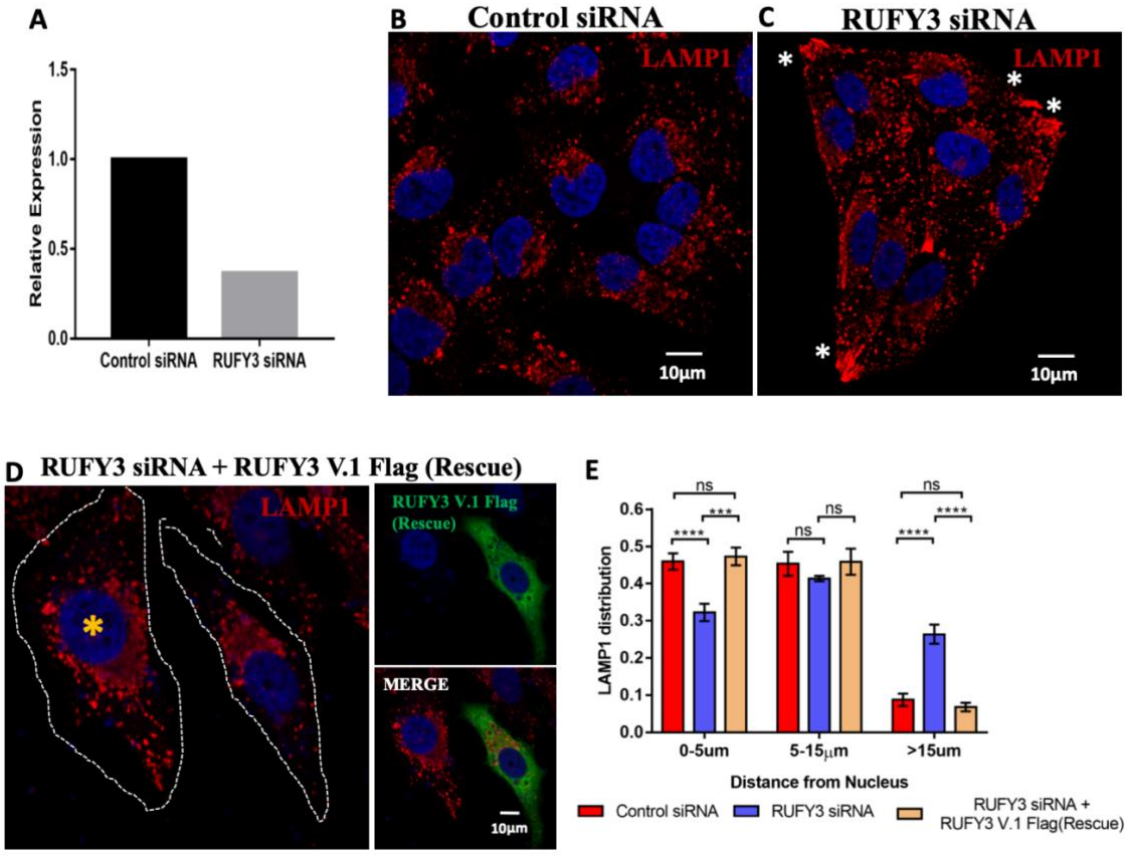

Figure 7: RUFY3 depletion causes peripheral repositioning of lysosomes (A) Relative quantitative real-time PCR analysis of RUFY3 mRNA transcript level (RUFY3 siRNA targets isoform 1 and 4) (B and C) Representative confocal panels of HeLa cells treated with control siRNA (B) and RUFY3 siRNA (C), stained with LAMP1 (D) Confocal panels of HeLa cells treated with control and RUFY3 siRNA for 48 hours followed by transfection with RUFY3 V.1 Flag (Rescue) (E) Quantification of LAMP1 distribution over distance (n=3, 25-30 cells per experiment)

3.3 RUFY3 V.1 is sufficient to mediate retrograde transport

To further validate the role of RUFY3 V.1 in mediating perinuclear clustering in cells, we used the FRB-FKBP rapamycin-induced heterodimerization system (Figure 8A) to localize RUFY3 to different intracellular compartments. In this approach, GFP tagged RUFY3 V.1 was fused to FKBP12 (FK506 Binding protein) tag and Tom-70p (Mito) (localized to outer mitochondrial membrane) was fused to FRB (FKBP-Rapamycin Binding) protein tag. On addition of rapamycin (working conc. 200nM), FKBP tagged RUFY3 V.1 was rapidly translocated to the mitochondrial membrane where Mito-FRB was targeted (Figure 9A to 9D). Interestingly, we observed dramatic clustering of mitochondria (stained with Tom-20) near MTOC. Tom-20 distribution over distance (from the nuclear rim to the cell periphery) was quantified for three independent experiments (20-25 cells/experiments) and we observed increased perinuclear distribution in FKBP tagged RUFY3 V.1 expressing cells treated with rapamycin (Figure 9E).

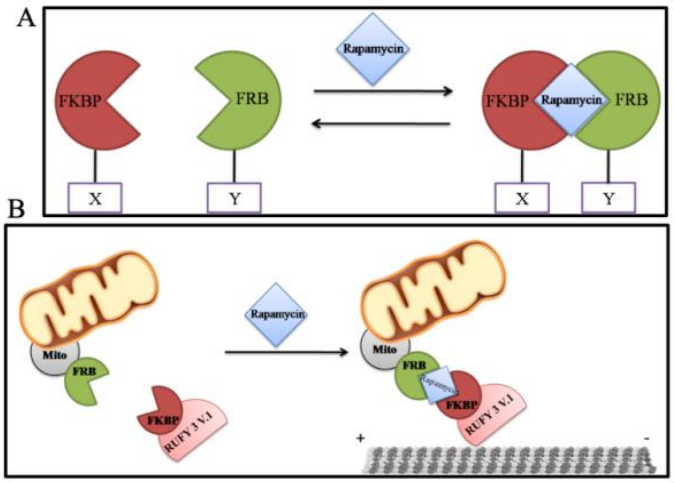

Figure 8: FKBP-FRB Rapamycin Induced Heterodimerization System (A) Representatitive image of FKBP-FRB heterodimerization system (B) Representative image of the methodology used for mislocalising RUFY3 V.1 on mitochondria

Furthermore, previous studies have established that functional dynein-dynactin machinery is necessary to mediate retrograde transport. We were therefore intrigued to elucidate if RUFY3 V.1 mediated perinuclear clustering was dependent on the dynein-dynactin machinery. On addition of rapamycin, compared to a predominant perinuclear clustering in control cells, dynein depleted cells showed a typical dispersed pool of mitochondria, confirming that RUFY3 V.1 mediated perinuclear clustering is dynein dependent (Figure 10A and 10B).

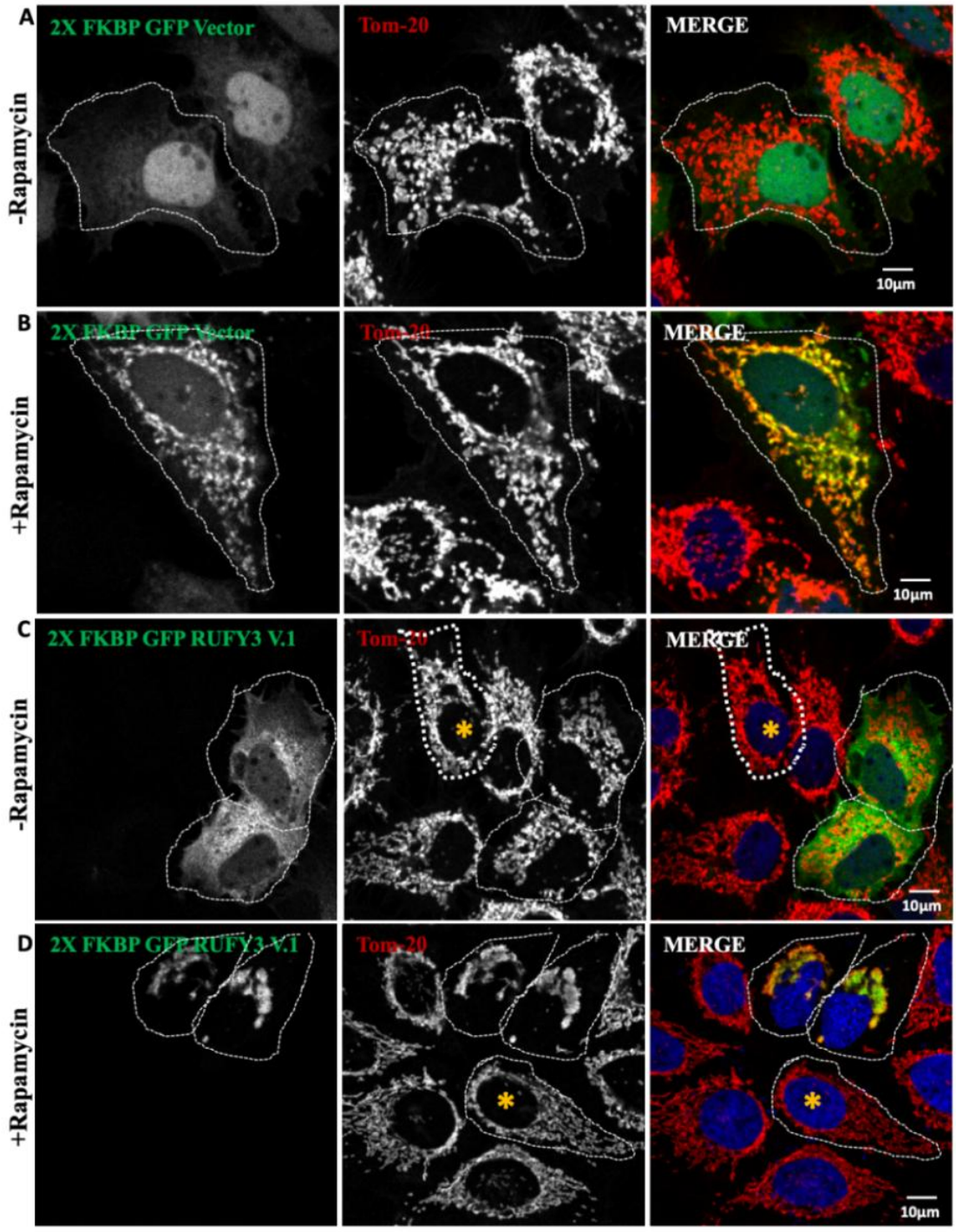

Figure 9: **Mislocalized RUFY3 V.1 mediates perinuclear clustering** (A and D) Representatitive confocal panels of HeLa cells co-transfected with FKBP tagged RUFY3 V.1 and FRB tagged Tom70p, co-stained with endogenous Tom-20 (in the presence and absence of rapamycin) (E) Quantification of Tom-20 distribution over distance (n=3;20-25 cells/experiment)

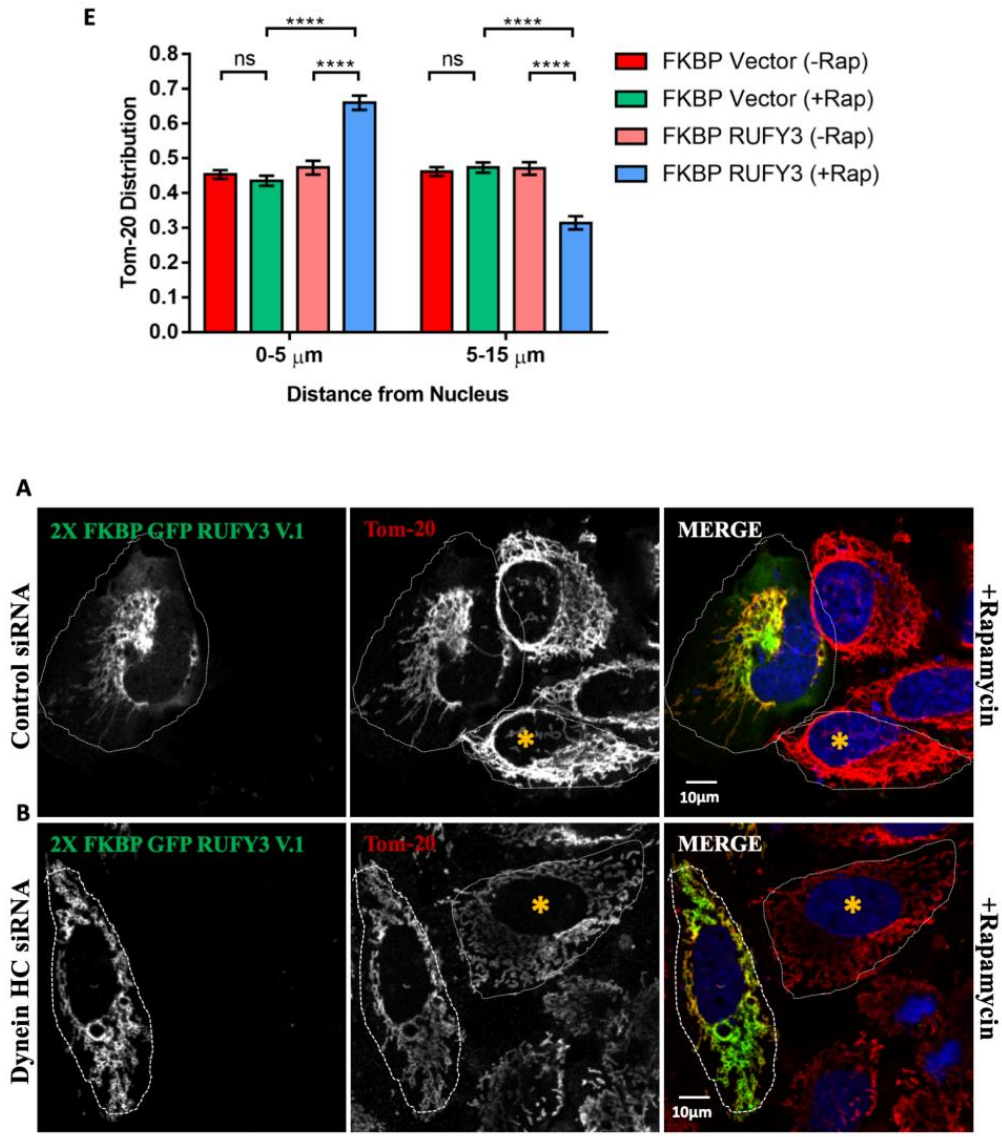

Figure 10: **RUFY3 mediated perinuclear clustering is dynein dependent** (A and B) Representatitive confocal panels of HeLa cells treated with control and dynein heavy-chain siRNA. After 48 hours, FKBP tagged RUFY3 V.1 and Mito-FRB were co-transfected and stained with endogenous Tom-20 (untransfected cells represented with *).

3.4 Members of JNK interacting protein family and dynein-dynactin machinery as interaction partners for RUFY3 V.1

↓ Unique	Total	reference	AVG	Gene Syml	MWT(kDa)
55	684	sp|Q7L099|RUFY3_HUMAN	2.8205	RUFY3	52.93
29	167	SjGST_Schistosoma	2.8122		25.48
15	18	sp|Q9H6S0|YTDC2_HUMAN	2.5041	YTHDC2	160.15
13	13	sp|P41252|SYIC_HUMAN	2.8951	IARS	144.41
8	8	sp|Q14683|SMC1A_HUMAN	2.7217	SMC1A	143.14
7	9	sp|Q14203|DCTN1_HUMAN	2.9451	DCTN1	141.61
7	7	sp|O75533|SF3B1_HUMAN	3.2119	SF3B1	145.74
7	7	sp|Q00341|VIGLN_HUMAN	2.6022	HDLBP	141.37
6	6	sp|P16152|CBR1_HUMAN	3.6753	CBR1	30.36
6	6	sp|O60271|JIP4_HUMAN	3.4147	SPAG9	146.11
6	6	sp|P27708|PYR1_HUMAN	2.9191	CAD	242.83
5	5	sp|O95347|SMC2_HUMAN	2.7286	SMC2	135.57

Figure 11: Results of mass spectrometry Representatitive image of mass spectrometry studies conducted using GST tagged delta RUN RUFY3 as bait, incubated with HEK293T cell lysate.

Our findings have confirmed the role of RUFY3 V.1 in regulating distribution of lysosomes. Next, we sought to identify potential mediators which might be facilitating the process. We conducted a pull-

down assay using GST tagged ΔRUN RUFY3 V.1 as the bait protein and incubated it with HEK293T cell lysate (Figure 11). The samples were electrophoresed on SDS PAGE gel and the requisite bands (obtained after silver staining) were cut and sent for mass spectrometry (Conducted by Ms. Rituraj Marwaha, IISER Mohali). Interestingly, we obtained JIP3/JIP4 and members of dynein-dynactin machinery as one of the potential interaction partners for RUFY3 V.1. Furthermore, in a co-immunoprecipitation assay, we observed that RUFY3 V.1 could co-immunoprecipitate JIP4, dynactin p150 subunit and dynein intermediate chain (DIC) (data not shown). Thereby, we speculated RUFY3 V.1 mediated lysosome positioning to be JIP4-dynein dependent.

3.5 RUFY3 V.1 complexes with the JIP4-dynein machinery

To further confirm the role of JIP4 in lysosome dynamics, we transfected HeLa cells with siRNA against JIP4 and analyzed lysosome distribution. As expected, we observed dramatic peripheral repositioning of lysosomes, similar to RUFY3 depleted cells (Figure 12A and 12B). LAMP1 distribution over distance (from the MTOC to the cell periphery) was quantified over three independent experiments (30-40 cells/experiments) (Figure 12C). However, this effect was not rescued on expressing RUFY3 V.1 in JIP4 depleted background (Figure 13A and 13B). This further validated our speculation that RUFY3 V.1 complexes with JIP4-dynein machinery to mediate perinuclear clustering of lysosomes.

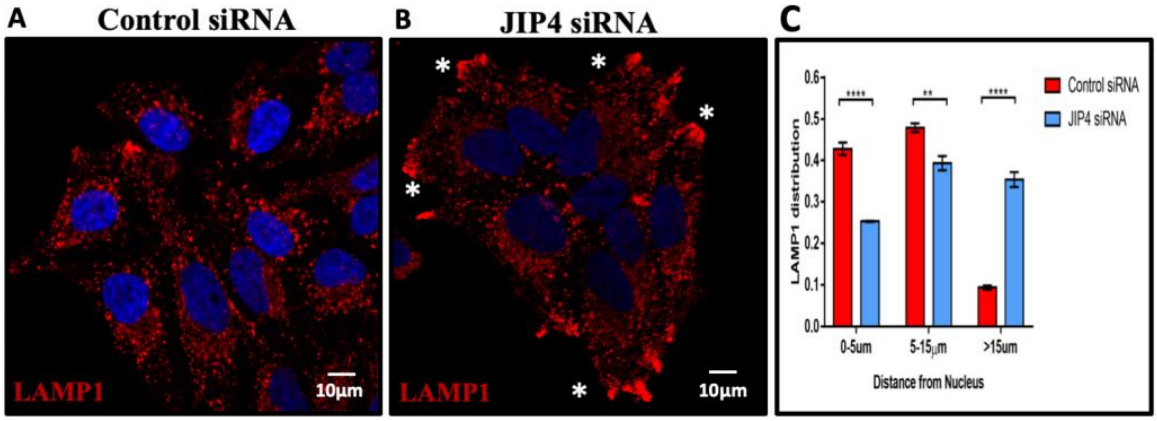

Figure12:JIP4 knock-down causes peripheral redistribution of lysosomes (A and B) Representatitive confocal panels of HeLa cells treated with control siRNA (A) and JIP4 siRNA (B) stained with LAMP1 (C) Quantification of LAMP1 distribution over distance (n=3; 25-30 cells/experiment)

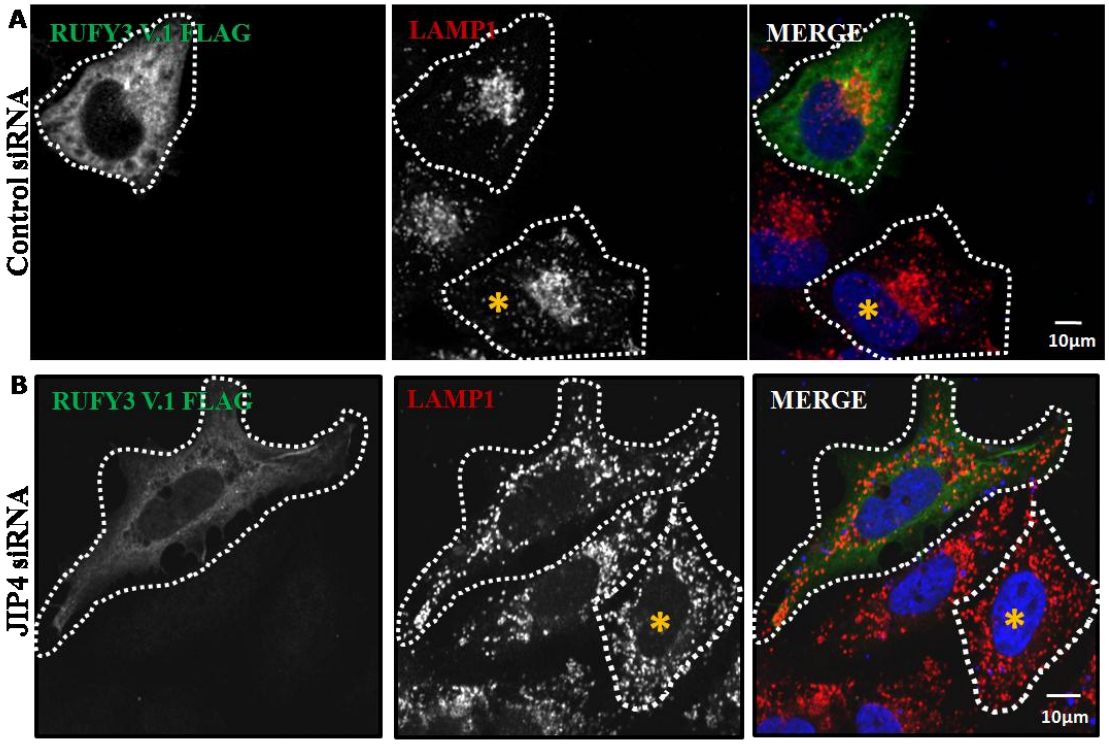

Figure 13: RUFY3 V.1 complexes with JIP4-dynein machinery (A and B) Representatitive confocal panels of HeLa cells treated with control siRNA (A) JIP4 siRNA (B) and dynein HC siRNA (C) for 48 hours followed by transfection with Rufy3 V.1 Flag and stained with LAMP1

3.6 Conclusion and Future Directions

In summary, our findings suggest that RUFY3 V.1, via its N-terminal RUN domain, interacts with active GTP bound Arl8b and regulates dynein-dependent positioning of lysosomes. Furthermore, we also speculate that RUFY3 V.1 complexes with JIP4-dynein machinery and thereby mediate retrograde transport of lysosomes along microtubule tracks.

However, a plethora of questions still remain unaddressed, detailed knowledge of which will improve our understanding of RUFY3 V.1 mediated distribution of lysosomes. Firstly, we want to determine the binding sites of RUFY3 V.1 and JIP4-dynein machinery. We have made various domain-deletion mutants of RUFY3 V.1 and are trying to confirm their binding to JIP4-dynein machinery by western-blot assays and confocal studies (in the process). Also, as mentioned previously, TMEM55B-JIP4-dynein complex mediates the transport of lysosomes to the MTOC. In addition, our findings strongly suggest that RUFY3-JIP4-dynein complex also regulates retrograde transport of lysosomes. However, whether RUFY3 V.1 complexes with TMEM55B-JIP4 or forms an independent complex with the Arl8b

and JIP4-dynein (in the process) needs to be explored. Additionally, co-immunoprecipitation assays and confocal studies suggest that RUFY3 V.1 complexes with JIP4 and Arl8b. We thereby speculate that RUFY3 V.1 acts as an adaptor for Arl8b and JIP4-dynein, thereby mediating the transport of Arl8b-positive compartments to MTOC (Figure 14). We would like to validate these findings further.

Finally, we are keenly interested to determine the significance of RUFY3 mediated lysosome positioning. Previous studies have noted that two key cellular processes, namely autophagy and cell migration, are regulated by the cellular distribution of lysosomes. Lysosome transport to the perinuclear region following starvation is critical to facilitate autophagosome-lysosome fusion and control the rate of autophagosome degradation. Additionally, peripheral lysosomes play an essential role in regulating cell migration by either mediating focal adhesion turnover or by the release of various matrix metalloproteinase (MMPs) which degrade extracellular matrix (ECM) facilitating cell spreading and invasion. Our preliminary findings with RUFY3 suggest that it regulates autophagy as well as cell migration. We observed that RUFY3 depletion in HeLa cells impaired autophagic flux. However, whether RUFY3 regulates autophagosome-lysosome fusion or mediates autophagosome biogenesis remains to be determined. Also, RUFY3 depletion in HeLa cells slowed down the migration of cells and increased the levels of actin stress fibers. However, the mechanism of RUFY3 mediated cell migration needs to be explored further.

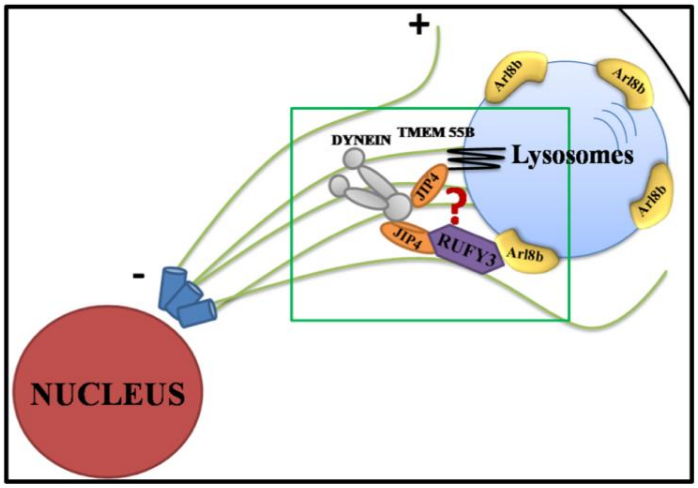

Figure 14: Proposed model for RUFY3 V.1 mediated retrograde transport of lysosomes Representatitive image of hypothesized mechanism of RUFY3 V.1 mediated lysosome positioning in mammalian cells.

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