Role of small GTPase Arl8b and its effector proteins in regulating cargo trafficking to lysosomes

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Certificate

The work presented in this thesis has been carried out by me under the supervision of Dr. Mahak Sharma at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, 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 bonafiderecord of original work done by me and all sources listed within have been detailed in the bibliography.

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

Dr. Mahak Sharma

(Supervisor)

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List of Publications

1. Khatter D, Raina VB, Dwivedi D, Sindhwani A, Bahl S,Sharma M. The small GTPase Arl8b regulates assembly of the mammalian HOPS complex on lysosomes. **J Cell Sci** 2015; 128:1746-61; PMID: 25908847; http://dx.doi.org/10.1242/jcs.162651

2.Khatter D, Sindhwani A, Sharma M. Arf-like GTPase Arl8: Moving from the periphery to the center of lysosomal biology. **Cell Logist.**2015 Sep21;5(3):e1086501; PMID: 27057420; http://dx.doi.org/10.1080/21592799.2015.1086501

Thesis Abstract

Eukaryotic cells have an elaborate endocytic system that is specialized totake up materials from the environment and route it to the lysosomes fordegradation. The endocytic pathway is marked by multiple fusion andfission events whose regulation involves an interplay of small GTPases,tethering factors and SNAREs. HOmotypic fusion and Protein Sorting (HOPS)complex is an evolutionarily conserved multisubunit tethering factor thatmediates vesicle fusion with lysosomes. The mechanism of mammalian HOPSaction and its crosstalk with other lysosome proteins is only beginningto be understood. In the first part of this thesis, we demonstrate thatthe small GTPase Arl8b interacts with, and recruits HOPS complex tolysosome membranes. Depletion of HOPS subunit Vps41 results in defects in cargo trafficking to lysosomes that were rescued upon expression ofwild-type but not an Arl8b-binding-defective mutant, suggesting thatArl8b-dependent localization of HOPS complex to lysosomes is required for cargo degradation. Since the discovery of Arl8b, an ever-increasing numberof its interaction partners have come into light, inclusive of the RUNdomain-containing proteins. In the second section of the thesis, we haveidentified that Arl8b interacts with the RUN and FYVE (RUFY)domain-containing proteins, Rabip4' and Rabip4/RUFY1, via their RUNdomains. Arl8b depletion results in striking displacement of endogenousRabip4(s) from the endosomal membranes to the cytosol that can be rescuedupon expression of siRNA-resistant Arl8b. Future studies will be useful togain insights into how Arl8b regulates the membrane localization ofRabip4(s) and significance of Rabip4(s) interaction with Arl8b in membranetrafficking.

Thesis Synopsis

Title – Role of small GTPase Arl8b and its effector proteins in regulating cargo trafficking to lysosomes

Advisor – Dr. Mahak Sharma

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Chapter 1: Introduction

The endocytic pathway in eukaryotic cells is a complex network of membrane-bound compartments that constantly exchange material through vesicular or tubular carriers. Vesicle fusion is a tightly regulated process that involves interplay of small GTPases, tethering factors and SNARE proteins. The Rab and Arf families of small GTPases are important regulators of endocytic trafficking that recruit their numerous effectors to intracellular membranes in a GTP-dependent manner and mediate vesicle budding, tethering, and subsequent fusion. Recent studies have also characterized the Arf-like (Arl) family of small GTPases, which are implicated in diverse cellular processes including vesicular trafficking, cytoskeletal organization and ciliogenesis. Small GTPases in their GTP-bound form (active) recruit effector proteins/protein complexes (such as tethering factors) that bridge the two membranes destined for fusion and proofread SNARE complex assembly, thereby imparting specificity to the fusion process. Two multi-subunit tethering factors that regulate trafficking through the endocytic pathway are Class CcOReVacuole/Endosome Tethering (CORVET) complex, required for homotypic early endosome fusion and maturation of early endosomes to late endosomes, and HOmotypic fusion and Protein Sorting (HOPS) complex, which mediates homotypic late endosome fusion and their fusion with lysosomes. Both the complexes are well studied in yeast and are conserved from yeast to mammals. However, the mechanistic insight into the mammalian counterparts of the vesicle fusion machinery is only beginning to be understood. With a focus on the lysosome biology, we steered our efforts to understand how HOPS complex is recruited to lysosomes, described in the first part of the thesis. By using a diverse array of microscopic and biochemical techniques, we have found that small GTPase Arl8b regulates HOPS complex assembly on lysosomes. We have also established a well-known Arl8b effector- PLEKHM2/SKIP, as a novel interaction partner of HOPS

subunit Vps39, and that the Arl8b-HOPS-SKIP tripartite complex governs cargo traffic to lysosomes.

Since the discovery of Arl8b, an ever-increasing number of its interaction partners have come into light, inclusive of the RUN domain-containing proteins. RUN domain-containing proteins have been implicated as effectors of small GTPases and as regulators of membrane polarity and trafficking. RUN and FYVE domain-containing protein RUFY1 localizes to early endosomes where it regulates transferrin receptor recycling to the plasma membrane. RUFY1 has two isoforms – Rabip4' containing 708 amino acids is a Rab4 and Rab5 effector, and Rabip4/RUFY1 containing 600 amino acids is a dual effector of Rab4 and Rab14. Previously, Rabip4' has been shown to regulate lysosome positioning in HEK293T cells. In the second part of the thesis, we have identified RUN and FYVE domain-containing protein Rabip4' as an interaction partner of Arl8b. Future studies will be instrumental in revealing the precise role of this novel Arl8b interaction partner in regulating endocytic traffic towards lysosomes and lysosome positioning.

Chapter 2: The small GTPase Arl8b regulates assembly of mammalian HOPS complex on lysosomes

HOPS complex is a multimeric tethering factor that was first described in *Saccharomyces cerevisiae*, where it is composed of six subunits, namely, Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41. Small GTPase Ypt7 binds to Vps39 and Vps41 and recruits HOPS to late endosomes in yeast where it mediates homotypic fusion of late endosomes, late endosome-vacuole fusion, and fusion of autophagosomes with the vacuoles. HOPS complex is conserved from yeast to mammals and metazoan HOPS consists of all the six subunits previously described for yeast. While the metazoan HOPS complex biology is slowly unraveling, it is now clear that metazoan HOPS functions to mediate biogenesis of late endocytic compartments and fusion of lysosomes with late endosomes, phagosomes and autophagosomes. However, little was explored how mammalian HOPS complex assembles on lysosomes and bring about these functions. Our results demonstrate that the small GTPase Arl8b, but not Rab7 (mammalian ortholog of yeast Ypt7), is essential for membrane localization of the human Vps41 (hVps41) subunit of the HOPS complex, followed by assembly of the entire complex on the lysosomes,

mediated by subunit-subunit interactions. A T146 SNP in hVps41, previously identified to impair its neuroprotective function against α-synuclein-induced neurodegeneration, exhibits an abrogated Arl8b binding and lysosome localization. RNA interference (RNAi)-mediated depletion of hVps41 results in the impaired degradation of EGFR that is rescued upon expression of wild-type but not an Arl8b-binding-defective mutant of hVps41, suggesting that Arl8b-dependent lysosome localization of hVps41 is required for its endocytic function (**Khatter D et al., *JCS* 2015**).

Chapter 3: Arl8b effector, SKIP, interacts with Vps39 subunit of HOPS complex and regulates cargo trafficking to lysosomes

Previous studies have reported a role for Arl8b and its effector SKIP in regulating kinesin-dependent plus-end-directed motility of lysosomes, while dynein-dependent retrograde positioning of lysosomes is largely attributed to coordinated action of Rab7 and its effector RILP. RILP has been shown to directly binds HOPS complex and mediate its localization to late endosomes. In this study, we have identified that the Arl8b effector SKIP directly binds to, and promotes association of hVps39 with Arl8b and kinesin-1-positive peripheral lysosomes, and possibly competes with RILP for association with the HOPS complex. Arl8b expression is found to be crucial for the association of HOPS subunits with SKIP-positive endosomes. SKIP binds to Arl8b via its RUN domain while binding to hVps39 does not require the presence of this domain. Accordingly, knockdown of SKIP reduces the recruitment of hVps39 to Arl8b-positive lysosomes significantly. Furthermore, RNAi-mediated depletion of SKIP impairs trafficking to lysosomes and degradation of EGFR. In conclusion, our results show that Arl8b orchestrates the membrane association and endocytic function of HOPS subunits by directly recruiting hVps41 to lysosomes and, indirectly, by

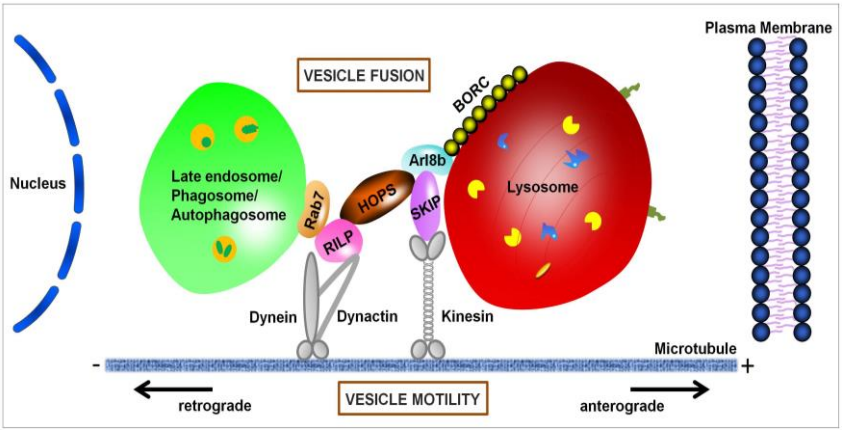

Figure 1: A model depicting the interplay of Rab7 and Arl8b effectors at endosome-lysosome junction (**Khatter D et al., *Cellular Logistics* 2015**)

association of its effector SKIP with hVps39 (**Figure 1**).

Chapter 4: Characterization of Rabip4' as a novel interaction partner of Arl8b

RUN domain-containing proteins act as effectors of Rab family of small GTPases and function as regulators of membrane traffic, polarity, motility and signaling. For instance, small GTPase Rab7, a well-known regulator of endosomal trafficking, interacts with RUN domain-containing protein FYCO1 and regulates anterograde movement of lysosomes. To date, at least one member of Arf-like (Arl) family, Arl8b, has been shown to interact with RUN-domain-containing protein and regulate lysosome motility. Early on after its discovery, Arl8b was recognized as a regulator of lysosome positioning in mammalian cells via its interaction with the RUN domain-containing protein SKIP, which in turn recruits anterograde microtubule motor kinesin-1 to lysosomes. Recent study from our laboratory has shown that Arl8b interacts with another RUN domain-containing protein PLEKHM1, and regulates cargo trafficking to lysosomes (Marwaha et al., JCB 2017). Previous studies have shed light on RUN and FYVE domain-containing (RUFY) protein family that comprises four members, namely, RUFY1, RUFY2, RUFY3 and RUFY4. The longer and shorter variant of RUFY1 (also known as Rabip4' and Rabip4, respectively) localizes to early endosomes and regulate receptor recycling. Surprisingly, a recent study demonstrated that Rabip4' regulates lysosome positioning, although the mechanism remains unknown. Here we find that Rabip4' interacts with Arl8b via its RUN domain and localizes to membranes that are positive for both LAMP1 and Arl8b. These findings suggest that besides early endosomes, Rabip4' also localizes to late endosomes/lysosomes. Notably, depletion of Arl8b results in partial displacement of endogenous Rabip4s from membrane to cytosol. Previous studies suggest that Rabip4'/Rabip4 can bind to the small GTPases Rab4 and Rab14. Our results show that Rabip4' colocalizes with both Rab4/Rab14 and Arl8b on the same endosomes. It would be important to determine whether Rabip4' can also serve as a dual effector for Rab and Arl family of small GTPases. Future studies will be useful to gain insights into coordinated regulation of lysosome distribution and cargo trafficking by Arl8b and Rabip4' in mammalian cells.

Abbreviations

LAMP1/2 – Lysosome-associated membrane protein 1/2

LSD – Lysosomal storage disorder

CHC – Clathrin heavy chain

CLC – Clathrin light chain

CME – Clathrin-mediated endocytosis

AP – Adaptor protein

GTP – Guanosine tri-phosphate

GDP – Guanosine di-phosphate

CCV – Clathrin coated vesicle

HSC – Heat Shock cognate

CIE – Clathrin-independent endocytosis

AMF – Autocrine motility factor

CTX – Cholera toxin

IL2 – Interleukin2

GPI - Glycosylphosphatidylinositol

GPI-AP – Glycosylphosphatidylinositol-anchored protein

SV40 – Simian virus 40

CD – Circular dichroism

GEEC – GPI-AP-enriched early endosomal compartments

CLIC – Clathrin-independent carriers

PI3K – Phosphatidylinositol-3-kinase

MHC – Major histocompatibility complex

PRR – Pattern recognition receptor

ER – Endoplasmic reticulum

PAS – Phagophore assembly site

ATG – Autophagy-related

SNARE - Soluble N-ethylmaleimide-sensitive factor attachment protein receptors

PLEKHM 1/2 – Pleckstrin Homology and RUN domain containing M1

HOPS – Homotypic fusion and protein sorting

VPS – vacuole protein sorting

APPL – Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif

EEA1 – Early endosome antigen 1

ERC – Endocytic recycling compartment

EHD – C-terminal Eps15 homology domain-containing protein

TGN – Trans-Golgi network

SNX – Sorting nexin

GPCR – G protein-coupled receptor

RTK – Receptor tyrosine kinase

ILV – Intra-luminal vesicle

EE – Early endosome

ESCRT - Endosomal Sorting Complex Required for Transport

MVB – Multi-vesicular body

GDI – Guanine dissociation inhibitor

GEF – Guanine exchange factor

GAP – GTPase activating protein

CORVET - Class C core vacuole/endosome tethering

RILP – Rab-interacting lysosomal protein

FYCO1 – FYVE and coiled-coil domain containing 1

EGFR – Epidermal growth factor receptor

CMT2B – Charcot-Marie-Tooth type 2B

SAR – Sar1p-like members of the Ras-family of small GTPases

ARF – ADP Ribosylation factor

ARL – ARF-like

RAB – Ras-like in rat brain

COP – Coatomer protein

GGA - Golgi-localized, gamma-ear containing, ADP-ribosylation factor binding

BORC – BLOC-1 related complex

BLOC-1 – Biogenesis of lysosome-related organelles complex 1

SKIP – SifA and kinesin interacting protein

NK – Natural killer

mTORC1 – Mammalian target of rapamycin complex 1

SCV – *Salmonella*-containing vacuole

SIF – *Salmonella*-induced filament

MTC – Multisubunit tethering complex

COG – Conserved oligomeric Golgi

GARP – Golgi-associated retrograde protein

TRAPP – Transport protein particle

VAMP – Vesicle associated membrane protein

PCCA2 – Progressive cerebello-cerebral atrophy type 2

SM – Sec1/Munc18

TGFBRAP1 – Transforming growth factor beta receptor associated protein 1

SDS-PAGE – Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

EM – Electron micrograph

α-SNAP – Soluble NSF attachment protein

NSF – N-ethylmaleimide-sensitive factor

RING – Really interesting new gene

ALPS – Amphipathic lipid packing sensor

VIPAR – VPS33B interacting protein, apical-basolateral polarity regulator, spe-39 homolog

SPE39 – Spermatogenesis-defective protein 39

MDV – Mitochondria-derived vesicle

SNK – Serum-inducible protein kinase

HPS – HermanskyPudlak syndrome

RUN – RPIP8, unc-14 and NESCA

RUFY – RUN and FYVE domain-containing

FYVE – Fab1, YOTB, Vac1and EEA1

PX domain – Phox homology domain

PtdIns – Phosphatidylinositol

SNP – Single nucleotide polymorphism

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

TfR – Transferrin receptor

PCR – Polymerase chain reaction

GFP – Green fluorescent protein

KLC – Kinesin light chain

GST – Glutathione S transferase

HA - Haemagglutinin

MTOC – Microtubule organizing center

CHCR – Clathrin heavy chain repeat

Table of Contents

Chapter I

Introduction

(Parts of section 1.4.4 are derived from Khatter et al, Cellular Logistics 2015)

Chapter 1

1.1 Lysosomes: the hub of cargo degradation

Lysosomes function as the digestive system in eukaryotic cells, serving to degradebiomolecules and unwanted materials taken up from the cellular environment and recycle cellular waste. They are membrane-bound organelles that were discovered by Christian de Duve in 1955when he discovered that the activity of acid hydrolases in the subcellular homogenate fractions remarkably increased upon insults to the membrane integrity. The interior of lysosomes is filled with over 60 different types of hydrolytic enzymes and maintained at an acidic pH (4.5-5),provided through the activity of the proton-pumping V-ATPases in the limiting membrane that ensures maximum degradative activity of the hydrolases(Xu and Ren 2015). Lysosomes have a specialized limiting membrane decorated with more than hundred proteins of which the most abundant are the type 1 transmembrane proteins LAMP-1 and LAMP-2. The inner face lined with a thick glycocalyx consisting primarily of the oligosaccharide side chains on LAMP-1 and LAMP-2protects the lysosome membrane from the degradative action of the lumenal acid hydrolases(Settembre, Fraldi, Medina and Ballabio 2013).Recent advancements have uncovered that lysosomes mediate a range of unconventional cellular processes in addition to their traditional role in degradation. Lysosomes can fuse with the plasma membrane and secrete their contents to the cell exterior known as lysosome exocytosis. Fusion of lysosomes with the plasma membrane is implicated in membrane repair, bone resorption and defense against pathogens. The cytosolic surface of the lysosome membrane is now recognized as a major site of action of that regulate cellular metabolism(Settembre, Fraldi et al. 2013). Mutations in genes encoding lysosome enzymes or other proteins involved in lysosome functions can cause lysosomal storage diseases (LSDs). LSDs

comprise a group of about 50 rare inherited metabolic disorders characterized by the accumulation of undegraded substrates inside the lysosome. Such mutations may also result in impaired degradation of autophagic substrates and an accumulation of misfolded proteins and protein aggregates, progressing to neurodegeneration. Additionally, lysosomes have been attributed important roles in several common human diseases such as cancer and obesity(Ballabio 2016).

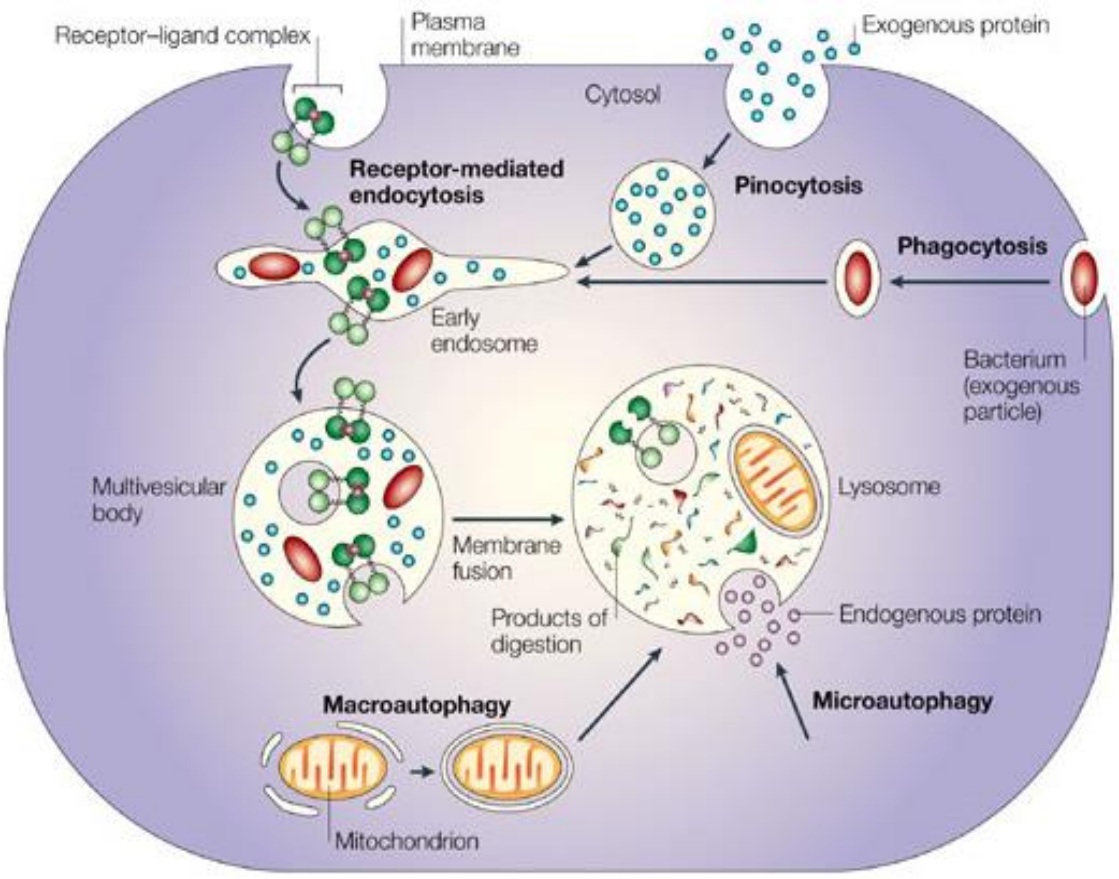

Figure 1.1 Endomembrane trafficking pathways leading to lysosomes. Three fundamental pathways that merge at the lysosomes comprise endocytosis, phagocytosis and autophagy. Cargo internalised at plasma membrane through either pathway reaches the early endosome. Early endosomes mature into late endosomes which fuse which lysosome compartments, where the cargo is degraded with the aid of hydrolytic enzymes. Adapted from Ciechanover 2005.

Even today the best understood role of lysosomes is degradation and recycling of cellular waste. Extracellular materials such as macromolecules, nutrients, receptors, solutes and integral membrane proteins are taken up by endocytosis in early endosomes that either recycle the cargo back to the plasma membrane or sort it

towards the late endocytic compartments which eventually fuse, transiently or completely with lysosomes. Transient or complete fusion results in the formation of degradative compartments called endolysosomes, which are hybrid organelles from which lysosomes are reformed(Luzio, Pryor and Bright 2007). In specialized cells such as macrophages and dendritic cells, another pathway, called phagocytosis, initiates at the plasma membrane and engulfs microbes in phagosomes that are again routed to the lysosome, an essential measure that protects cells from infectious organisms(Kinchen and Ravichandran 2008). A third pathway that leads to the lysosomes, known as autophagy, operates exclusively intracellularly,is activated by a broad range of cellular stress-inducing conditions and mediates the degradation of damaged organelles, protein aggregates, oxidized lipids and intracellular pathogens(Glick, Barth and Macleod 2010). The resulting breakdown products from all degradation processes are used to generate new cellular components and energy in response to the nutritional needs of the cells (Fig 1.1). The above-mentioned pathways are discussed in detail in the following section.

1.2 Endo-membrane trafficking pathways leading to lysosomes

1.2.1 Endocytosis

Endocytosis is a fundamental process by which eukaryotic cells internalize extracellular substances, plasma membrane components (macromolecules, plasma membrane receptors and proteins, transporters etc.) and soluble molecules in endocytic vesicles that are delivered to early endosomes. Endocytosis is necessary fornutrientsto reach the cells where they act as building blocks, regulation of receptor activity and maintenance of plasma membrane homeostasis, each crucial for maintaining healthy functioning of cells and tissues(Kumari, Mg and Mayor 2010). There are multiple modes of internalization that operate, concurrently, at the cell

surface and may be classified as either clathrin-dependent or clathrin-independent mechanisms (Fig. 1.2) described as follows:

1.2.1.1 Clathrin-dependent endocytosis

Clathrin-dependent endocytosis is the best characterized mechanism for mediating the internalization of cargo such as membrane receptors, viruses and toxins in cells(Zhu, Zhuang, Ben, Qian, Huang et al. 2011). It is a process where the endocytic vesicles carrying the cargo are coated on the outside with a lattice consisting of polymerized clathrin. (Popova, Deyev and Petrenko 2013).Clathrin assembles into triskelia composed of three clathrin heavy chains (CHCs) and three clathrin light chains (CLCs), whose three-dimensional arrangement forms the clathrin coat. Both the clathrin heavy chains and light chains are highly conserved across different species. Clathrin-mediated endocytosis (CME) can be divided into five steps, namely, nucleation, cargo selection, clathrin coat assembly, membrane scission and uncoating(Doherty and McMahon 2009). The formation of clathrin-coated pit initiates with the recruitment of adaptor and accessory proteins which interact with membrane lipids and sorting motifs in cargo proteins and subsequently recruit clathrin from the cytoplasm to the initiation site at the plasma membrane.Five adaptor protein complexes have been identified: AP-1, AP-2, AP-3, AP-4 and AP-5, each comprising of four subunits : α, β, μ and σ. This complex forms a structure resembling Mickey Mouse's head, where the center is formed by the μ and δ subunits, and the two "ears" are composed of the C-terminal domains of the two large subunits, α and β, connected to the "head" via a flexible neck(Popova, Deyev et al. 2013). AP-1, AP-3, AP-4 and AP-5 localize majorly to the trans-Golgi network and/orendosome membranes. AP-2 is the main adaptor protein present at plasma membrane that plays a role in the formation of clathrin-coated vesicles during endocytosis. To aid cargo sorting into

clathrin-coated vesicles, different kinds of endocytic sorting signals such as the di-Leucine – based motif [DE]xxxL[LI] and the Tyrosine-based motif Yxxφ (where φ is a bulky hydrophobic amino acid) are present in the cargo proteins that are recognized by adaptor-protein complexes(Staudt, Puissant and Boonen 2016). As AP-2 or other adaptor proteins recognize the cargo, clathrintriskelia are recruited from the cytosol and the clathrin coat starts to assemble. After cargo selection and clathrin coat assembly, dynamin-dependent membrane scission occurs at the neck of nascent vesicle that releases the vesicle in the cytoplasm. Dynamin is a large GTPase that assembles intomultimeric helical arrays, wrapping the necks of clathrin-coated pits, and its GTP hydrolysisis necessary for membrane fission, releasing the CCV into the cell interior(Doherty and McMahon 2009). Post-detachment from the plasma membrane, the clathrin-coated vesicle loses its coat and fuses with early endosomes. The disassembly of the clathrin coat is mediated by auxilin, which is recruited to the coat once the vesicle has pinched off from the membrane. Auxilin then recruits the ATPase HSC70 (Heat Shock Cognate 70), which initiates the disassembly of clathrin coat.

1.2.1.2 Clathrin – independentendocytosis

Lesser understoodendocytic mechanisms that do not utilize clathrin operate in both animals and plants. These pathways exploit lateral heterogeneity in plasma membrane lipid and protein composition to select cargo into dynamic membrane microdomains that bud into the cell (Nichols and Lippincott-Schwartz 2001). While previous reports have shown that CIE accounts for about 70% of fluid uptake and 60-85% of membrane uptake, recent studies suggest little significance of this pathway in mammalian cells. CI mechanisms can be further classified as:

1.2.1.2.1 Caveolae-dependent endocytosis

Lipid rafts are sub-domains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids(Kumari, Mg et al. 2010). Caveolae are small plasma membrane invaginations of 60-80 nm in diameter that can be viewed as a subset of lipid rafts with the presence of the protein caveolin-1. Caveolae are formed by assembly of caveolins, integral membrane proteins that directly bind to membrane cholesterol, of which there are three subtypes – Caveolin-1, 2 and 3(Doherty and McMahon 2009). Caveolin 1 and 2 are responsible for caveolae formation in endothelial cells, adipocytes and fibroblasts while Caveolin 3performs this function in skeletal and cardiac muscle cells. Caveolin-1 is enriched in caveolae, with 100-200 molecules per caveola, and cells that do not express this protein are devoid of morphologically evident caveolae(Doherty and McMahon 2009). The sensitivity of caveolae-dependent endocytosis to cholesterol depletion distinguishes these pathways from clathrin-dependent and constitutive pinocytotic pathways (Nabi and Le 2003). Endocytic ligands can be sorted at the plasma membrane to different caveolae domains for internalization to distinct intracellular compartments. Caveolaepathwayis dynamin-dependent and has been shown to mediate internalization of sphingolipids and sphingolipid binding toxins (cholera toxin [CTX]*and shiga toxin), GPI-anchored proteins, the autocrine motility factor (AMF), endothelin, growth hormone, and IL2 receptors, viruses (including SV40), and bacteria(Doherty and McMahon 2009).

1.2.1.2.2 Flotillin – dependent endocytosis

Flotillins are highly conserved and ubiquitously expressed proteins that are oligomerized in membrane microdomains distinct from the caveolae. Flotillins predominantly localize to the plasma membrane and endosome structures, *i.e.*, late endosomes, recycling endosomes and exosomes. The two flotillin proteins, flotillin 1 (also known as reggie 2) and flotillin 2 (also called reggie 1) share ~50% amino

acidsequence identity and constitutively associate with cholesterol-associated lipid microdomains by virtue of acylation, hetero-oligomerization and binding to cholesterol(Kumari, Mg et al. 2010). Each flotillin protein regulates the stability of the other and both are required for induction of membrane invaginations in a dose-

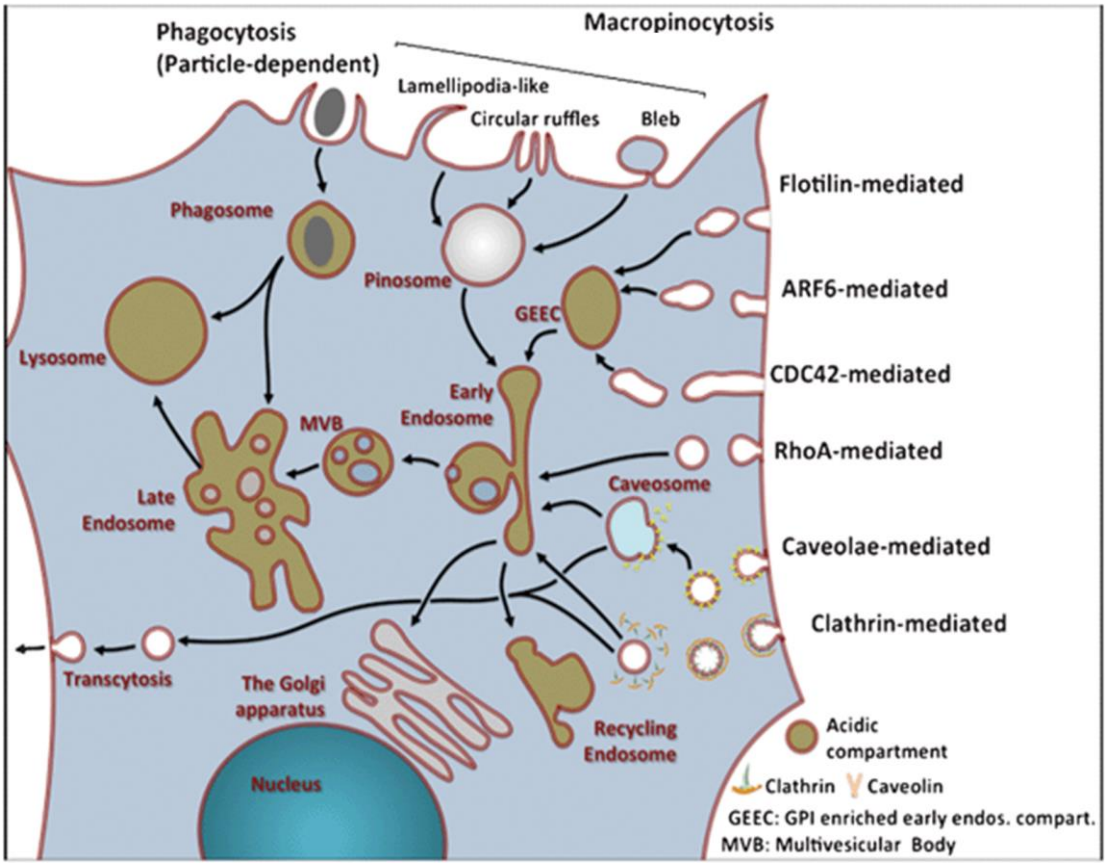

Figure 1.2 Mechanisms of endocytosis in a typical eukaryotic cell. Endocytosis is a process by which cells take up content from extracellular environment in endocytic vesicles. If the budding process is aided by clathrin coat decorating these vesicles, then the mechanism is called clathrin-dependent. Clathrin-independent mechanisms rely on plasma membrane lipid and protein composition to select cargo into vesicles budding into the cell. Adapted from Canton and Battaglia 2012.

dependent manner.While, on one hand, flotillin-mediated endocytosis of some cargo molecules is dynamin-independent, growth factor induced internalization by flotillins clearly depends on dynamin. Both flotillin proteins can be phosphorylated by Fyn kinase at a tyrosine residue that leads to internalization of the microdomains and a redistribution of both flotillin proteins from the plasma membrane to late endosomes and lysosomes. Several cargo molecules have been suggested to utilize flotillin-mediated internalization pathway, such as the GPI-anchored protein CD59, cholera

toxin B subunit, cationic molecules and polyplexes, proteoglycans and proteoglycan-bound ligands, as well as Niemann-Pick C1-like protein(Doherty and McMahon 2009). Studies have also shown thatflotillindepletion reduces the uptake of cargo such as amyloid precursor protein, dopamine receptor, and epidermal growth factor receptor at the plasma membrane, indicating that they all depend upon flotillins for preassembly prior to clathrin-mediated endocytosis.

1.2.1.2.3 The CLIC/GEEC pathway

Endocytosis of lipid-anchored proteins such as glycosylphosphatidylinositol-anchored proteins (GPI-APs) occurs via a dynamin-independent endocytic route into specialized early endosome compartments called GEECs (GPI-AP enriched Early Endosomal Compartments). GEECsresult from fusion of primary uncoated CI tubulo-vesicular carriers called CLICs (Clathrin-independent carriers) which are directly derived from cell surface(Kumari, Mg et al. 2010). These membrane carriers
are highly dynamic and are associated with the activity of small G-proteins Cdc42 and Arf1, but mechanistic insights into their biogenesis is lacking. The GEECs fuse with sorting endosomes in Rab5- and PI3K-dependent manner. This endocytic pathway, termed the CLIC/GEEC pathway, was first identified for its selective internalization of various GPI-APs like the folate receptor(Doherty and McMahon 2009). Additionally, this pathway is characterized by its ability to internalize bacterial toxins and large amounts of extracellular fluid.

1.2.1.2.4 Arf6-associated pathway

Small GTPase Arf6 localizes to plasma membrane in its GTP-bound state where it recruits its effector exocyst complex and regulates membrane recycling to plasma membrane(Prigent, Dubois, Raposo, Derrien, Tenza et al. 2003). An Arf6-dependent endocytic pathway, independent of clathrin and dynamin, but cholesterol-

dependentoperates at the cell surface for the uptake of proteins involved in nutrient transport(Glut1, CD98, Lat1), in extracellular matrix interaction(CD44, CD147), in immune function (MHCClass I, CD1a), and GPI-anchored proteins (CD55 and CD59). In epithelial cells, the small GTPaseArf6 is associated with a distinct endosome compartment that contains integral membrane proteins endocytosed into cells independent of AP-2 and clathrin(Donaldson 2003). Post-internalization, these proteins can either recycle back to the plasma membrane or fuse with the Rab5-associated endosome system. Activation of Arf6 results in increased membrane uptake by macropinocytosis, which is then recycled back to the plasma membrane upon Arf6 inactivation(Radhakrishna and Donaldson 1997). Membrane trafficking in this pathway is also modulated by Arf-6 – mediated activation of phosphatidylinositol-4-phosphate-5-kinase and actin remodeling.

1.2.2 Phagocytosis

Phagocytosis is the active process by which cells take up large particulate matter such as bacteria, fungi, parasites, viruses, dead cells and assorted debris. Uptake of foreign particles is specialized by phagocytic cells such as macrophages, monocytes, neutrophils and dendritic cells as an important measure in regulating innate and adaptive immune responses. Phagocytosis differs from other mechanisms of cellular uptake such as endocytosis and macropinocytosis in that phagocytosis can accommodate a wide variety of particle sizes from hundreds of nanometers to tens of micrometers progressively engaged with phagocyte surface receptors, unlike the former where a limited number of receptors need to be activated by soluble ligands. A plethora of different phagocytic receptors exist, such as Pattern Recognition Receptors (PRRs), opsonic receptors and receptors of apoptotic corpses,which have various degrees of ligand specificity. Also, phagocytosis involves local remodeling of

the actin cytoskeleton, which drives the deformation of the plasma membrane(Fletcher and Mullins 2010). The formation of a phagosome initiates with the binding of ligand to surface receptors followed by activation of receptor-meditated signaling cascades. This activation triggers remodeling of actin cytoskeleton and progressive engagement of additional receptors around the particle, thereby leading to closure of the phagosome. In addition to the clearance of foreign particles, phagocytosis is also critical for cell turnover within an organism. Billions of cells that die by apoptosis everyday are taken up by phagocytosis and disposed off. Defects in apoptotic cell clearance, usually associated with impaired phagocytosis, often results in autoimmune disorders(Munoz, Lauber, Schiller, Manfredi and Herrmann 2010). Thus pharmacological potentiation of phagocytosis has a medical relevance in treatment of certain forms of autoimmune disorders.

1.2.3 Autophagy

Autophagy or macroautophagy is a degradative process that delivers cytoplasmic contents such as worn-out organelles, long-lived unwanted proteins and protein aggregates to lysosomes for degradation. In addition to maintaining the regular organelle turnover and cellular homeostasis, autophagy also sets in under conditions of energy stress and nutrient starvation(Feng, He, Yao and Klionsky 2014). As a survival strategy/response, cellular membranes elongate and sequester portions of cytoplasm inclusive of proteins, organelles and foreign matter leading to formation of a nascent vesicle called autophagosome. The process begins with an isolation membrane (also known as phagophore) derived from ER or Golgi or endosomes at Phagophore Assembly Site (PAS) or omegasome, followed by its elongation, cargo engulfment and sealing to form the autophagosome(Yang and Klionsky 2010). A conserved group of 18 autophagy-related (ATG) proteins has been identified as the

core machinery for autophagosome biogenesis in yeast.Homologues of proteins of the core machinery have been identified from yeast to mammals, and most of them participate in autophagy(Xie and Klionsky 2007). Much of the basis of our current understanding of autophagy is derived from the seminal work of the Japanese researcher, Yoshinori OhsumiHis key contributions include the identification of autophagy induction upon nutrient deprivation, autophagosome formation and most proteins and pathways involved in autophagy (Tsukada and Ohsumi 1993; Mizushima, Noda, Yoshimori, Tanaka, Ishii et al. 1998; Noda and Ohsumi 1998; Nakatogawa, Ishii, Asai and Ohsumi 2012). Autophagy is accompanied by processing of ubiquitously present, microtubule-associated protein light chain 3 (LC3B) by a cysteine protease ATG4 that generates LC3B-I, a hallmark of onset of autophagy. LC3B-I is conjugated to phosphatidylethanolamine to form LC3B-II that is recruited and integrated into the growing phagophore, where it plays a role in selecting cargo for degradation. Autophagosomes then fuse with lysosomes and form autolysosomes where the intra-autophagosomal components are degraded by lysosome hydrolases. This occurs concurrently to LC3B-II degradation in the autolysosomal lumen, making it a useful marker for detection of starvation-induced autophagic activity(Feng, He et al. 2014). The machinery required for autophagosome-lysosome fusion is similar to that required for any other fusion process in mammalian cells, comprising of small GTPases, tethering factors and SNARE proteins. These fusion events are preceded by transport of these two organelles to the perinuclear area as a consequence of increased intracellular pH caused by starvation. A crosstalk between small GTPase Rab7, adaptor protein PLEKHM1, tethering factor HOPS complex and SNAREs, then completes the fusion process(McEwan, Popovic, Gubas, Terawaki, Suzuki et al. 2015;

Wartosch, Gunesdogan, Graham and Luzio 2015).Syntaxin17 is an autophagosomal SNARE implicated in HOPS-mediated, direct autophagosome-lysosome fusion.

1.3 Endosome maturation and cargo sorting

Endocytic cargo containing vesicles internalized from the plasma membrane via clathrin-dependent or –independent pathwaysfirst encounter and fuse with early or sorting endosomes that are the main sorting station in the endocytic pathway (Fig. 1.3). As a result of sorting, incoming cargo destined for degradation in lysosomes is retained and accumulated over time in early endosomes,while cargo directed for recycling either traffics directly to the plasma membrane or via the endocytic recycling compartment(Jovic, Sharma, Rahajeng and Caplan 2010). Early endosomes are heterogeneous in terms of morphology, localization, composition and function. They have a complex structure with vacuolar domains that sequester the endocytic cargo and tubular domains from where endosomes targeted for plasma membrane or Golgi complex emanate. Early endosomes are weakly acidic (pH=6.8-5.9) and contain a relatively low Ca^{2+} concentration. Small GTPaseRab5 is the master regulator for the biogenesis of endolysosome system (Zeigerer, Gilleron, Bogorad, Marsico, Nonaka et al. 2012). Rab5, together with its effectorVPS34/p150, a phosphatidylinositol 3-kinase (PI(3)K) complexthat generates the phosphoinositide (PI) PtdIns(3)P, helps to manifest the identity of early endosomes(Huotari and Helenius 2011).In mammalian cells, distinct subpopulations of early endosomes labeled by Rab5 effectors, APPL1/APPL2 and EEA1, are present(Perini, Schaefer, Stoter, Kalaidzidis and Zerial 2014). APPL1/APPL2-positive endosomes lack PI3P and are localized in cells more peripherally than the canonical EEA1-positive early endosomes. Both EEA1- and APPL-positive endosomes are involved in cargotrafficking and signal transduction processes in the cells(Urbanska, Sadowski, Kalaidzidis and Miaczynska 2011).

Sorting events at the early endosomesenable recycling of the internalized cargo such as receptors, adhesion molecules, ion channels and pumps back to the plasma membrane through either fast or slow recycling pathways(Maxfield and McGraw 2004). Fast recycling pathway involves sorting of proteins and lipids directly from the early endosomes to the plasma membrane. Fast recycling accounts for about 33-60% of the recycling cargo population and determines important consequences for processes such as nutrient uptake, cholesterol efflux and regulation of surface expression of receptors. On the other hand, a prior transfer of the sorted cargo to a juxtanucleartubulo-vesicular compartment called the endocytic recycling compartment (ERC) from which recycling endosomes emanate and reach out to the plasma membrane, leads to slow recycling pathways(Grant and Donaldson 2009). An important function proposed for the transport of cargo from the early endosome to the ERC is to prevent its entry into the degradative compartments. Whereas the fast recycling route returns glycosphingolipids to the plasma membrane and is regulated by small GTPase Rab4, many receptors (eg. transferrin receptor) recycle via Rab11-mediated slow recycling pathway that traverses the ERC. C-terminal EHD proteins specifically EHD1 also regulate cargo recycling to the plasma membrane via the slow recycling pathway(Sharma, Giridharan, Rahajeng, Naslavsky and Caplan 2009). There exists yet another recycling pathway emerging from early endosomes that carries cargo towards the TGN and plasma membrane. The major regulator of this pathway is a conserved multimeric protein complex known asRetromer that is recruited to endosomes via small GTPase Rab7a and early endosome protein sorting nexin 3(Seaman 2012).Retromer is highly conserved across all eukaryotes, comprising of Vps26, Vps29, Vps35, SNX1, SNX2, SNX5 and SNX6 proteins in mammals(Trousdale and Kim 2015). Retromer cargoes include mannose 6-phosphate

receptor, receptors of the G-protein coupled receptor (GPCR) and receptor tyrosine kinase (RTK) families, glucose and metal ion transporters and polarity proteins(Liu 2016).

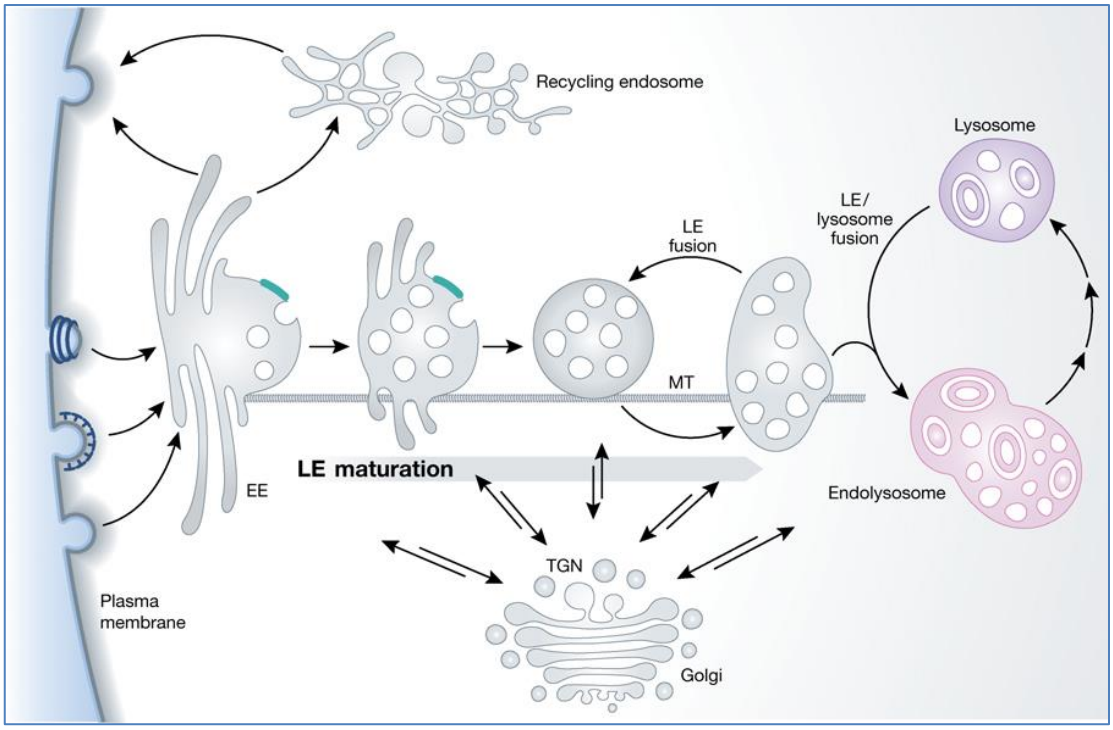

Figure 1.3 Endosome maturation and cargo sorting. Early endosomes act as a sorting station for the incoming cargo, either recycling it back to plasma membrane or routing it to lysosomes for degradation. As the endosomes mature from early to late, they undergo various morphological and physiological changes, sorting the cargo into the intra-luminal vesicles. Fusion with lysosomes generates an endo-lysosomal compartment suited for the function of hydrolytic enzymes under low pH conditions. Adapted from Huotari and Helenius 2011.

The formation of intra-luminal vesicles (ILVs), a characteristic of late endosomes, begins at the stage of early endosomes. For this thecytosolic surface of the EE membrane has characteristic‘plaques’ containing clathrin and components of the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, recruited there byubiquitinated membrane proteins, that induce inward budding of the limiting membrane leading to formation of intra-luminal vesicles(Huotari and Helenius 2011). The ESCRT machinery was first identified in yeast by genetic isolation of mutants termed ‘Class-E mutants’ that were defective in protein sorting to the vacuole resulting in enlarged pre-vacuolar endosome-like compartments containing

undegraded proteins. Studies have revealed that Class E genes act in succession to concentrate trafficking cargoes and include them in late endosomes(Alfred and Vaccari 2016). ESCRT machinery is organized into five distinct protein complexes: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and Vps4 AAA-ATPase complex. Ubiquitination of cargoes such as EGF receptor provides the key signal for initial cargo binding by ESCRT-0. ESCRT-0 recruits ESCRT-I, followed by assembly of ESCRT-II complex, and together they recruit and activate the ESCRT-III complex. ESCRT-III forms spirally-organized multimeric filaments that bend the endosome membrane away from cytoplasm to form invaginated buds, trapping the sorted cargoes in nascent intra-luminal vesicles of the MVBs, which eventually pinch off in the endosome lumen. Furthermore, ESCRT-III recruits the deubiquitinating enzyme Doa4 to remove ubiquitin from cargoes that are included in the ILVs. Finally, the Vps4 ATPase complex binds and fully unfolds the ESCRT-III complex in an ATP-dependent manner and favors pinching off the ILV neck, the final step of ILV biogenesis(Hu, Dammer, Ren and Wang 2015; Schoneberg, Lee, Iwasa and Hurley 2017). A mature late endosome generally has ≥30 ILVs. The formation of a new late endosome is preceded by the generation of a Rab7 domain leading to transient formation of a hybrid endosome labeled by small GTPases Rab5 and Rab7. The Rab5 to Rab7 switch reprograms the association of effector proteins from the cytosol and redefines many of the properties of the endosomes(Rink, Ghigo, Kalaidzidis and Zerial 2005). The endosomes can no longer fuse with early endosomes, instead they acquire the requisite machinery to fuse with themselves and lysosomes. Other critical changes that mark the maturation of early to late endosomes are drop in lumenal pH (6.0 – 4.9), conversion of PtdIns(3)P to PtdIns(3,5)P(2) with sorting of some PtdIns(3)P into ILVs and acquisition of lysosome content such as lysosome

hydrolases from the Trans-Golgi Network (TGN)(Piper and Katzmann 2007). Additionally, there is a loss of tubular extensions present on early endosomes that now move into the perinuclear region of the cell. Here they undergohomotypic fusion with other late endosomes and fusion with lysosomes and form endolysosomes where most of the content is degraded with the aid of hydrolytic enzymes in the endosomes(Scott, Vacca and Gruenberg 2014).

1.4Molecular players involved in vesicle fusion

Vesicle fusion is a tightly regulated process that involves an interplay of small GTPases, tethering factors and SNAREs.Broadly, it initiates by vesicle budding from the donor compartment which is aided by dynamin and coat proteins. Following this, the budded vesicle traverses along microtubules with the help of motor proteins such as dynein and kinesin until it reaches in close proximity to the acceptor compartment. There the small GTPase recruits tethering factors that mediate reversible bridging of two vesicles destined for fusion. Next the two vesicles dock onto each other and v- and t-SNAREs bundle up to form the trans-SNARE complex further leading to water displacement and fusion of two membrane bilayers (Fig. 1.4). The aforementioned proteins comprising the vesicle fusion machinery are discussed below in further detail.

1.4.1 Small GTPases

Small GTPases play a central role in regulating membrane traffic between various compartments in eukaryotic cells by acting as molecular switches. These low molecular weight proteins are spatially regulated by cycling between their GTP- and GDP-bound forms, which activate and inactivate the G-protein, respectively (Fig. 1.5)(Takai, Sasaki and Matozaki 2001).GDP-bound small GTPase localizes to cytoplasm where a protein called Guanine-Dissociation Inhibitor (GDI) sequesters it.

GTP binding, aided by Guanine Exchange Factor (GEF), brings the GTPase to the membrane where it recruits its downstream effectors that mediate cargo selection, vesicle tethering, motility and fusion. Subsequent hydrolysis of GTP facilitated by a

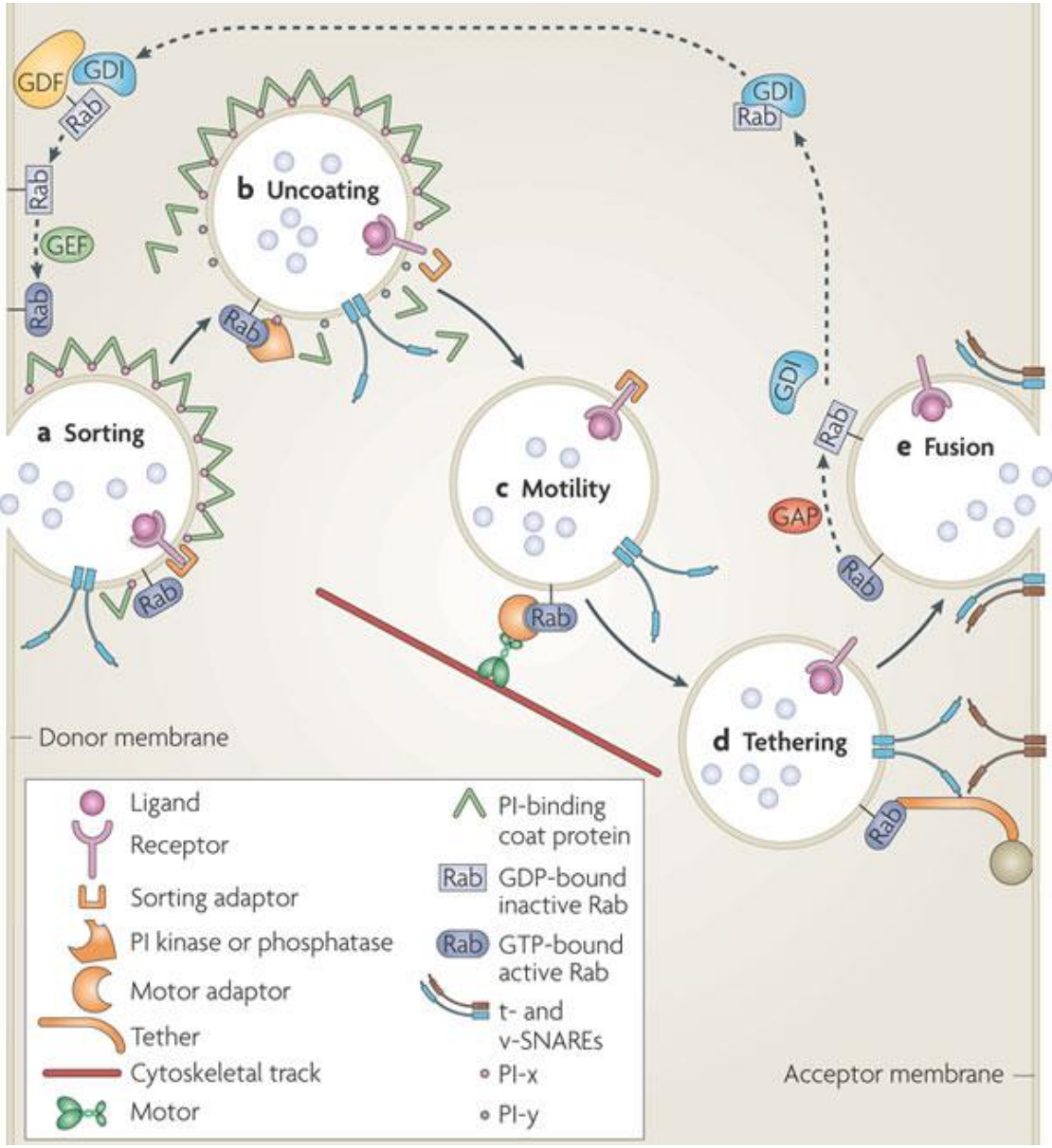

Figure 1.4 Molecular players in vesicle fusion. Vesicle fusion involves an interplay of small GTPases, tethering factors and SNAREs. Vesicle budding initiates at the plasma membrane with the aid of dynamin and coat proteins, following which the vesicle travels along a microtubule with the help of motor proteins to come in promixity to the acceptor membrane. Here the small GTPase recruits the tethering factor that bridges the two membranes followed by SNARE complexing and fusion of these membranes. Adapted from Stenmark

GTPase Activating Protein (GAP) renders the GTPase inactive(Cherfils and Zeghouf 2013).

Members of Ras superfamily, primarily the Rab and Arf family of GTPasesare well-

characterized regulators of vesicle fusion. RabGTPases are ubiquitously expressed family of small GTP-binding proteins constituting more than 60 members inmammals. Membrane anchorage of Rabs is dependent upon their C-terminal geranylgeranyl post-translational modification, that requires GTP binding to the Rab protein(Park 2013). Rab proteins are widely appreciated as regulators of various steps in the vesicle fusion pathway. The endocytic system in mammalian cells is decorated with multiple Rabs such as Rab4 and Rab5 on the early endosomes, Rab11 on the recycling endosomes and Rab7 and Rab9 on the late endosomes. GTP-bound Rab5 binds to its downstream effectors such as EEA1 and Rabaptin-5 that regulate early endosome fusion events. Activated Rab5 also recruits multi-subunithexamericCORVET complex to the early endosome membranes that brings about fusion of early endosomes with late endosomes(Mizuno-Yamasaki, Rivera-Molina and Novick 2012).

In mammals, there are two Rab7 proteins, Rab7a and Rab7b that are differentially localized and control distinct steps of transport. Rab7a is localizedmainly to the late endosomes and regulates transport to the late endocytic compartments, while Rab7bcontrols endosomes to Golgi transport, being localized both to the TGN and late endosomes(Progida, Cogli, Piro, De Luca, Bakke et al. 2010). Rab7a (hereafter referred as Rab7) interacts with several proteins and servesvarious important functions, besides its established role in endocytosis. Rab7 participates in multiple regulation mechanisms in endosome sorting, biogenesis and positioningof lysosomes and lysosome-related organelles, trafficking and degradation of several molecules internalized at plasma membrane, phagocytosis and autophagy(Guerra and Bucci 2016). For instance, Rab7 interacts with its effectors RILP and FYCO1 for retrograde and anterograde movement of late endosomes on microtubules, respectively(Wang,

Ming, Xiaochun and Hong 2011). Rab7 interacts with its effector PLEKHM1 and together they regulate degradation of EGF-receptor in the lysosomes and autophagosome-lysosome fusion(Hyttinen, Niittykoski, Salminen and Kaarniranta 2013). Rab7, by virtue of its interaction withVps26 and Vps35 subunits of the retromer complex, regulates retrograde transport of the transmembrane cargo from endosomes to the trans-Golgi network. Rab7 is essential for the fusion of phagosomes with late-endosomes and lysosomes, for functional phagosomal acidification and for the centripetal displacement of phagosomes(Zhang, Chen, Wang and Wang 2009). Studies have also linked Rab7 to the actin cytoskeleton organization and assembly of intermediate filaments. In neuronal cells, Rab7 regulatesneurotrophin receptor trafficking and promotes neurite outgrowth via its interaction with its newly identified effector, protrudin(Bucci, Alifano and Cogli 2014). Notably, mutations in Rab7 cause the Charcot-Marie-Tooth type2B (CMT2B) peripheral neuropathy, marked by inhibited neurite growth(Spinosa, Progida, De Luca, Colucci, Alifano et al. 2008). Rab7 is also necessary for the biogenesis and progression of axonal retrograde transport carriers in motor neurons and for the neuronal migration during development of cerebral cortex into its specific layered structure.

ArfGTPases are classified into Arf, Arf-like (Arl) and SAR group of proteins. There are six mammalian ARF proteins, namely, ARF1-6 that are highly conserved across evolution and have orthologs in diverse species. Arf proteins are characterized by the presence of myristoylated N-terminal amphipathic helix that inserts into the membrane upon GTP-binding(Jackson and Bouvet 2014). GTP-bound Arfs recruit coat proteins (COPI, COPII, clathrin, clathrin adapters and GGA proteins), lipid-modifying enzymes, tethers and other effector molecules that influence membrane trafficking(Donaldson and Jackson 2011). ARF1 and ARF6 are two of the best

characterized ARF proteins and molecular structures of the mammalian proteins have been elucidated by crystallography studies. While ARF1 localizes to Golgi and regulates budding of clathrin-coated vesicles from TGN, ARF6 localizes to plasma membrane and endosome compartments, where it regulates endocytic membrane

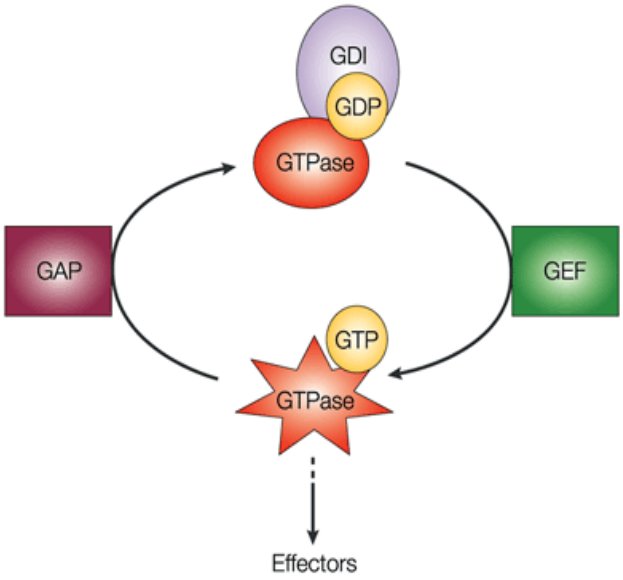

Figure 1.5 The GTPase cycle. Vesicular trafficking by small GTPases is a highly regulated process. **S**mall GTPases cycle transiently between an inactive GDP-bound state and an active GTP-bound form. In the active state, they can recruit their downstream effectors to bring about vesicle motility, trafficking and signaling. Adapted from Coleman et al 2004.

trafficking and actin remodeling (D'Souza-Schorey and Chavrier 2006).Early studies to identify genes encoding ArfGTPases in evolutionary divergent organisms led to the discovery of related proteins that have been termed Arf-like (Arl) GTPases. More than 20Arl proteins have been identified to date, that are also highly conserved throughout eukaryotic evolution. Some Arl proteins are myristoylated while most seem to lack this modification. For example, Arl8a and Arl8b are N-terminally acetylated that favors their association with lysosome membranes(Burd, Strochlic and Setty 2004). Arls have a wide array of functions ranging from microtubule biogenesis to ciliogenesis to vesicle motility and fusion. For instance, Arl1, enriched on the TGN, regulates the structure and function of Golgi apparatus (Lu, Horstmann, Ng and Hong 2001).Arl1 mediates Rab4-dependent formation of endosomal sorting domains with downstream assembly of adaptor protein complexes that constitute the endosome sorting machinery. Arl1 is also required for secretory granule biogenesis in larval

salivary glands of *Drosophila*(Torres, Rosa-Ferreira and Munro 2014). Arl2 is a regulator of tubulin folding, microtubule polymerization, integrity and dynamics (Zhou, Cunningham, Marcus, Li and Kahn 2006; Beghin, Honore, Messana, Matera, Aim et al. 2007). *Trypanosoma brucei*Arl2 plays a role in tubulin acetylation and cytokinesis (Price, Peltan, Stark and Smith 2010). Arl3 and its GEF Arl13 act antagonistically andmediate sorting of the lipidated cargo into the ciliary compartment during ciliogenesis(Li, Wei, Zhang, Ling and Hu 2010; Gotthardt, Lokaj, Koerner, Falk, Giessl et al. 2015; Fansa and Wittinghofer 2016; Zhang, Li, Zhang, Torres, Harris et al. 2016). Arl6 has been implicated in recruitment of coat-like ciliary trafficking complex called BBsome to the ciliary membranes (Mourao, Nager, Nachury and Lorentzen 2014).BBsome is involved in the transport of ciliary membrane proteins such as G protein-coupled receptors and components of the hedgehog signaling pathway (Berbari, Lewis, Bishop, Askwith and Mykytyn 2008; Seo, Zhang, Bugge, Breslow, Searby et al. 2011). Arl6 also modulates the mammalian ciliary disassembly and the Wnt signaling pathway (Wiens, Tong, Esmail, Oh, Gerdes et al. 2010). Arl7 has been implicated as a promoter of transferrin transport from early endosomes to recycling endosomes, possibly via its interaction with microtubules(Wei, Xie, Abe and Cai 2009).

Arl8 is a primitive GTPase that appeared early during evolutionand has been highly conserved from protozoans to metazoansas well as in plants(Pasqualato, Renault and Cherfils 2002). Interestingly, no Arl8 homolog is found in yeast, indicating its loss over the course of evolution of these species(Sebald, Krueger, King, Cohn and Krakow 2003; Li, Kelly, Logsdon, Schurko, Harfe et al. 2004). In vertebrates, there are 2 closely related paralogs, Arl8a andArl8b, which share 91% sequence identity to each other(Hofmann and Munro 2006). Like Arfs, Arl8 also contains a conservedN-

terminal amphipathic helix that acts as a membraneanchor and aids in the firm association of the active GTP-boundform of Arl8 to the lipid bilayer(Donaldson and Jackson 2011). Arl8b membranelocalization is dependent upon both the acetylated methionineat the N-terminus and the hydrophobic residues of theamphipathic helix(Hofmann and Munro 2006; Nakae, Fujino, Kobayashi, Sasaki, Kikko et al. 2010). Recent studies have identified BORC (BLOC-1 Related Complex), a multisubunit protein complex implicated in biogenesis of lysosome-related organelles, as a regulator of Arl8b recruitment to lysosomes(Pu, Schindler, Jia, Jarnik, Backlund et al. 2015). Arl8b has recently emerged as a crucial regulator oflysosome positioning and membrane trafficking towardlysosomes. Through interaction with its effector SKIP (also known as PLEKHM2), thehuman Arl8 paralog (Arl8b) mediates kinesin-1 dependentmotility of lysosomes on microtubule tracks toward the cellperiphery(Rosa-Ferreira and Munro 2011). Arl8b-mediated kinesin-driven motility is alsoimplicated in regulating lytic granule polarization in NK cells,lysosome tubulation in macrophages, cell spreading, andmigration(Kaniuk, Canadien, Bagshaw, Bakowski, Braun et al. 2011; Mrakovic, Kay, Furuya, Brumell and Botelho 2012; Tuli, Thiery, James, Michelet, Sharma et al. 2013). A downright implication of anterograde lysosome trafficking is reported in invasive growth of prostate cancer. It facilitates the release of lysosome proteases called matrix metalloproteinases that promote degradation of extracellular matrix required for cancer progression. Arl8b facilitates lipid hydrolysis and maintains efficient metabolism for a proliferative capacity in low nutrient environments(Dykes, Gray, Coleman, Saxena, Stephens et al. 2016). Arl8b-dependent lysosome positioning also regulates mammaliantarget of rapamycin complex 1 (mTORC1) activity andautophagosome-lysosome fusion(Korolchuk, Saiki, Lichtenberg, Siddiqi, Roberts et al. 2011). Moreover, Arl8b regulates membrane

traffictoward lysosomes by recruiting subunits of the HOPScomplex, a multi-subunit tethering complex that mediatesendo-lysosome fusion(Garg, Sharma, Ung, Tuli, Barral et al. 2011; Khatter, Raina, Dwivedi, Sindhwani, Bahl et al. 2015). Concurrently, Arl8b silencing impedes the display of MHC-II antigen presentation complexes on the surface of dendritic cells and macrophages, thereby impairing the activation of immune response against these antigens(Garg, Sharma et al. 2011; Michelet, Garg, Wolf, Tuli, Ricciardi-Castagnoli et al. 2015). Arl8b is an important host factor that regulates phago-lysosome fusion and clearance of phagocytosed microbes in macrophages, both in mammals and *C.elegans*(Garg, Sharma et al. 2011; Sasaki, Nakae, Nagasawa, Hashimoto, Abe et al. 2013).Arl8b is exploited by a facultative intracellular pathogen, *Salmonella enterica*serovar*typhimurium*, to establish a replicative niche in the host cells called SCV (*Salmonella*-containing vacuole). Arl8b associates with SCVs and the emanating SIFs (*Salmonella*-Induced Filaments) and regulates*Salmonella's* virulence and pathogenesis(Gillingham, Sinka, Torres, Lilley and Munro 2014). Given the widespread potential roles of Arl8b in various processes, it would be interesting to explore the propensityof Arl8b and its effector proteins to serve as therapeutictargets for various infections, neurodegenerative diseases and cancer progression.

1.4.2 Tethering factors

Tethering factors are multi-subunit or coiled-coil proteins that bridge intracellular transport vesicles with acceptor membranes and ensure correct docking and fusion. Tethering factors are recruited by activated small GTPases to distinct compartments in the cells that promotes initial specific interaction of two compartments destined for fusion Fig. 1.6). They are also responsible for SNARE proofreading i.e. they facilitate interaction of only the cognate SNAREs, adding another layer of specificity to vesicle

fusion(Chia and Gleeson 2014). The tethers can be divided into two classes:homodimeric coiled-coil proteins and multi-subunit tethering complexes (MTCs). Coiled-coil proteins are large, hydrophilic proteins comprising two globular heads connected by long coiled-coil domains. They can tether vesicles that are separated by long distances (>200 nm) in the cell(Brocker, Engelbrecht-Vandre and Ungermann 2010; Chia and Gleeson 2014).Most coiled-coil tethers are found associated with Golgi and are called Golgins, e.g., GM130, Giantin, p115. Some others such as EEA1 function in the endosomalpathway, which are recruited by RabGTPasesand bring about tethering and fusion (homotypic and heterotypic) of these compartments. On the contrary, multi-subunit tethering complexes comprise members that constitute 3-10 subunits and can interact with vesicles over short range of distances (upto 30 nm).MTCs function either along the secretory pathway, e.g. Dsl1, COG, GARP and Exocyst or in the endo-lysosome pathway, e.g., CORVET and HOPS complexes. There are also MTCs called TRAPP (Transport protein particle) complexes (TRAPP1-3) that function in both secretory and endo-lysosome pathways. All these tethering factors are highly conserved between species and have been characterized for both yeast and mammals in parallel.(Brocker, Engelbrecht-Vandre et al. 2010; Chia and Gleeson 2014).Various multi-subunit tethering complexes are discussed briefly below.

1.4.2.1 DSL1 complex

Dsl1 complex is an ER-localized MTC that is required for specific recognition of COPI-coated Golgi-derived vesicles enroute to ER. It is the smallest of all the MTCs with only three subunits – Dsl1, Dsl3 and Tip20 in yeast and NAG, RINT1 and ZW10

in mammals(Spang 2012; Tagaya, Arasaki, Inoue and Kimura 2014). The yeast subunits form a stable complex with three Q-SNAREs, namely, Use1, Ufe1 and Sec20, that serves as membrane anchor for the Dsl1p complex at the ER membrane. The mammalian complex (also known as NRZ complex) is associated with the ER

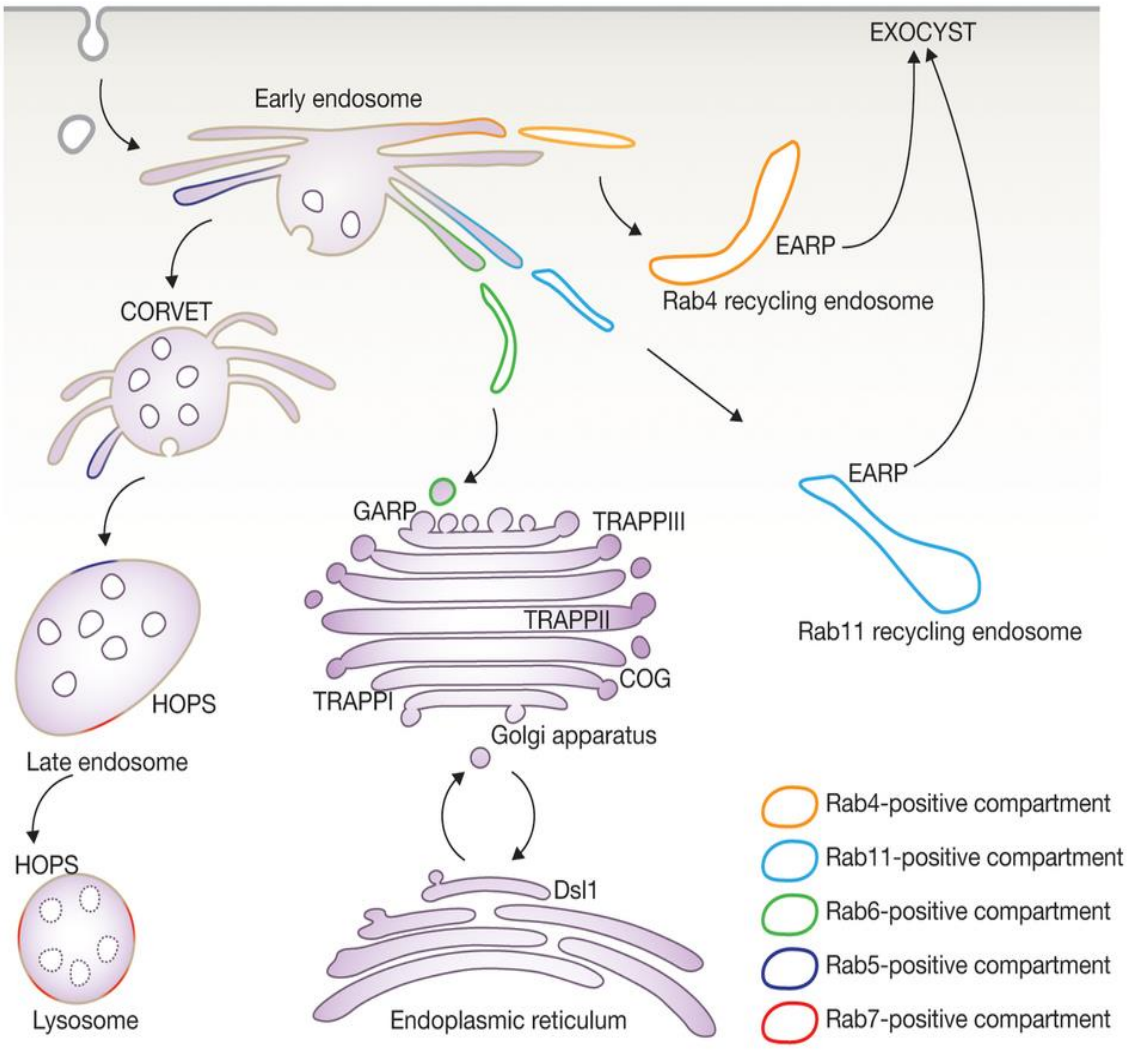

Figure 1.6 Multi-subunit tethering factors in a mammalian cell. Tethering factors are multi-subunit or coiled-coil protein complexes that bridge the two membranes in question prior to fusion. These proteins are recruited to distinct compartments in the cells by activated small GTPases and together they provide specificity to vesicle fusion process. Adapted from Desfougeres et al 2015.

SNAREs Syntaxin18, BNIP1, p31 and Sec22b(Meiringer, Rethmeier, Auffarth, Wilson, Perz et al. 2011).So far, this is the only complex that is known to bind ER membranes independent of any interaction with aGTPase. Dsl1 is an example of a tethering complex that is stabilized at the organelle membrane by binding to

SNAREs, recognizes the incoming vesicle via its coat and then promotes SNARE-mediated fusion(Sztul and Lupashin 2009).

1.4.2.2 GARP complex

The Golgi Associated Retrograde Protein (GARP) complex is required for protein sorting at late Golgi and in tethering endosome-derived vesicles to the trans-Golgi network. This complex contains four core subunits (Vps51 through Vps54) and is recruited by small GTPase Ypt6/Rab6 to TGN(Benjamin, Poon, Drysdale, Wang, Singer et al. 2011; Chia and Gleeson 2011). GARP complex interacts with the yeast SNARE Tlg1 or in mammals, with SNAREs including Syntaxin 6, Syntaxin 16 and VAMP 4. GARP is essential for cellular sphingolipid homeostasis and its deficiency leads to accumulation of sphingolipid synthesis intermediates, changes in sterol distribution and lysosome dysfunction. Further, mutations in this complex cause progressive autosomal recessive cerebello-cerebral atrophy type 2 (PCCA2) in humans(Feinstein, Flusser, Lerman-Sagie, Ben-Zeev, Lev et al. 2014).

1.4.2.3 COG complex

The Conserved Oligomeric Golgi (COG) complex is composed of eight subunits that form two lobes consisting of COG2-COG3-COG4 and COG5-COG6-COG7, linked by a heterodimer of COG1 and COG8(Lees, Yip, Walz and Hughson 2010). COG complex interacts with Ypt1/Rab1 and Ypt6/Rab6 and is necessary for the retrograde transport between Golgi compartments. Moreover, the COGcomplex binds directly to the Sec1/Munc18 (SM) -homologousprotein Sly1 and to the SNARE Sed5 (yeast homologueof mammalian syntaxin 5)for facilitating endosome-to-TGN and intra-Golgi retrograde transport(Laufman, Hong and Lev 2013). Mutations in the COGcomplex are associated with impaired protein sorting and glycosylation defects (Smith and Lupashin 2008; Willett, Ungar and Lupashin 2013).

1.4.2.4 Exocyst complex

The Exocyst complex is again composed of eight subunitsand is known to tether secretory vesicles to plasma membrane. The eight subunits are Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84(Heider and Munson 2012). Several lines of evidence suggest that the exocystassembles at the sites of exocytosis by binding to Rab andRho GTPases. The yeast exocyst binds to the RabGTPase Sec4 on secretoryvesicles via its subunit Sec15, and to the Rho GTPasesRho1, Rho3 and Cdc42 at the plasma membrane via Sec3 subunit(Kee, Yoo, Hazuka, Peterson, Hsu et al. 1997). In mammalian cells, Sec15 interacts with Rab11, which is involved in generation of vesicles at the TGN or recycling endosomes for subsequent delivery to the plasma membrane(Wu and Guo 2015). Furthermore, Sec3 competitively binds to the Rho GTPases Rho1 and Cdc42 and functions at different stages of cell growth.Knockout of Exocyst subunits results in early embryonic lethality at the organismal level, inhibited neurite outgrowth in neuroendocrine cells and hippocampal neurons and defective protein translation and filopodia formation.Recent studies have implicated exocyst in several other crucial functions like cell migration, tumor invasion, autophagy and cytokinesis(Martin-Urdiroz, Deeks, Horton, Dawe and Jourdain 2016).

1.4.2.5 TRAPP complexes

The Transport Protein Particle (TRAPP) complexes are tethering factors that play a role in ER to Golgi transport (TRAPPI), intra-Golgi trafficking (TRAPPII) and autophagosome formation (TRAPPIII). TRAPP complexes have multiple subunits that are conserved from yeast to humans. Seven subunits are small, 140-283 amino acids, and the rest are larger, 417-1435 residues. These subunits are termed TrsN (N = size in KDa) in yeast and TrappC or TCP 1-13 in mammals(Kim, Lipatova and Segev

2016).Physical, genetic and cellular studies support a role for yeast TRAPPI and TRAPPII as GEFs for Ypt1p and Ypt31/32pGTPases, respectively. However, no reports have shown Rab-GEF activity for mammalian TRAPP complexes. In yeast, TRAPPI directly interacts with vesicles derived from ER and facilitates their transport to the Golgi, from where they are routed to the plasma membrane(Kim, Raunser, Munger, Wagner, Song et al. 2006). TRAPPII regulates traffic within the Golgi and in the retrograde pathway from early endosomes to the late Golgi for the retrieval of Golgi-resident proteins(Sacher, Kim, Lavie, Oh and Segev 2008).TRAPP complexes interact with coat proteins and specific subunits are able to differentiate between Golgi-derived COPI vesicles (TRAPPII) and ER-derived COPII vesicles (TRAPPI)(Barrowman, Bhandari, Reinisch and Ferro-Novick 2010).The TRAPIII specific subunit, Trs85, has been suggested to play a role in selective autophagy where it is required for the organization of pre-autophagosomal structure in the cytoplasm, andin vacuole targeting (Cvt) pathway that delivers the hydrolase aminopeptidase I to the vacuole(Meiling-Wesse, Epple, Krick, Barth, Appelles et al. 2005). Mammalian TRAPII is likely to function in ER-to-Golgi transport based on the localization and RNAi phenotype of one of its subunits. In contrast to yeast TRAPPIII, a role for mammalian TRAPIII is proposed in ER-to-Golgi transport of COPII vesicles(Bassik, Kampmann, Lebbink, Wang, Hein et al. 2013; Kim, Lipatova et al. 2016). Moreover, it was shown to be required for ciliogenesis and for cell entry of the human papilloma virus, implicative of its role in endocytosis.

1.4.2.6 Tethering complexes of Endocytic pathway – CORVET and HOPS

The multi-subunit tethering complexes that function in the endocytic pathway areClass CcOReVacuole/Endosome Tethering (CORVET) and HOmotypic fusion and Protein Sorting (HOPS) complexes (Fig. 1.7). CORVET is required for the early

endosomehomotypic fusion and early to late endosome fusion, while HOPS complex regulates fusion events at the lysosomes. In addition to tethering membranes, the two complexes activate and proof-read SNARE assembly to drive membrane fusion(Balderhaar and Ungermann 2013). Both are hexameric complexes that are

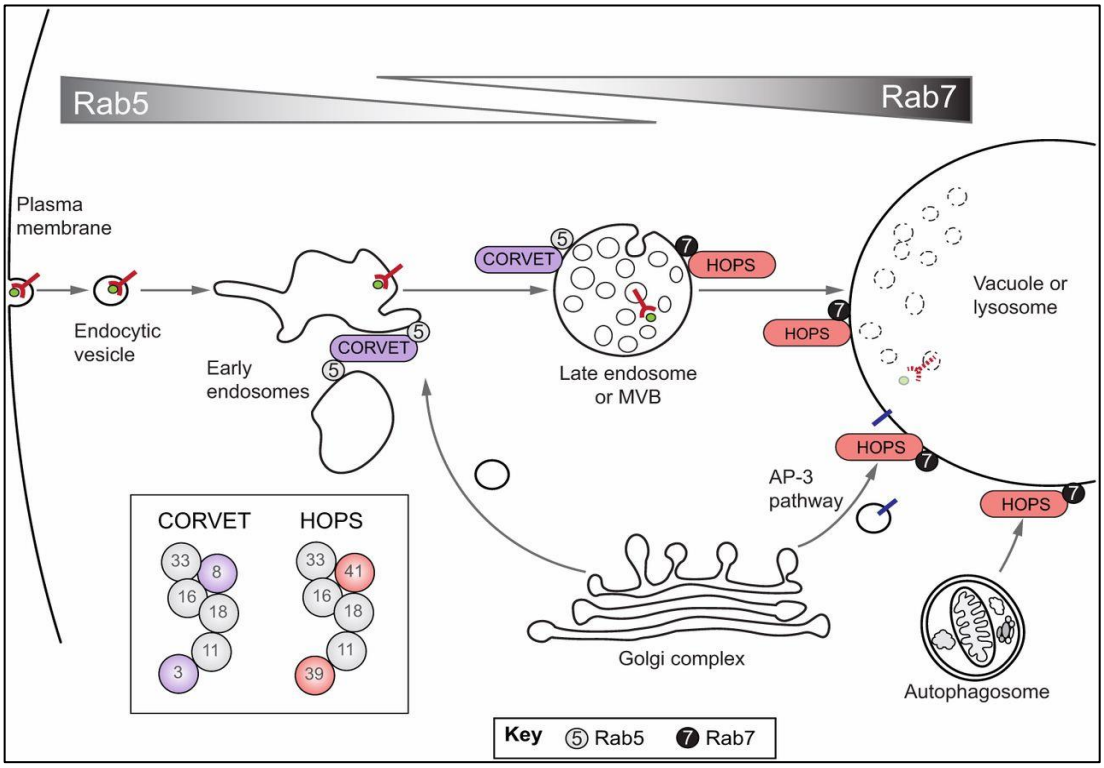

Figure 1.7 Multi-subunit tethering complexes of endocytic pathway in yeast. Two hexameric tethering factors function in the endocytic pathway - CORVET and HOPS. CORVET binds to small GTPase Rab5 and is required for early endosomal homotypic fusion and early to late endosomal fusion. At the late endosome Rab5 is replaced by Rab7, which then interacts with HOPS to promote homotypic vacuole and late endosome to vacuole fusion events. Adapted from Balderhaar and Ungermann 2013.

composed of a shared core of four subunits while the two accessory subunits are distinct and mediate targeting to specific membranes by association with distinct small GTPases. Both the complexes are well characterized in yeast and sequence alignment has shown theconservation of all subunits from yeast to mammals. (Dubuke and Munson 2016). Class CcOReVacuole/Endosome Tethering (CORVET)acts upstream of HOPS complex in the endocytic pathway and regulates fusion events at the early endosome. Four subunits that form the core complex are Vps11, Vps16, Vps18 and Vps33 and the accessory subunits are Vps8 and Vps3 in yeast,andVps8and

TGFBRAP1 in mammals(Solinger and Spang 2013; Perini, Schaefer et al. 2014). CORVET complex was identified as an interaction partner of Vps21 (yeast homolog of Rab5) necessary for endolysosome biogenesis (Kuhlee, Raunser and Ungermann 2015). It has been shown that purified CORVET is able to tether vacuole-associated endosomes efficiently in a Rab5/Vps21 – dependent manner, both *in vivo* and *in vitro*(Balderhaar, Lachmann, Yavavli, Brocker, Lurick et al. 2013). Like its yeast counterpart, mammalian CORVET is also a Rab5 effector that regulates biogenesis of early endosomes and their maturation to late endosomes(Perini, Schaefer et al. 2014). HOPS complex is better characterized in terms of its structure and function thanCORVET complex, where the two accessory subunits present are Vps39/vam6 and Vps41/vam2. HOPS mediates fusion events at the vacuole/lysosome,which is a coordinated action of its well-regulated interaction with small GTPases and SNAREs(Spang 2016). The structure, subunit composition and mechanism of action of HOPS complex is discussed in detail in the section below.

1.4.3 SNAREs and SNARE-associated proteins

SNAREs (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptors) are highly conserved membrane-associated proteins that have been implicated in all intracellular membrane fusion events. Syntaxin and Vesicle Associated Membrane Protein (VAMP)were the first SNARE proteins to be discovered with more than 60 members discovered in mammals so far. Both of these are anchored to the membrane by a carboxy-terminal transmembrane domain(Chen and Scheller 2001). A characteristic feature of all SNAREs is that they contain conserved heptad repeat sequences in their membrane-proximal regions called SNARE motifsthat form coiled-coil structures. These coiled-coil forming domains constitute the core SNARE complex that has been found to be resistant to SDS denaturation, protease digestion

and temperatures as high as 90^0C *in vitro*(Chen and Scheller 2001). SNAREs are divided as R-SNAREs (arginine-containing SNAREs) or Q-SNAREs (glutamine-containing SNAREs), based on the identity of a highly conserved residue present in the central ionic interaction layer (the zero layer) in the otherwise hydrophobic core of the SNARE complex. SNAREs have also been classified as v-SNAREs and t-SNAREs according to their vesicle or target membrane localization, respectively(Jahn and Scheller 2006). A working model proposed in 1993 to explain SNARE-mediated fusion postulated that vesicle-anchored SNARE associates with the cognate membrane-anchored SNARE to forms a reversible quarternarytrans-SNARE complex (also known as SNAREpin)(Hong and Lev 2014). There is now sufficient evidence that the SNARE complex forms in a zipper-like fashion, proceeding from the N-terminal region towards the C-terminal region that progressively narrows the gap between the two membranes(Lou and Shin 2016).The SNAREs involved in the complex formation prior to the merging of two membranes form a part of what is now called a cis-SNARE complex post-fusion, because they now reside on a single resultant membrane. Following membrane fusion, the cis-SNARE complex is bound and disassembled by an adaptor protein called α-SNAP (Soluble NSF Attachment Protein)/Sec17. Next the hexameric AAA-ATPase NSF (N-ethylmaleimide-SensitiveFactor)/Sec18catalyzes the ATP-dependent unfolding of the SNARE proteins and releases them into the cytosol for recycling(Ryu, Jahn and Yoon 2016).

The assembly of functional SNARE complexes is also positively regulated by tethering factors that add specificity, speed and fidelity to the fusion events. Tethering factors appear to influence SNAREpin assembly in several ways including stabilization of the SNARE proteins, gathering of t-SNAREs on target membrane or activating the assembly process by interacting with/incorporating SM proteins(Hong

and Lev 2014). SM proteins are evolutionary conserved soluble membrane proteins, of which four classes are known – Sly1, VPS45, VPS33 and Munc18(Jahn 2000). It is now beginning to be understood how SM proteins cooperate with SNARE complexes for fusion.X-ray structures of yeast Vps33 bound to two individual SNAREs revealed that SM proteins could act as templates for generating partially zipped SNARE assembly intermediates through specific SNARE motif recognition (Baker, Jeffrey, Zick, Phillips, Wickner et al. 2015). Two distinct SNARE complexes are involved in the lysosome degradation pathway. Both complexes include Syntaxin-7, Vti1b and Syntaxin8, but differ in R-SNARE, VAMP7 and VAMP8; the former acts in late endosome to lysosome fusion while the latter governs early to late endosome fusion and homotypic fusion of late endosomes(Pryor, Mullock, Bright, Lindsay, Gray et al. 2004). The autophagosomal SNARE Syntaxin 17 interacts with the lysosome SNARE VAMP8 and catalyzes HOPS-dependent fusion of autophagosomes with lysosomes(Itakura and Mizushima 2013).

1.5 HOPS complex– a multisubunit tethering factor that regulates cargo delivery to lysosomes

HOmotypic fusion and Protein Sorting (HOPS) complex is a multimeric tethering factor that was first described in *Saccharomyces cerevisiae*. A classification of yeast mutants revealed that the deletion of four proteins, Vps11, Vps16, Vps18 and Vps33 resulted in the most severe defect in endosome morphology(Wada, Ohsumi and Anraku 1992). Unlike wild-type cells, which have one to three vacuoles (termed Class A), these mutants lacked a vacuolar compartment, and the mutants were called Class C mutants. A deletion of the remainder two subunits, Vps39 and Vps41,resulted in a less dramatic phenotype that was marked by highly fragmented vacuoles (Class B mutants)(Raymond, Howald-Stevenson, Vater and Stevens 1992; Arlt, Perz and

Ungermann 2011). It later turned out that all the six proteins present in yeast function together as a complex along the endocytic pathway and the six subunits are conserved from yeast to metazoans. The four subunits that form the core part of the complex are sharedwith another multimeric tethering factor called CORVET that functions upstream in the same pathway. Structurally, most HOPS subunits are predicted to have a similar secondary structure with N-terminal β-propeller and C-terminal α-solenoid domains(Graham, Wartosch, Gray, Scourfield, Deane et al. 2013; Behrmann, Lurick, Kuhlee, Balderhaar, Brocker et al. 2014). Furthermore, two of the six yeast complex subunits, namely, Vps11 and Vps18 have C-terminal RING domains that can function as E3 ubiquitin ligases(Nickerson, Brett and Merz 2009). Although the mechanism is yet not clear, the deletion of Vps11 and Vps18 RING domains has been linked to altered/defective protein sorting(Balderhaar and Ungermann 2013). Vps33, on the other hand, belonging to the Sec1/Munc18 (SM) family stands out with mostly α-helical secondary structure. SM family members are known to be SNARE-interaction partners and structural (yeast) and functional (mammals) studies have shed light on the interaction of Vps33 with SNARE partners(Starai, Hickey and Wickner 2008).

Recent structural data combined with the previous subunit-interaction analyses now provide a first glimpse into the organization of yeast HOPS complex Fig. 1.8). Electron microscopy combined with single-particle analysis and tomography studies has revealed a flexible ~30 nm elongated, seahorse-like structure, which can adopt contracted and elongated shapes or different conformations without altering the overall structure and subunit arrangement(Brocker, Kuhlee, Gatsogiannis, Balderhaar, Honscher et al. 2012). By combining antibody labeling with the EM structures of previously characterized subunit dimers (Vps11-Vps39 and Vps16-Vps33) and

subunit trimers (Vps11-Vps39-Vps18), the positions of selected subunits were assigned, thus identifying their relative position in the complex.The large head is composed of Vps41 and Vps33 subunits while Vps39 is found in the bulky tip of the tail. Vps11 and Vps18 connect head and tail. The two Rab-binding subunits

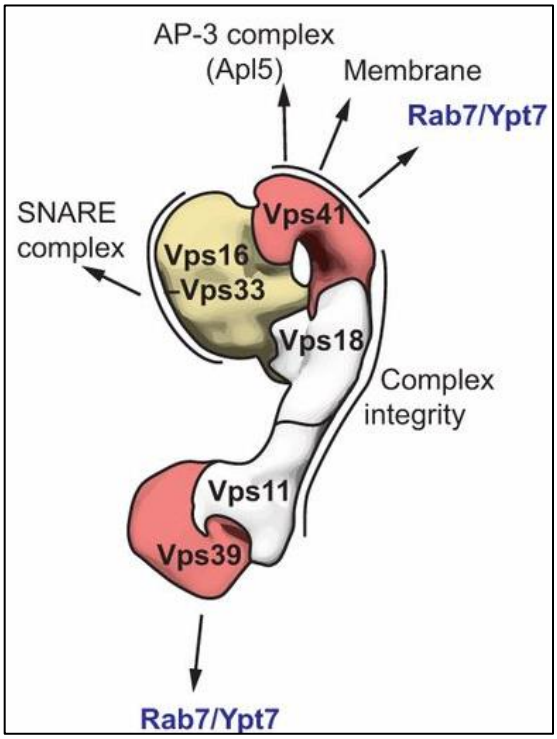

Figure 1.8 The organization of yeast HOPS complex. Cryo-electron structure of yeast HOPS complex depicting the sea-horse like arrangement of all subunits. The two Rab7-binding subunits, Vps39 and Vps41 lie on opposite ends of the complex and are responsible for membrane-binding. Adapted from Balderhaar and Ungermann 2013.

Vps39 and Vps41 are present on the opposite ends of the elongated complex(Brocker, Kuhlee et al. 2012). The structural analysis of the yeast HOPS complex provides a major advance in understanding the Rab-dependent tethering at the molecular level (Fig. 1.9). Small GTPase Ypt7 binds to Vps39 and Vps41 and recruits HOPS to late endosomes(Wurmser, Sato and Emr 2000; Brett, Plemel, Lobingier, Vignali, Fields et al. 2008). Vps39 is a stronger interaction partner of Ypt7 than Vps41 however, a cooperation of the two Rab-binding sites is essential for HOPS function. Vps39 was earlier reported to act as Ypt7 GEF, but was later disproved to be so(Wurmser, Sato et al. 2000; Peralta, Martin and Edinger 2010). Instead, the dimericMon1-CCZ1 complex was evidenced to promote nucleotide exchange on Ypt7 independently of

Vps39/HOPS (Nordmann, Cabrera, Perz, Brocker, Ostrowicz et al. 2010).At the endosome membranes, HOPS may be reoriented as Vps41 also binds to lipids via its ALPS (Amphipathic Lipid Packaging Sensor) motif, such that one Ypt7 binding site on the late endosome is free that can now engage with Ypt7 present on the vacuolar membrane. In agreement with this model, HOPS requires Ypt7 to tether proteoliposomes*in-vitro* and promote their fusion(Stroupe, Hickey, Mima, Burfeind and Wickner 2009). Furthermore, elongated structures between endosomes and

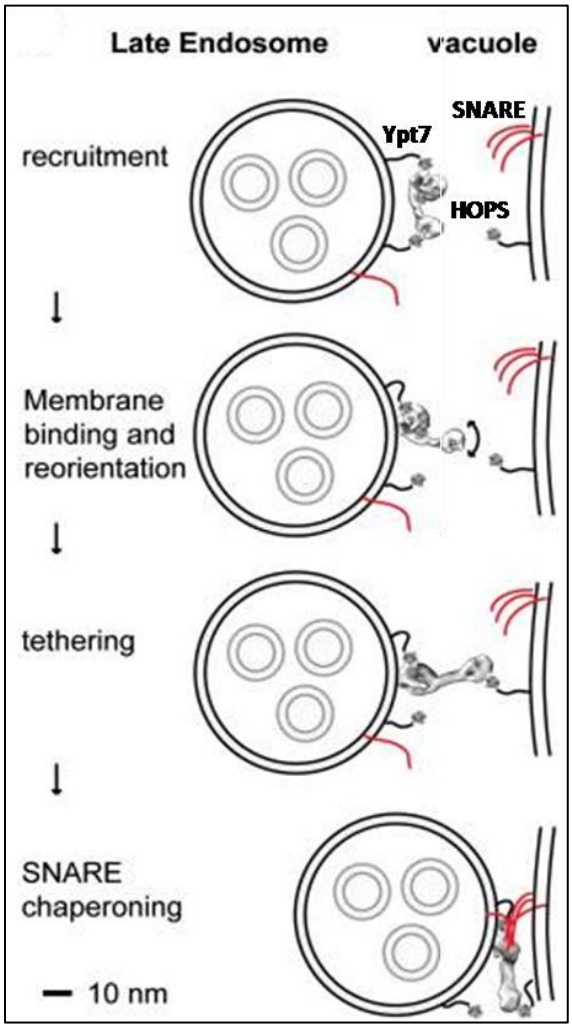

Figure 1.9 The function of HOPS in fusion at the vacuole. Small GTPase Ypt7 binds to Vps39 and Vps41 and recruits HOPS to late endosomes. At the endosomal membranes, HOPS may be reoriented such that one Ypt7 binding site on the late endosome is free that can now engage with Ypt7 present on the vacuolar membrane, thus bridging the two membranes before fusion. Adapted from Brocker 2012.

lysosomes have been observed in ultrastructural studies, which could represent HOPS tethering the two membranes. Closer apposition of the membranes would then allow Vps33 to facilitate and proofread SNARE assembly prior to membrane mixing. Vps33

binds to the SNARE motifs of the two vacuolar SNAREs, R-SNARE Nyv1 and Qa-SNARE Vam3, as well as to the complete vacuolar SNARE complex that additionally comprises the Qb-SNARE Vti1 and Qc-SNARE Vam7(Brocker, Kuhlee et al. 2012).In yeast, HOPS is required for most fusion events at the vacuolar lysosome, including homotypic fusion between vacuoles and heterotypic fusion with autophagosomes, late endosomes and AP-3 vesicles(Rieder and Emr 1997; Seals, Eitzen, Margolis, Wickner and Price 2000). Vacuolar kinase Yck3-mediated phosphorylation of Vps41 ALPS motif is required for the fusion of Golgi-originating AP-3 vesicles with the vacuole(LaGrassa and Ungermann 2005; Cabrera, Ostrowicz, Mari, LaGrassa, Reggiori et al. 2009). Phosphorylation at the membrane-interacting ALPS motif releases Vps41 from its close membrane apposition enabling it to bind the δ-ear domain of the AP-3 subunit Apl5(Cabrera, Langemeyer, Mari, Rethmeier, Orban et al. 2010). Phosphorylation of Vps41 also regulates its interaction with Ypt7 where the non-phosphorylatable Vps41 mutant showed a stronger interaction with Ypt7. Consequently, when HOPS was phosphorylated by purified Yck3 *in vitro* or phosphomimetic mutants of Vps41 were used *in vivo*, membrane fusion became strongly Ypt7-dependent (Brett, Plemel et al. 2008).

*S.cerevisiae*studies have been instrumental in understanding the role of HOPS complex in tethering, SNARE-pair formation and fusion of organelles with the vacuolar compartment. While the metazoan HOPS complex biology is slowly unraveling, it is now clear that metazoan HOPS mediates biogenesis of the late endocytic compartments, late endosome-lysosome fusion, phagosome-lysosome fusion andautophagosome-lysosome fusion.Metazoan HOPS consists of all the six subunits previously described for yeast and their homologs have been discovered in various kingdoms/genera across evolution(Balderhaar and Ungermann 2013; Solinger

and Spang 2013). Interestingly, metazoan Vps16 and Vps33have two isoforms each – A and B. Vps16A interacts with Vps33A and both function in the endo-lysosome fusion, whereas, Vps16B (VIPAR or SPE-39) interacts with Vps33B and required for phagocytosis and earlier endosome fusion reactions(Tornieri, Zlatic, Mullin, Werner, Harrison et al. 2013; Gautreau, Oguievetskaia and Ungermann 2014). Previously, it was speculated that both the Vps33 isoforms function as part of HOPS complex in metazoans. However, crystallography studies have recently revealed that Vps16 exists in complex with Vps33a(Baker, Jeffrey and Hughson 2013; Graham, Wartosch et al. 2013). In a separate study it was shown that there was no effect of deleting either VIPAR or Vps33B on fusion of endosomes and autophagosomes with lysosomes. The topic is likely a matter of deeper investigation with upcoming reports suggesting that Vps33B depletion leads to accumulation of late endosomes and impaired degradation of endocytosed cargo(Galmes, ten Brink, Oorschot, Veenendaal, Jonker et al. 2015). Another study provides evidence that *Drosophila* and mammalian Vps33B proteins play critical roles in the maturation of phagosomes and endosomes following microbial recognition(Akbar, Mandraju, Tracy, Hu, Pasare et al. 2016).

Defects in HOPS subunits result in strong deficiencies in various invertebrates. For instance, loss of Vps11 causes retinal pathogenesis in zebra fish model of syndromic albinism(Thomas, Vihtelic, denDekker, Willer, Luo et al. 2011). Zebra fish Vps11 also functions to maintain mature melanophore properties, including cell morphology, organelle integrity and survival(Clancey, Beirl, Linbo and Cooper 2013). Similarly, a Vps18 zebra fish mutant has reduced retina pigment epithelium and body pigmentation.Also, Vps18 and Vps33 have been linked to development and maintenance of normal eye color pigmentgranules in *Drosophila*(Peng, Ye, Yan, Kong, Shen et al. 2012). Loss of *Drosophila* Vps16A also causes deficits in lysosomal

delivery, accumulation of autophagosomes, as well as eye color defects due to the loss of pigment granules(Pulipparacharuvil, Akbar, Ray, Sevrioukov, Haberman et al. 2005). In zebrafish, mutation of Vp18 results in accumulation of pre-lysosomal vesicles in the cell cytoplasm causing cell enlargement and hepatomegaly(Sadler, Amsterdam, Soroka, Boyer and Hopkins 2005). Vps18 mutation results in strong defects in the biogenesis of endosomes and lysosomes in *C.elegans*. Evidence suggests that phagosomes containing internalized cell corpses are unable to fuse with lysosomes in Vps18 mutants indicating the important role of Vps18-mediated lysosomal degradation in proper clearance of apoptotic cells during programmed cell death(Xiao, Chen, Fang, Xu, Sun et al. 2009). Furthermore, mutating Vps18 homolog, *dor*, in *Drosophila* causes the accumulation of exaggerated multivesicular structures in retinal cells, blockage of autophagosome-lysosome fusion in larval fat body, and promotion of tumor metastasis(Peng, Ye et al. 2012). While *C.elegans*Vps39 is important for degradation of autophagosomes(Manil-Segalen, Lefebvre, Jenzer, Trichet, Boulogne et al. 2014), Vps41 protects*C.elegans* dopaminergic neurons from α-synuclein-induced degeneration by augmenting clearance of misfolded protein aggregates(Harrington, Yacoubian, Slone, Caldwell and Caldwell 2012). Zebrafish Vps39 mutants display defects in several tissues and cell types as well as compromised vision and innate immunity, largely resulting from defects in lysosomes and lysosome-related organelles(Schonthaler, Fleisch, Biehlmaier, Makhankov, Rinner et al. 2008). *Trypanosomabrucei*Vps41 plays an important role in intracellular iron utilization and maintenance of normal cellular morphology(Lu, Suzuki, Iizuka, Ohshima, Yabu et al. 2007). All six subunits of the HOPS complex via interaction with autophagosomal SNARE Syntaxin-17 are important for autophagosome-lysosome fusion in *Drosophila*(Takats, Pircs, Nagy, Varga, Karpati et al. 2014).

Moreover, a recent study has shown*Drosophila*Rab2 localizes to completed autophagosomes followed by recruitment of HOPS complex through coincident interactions with Syntaxin17 and Rab2. This machinery is central to autophagy required for transverse-tubule disassembly and remodeling(Kajiho, Kajiho, Frittoli, Confalonieri, Bertalot et al. 2016).

In recent years there has been a growing interest in the biology of mammalian HOPS complex in which direction successive studies have unfolded its role across multiple trafficking/degradation pathways merging at lysosomes. Human HOPS-specific subunits Vps39 and Vps41 localize to late endocytic compartments and their depletion results in accumulation of late endosomes, depletion of lysosomes and a block in degradation of endocytic, phagocytic and autophagic cargo(Pols, ten Brink, Gosavi, Oorschot and Klumperman 2013). In agreement with this, previous studies have reported increased lysosome clustering and endo-lysosome fusion upon overexpression of HOPS subunits(Caplan, Hartnell, Aguilar, Naslavsky and Bonifacino 2001; Poupon, Stewart, Gray, Piper and Luzio 2003). Recent studies have shown that, similar to other homologs, mammalian HOPS subunits also regulate phagocytic and autophagic traffic towards lysosomes. Fusion with the lysosome in either pathway utilizes interaction of HOPS with SNAREs. Syntaxin7, Syntaxin8, Vamp7, Vamp8 and Vti1b are the SNAREs that drive fusion of late endocytic compartments (Antonin, Holroyd, Fasshauer, Pabst, Von Mollard et al. 2000). In mammalian system, the SNARE dependent fusion in this pathway has been best understood in the context of the autophagosomal SNARE Syntaxin17. Syntaxin17 localizes to the outer membrane of completed autophagosomes in LAMP2-dependent manner, where it interacts with the lysosome SNARE Vamp8(Hubert, Peschel, Langer, Groger, Rees et al. 2016). All HOPS subunits co-immunoprecipitate with

Syntaxin17 and maximum affinity is found for endogenous Vps33A and Vps16. Depletion of Syntaxin17 causes accumulation of autophagosomes without any degradation(Takats, Pircs et al. 2014).A missense point mutation in the murine *Vps33a* gene gives rise to the *buff* mouse phenotype, characterized by pigmentation, platelet activity, and motor deficiencies(Zhen and Li 2015). Except for Vps16, this mutation enhances interactions of other subunits of HOPS complex with Syntaxin17, which adversely affects the autophagosome-lysosome fusion. Syntaxin17 also mediates HOPS-dependent fusion of Mitochondrial-Derived Vesicles (MDVs) with endolysosomes, promoting the delivery of damaged mitochondrial content to lysosomes for degradation(McLelland, Lee, McBride and Fon 2016).Mammalian Vps18 is indeed able to bring about ubiquitylation of GGA3 (Golgi-localized, Gamma-ear containing, ADP-ribosylation factor binding) adaptor protein that functions between Golgi and endosome (Yogosawa, Kawasaki, Wakatsuki, Kominami, Shiba et al. 2006)and Serum-inducible kinase (SNK), a polo-like kinase that regulates entry into S-phase during cell division(Yogosawa, Hatakeyama, Nakayama, Miyoshi, Kohsaka et al. 2005). Mammalian genetic deficiencies are consistent with the role of HOPS complex in the delivery of vesicle contents to lysosomes and lysosome-related organelles. Defects in Vps39 and Vps41 results in embryonic lethality as early as gastrulation(Messler, Kropp, Episkopou, Felici, Wurthner et al. 2011).Loss of Vps33A results in Hermansky-Pudlak Syndrome (HPS) characterized by oculo-cutaneous albinism and plateletstorage disease, closely resembling the clinical presentation of *buff* mouse. Mutations in multiple HOPS subunits impair infection by Ebola virus and export of HIV virions(Carette, Raaben, Wong, Herbert, Obernosterer et al. 2011). Ablation of Vps18 leads to neurodegeneration, impaired neuronal migration and inhibits dendritogenesis in

mice(Peng, Ye et al. 2012). Interestingly, additional specific functions of mammalian/human HOPS subunits have also been reported. Vps41 is involved in the fusion of carriers that deliver LAMP1 membrane protein to the lysosome(Pols, van Meel, Oorschot, ten Brink, Fukuda et al. 2013). Vps41 self-assembles into a lattice acting as a coat protein for AP3for sorting to the regulated secretory pathway(Asensio, Sirkis, Maas, Egami, To et al. 2013). Vps41 binds to caspase 8, and its overexpression promotes Fas-induced apoptosis(Wang, Pan, He, Zhang, Chen et al. 2013).

1.6 Thesis objective

We are only beginning to comprehend membrane tethering at molecular level. The study of yeast HOPS complex as a vesicle tether has contributed significant advances to the understanding of vesicle fusion in endocytic pathway. Both *in vitro* and *in vivo* experimental approaches have revealed the interplay of different components of vesicle fusion machinery employed in yeast. An extrapolation to the metazoan system has unraveled conservation of all HOPS subunits and their functional relevance in cargo degradation. Briefly, we know yeast HOPS localizes to late endosome and vacuole membranes with the aid of small GTPase Ypt7(Wurmser, Sato et al. 2000; Brett, Plemel et al. 2008). Once there, HOPS catalyzes membrane fusion by bridging two compartments and recruiting and proofreading SNARE assembly at the fusion site. Multiple studies have shown that mammalian HOPS regulates endocytic, autophagic and phagocytic traffic towards lysosomes(Pols, ten Brink et al. 2013; Jiang, Nishimura, Sakamaki, Itakura, Hatta et al. 2014; McEwan, Richter, Claudi, Wigge, Wild et al. 2015). Although this knowledge is persistent in the field for over a decade now, insights into the molecular arrangement of its subunits and its mechanism of action are still lacking. Rab7, the mammalian homolog of Ypt7, has

long been considered a regulator of HOPS assembly and function. Unfortunately, to date, no direct interaction approaches or effector-binding assayshave supported this idea, which begs the question what regulates assembly of the mammalian HOPS complex on lysosomes. A stepping stone towards discerning the actual molecular cue was the study by Garg and Sharma et al where the authors had shown that small GTPase Arl8b directly interacts with the HOPS subunit Vps41. With it as the starting point, I directed my efforts to elucidate the mechanism of HOPS assembly on lysosomes. The specific questions that I address in the first part of thesis are:

1. Investigate the role of small GTPases, Rab7 and Arl8b, in recruitment of mammalian HOPS complex to lysosomes.
2. Characterize the protein-protein interactions between the different subunits of the HOPS complex to build a model of their assembly on lysosome membranes.
3. Analyze the significance of Arl8b-Vps41 interaction in regulating cargo traffic to lysosomes.

Arl8b is a crucial regulator of lysosome positioning and cargo degradation in mammalian cells. It serves these functions in conjunction with various interaction partners that are slowly emerging. A class of proteins comprising RUN-domain containing proteins (e.g. PLEKHM2, PLEKHM1) have recently surfaced as Arl8b effectors. They bind to motors in an Arl8b-dependent manner and bring about lysosome movement in eukaryotic cells. In the second part of this thesis, I have characterized a previously reported coordinator of lysosome positioning, RUN and FYVE domain-containing protein 1 (RUFY1), as a novel interaction partner of Arl8b.

Chapter II

Materials and Methods

Chapter 2

2.1 Plasmids

Vps expression constructs were kind gifts from DrsChengyu Liang (University of Southern California, Los Angeles, USA), J. Wade Harper (Harvard Medical School, Boston, USA) and Victor Faundez (Emory University, Atlanta, USA). GFP–Rab7, GFP–Rab7-T22N and GFP-LAMP1 expression constructs were kind gifts from Dr Steve Caplan (University of Nebraska Medical Center, Omaha, USA). The GFP–RILP plasmid was a kind gift from Dr Jacques Neefjes (The Netherlands Cancer Institute, Amsterdam, Netherlands). GFP–KLC2 plasmid was a kind gift from Dr Michael Way (London Research Institute, London, UK). GFP-Rab4 dominant-active and GFP-Rab14 are kind gifts from DrsMireille Cormont(University of Nice Sophia Antipolis, France) and Isabella Coppens (Johns Hopkins University, U.S.A), respectively. GFP-PX domain of p40PHOX was a kind gift from Dr John Brumell (University of Toronto, Canada). The following constructs were described previously: wild-type Arl8b, Q75L and T34N in pcDNA3.1, GFP-Arl8b, Arl8b-pGEX-4T3, Arl8b-T34N-pGEX-4T3, Rab7-pGEX-4T3, Arl8b-pGBKT7, Vps41-pGADT7 (Garg, Sharma et al. 2011) and GST-PLEKHM1 1-198 (Marwaha, Arya, Jagga, Kaur, Tuli et al. 2017). Arl8b with C-terminal HA, Myc or Flag epitopes, Flag-SKIP-WT, NΔ300, Flag-SKIP-RUN (1–300) and HAVps41-NΔ450 were cloned in pcDNA3.1(-) (Invitrogen). Arl8b-tomato expression constructs were cloned in ptd-Tomato-N1 (Clontech) vector. The human HOPS subunits and SKIP yeast two-hybrid plasmids were cloned in pGBKT7 and pGADT7 vectors (Clontech). All the point mutants, truncation mutants and siRNA-resistant constructs were constructed using Stratagene site-directed mutagenesis kit (Agilent). HA-Vps41-ΔTPR was made by overlap extension PCR. Human Rabip4s were cloned in mammalian expression vectors,

pCDNA3.1(-) and pEGFPN1. For protein purification, the RUN-containing fragments were cloned in pGEX4T1 vector.

2.2 Reagents and antibodies

The following reagents were used in this study: Unlabelled EGF, Rhodamine-EGF and dextran-oregon green (Life Technologies), MG132 (Sigma).

The following antibodies were used in this study: rabbit polyclonal antibodies against LAMP1 (Novus Biologicals, Littleton, CO), Rab7 (Cell Signaling Technologies), Vps11 (Abcam, Cambridge, MA), Vps33a (Proteintech, Chicago, IL) and HA epitope (Sigma) and mouse monoclonal antibodies directed against Vps41 (Santa Cruz Biotechnology, Santa Cruz, CA), LAMP1 (BD Biosciences, San Jose, CA), Epidermal Growth Factor (EGF)-receptor (Life technologies), EEA1 (BD Biosciences, San Jose, CA), Transferrin (Tf)-receptor (Life technologies), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Abcam, Cambridge, MA), Tubulin (Sigma), HA epitope (Covance, Princeton, NJ), RUFY1 (Santa Cruz Biotechnology, Dallas, Texas)Myc epitope (Life technologies), Flag epitope (Sigma). Arl8b antisera was generously gifted by Dr. Michael Brenner. Secondary goat anti-mouse Alexa 488, 568, 647 and 405 and goat anti-rabbit Alexa 488, 568, 647 and 405 antibodies were purchased from Life technologies. Goat anti-mouse and anti-rabbit horseradish peroxidase (HRP) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.3 Cell culture

HeLa cells and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Lonza) supplemented with 10% fetal bovine serum (FBS; Life Technologies), glutamine, HEPES, non-essential amino acids and antibiotics at 37^0C under an atmosphere of humidified 5% CO_2 in a cell-culture

chamber. Each cell line was regularly screened for absence of mycoplasma contamination by using MycoAlert^{TM} Mycoplasma detection kit (Lonza) and was passaged for no more than fifteen passages.

2.4 Transfections and Immunofluorescence

HeLa cells were grown on coverslips to ~60% confluence and transfected with desired constructs using Xtremegene HP transfection reagent (Roche). The plasmid DNA to be transfected was mixed with the transfection reagent in 1:1 ratio in reduced serum media (OptiMeM), incubated at room temperature for 30 minutes followed by incubation of DNA-lipid complexes with the cells for 18-20 hours at 37^0C. Following this, cells were washed with 1X PBS (pH=7.4) and fixed with 4% paraformaldehyde made in PHEM buffer (60mM PIPES, 10mM EGTA, 25mM HEPES, 2mM $MgCl_2$, pH=6.8) for 10 minutes. Next, cells were incubated with blocking solution (5% fetal bovine serum (FBS) + 0.2% saponin made in PHEM buffer) for 1 hour. Cells were then incubated with appropriate primary antibodies in PHEM buffer containing 0.2% saponin for 45 minutes at room temperature. Further, they were immunostained with secondary antibodies conjugated with Alexa-fluor conjugates, in PHEM buffer containing 0.2% saponin, for 30 minutes at room temperature. Following this, cells were washed thrice with PBS and mounted using Fluoromount-G (Southern biotech) mounting medium. Images of immunostained cells were acquired with a Zeiss 710 Confocal Laser Scanning Microscope using a Plan-Apochromat 63×/1.4 NA objective with appropriate filters. The Zen 2012 software was used for data acquisition; linear adjustments (contrast and brightness) were done using Adobe Photoshop.

2.5 Protein purification and purified-protein interaction assay

Arl8b, Arl8bT34N, Rab7and Rabip4 1-194 were cloned in pGEX4T series vectors and overexpressed in *E.coli* BL-21 Star DE3 cells (Life technologies) and induced

with 0.5mM IPTG for 5 hours at 30^0C. The bacterial cells were harvested and the pellet was either resuspended in PBS in case of Rab7, or Prep buffer (20mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 0.5mM DTT, 0.5% TritonX-100, 5% glycerol) in case of Arl8b, Arl8bT34N, PLEKHM1 1-198 and Rabip4 1-194 containing protease inhibitor cocktail (Roche). The bacterial cells were lysed by sonication and the lysate was cleared by centrifugation. The supernatant obtained was incubated with glutathione-Sepharose beads (Pierce) for 3 hours. The resin was washed extensively with either PBS or Prep buffer (as indicated above), and incubated with 30mM glutathione overnight to elute the protein.

His-tagged Vps41 WD40 and Vps41 WD40 (T146P) were overexpressed in *E.coli* BL-21 Star DE3 cells (Life technologies) and the proteins were purified from the pellet fraction post-sonication.

For purified protein interaction assay, 10µg of each bait protein was bound to the beads in 1X PBS for 2 hours at 4^0C, following which the beads were blocked with 5% BSA for 2 hours at 4^0C. The beads were then washed thrice with TAP buffer (20mM Tris-Cl pH 7.4, 150mM NaCl, 0.5% NP-40, 1mM sodium orthovandate) and incubated with 4-5µg of purified prey protein (in TAP buffer) with rotation for 1 hour at 4^0C. Next, the beads were washed with TAP buffer multiple times, and the proteins were eluted by boiling in reducing 4X Laemelli buffer. The eluates were then subjected to 10% SDS-PAGE and analyzed by immunoblotting.

2.6 GST-pulldown assay

Purified GST, GST-Arl8b, GST-Arl8bT34N, GST-Rab7, GST-PLEKHM1 1-198 or GST-Rabip4 1-194 were incubated with glutathione-Sepharose beads (Pierce) in PBS-0.2% CHAPS for 2 hours, following which the beads were blocked with 5% BSA for 1 hour. HeLa or HEK293T cells overexpressing various prey proteins (as indicated in

the text) were lysed in TAP buffer (20mM Tris-Cl pH 7.4, 150mM NaCl, 0.5% NP-40, 1mM sodium orthovandate) containing protease inhibitor cocktail (Sigma) for 20 minutes on ice. Following this, the lysates were cleared by centrifugation and incubated with purified protein-bound beads for 3 hours. The beads were then washed with TAP buffer multiple times, and the proteins were eluted by boiling in reducing 4X Laemelli buffer. The eluates were then subjected to 10% SDS-PAGE and analyzed by immunoblotting.

2.7 Cell lysates and Immunoblotting

HeLa cells grown on tissue-culture dishes were washed thrice in 1X PBS (pH 7.4) and scraped off with a cell scraper. The cells were pelleted and lysed in lysis buffer (25mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X 100 and protease inhibitor cocktail), as mentioned previously. Lysates were then clarified by centrifugation and protein levels were quantified using the Bradford assay. The proteins were resolved on 10% SDS-PAGE and transferred onto nitrocellulose/PVDF membranes. Membranes were blocked with 5% skim-milk made in PBST (PBS with 0.3% tween-20) for an hour at room temperature, following which they were incubated with primary antibodies diluted in PBS-0.3% tween-20, for one hour at room temperature. Membranes were washed with PBST and then incubated with HRP-conjugated goat anti-mouse- or goat anti-rabbit-IgG secondary antibodies, diluted in PBS-0.3% tween-20, for 30 minutes at room temperature. The blots were washed with PBST multiple times and developed by ECL (Enhanced Chemi-Luminiscence).

2.8 Co-immunoprecipitation

HEK293T cells transfected with indicated constructs (in the text) were lysed in TAP buffer containing protease inhibitor cocktail, as mentioned previously, and post-

nuclear supernatants were incubated with anti-HA conjugated beads (Sigma) for 3 hours at 4^0C. The beads were then washed with TAP buffer multiple times, and the eluted proteins were then resolved by 10% SDS-PAGE and analyzed by immunoblotting.

2.9 Membrane-cytosol fractionation

HeLa cells were harvested in 150μL ice-cold homogenization buffer (25mM HEPES, 100mM NaCl, 1mM EDTA, pH 7.4 and protease inhibitor cocktail) by 50 vertical strokes in a glass Dounce homogenizer (Thomas) at 4°C. After centrifugation at 800 × *g* for 10 minutes to remove unbroken cells and cellular debris, the supernatant was then ultracentrifuged at 1,08,000 ×*g* for 1 hour at 4°C to yield a pellet of total cellular membranes and a supernatant representing the cytosolic fraction. The membrane pellet was dissolved in urea buffer (70mM Tris–HCl, pH 6.8, 8M urea, 10mM N-ethylmaleimide, 10mM iodoacetamide, 2.5% SDS and 0.1M DTT) by incubating at 37°C for 15 minutes. Equal volumes of both fractions were mixed with 4X Laemmli buffer to a final concentration of 1X and subjected to SDS–PAGE and immunoblot analysis.

2.10 Yeast-two-hybrid analysis

The *Saccharomyces cerevisiae* strain AH109 was maintained on Yeast extract, Peptone, Dextrose (YPD) agar plates. The yeast were co-transformed by the lithium acetate procedure, and then plated on double-dropout plates, lacking leucine and tryptophan to test viability. Following 72 hours incubation at 30^0C, three to four colonies were picked and suspended in water, equilibrated to the same optical density at 600 nm, and replated on double dropout plates (+His) as well as plates also lacking histidine (−His) to score the interactions.

2.11 Gene silencing by siRNA and shRNA

HeLa cells were treated with non-targeting siRNA (On-Target Plus smart pool, Dharmacon; D-001810-10-05) or oligonucleotides against Rab7 (On-Target Plus smart pool, Dharmacon; L-010388-00-0005), Arl8b (Dharmacon;(siRNA-1) 5'-AGGUAACGUCACAAUAAAGAU-3'; 5'-TGTCATCGCGTCAGGTCAATTTT-3' (siRNA-2);5'-GCTGAAGATGAATATCCCTAA-3'(siRNA-3)), Vps41 (Dharmacon; 5'-CCATTGACAAACCACCATTTA-3') and SKIP (ON-TARGETplusSMARTpool) using Dharmafect-1 for 72 hours. After 48 hours of siRNA treatment, cells were tranfected with either HA-Vps41 or Arl8b-tomato rescue constructs in separate experiments (as indicated), and incubated for another 24 hours. Cells were then fixed with 4% paraformaldehyde for 10 minutes and immunostained using appropriate antibodies.

For testing the knockdown efficiency, cells were harvested 72 hours post-siRNA treatment with desired oligonucleotides and lysed in lysis buffer (25mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X100 and protease inhibitor cocktail). The protein levels were quantified using the Bradford assay, and equal amounts of proteins were subjected to SDS-PAGE and immunoblotting (as mentioned previously).

2.12 EGF-receptor (EGFR) degradation and pulse-chase assay

Control- or Vps41-shRNA or -siRNA silenced cells were plated on glass coverslips at 30% confluence. The cells were starved in serum-free DMEM for 4 hours, following which they were pulsed with 500ng/mL Rhodamine-EGF (Life technologies) for 7 minutes at 37^0C. Further, the cells were chased in complete medium at 37^0C for the indicated time points, after which they were fixed with 4% paraformaldehyde and immunostained, as described previously. The images were acquired using Zeiss 710

Confocal Laser Scanning Microscope, and the EGFR intensity was measured using the ImageJ software. Using the software, boundary of each cell quantified was drawn, and the obtained parameters (area of cell, mean fluorescence and Integrated density) were used to calculate Corrected Total Cell Florescence (CTCF), by the formula :

CTCF = Integrated density - (area x mean fluorescence of background)

For rescue experiments, HeLa cells plated on glass coverslips at 30% confluence were treated with control or Vps41 siRNA for 72 hours. 48 hours post-siRNA treatment, cells were transfected with either HA-Vps41 wild-type rescue construct or HA-Vps41 T146P rescue construct and incubated for another 24 hours. EGFR degradation experiments were carried out as described above.

Alternatively, HeLa cells were treated with either control or Vps41 siRNA duplexes, starved in serum-free medium and pulsed with unlabelled EGF for indicated times. Next, cells were harvested and lysed in RIPA buffer (10mM Tris-Cl pH 8, 140mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% Triton-X 100, 0.1% SDS, 0.1% sodium deoxycholate and protease inhibitor cocktail) as described previously. The protein levels were quantified using the Bradford assay, and equal amounts of proteins were resolved on 8% SDS-PAGE gel, and immunoblotted using either anti-EGFR or anti-GAPDH (loading control) antibodies.

For EGFR pulse-chase assay, control- or Vps41-shRNA silenced cells were either incubated with dextran Oregon-green overnight or directly subjected to unlabeled or Rhodamine-labeled EGF for 7 minutes at 37^0C. Cells were then chased in complete medium for indicated time points, fixed and immunostained as described previously.

2.13 Statistics

Data from ImageJ was imported into Microsoft Excel. The mean and the standard deviation of the mean were calculated from data obtained by counting 100 cells, for

each time-point, from three independent experiments. Statistical significance was calculated using paired Student's t-test. $P<0.05$ was considered to be statistically significant.

Chapter III

The small GTPase Arl8b regulates assembly of the mammalian HOPS complex on lysosomes

The following chapter has been published in Journal of Cell Science, 2015128:1746-61

Chapter 3

3.1 Introduction

The endocytic system in eukaryotic cells is a complex and elaborate network of membrane-bound compartments/organelles that constantly exchange material via vesicular or tubular carriers. The Rab and Arf families of small GTPases are important regulators of endocytic trafficking that recruit their numerous effectors to intracellular membranes in a GTP-dependent manner and mediate vesicle budding/formation, tethering and fusion (Grosshans, Ortiz and Novick 2006; Itzen and Goody 2011). Recent studies have also characterized the Arf-like (Arl) family of small GTPases, which are implicated in diverse cellular processes including vesicular trafficking, cytoskeletal organization, and ciliogenesis(Burd, Strochlic and Gangi Setty 2004; Donaldson and Jackson 2011).

Rab proteins, in their GTP-bound form, facilitate membrane recruitment of tethering factors that are either long coiled coil proteins or multi-subunit protein complexes (Kummel and Heinemann 2008; Brocker, Engelbrecht-Vandre et al. 2010; Brown and Pfeffer 2010). For instance, the vacuolar protein sorting pathway in yeast depends upon multi-subunit tethering complexes (MTCs): Class C core vacuole endosome tethering (CORVET) and homotypic fusion and protein sorting (HOPS) which are effectors of RabGTPases, the Rab5-homolog Vps21 and the Rab7-homolog Ypt7, respectively. Moreover, Rab binding regulates their association with target membranes (Seals, Eitzen et al. 2000; Wurmser, Sato et al. 2000; Peplowska, Markgraf, Ostrowicz, Bange and Ungermann 2007; Ostrowicz, Brocker, Ahnert, Nordmann, Lachmann et al. 2010). Both HOPS and CORVET are hexameric complexes that share four of the six subunits known as the “core” subunits. The core subunits shared by the two MTCs are encoded by the class C phenotypic class of

vacuole protein sorting (VPS) genes and include Vps11p, Vps16p, Vps18p and the Sec1-like protein Vps33p. The two "accessory" subunits of HOPS are Vps39 and Vps41 while their corresponding homologous counterparts in CORVET are Vps3 and Vps8 (Wada, Ohsumi et al. 1992; Nakamura, Hirata, Ohsumi and Wada 1997; Rieder and Emr 1997; Nickerson, Brett et al. 2009).

The role of the yeast HOPS complex as a tethering factor required for vacuolar fusion has been extensively studied using both *in vivo* knockout approaches and *in vitro*proteoliposome assays (Seals, Eitzen et al. 2000; Stroupe, Hickey et al. 2009; Hickey and Wickner 2010; Ostrowicz, Brocker et al. 2010). Vacuolar localization of the yeast HOPS is mediated by the small GTPase Ypt7 that directly binds to and recruits Vps41 and Vps39 subunits to vacuolar membrane (Ostrowicz, Brocker et al. 2010; Brocker, Kuhlee et al. 2012). Once HOPS is targeted to membranes, it catalyzes membrane fusion by recruiting and proofreading SNAREs at the fusion site via its Vps33 subunit (Starai, Hickey et al. 2008; Wickner 2010). Homologs of all six HOPS subunits have also been identified in higher eukaryotes, with more than one homologue of HOPS subunits Vps33 in metazoan cells, Vps33a and Vps33b, respectively (Peterson and Emr 2001; Richardson, Winistorfer, Poupon, Luzio and Piper 2004; Gissen, Johnson, Gentle, Hurst, Doherty et al. 2005; Pulipparacharuvil, Akbar et al. 2005; Zlatic, Tornieri, L'Hernault S and Faundez 2011). Studies in higher eukaryotes have suggested an evolutionarily conserved function of HOPS subunits in regulating fusion of endocytic, phagocytic and autophagic cargo with lysosomes and biogenesis of lysosome-related organelles (Sevrioukov, He, Moghrabi, Sunio and Kramer 1999; Maldonado, Hernandez, Lozano, Castro and Navarro 2006; Akbar, Tracy, Kahr and Kramer 2011; Swetha, Sriram, Krishnan, Oorschot, ten Brink et al.

2011; Zlatic, Tornieri et al. 2011; Delahaye, Foster, Vine, Saxton, Curtin et al. 2014; Manil-Segalen, Lefebvre et al. 2014; Takats, Pircs et al. 2014).

More recently, several reports have elucidated the role of mammalian HOPS subunits in regulating trafficking towards lysosomes (Aoyama, Sun-Wada, Yamamoto, Yamamoto, Hamada et al. 2012; Pols, ten Brink et al. 2013; van der Kant, Fish, Janssen, Janssen, Krom et al. 2013). In summary, human HOPS subunits hVps41 and hVps39 localize to late endosomes and lysosomes and their depletion results in accumulation of late endosomes, depletion of lysosomes, and a block in degradation of endocytosed cargo (Pols, ten Brink et al. 2013). In agreement with this, previous studies have reported increased endo-lysosome fusion upon overexpression of HOPS subunits (Caplan, Hartnell et al. 2001; Poupon, Stewart et al. 2003; Pols, ten Brink et al. 2013). Furthermore, Vps41/Vam2 knockout mouse embryonic fibroblasts exhibit morphological and functional alteration of late endosomes and lysosomes and defective attenuation of developmentally important signaling pathways (Aoyama, Sun-Wada et al. 2012). Other mammalian HOPS subunits, Vps11 and Vps18, also regulate cargo trafficking towards late endosomes and lysosomes (Chirivino, Del Maestro, Formstecher, Hupe, Raposo et al. 2011; Peng, Ye et al. 2012). Recent studies have shown that similar to its other homologs; mammalian HOPS subunits not only regulate endocytic traffic but also phagocytic and autophagic traffic towards lysosomes (Barry, Boucherit, Mottola, Vadovic, Trouplin et al. 2012; Jiang, Nishimura et al. 2014).

Although we now appreciate the importance of the mammalian HOPS complex in regulating cargo delivery to lysosomes, we still do not understand what factors regulate the recruitment of HOPS subunits to lysosome membranes, and how the HOPS complex assembles on these membranes. Previous reports have suggested that

similar to its yeast counterpart, mammalian HOPS subunits interact with Rab7 and that this interaction regulates recruitment of the HOPS complex to late endosomes and lysosomes. In support of this model, co-immunoprecipitation approaches have shown an association of Rab7 with Vps39 and Vps41 subunits of the mammalian HOPS complex (Rink, Ghigo et al. 2005; Poteryaev, Datta, Ackema, Zerial and Spang 2010; Sun, Westphal, Wong, Tan and Zhong 2010). However, thus far, direct binding to Rab7, and whether any mammalian HOPS subunit acts as a Rab7 effector has not been demonstrated. Recently, it was also reported that the Rab7 effector RILP (Rab-interacting lysosomal protein) directly interacts with multiple subunits of the HOPS complex and guides their localization to late endosomes (van der Kant, Fish et al. 2013; Lin, Yang, Wang, Wang, Yun et al. 2014).

Previously, we have found that hVps41 subunit of the HOPS complex directly interacts with a lysosome small GTPase of the Arl family, Arl8b and depletion of Arl8b prevents membrane localization of hVps41 (Garg, Sharma et al. 2011). To resolve the mechanism by which the HOPS complex associates with the late endosome and lysosome compartment, we tested binding of all the human HOPS subunits with the small GTPases Rab7 and Arl8b. Our results suggest that Arl8b, but not Rab7 or RILP, targets the HOPS subunit hVps41 to lysosomes. Moreover, depletion of Arl8b dramatically reduced membrane association of multiple HOPS subunits, suggesting that Arl8b is a critical factor required for membrane association of human HOPS complex. In addition, using siRNA-mediated depletion of hVps41 and rescue either by wild type hVps41 or an Arl8b-binding defective mutant, we demonstrate that association of hVps41 with Arl8b is required for its function in degradation of endocytic cargo. These results suggest that small GTPase Arl8b is

acritical factor that orchestrates the assembly of human HOPS complex on lysosomes and regulates the function of this tethering complex in membrane trafficking.

3.2 Results

3.2.1 Arl8b, but not Rab7, interacts with the hVps41 subunit of the HOPS complex in a GTP-dependent manner

In our previous study, we had identified hVps41 as a direct binding partner for Arl8b that is recruited to lysosomes in an Arl8b-dependent manner (Garg, Sharma et al. 2011). However, whether hVps41 interaction with Arl8b promotes the assembly of other HOPS subunits on lysosome membranes, and whether this interaction regulates the function of the HOPS complex in endocytic trafficking has not been established. Moreover, it is not known if the late endosome small GTPase Rab7 directly binds to human HOPS subunits and regulates their recruitment to lysosomes.

To gain insight into this, we first tested apparent direct interactions of late endosome-lysosome small GTPases Arl8b and Rab7 with the six subunits of the HOPS complex in a yeast two-hybrid assay (Fig. 3.1A). As reported previously, interaction of Arl8b with the hVps41 subunit was also observed in this assay (Fig. 3.1A, bottom panel) (Garg, Sharma et al. 2011). No interaction of Arl8b was observed with the other five subunits of the HOPS complex. Interestingly, while a strong interaction of Rab7 with its known interaction partner, RILP (Cantalupo, Alifano, Roberti, Bruni and Bucci 2001) was observed in this assay, no direct interaction was seen with any subunits of the HOPS complex (Fig. 3.1A, bottom panel). These results were further corroborated using a GST pull-down approach in which GST tagged proteins: wild type (WT) Arl8b or Arl8b T34N (constitutively GDP-bound mutant) or Rab7 were used as bait to pull-down hemagglutinin (HA)-hVps41 from transfected HeLa cell lysates (Fig. 3.1B, upper panel). The biochemical pulldown data independently confirmed the

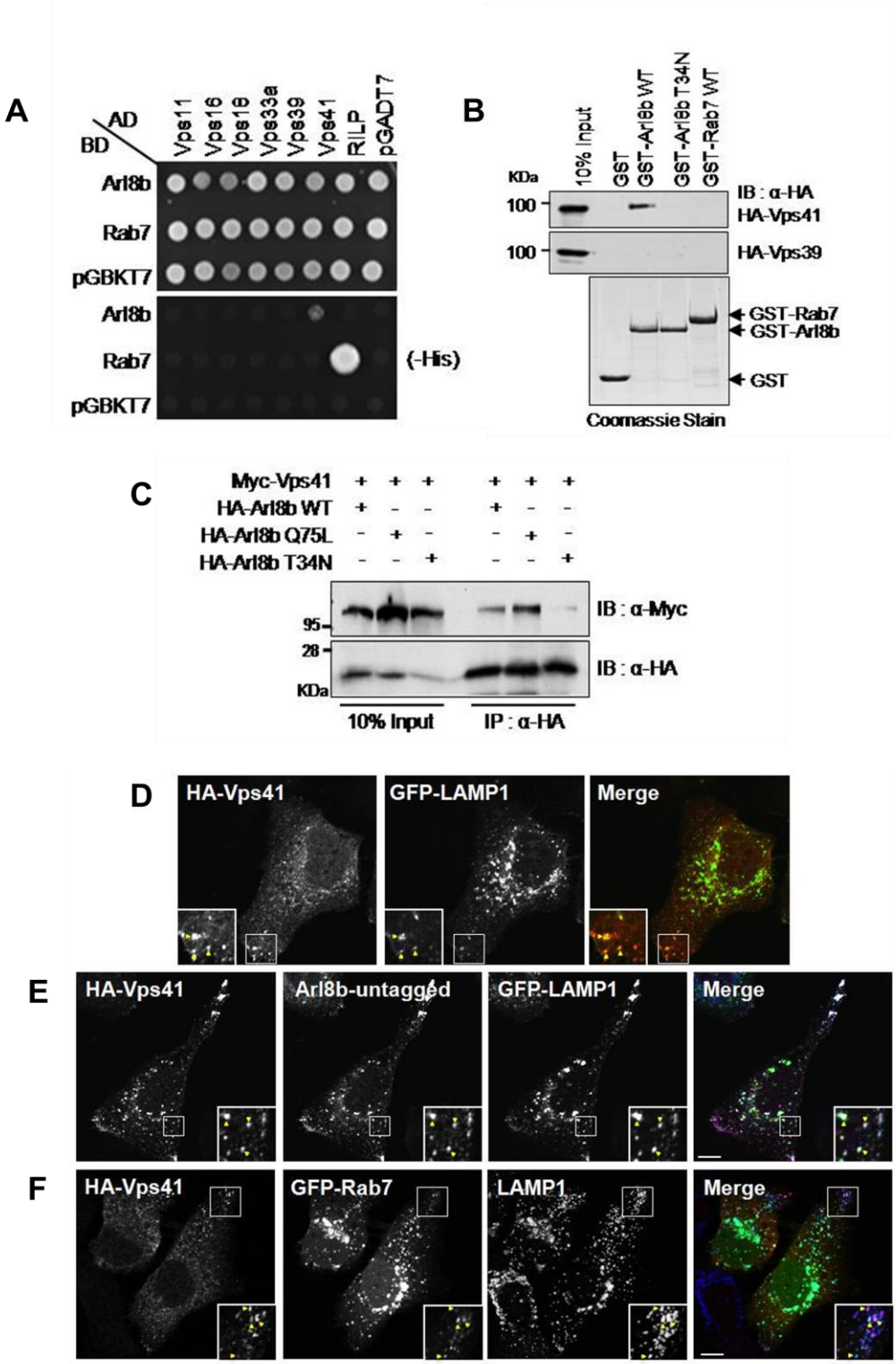

Figure 3.1. Arl8b, but not Rab7, interacts with hVps41 and recruits it to lysosomes. (A) Interaction of the human HOPS subunits with Arl8b and Rab7 was tested in a yeast two-hybrid system by assaying for growth of co-transformants on non-selective medium, to confirm viability, and on selective medium, to detect interactions. BD, binding domain; AD, activating domain. (B) A representative immunoblot (IB) of a GST pulldown assay using HeLa cell lysates expressing either HA–hVps41 or HA–hVps39 incubated with glutathione-conjugated beads bound to the indicated GST proteins. GST-purified proteins were visualized with Coomassie Blue dye staining. WT, wild type. (C) Western blot showing HEK293T cell lysates expressing Myc–hVps41 in combination with different forms of Arl8b, immunoprecipitated (IP) with anti-HA-conjugated beads. (D–F) HeLa cells transfected with HA–hVps41 and GFP–LAMP1 (D), or HA–hVps41, untagged Arl8b and GFP–LAMP1 (E), or HA–Vps41 and GFP–Rab7 (F) were analyzed for lysosomal localization by confocal microscopy. Colocalized pixels are indicated in the inset. Colocalized puncta are indicated by arrowheads. Scale bars: 10 μm.

conclusions from the yeast two-hybrid assay where interaction of hVps41 was observed with GST-Arl8b but not with GST-Arl8b T34N or GST-Rab7. GST pull-down also revealed that both Arl8b and Rab7 do not interact with other HOPS subunits (hVps39, hVps16 and hVps18) transfected alone into HeLa cells (Fig. 3.1B, middle panel and data not shown). To analyze whether Arl8b and hVps41 interact *in vivo,*we performed co-immunoprecipitation experiments. Our results clearly indicate that Myc-tagged hVps41 co-immunoprecipitated in cells transfected with wild type and putative GTP-locked Arl8b mutant (Arl8b Q75L) but not with putative GDP-locked Arl8b mutant (Arl8bT34N) indicating that Arl8b and hVps41interact *in vivo* (Fig. 3.1C).

Next, to understand the role of Arl8b versus Rab7 in regulating association of hVps41 with lysosomes, we overexpressed hVps41 with either of the two GTPases and quantified the number of Vps41 endosomes co-localizing with the late endosome/lysosome marker LAMP1. HA-tagged hVps41 localizes both to cytoplasm and peripheral LAMP1 positive vesicles in HeLa cells, as has been previously reported (Fig. 3.1D). Interestingly, there was a significant increase in the number of Vps41 endosomes colocalizing with LAMP1 in Arl8b-overexpressing cells (Fig. 3.1E; quantification shown in Fig. 3.2A). On the other hand, no significant change in Vps41 localization was observed upon Rab7 overexpression and only a few Vps41-positive structures were present on Rab7/LAMP1 positive compartment (Fig. 3.1F; quantification shown in Fig. 3.2A). hVps39, another lysosome-localized subunit of the HOPS complex (Caplan, Hartnell et al. 2001; Pols, ten Brink et al. 2013), also showed increased recruitment to LAMP1 endosomes upon overexpression of Arl8b but not Rab7 (quantification shown in Fig. 3.2A). We also noticed that lysosomes (LAMP2-positive) in Vps41/Arl8b co-transfected cells appeared more clustered and

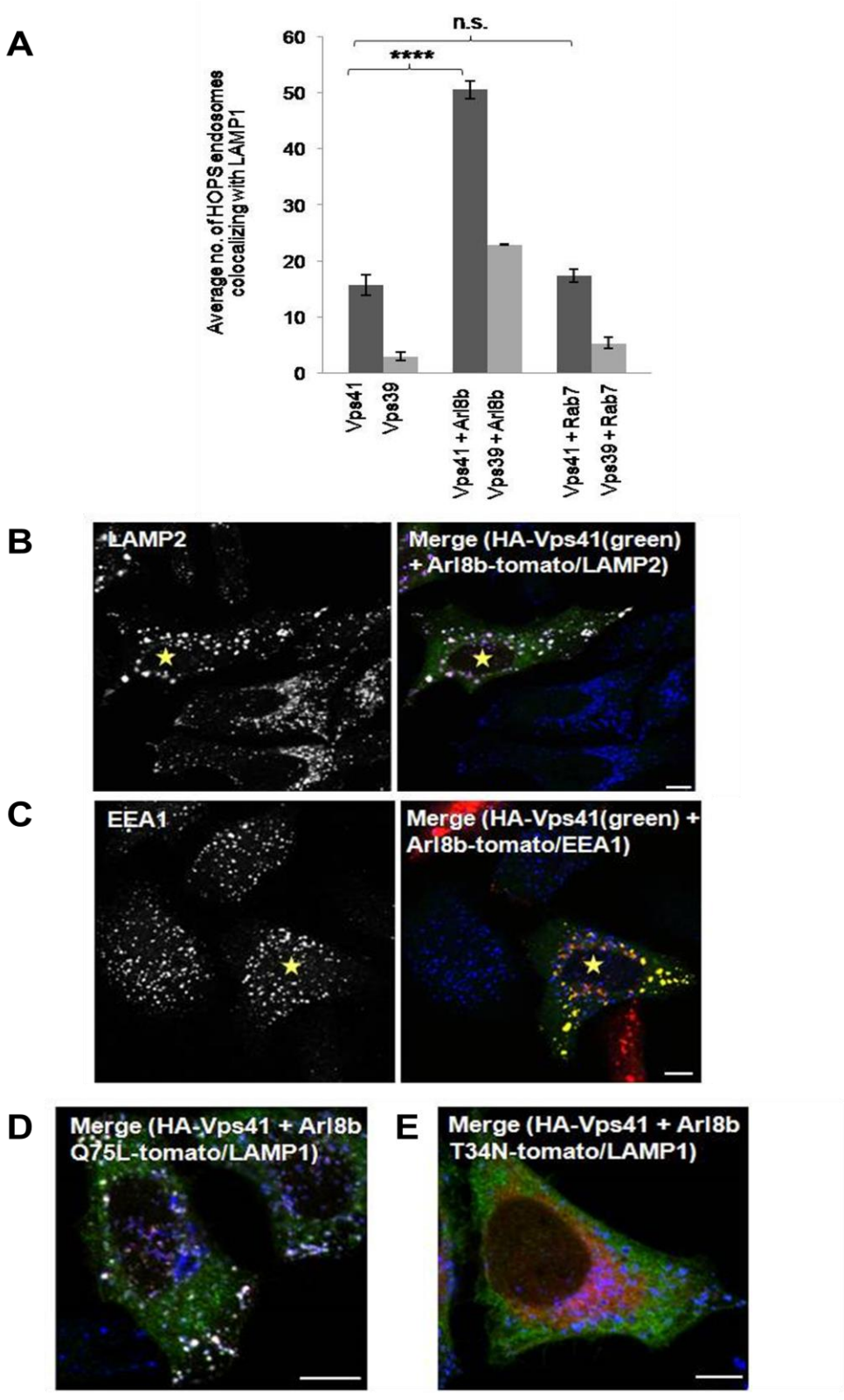

Figure 3.2. Vps41 localizes to lysosomes dependent upon GTP-bound Arl8b, but not Rab7. (A) Quantification representing average number of HOPS-positive endosomes colocalizing with LAMP1 (confocal data in Figure 3.1(D-F)). Values plotted correspond to the mean±s.d. of three independent experiments (n=25 cells per experiment); ****P=0.0001; n.s., not significant. (B-C) HeLa cells co-transfected with HA-Vps41and Arl8b were co-stained with either LAMP2 (B) or EEA1 (C). Merge image shows colocalization of Vps41 and Arl8b with LAMP2 but not EEA1. (D-E) HeLa cells were co-transfected with HA-Vps41 and either Arl8b Q75L (D) or Arl8b T34N (E), and analyzed for their lysosomal localization by confocal microscopy. Scale bars: 10 μm.

larger than in the untransfected cells, while no obvious difference was observed in the distribution of early endosomes (EEA1-positive, Fig. 3.2B,C).

Next, to test if the GTP-bound form of Arl8b is responsible for hVps41 localization to lysosomes, we co-expressed putative GTP-locked (Q75L) and putative GDP-locked (T34N) mutants of Arl8b with hVps41. As predicted, Arl8b T34N was present in the cytosol and failed to associate with the LAMP1 compartment (Fig. 3.2E). Unlike the cells transfected with Arl8b Q75L mutant where Vps41 was present on punctate structures, a complete redistribution of Vps41 to the cytoplasm was observed in cells co-expressing the Arl8b T34N mutant suggesting that GTP-Bound Arl8b is required for membrane localization of hVps41 (merge images, Fig. 3.2D,E).

Finally, localization of hVps41 was also analyzed in cells treated with control-, Arl8b, or Rab7-siRNA. The efficiency of gene silencing was confirmed by immunoblotting and was found to be >90% for each protein (Fig. 3.3A). Previous studies have reported that Arl8b silencing prevents kinesin-1 dependent motility of lysosomes to the cell periphery leading to an accumulation of lysosomes at the MTOC (Hofmann and Munro 2006; Rosa-Ferreira and Munro 2011; Tuli, Thiery et al. 2013). Rab7 silencing causes formation of an enlarged late endosome compartment with an increased number of intra-luminal vesicles (ILVs) and a modest reduction in the size and number of dense lysosomes as visualized by electron microscopy (Vanlandingham and Ceresa 2009). Both of these phenotypes were also observed in our experiment upon LAMP1 staining of cells silenced for Rab7 and Arl8b (Fig. 3.3C,D red in merge images). Upon analyzing the distribution of HA-hVps41 in the siRNA-treated cells, we found that Vps41 was completely cytosolic in Arl8b-silenced cells (Fig. 3.3C), but in cells depleted of Rab7 it continued to localize to the LAMP1-positive vesicles (Fig. 3.3D). To test the specificity of Arl8b depletion, we rescued the

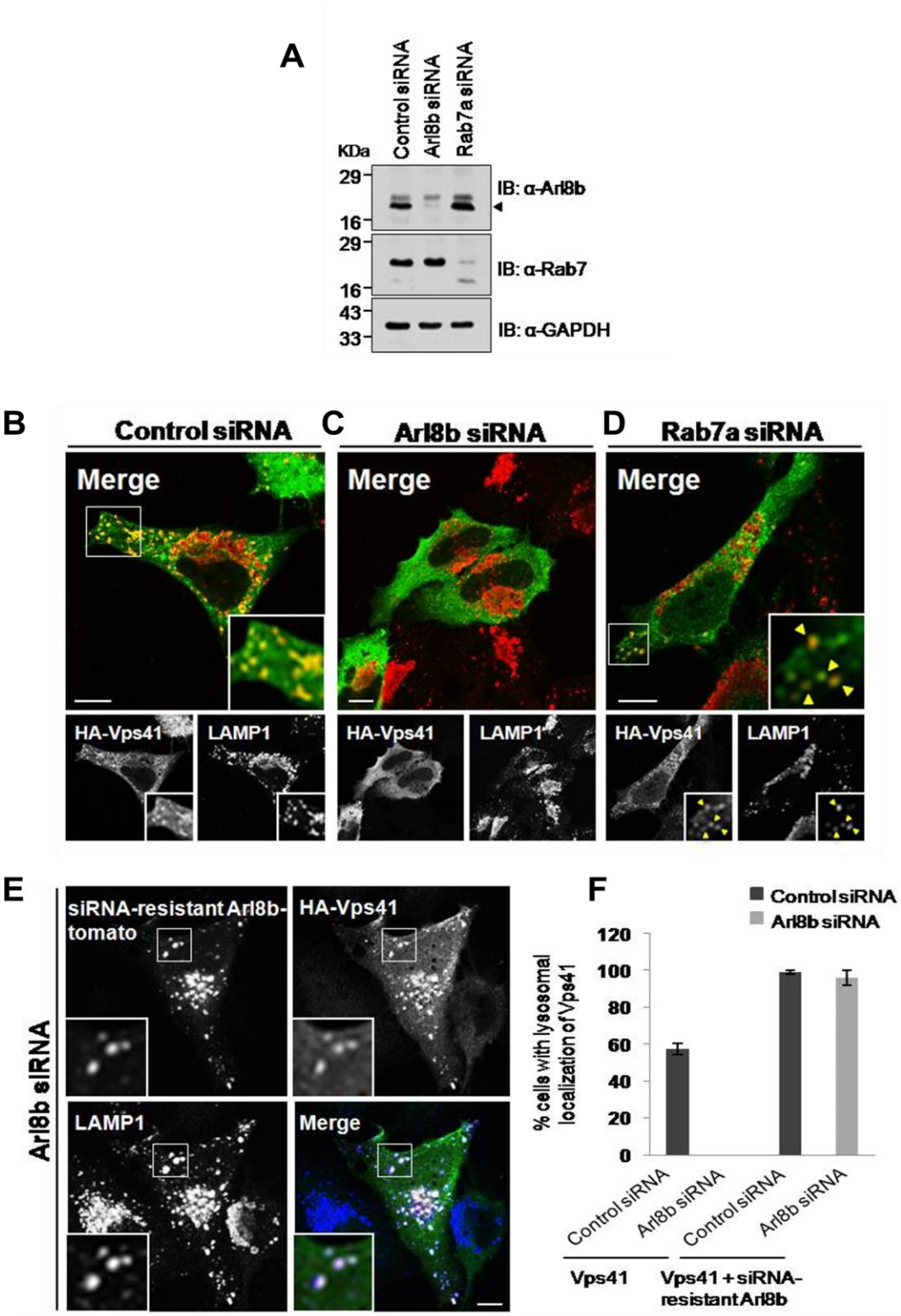

Figure 3.3. Arl8b expression is required for localization of hVps41 to lysosomes. (A) An immunoblot (IB) of control-, Arl8b- or Rab7-depleted HeLa cell lysates depicting levels of the two proteins. GAPDH was used as the loading control. In the top panel, the arrowhead indicates the position of the dominant band inferred to be Arl8b (B–D) Immunofluorescence depicting the localization of HA–Vps41 to LAMP1^{+} compartments in control (B), Arl8b- (C) or Rab7- (D) depleted cells. (E) Immunofluorescence depicting the localization of HA–Vps41 to LAMP1^{+} compartments in Arl8b-silenced cells co-expressing HA–Vps41 and siRNA-resistant Arl8b–tomato. (F) The percentage of cells with lysosomal localization of Vps41 was quantified following different treatments as indicated. Values plotted are the mean±s.d. of three independent experiments (n=100 cells for each experiment).

effect of Arl8b-siRNA by expressing a siRNA-resistant version of Arl8b, and analyzed localization of Vps41 in the transfected cells. As demonstrated, in siRNA-resistant Arl8b-transfected cells, a complete restoration of Vps41 localization to lysosomes was observed (Fig. 3.3E; quantification is shown in Fig. 3.3F). We next investigated if the lack of Vps41 membrane localization observed in Arl8b-siRNA treated cells could be rescued by co-expressing either Rab7 or RILP, a known Rab7 effector that has previously been shown to directly bind to Vps41 and recruit Vps41 and other HOPS subunits to late endosomes (van der Kant, Fish et al. 2013; Lin, Yang et al. 2014). In accordance with these studies, we also found strong recruitment of multiple HOPS subunits (including Vps41) to RILP-decorated late endosomes that were clustered near the perinuclear region (Fig. 3.4A,C). We anticipated that RILP should be able to rescue Vps41 membrane association in the absence of Arl8b due to its reported direct interaction with Vps41 C-terminal domain (Lin, Yang et al. 2014). Surprisingly, while both Rab7 and RILP colocalized with LAMP1 in both control and Arl8b-siRNA treated cells (Fig. 3.4C-F arrowheads in bottom panels), they failed to recruit Vps41 to these endosomes upon Arl8b depletion (Fig. 3.4D,F). This suggests that Arl8b is required for Vps41 association with lysosomes and neither Rab7 nor RILP expression could rescue this effect. On the contrary, in Rab7-siRNA treated cells, while RILP was completely cytosolic (Fig. 3.4B), both Vps41 and Arl8b continued to colocalize with LAMP1 compartment, similar to their localization in control-siRNA treated cells (Fig. 3.4G,H). Collectively, our findings suggest that Arl8b, but not Rab7 or Rab7 effector RILP regulates the localization of hVps41 to lysosomes. Our results also highlight the differences in the mammalian and yeast systems wherein direct binding of Vps41p with Ypt7 (the Rab7 homolog in yeast) is well-established and is essential for vacuolar (yeast equivalent of lysosomal)

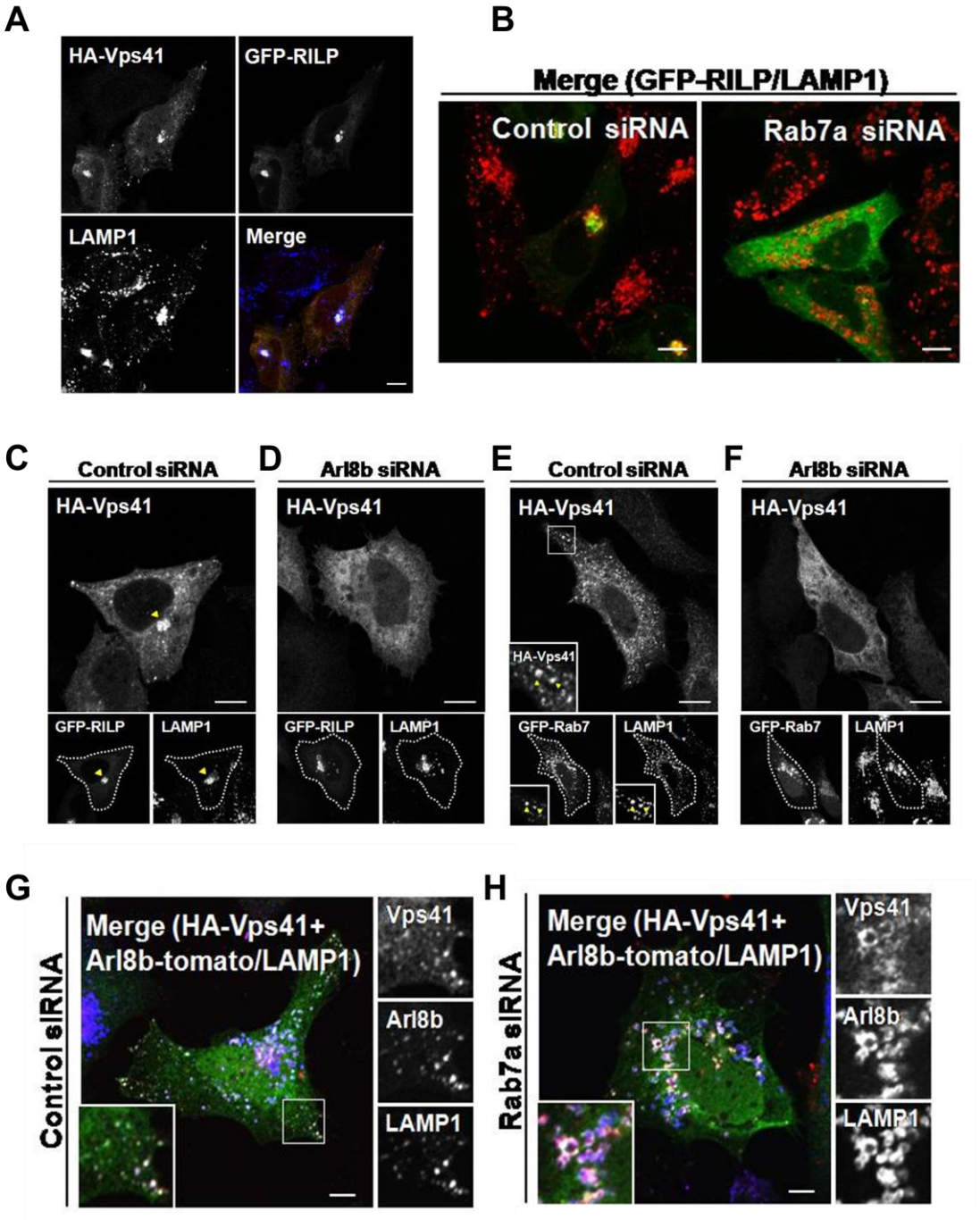

Figure 3.4. Arl8b expression is required for hVps41 association with lysosomes and RILP- or Rab7-containing late endosomes. (A) Confocal micrograph depicting the colocalization of HA-Vps41 with GFP-RILP. (B) Control or Rab7-silenced HeLa cells were transfected with GFP-RILP and immunostained with LAMP1, to visualize its localization by immunofluorescence. (C-F) Control (C,E) and Arl8b-siRNA-treated cells (D,F) were co-transfected with HA–Vps41 and either GFP–RILP (C,D) or GFP–Rab7 (E,F), stained with LAMP1 and analyzed by confocal microscopy. (G-H) Control- (G) or Rab7- (H) silenced HeLa cells were co-transfected with HA-Vps41 and Arl8b-tomato and analyzed for lysosomal localization by confocal microscopy. Colocalized puncta are indicated in the inset. Scale bars:10 μm.

localization of Vps41p (Hickey, Stroupe and Wickner 2009; Brocker, Kuhlee et al. 2012), while in mammalian cells, small GTPase Arl8b performs this function.

3.2.2 Interaction of hVps41 with Arl8b requires the N-terminal WD40 domain

To gain insight into the mechanisms by which hVps41 interacts with Arl8b, we created several C-terminal domain deletion mutants of hVps41 and analyzed their binding to Arl8b by GST-pulldown assays. Bioinformatics analysis predicts three domains within hVps41, namely WD40, Clathrin Heavy Chain Repeat (CHCR) and RING-H2 Zinc finger domain. A recent study by Harrington et al. has predicted that the amino acids 393-531 of hVps41 are a tetratricopeptide repeat (TPR)-like domain that is structurally similar to the CHCR domain (Ybe, Brodsky, Hofmann, Lin, Liu et al. 1999; Harrington, Yacoubian et al. 2012). We found that truncation of the C-terminal 142 amino acids (Vps41 L713X) containing the RING-H2 Zinc finger domain or truncation of both the RING-H2 and CHCR domains (Vps41 R532X), did not alter the interaction with Arl8b, as observed in the GST-pulldown assays (Fig. 3.5A). Next, we created an internal deletion mutant of Vps41 lacking only the TPR-like domain (Vps41ΔTPR) and an N-terminal deletion mutant lacking only the WD40 domain (Vps41ΔWD40). Truncation of the complete WD40 domain made the Vps41 protein unstable and possibly a target for degradation in proteasomes. Consequently, expression of the Vps41ΔWD40 mutant was only found in HeLa cells treated with proteasome inhibitor MG132 (Fig. 3.5C). Interaction analysis using these mutants demonstrated that while deletion of the TPR-like domain had no effect on binding to Arl8b, Vps41 lacking only the WD40 domain failed to bind to Arl8b (Fig. 3.5B,C). The data was quantified by densitometric scanning of theseblots to further substantiate these results (Fig. 3.5D). Next, to confirm that WD40 domain of Vps41 is sufficient for binding to Arl8b, recombinantly-expressed, purified His-tagged Vps41

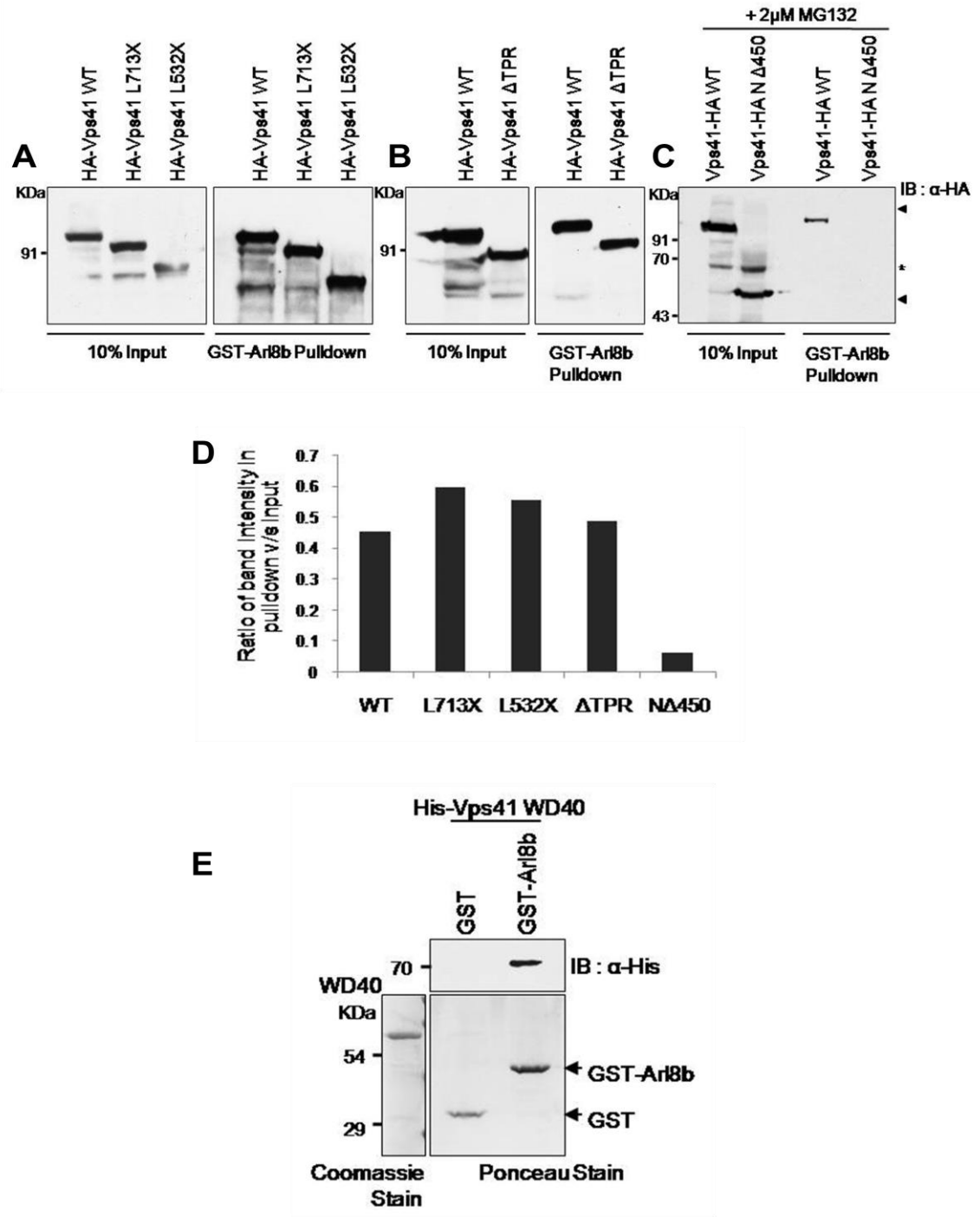

Figure 3.5. Interaction of hVps41 with Arl8b requires the N-terminal WD40 domain. (A–C) A representative immunoblot (IB) of a GST pulldown assay using GST–Arl8b as the bait incubated with HEK293T cell lysates expressing either wild-type (WT) or various domain deletion mutants of Vps41 (as labeled). Treatment with MG132 (2 μM) for 12 hours was performed to restore expression of Vps41-NΔ450. Arrowheads indicate the position of specific bands; the asterisk indicates the position of non-specific bands. (D) Densitometric quantification of two independent GST pulldown experiments with wild-type and mutant Vps41. (E) Purified GST or GST–Arl8b on glutathione–agarose beads were incubated with purified His–Vps41-WD40 domain, subjected to SDS-PAGE and immunoblotted using anti-His. Purified proteins were visualized with Coomassie Blue and Ponceau staining, as indicated.

WD40 domain and GST or GST-Arl8b proteins were incubated together in an *in vitro* binding assay. Our results indicate that Vps41 WD40 domain bound to GST-Arl8b but not GST alone, suggesting that Vps41 WD40 domain is both essential and sufficient for binding to Arl8b (Fig. 3.5E).

We next analyzed the localization of the Vps41 truncation mutants in the presence of overexpressed Arl8b to elucidate their recruitment to the Arl8b-positive compartment. As expected from our binding assays, colocalization with Arl8b-positive endosomes was observed with full-length HA-tagged hVps41 and domain deletion mutants lacking RING-H2 (Vps41 L713X) or both RING-H2 and CHCR domains (Vps41 R532X) or only the TPR-like domain (Vps41ΔTPR-like) (Fig. 3.6A-D). Quantification of the colocalization coefficients from several transfected cells with wild-type and mutants shows no significant difference indicating that association of Vps41 with Arl8b-positive endosomes is mediated via its WD40 domain (Fig. 3.6E). Vps41NΔ450 mutant was not evaluated in this assay due to its lack of expression in HeLa cells and rapid degradation by proteasome machinery. These results strongly support the importance of Vps41WD40 domain in regulating its interaction with Arl8b, and thereby its localization and function in endocytic trafficking.

3.2.3 T146 SNP in the Vps41 WD40 domain abrogates Arl8b binding and its localization to lysosomes

Previous studies have identified hVps41 as a candidate gene mediating neuroprotection of *C. elegans* dopaminergic neurons from α-synuclein-induced neurodegeneration. Interestingly, in this study two SNPs within the WD40 domain of hVps41 (A187T and T146P) were identified which abrogate the neuroprotective effect of Vps41 on dopaminergic neurons (Harrington, Yacoubian et al. 2012). Since we found that the WD40 domain of hVps41 is important for the interaction with

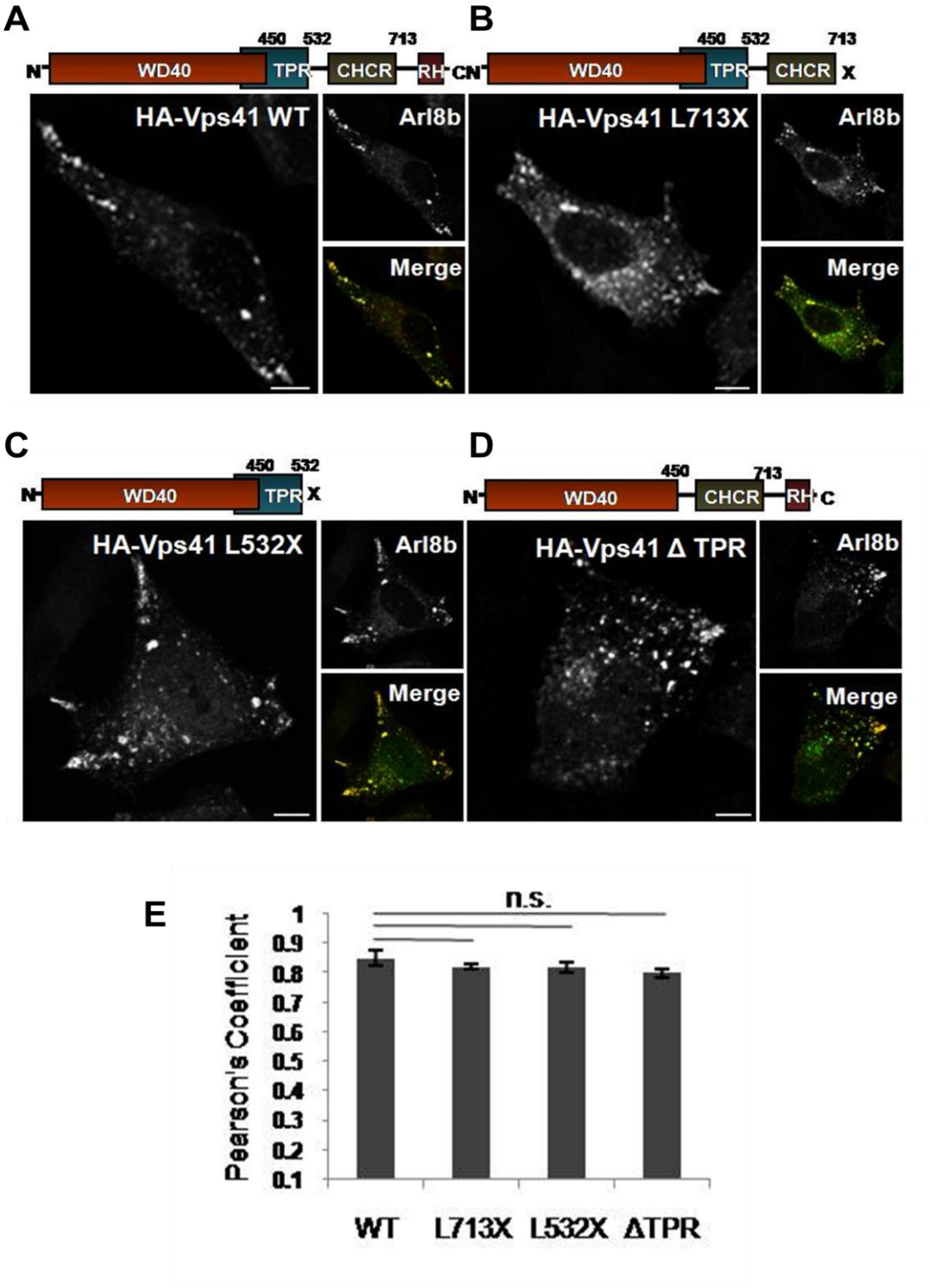

Figure 3.6. Colocalization of hVps41 with Arl8b requires the N-terminal WD40 domain. (A–D) HeLa cells were co-transfected with Arl8b and either wild-type HA–Vps41 (A) or HA-tagged domain deletion mutants (B–D, as indicated), and analyzed for their colocalization by confocal microscopy. Scale bars: 10 µm. (E) Pearson's coefficient depicting the overlap of Vps41 with Arl8b was calculated. Values plotted are the mean±s.d. of three independent experiments (n=30 cells per experiment). n.s., not significant.

Arl8b, we wanted to determine the consequence of these two SNPs in the hVps41 WD40 domain on Arl8b-binding and subsequent recruitment to lysosomes (Fig. 3.7A). To investigate this, we created Vps41 A187T and Vps41 T146P and tested their interactions with Arl8b using the GST-pulldown assay. While there was no difference observed in the interaction of Vps41 A187T with GST-Arl8b as compared to WT Vps41, the Vps41 T146P version showed a dramatic reduction in binding to GST-Arl8b (Fig. 3.7B). Further confirmation of these results was done using purified proteins where either His-tagged WD40 wild-type or WD40 T146P was incubated with GST and GST-Arl8b (Fig. 3.7C). Significant loss of binding of WD40 T146P to GST-Arl8b in comparison to the wild-type WD40 domain, further confirmed that this SNP in Vps41 abrogates its binding to Arl8b. To test if the impaired binding to Arl8b might be due to the unfolding of the Vps41 led by the threonine to proline conversion, circular dichroism experiments were carried out with recombinantly-expressed and purified wild-type WD40 and T146P WD40 domains. CD spectroscopy data indicated that this substitution led to structural changes in WD40 domain, but not a complete denaturation of the overall secondary structure of this domain (Fig. 3.7D). In support of this argument, the T146P substitution, while impaired binding to Arl8b did not change association of Vps41 with other HOPS subunits including Vps18, Vps11 and Vps33a as concluded by both yeast-two hybrid and co-immunoprecipitation approaches (Fig. 3.7E, 3.8A). We also expressed these hVps41 SNP transgenes to test their recruitment to Arl8b-positive lysosomes. As expected from loss of binding to Arl8b, Vps41 T146P also failed to be recruited to the Arl8b/LAMP1 compartment and continued to be cytosolic in the presence of overexpressed Arl8b, while both hVps41 WT and A187T showed strong colocalization with Arl8b (Fig. 3.8B-E). These observations explain why SNP T146P is deleterious to the neuroprotective

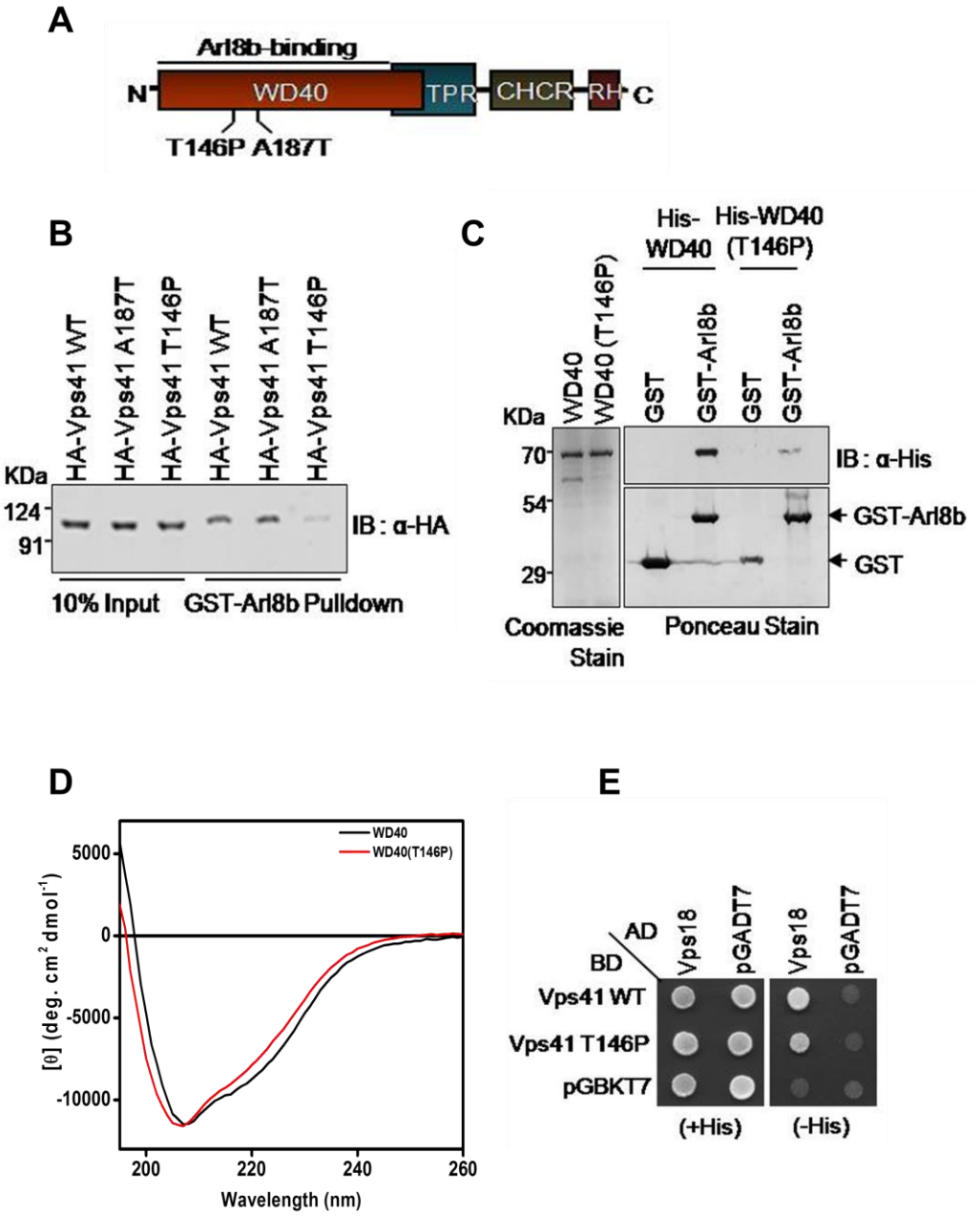

Figure 3.7. A T146 SNP in the hVps41 WD40 domain abrogates its binding to Arl8b. (A) Domain architecture of hVps41, showing the two SNPs in the WD40 domain. (B) A representative immunoblot (IB) of a GST pulldown assay using GST–Arl8b as bait incubated with HeLa cell lysates expressing wild-type (WT) HA–Vps41, HA–Vps41-A187T or HA–Vps41-T146P. (C) Western blot analysis of the purified proteins His–WD40 and His–WD40 (T146P) incubated with either purified GST or GST–Arl8b on beads. (D) CD spectroscopy depicting the secondary structures of WD40 domains of Vps41 WT (black) and Vps41 T14P (red). (E) Yeast two-hybrid interaction of Vps18 was tested with wild-type Vps41 and Vps41-T146P by assaying for growth of co-transformants on non-selective medium, to confirm viability, and on selective medium, to detect interactions. AD, activating domain; BD, binding domain.

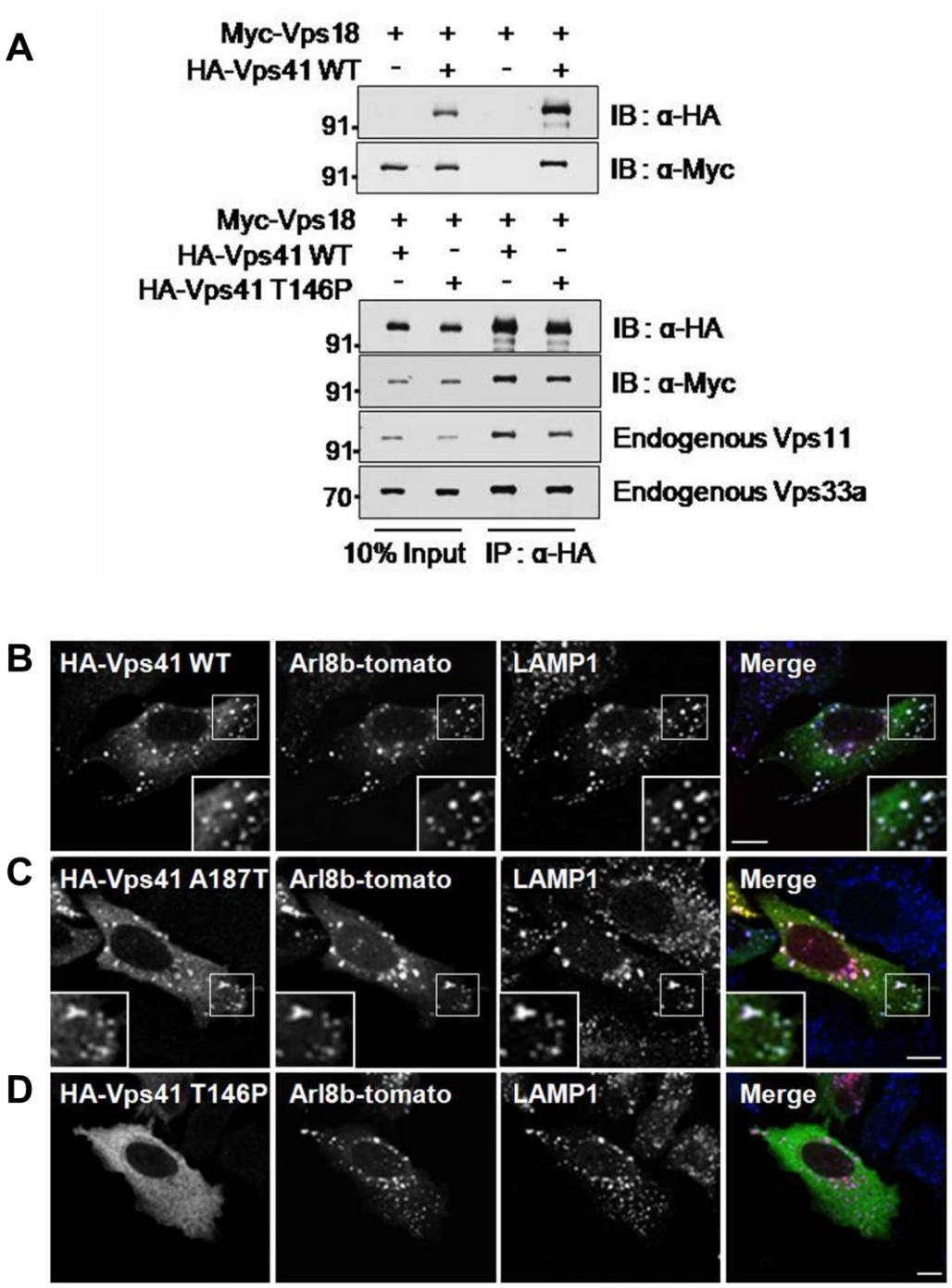

Figure 3.8. A T146 SNP in the hVps41 WD40 domain abrogates Arl8b binding and lysosomal localization. (A) Lysates of HEK293T cells transfected with Myc–Vps18 and either HA–Vps41 or HA–Vps41-T146P were immunoprecipitated (IP) using anti-HA antibody followed by anti-Myc immunoblotting. After stripping, the membranes were reprobed with anti-Vps11 and anti-Vps33a antibodies. (B-D) Immunostaining with LAMP1 of HeLa cells co-transfected with Arl8b–tomato and either wild-type HA–Vps41 (B) or HA–Vps41-A187T (C) or HA–Vps41-T146P (D). Colocalized pixels are shown in the inset. Scale bars: 10 μm.

function of hVps41, as this single amino acid change prevents localization of hVps41 to lysosomes in an Arl8b-dependent manner, even though it does not disrupt Vps41 association with other HOPS subunits.

3.2.4 Subunit-subunit interactions guide assembly of human HOPS subunits to Arl8b positive lysosomes

Given Arl8b's role in regulating association of hVps41 with lysosomes, we next investigated how other subunits of the human HOPS complex are recruited to Arl8b/hVps41 positive lysosomes. We hypothesized that the recruitment of other HOPS subunits must be guided by their topological arrangement within the complex. To address this question, we first tested interaction of human HOPS subunits with each other in a yeast two-hybrid assay (Fig. 3.9A). Our results suggest a model of human HOPS complex arrangement that is strikingly similar to its yeast counterpart (Ostrowicz, Brocker et al. 2010; Brocker, Kuhlee et al. 2012)(schematic in Fig. 3.9B). In particular, the interaction of hVps41 with hVps18, the interaction of hVps39 with hVps11, and the interaction of hVps16 with hVps33a were also conserved in mammalian cells (Fig. 3.9A). Also, hVps18 interacts with hVps16 and weakly with hVps11 subunits, suggesting that it might act as a central player that regulates integrity of the entire complex (Fig. 3.9A). Similar to a previous report (Graham, Wartosch et al. 2013), we did not find any interaction of the hVps33b subunit with the other five subunits of HOPS complex (data not shown and (Graham, Wartosch et al. 2013)).

Next, to test the hypothesis that assembly of other HOPS subunits to Arl8b- and hVps41-positive lysosomes is guided via protein-protein interactions, we overexpressed the human HOPS subunits either with Arl8b or in combination with Arl8b and Vps41 (Fig. 3.10). We first tested recruitment of the hVps18 subunit of the

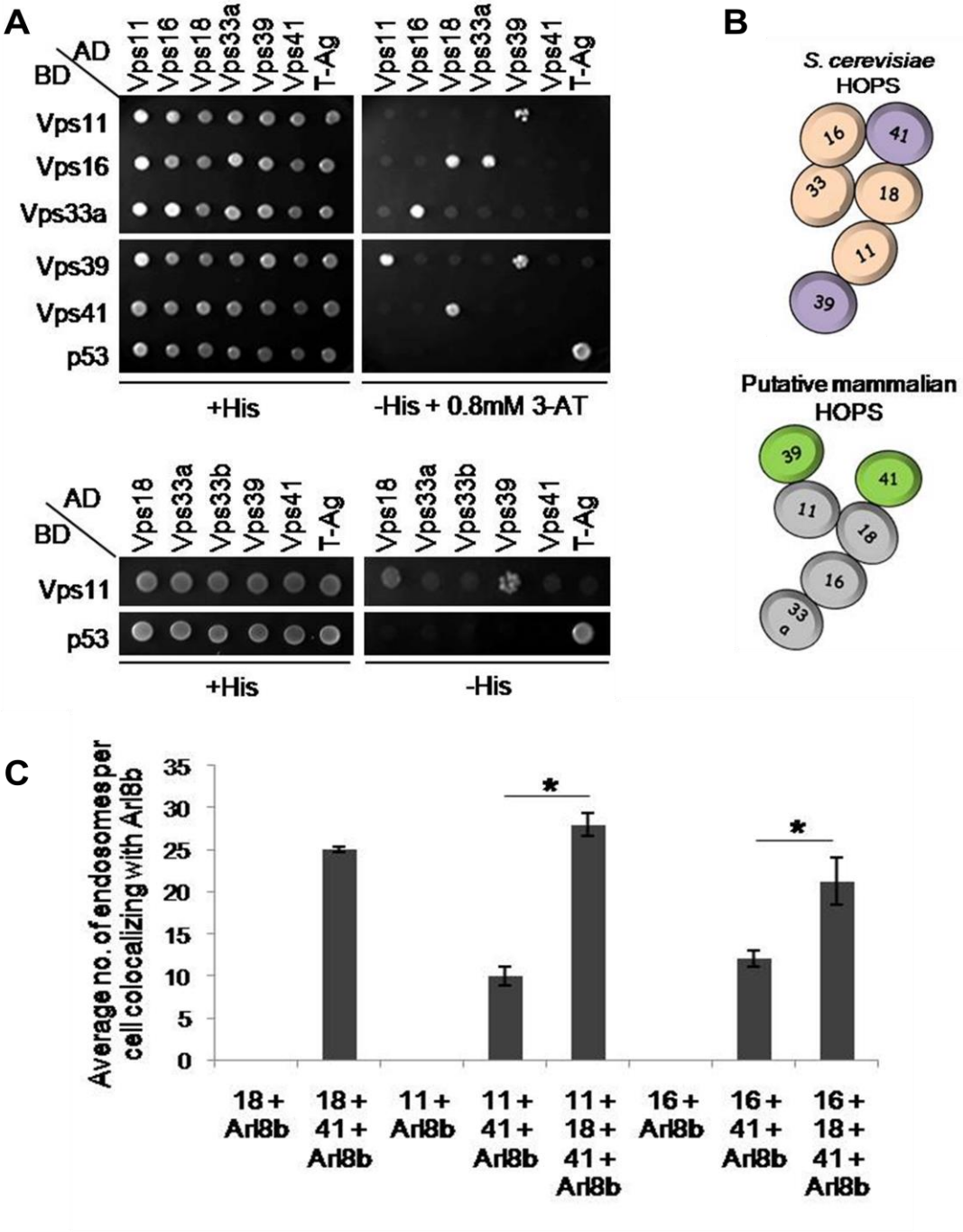

Figure 3.9. Subunit-subunit interaction analysis of the human HOPS complex. (A) Subunit-subunit interactions of all HOPS subunits was tested in a yeast-two-hybrid system by assaying for growth of co-transformants on non-selective medium, to confirm viability, and on selective medium containing 0.8mM 3-AT, to detect interactions. Yeast two hybrid interaction of Vps11 was also tested in a separate experiment, where yeast were plated on selective medium (-His) without 3-AT so as to detect weaker interactions of Vps11 with other HOPS subunits. p53 (pVA3) and SV40 T-antigen (pTD1) were used as positive control. (B) A model depicting the similar topological arrangement of *S.cerevisiae* HOPS and mammalian HOPS complex (based on the yeast-two-hybrid data). (C) HeLa cells were transiently co-transfected with Arl8b and multiple HOPS subunits in various combinations (label on x-axis), and number of co-localized endosomes of HOPS subunits with Arl8b+ were quantified. Values plotted are mean ± s.d. of three independent experiments (n=25 cells per experiment, * indicates $p<0.05$).

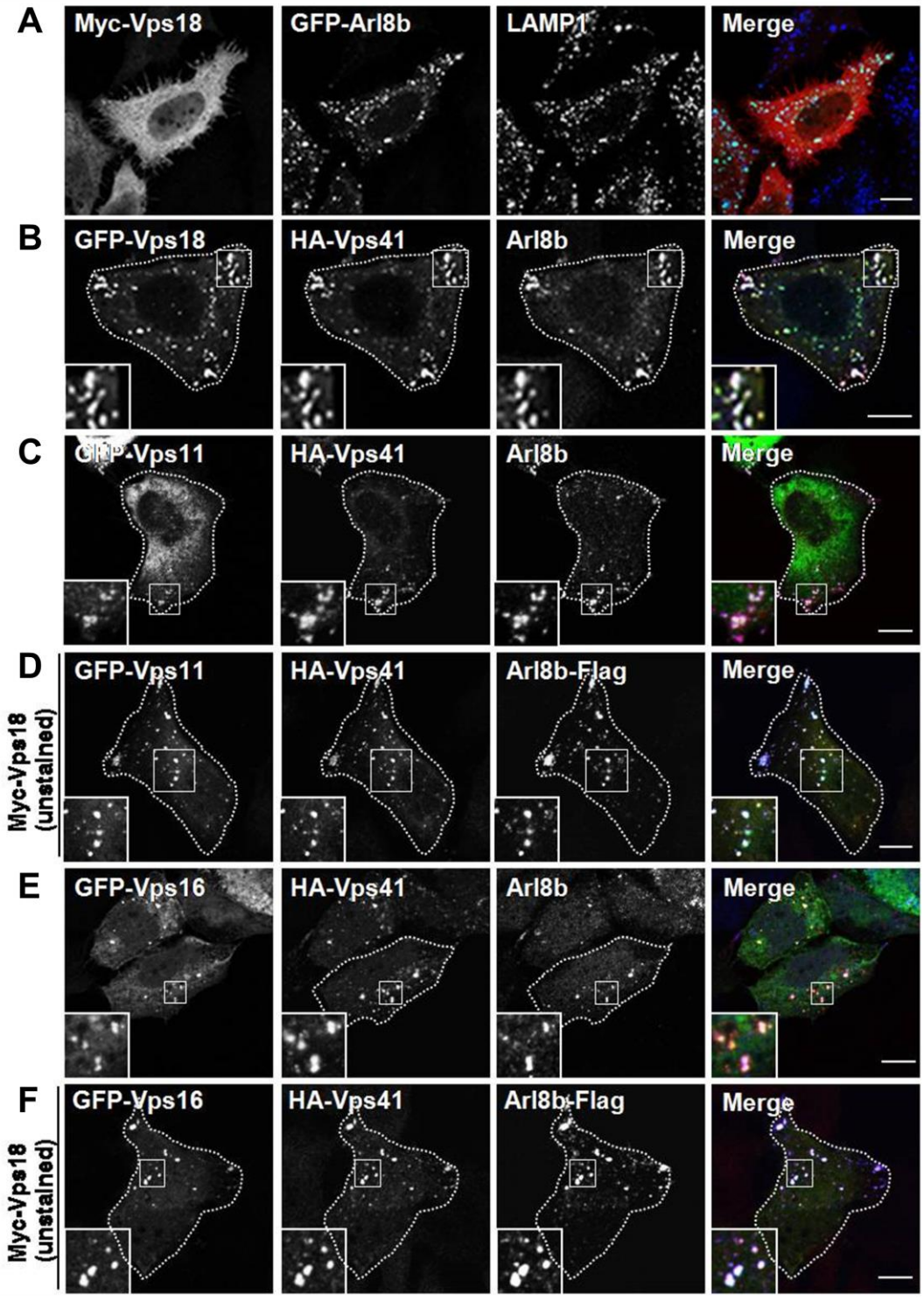

Figure 3.10. Subunit–subunit interactions guide assembly of the human HOPS complex to Arl8b-positive lysosomes. (A–F) Confocal micrographs depicting the localization of the indicated HOPS subunits to Arl8b^{+} and LAMP1^{+} compartments upon overexpression in multiple combinations in HeLa cells. Colocalized pixels are shown in the inset. Scale bars: 10 μm.

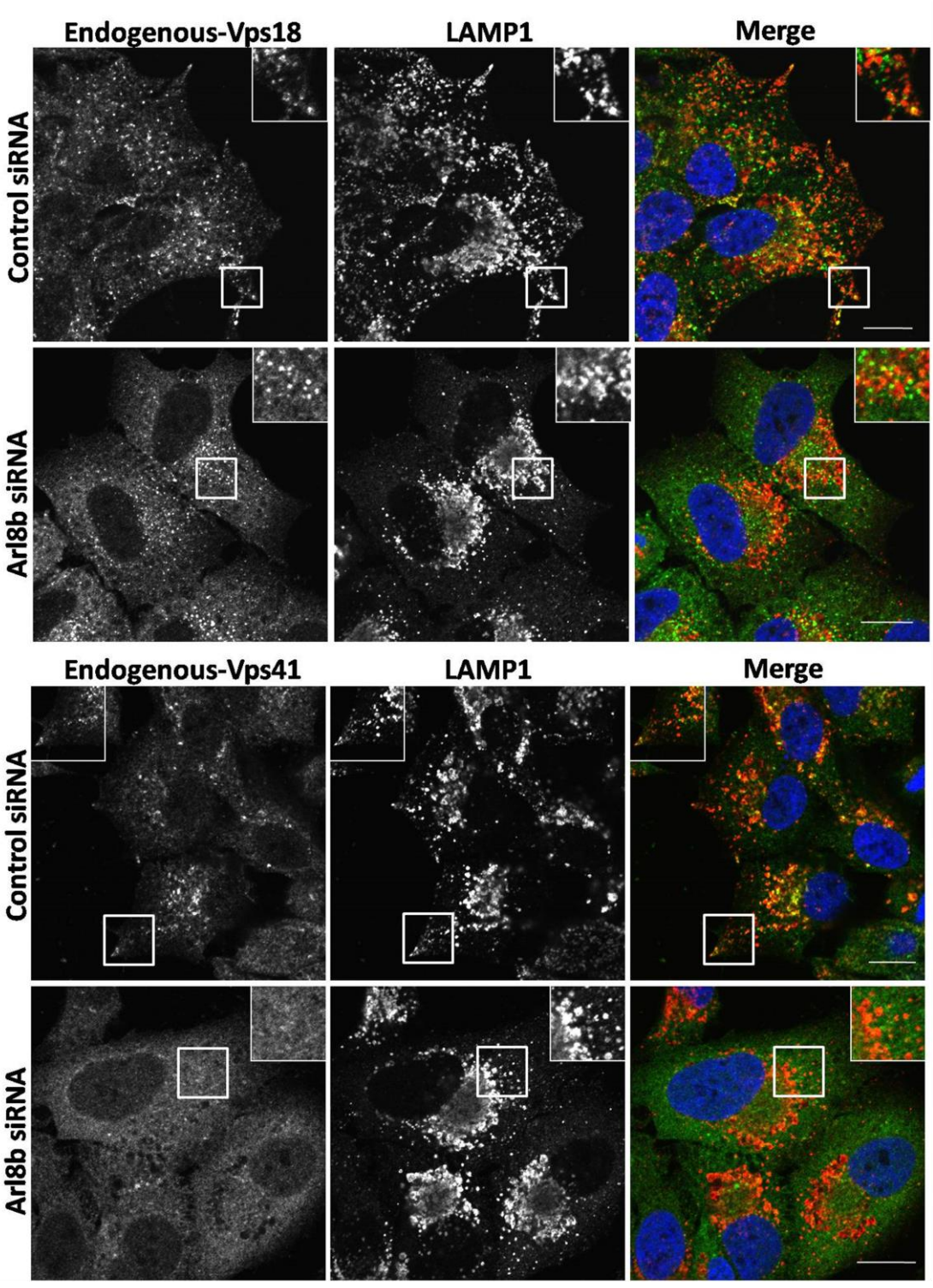

Figure 3.11. (A-D) Immunofluorescence depicting control (A,C) or Arl8b-silenced (B,D) HeLa cells stained for either endogenous Vps18 (A,B) or endogenous Vps41 (C,D). Scale bars: 10 µm.

HOPS complex to Arl8b- and hVps41-positive endosomes. While hVps18 had no significant co-localization with Arl8b when expressed alone, it was completely recruited to Arl8b structures upon co-expression with hVps41, suggesting that hVps41 recruits hVps18 to lysosome membranes (Fig. 3.10A,B). Unlike hVps18, hVps11 and hVps16 subunits were not efficiently recruited to Arl8b structures even in the presence of overexpressed hVps41 and continued to be largely cytosolic (Fig. 3.10C,E). Only a few punctate structures that showed colocalization with Arl8b and hVps41 were present in these cells. Our yeast two-hybrid interaction analysis agrees with these observations, as neither hVps11 nor hVps16 show direct interaction with hVps41, but rather interaction with hVps18 (Fig. 3.9A). Thus, as predicted from our yeast two-hybrid interaction data, co-expression of hVps18 along with hVps41 and Arl8b led to dramatic recruitment of both hVps11 and hVps16 to Arl8b-positive lysosomes (Fig. 3.10D,F). We further confirmed these findings by quantitative analysis of the number of endosomes showing colocalization for both HOPS subunits and Arl8b (Fig. 3.9C). In line with our published data, we observe that Vps41 and Vps18 fail to localize to lysosomes in Arl8b-depleted cells (Figure 3.11 (unpublished observations)). Further, we mapped the interaction of hVps18 with various domain deletion mutants of hVps41 and found that hVps18 interacts with C-terminal RING-H2 domain of Vps41, as no interaction of hVps18 was found with a mutant of Vps41 lacking RING-H2 domain (hVps41 C791X) (Fig. 3.12A, B (unpublished observations)). RING domains are protein-protein interaction domains defined by a consensus sequence of conserved cysteine and histidine residues that coordinate two zinc ions (C3HC4) (Fig. 3.12C,E). RING-H2 (C3H2C3) is a type of RING domain where first cysteine of the second zinc-coordination site is replaced by histidine (Fig. 3.12D). We mutated these residues sequentially and found that the residues

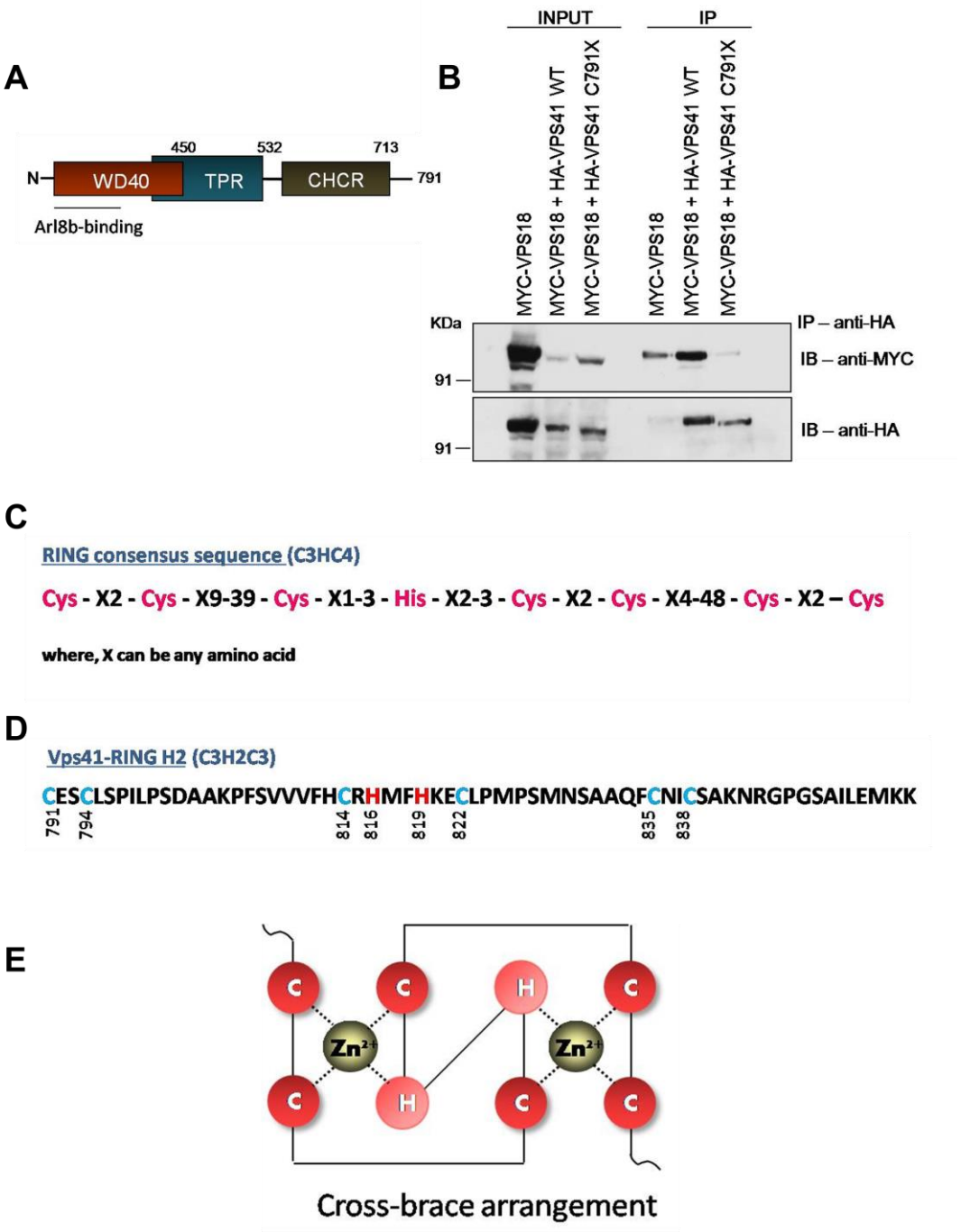

Figure 3.12. RING-H2 domain of hVps41 is essential for its interaction with hVps18. (A) Domain architecture of hVps41 lacking the RING-H2 domain (hVps41 C791X). (B) Western blot showing HEK293T cell lysates expressing Myc–hVps18 in combination with wild-type or RING-H2 deleted mutant of hVps41, immunoprecipitated (IP) with anti-HA-conjugated beads. (C) RING domain consensus sequence marked by cysteine and histidine residues, as indicated (C3HC4). (D) RING-H2 domain of hVps41 highlighting the conserved cysteine and histidine residues (C3H2C3). (E) Cross-brace arrangement of the RING-H2 domain conserved residues coordinating two zinc-ions.

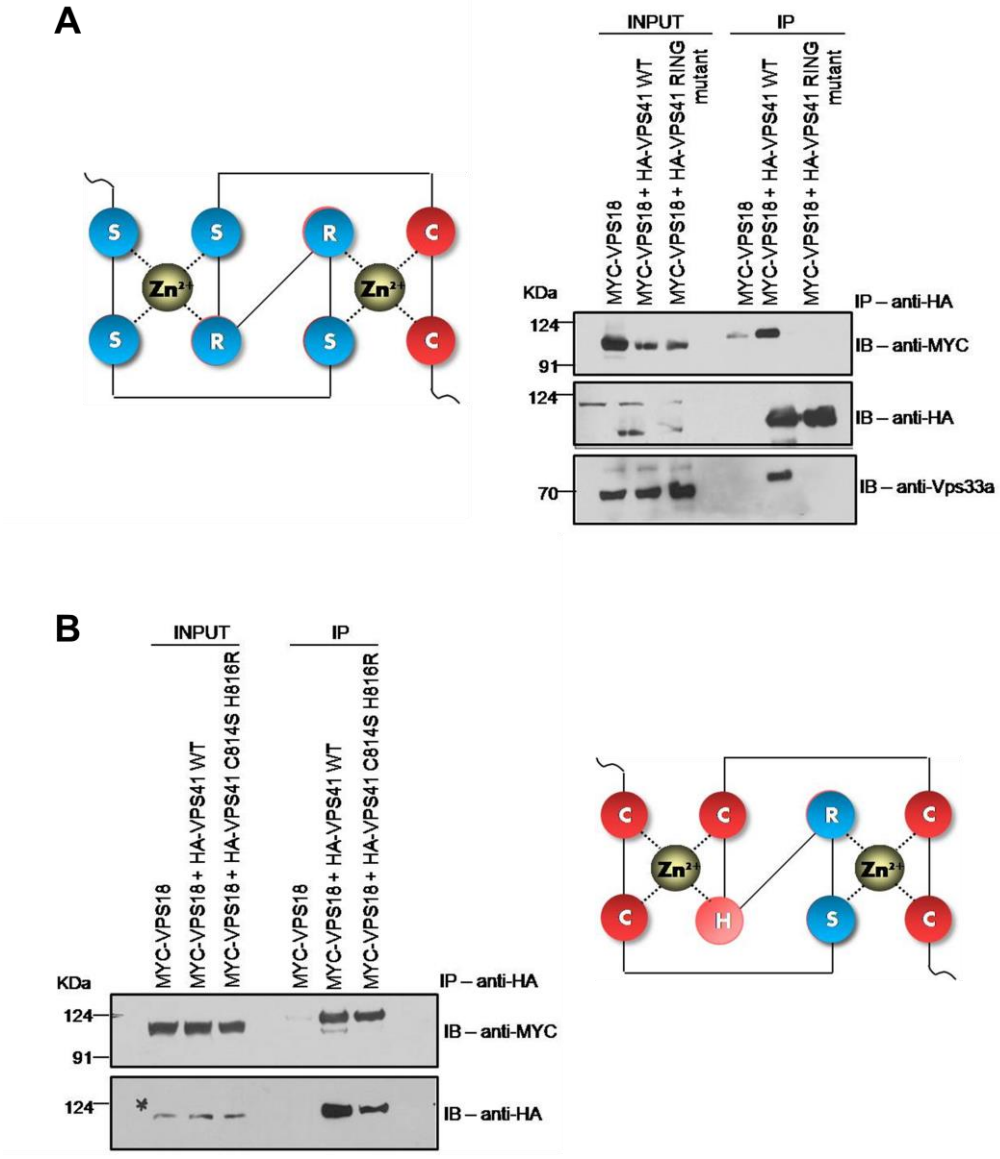

Figure 3.13. Conserved residues comprising the second Zn^{2+} coordination site in the RING-H2 domain of hVps41 are not essential for interaction with hVps18. (A-B) A representative immunoblot (IB) of HEK293T cell lysates expressing either Myc-Vps18 alone or in combination with hVps41 wild-type or its mutants, as indicated, immunoprecipitated (IP) with anti-HA-conjugated beads.

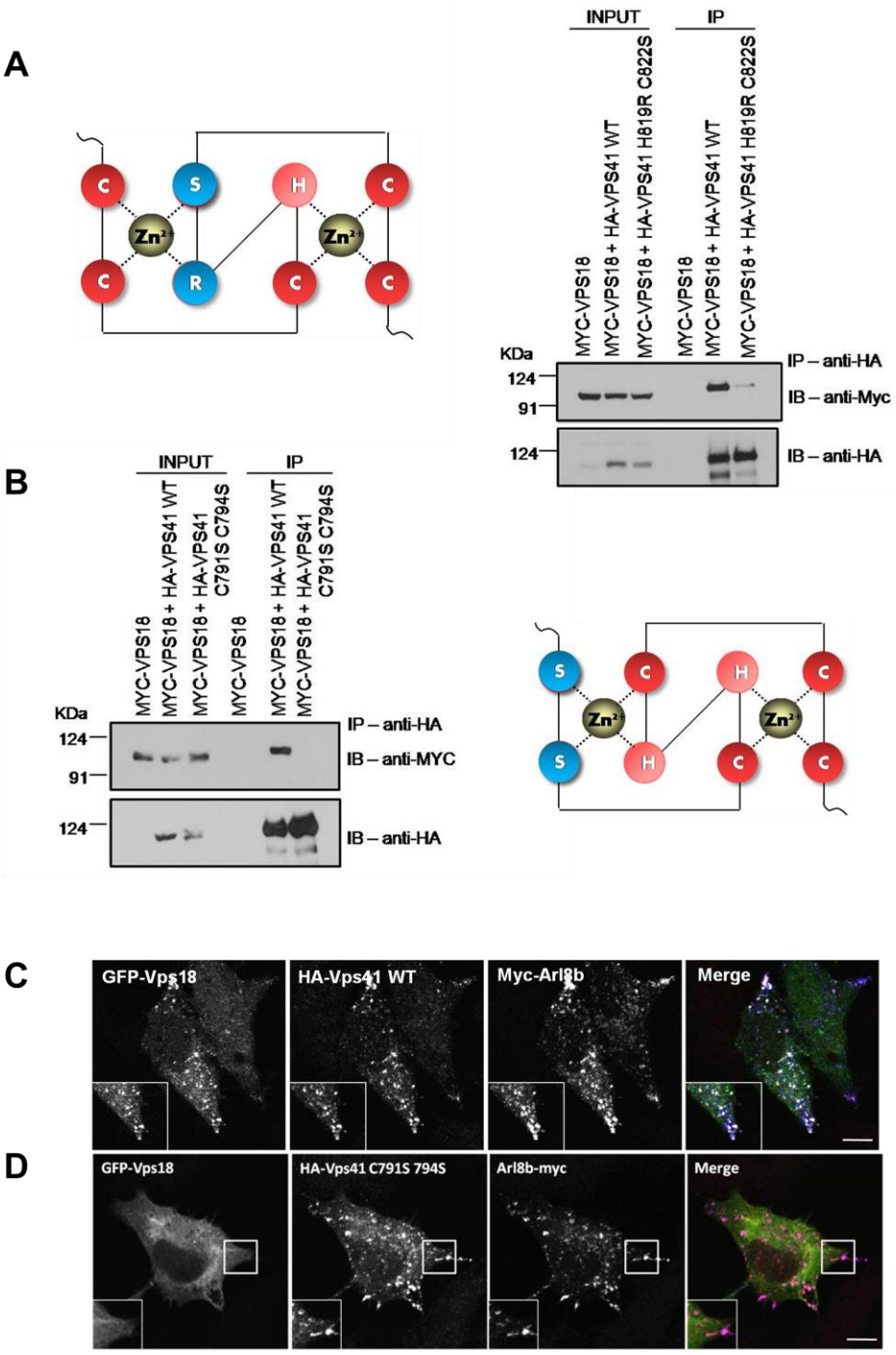

Figure 3.14. Conserved residues comprising the first Zn^{2+} coordination site in the RING-H2 domain of hVps41 determine its interaction with hVps18. (A-B) A representative immunoblot (IB) of HEK293T cell lysates expressing either Myc-Vps18 alone or in combination with hVps41 wild-type or its mutants, as indicated, immunoprecipitated (IP) with anti-HA-conjugated beads. (C-D) Confocal micrographs depicting the localization of hVps18 in HeLa cells transfected with Myc-Arl8b and either wild-type (C) or RING-H2 deleted mutant of hVps41 (D), as indicated. Scale bar = 10μm.

comprising the first coordination site were responsible for interaction with Vps18 (Fig. 3.13A-B, 3.14A-B). Unlike wild-type hVps41, this mutant of hVps41 (hVps41 C791S C794S) was deficient in recruiting hVps18 to lysosomes, suggesting that the lysosome localization of Vps18 is dependent upon its interaction with Vps41 (Fig. 3.14C-D(unpublished observations)). Further quantification of the data is needed to conclusively demonstrate that this mutant continues to localize to Arl8b-positive endosomes but does not recruit other HOPS subunits. Together, these results support our hypothesis that assembly of HOPS complex to Arl8b-containing lysosomes in the cell is guided by their subunit-subunit interactions.

3.2.5 Arl8b is required for membrane localization of human HOPS subunits

Our observations until now indicate that Arl8b regulates hVps41 localization to lysosomes, and other human HOPS subunits localize to Arl8b-positive endosomes guided by their subunit-subunit interactions. To further investigate if Arl8b is required for localization of multi-subunit human HOPS complex on lysosomes, we expressed HOPS subunits hVps41, hVps18 and hVps11 in Arl8b-siRNA transfected cells that were either also transfected with vector control or with siRNA-resistant Arl8b, and analyzed their co-localization with LAMP1. Similar to our results with Vps41, we found that in Arl8b-depleted cells transfected with vector control, multiple HOPS subunits were localized to cytoplasm (Fig. 3.15A). While this cytoplasmic localization of HOPS subunits was not rescued by expression of vector control, it was completely rescued in cells transfected with the siRNA-resistant Arl8b construct (Fig. 3.15B). To confirm these observations under endogenous conditions, we also performed membrane/cytosol fractionation to test membrane association of endogenously expressed HOPS subunits in control- versus Arl8b-siRNA treated cells. As depicted, HeLa cells treated with Arl8b-siRNA showed a profound decrease in

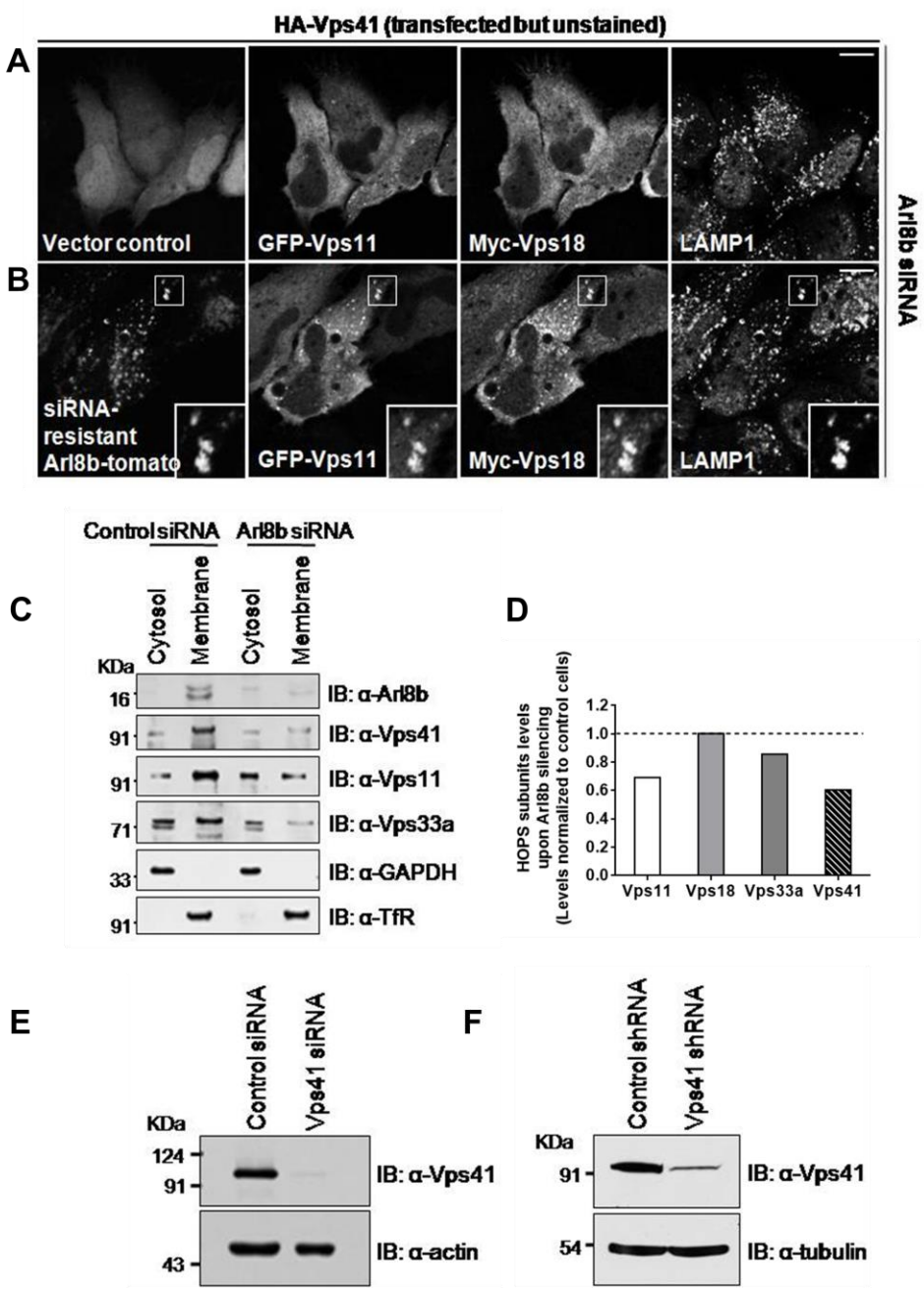

Figure 3.15. Arl8b is required for membrane localization of human HOPS subunits. (A,B) Arl8b-silenced HeLa cells were transfected with either ptd-Tomato-N1 vector (A) or siRNA-resistant Arl8b–tomato construct (B) together with the HOPS subunits GFP–Vps11, Myc–Vps18 and HA–Vps41 (unstained), and analyzed for their localization to Arl8b^{+} lysosomal compartments. Scale bars: 10 μm. Colocalized pixels are shown in the inset. (C) Immunoblotting (IB) depicting the presence of endogenous HOPS subunits in membrane and cytosol fractions of control- and Arl8b siRNA-treated HeLa cells. GAPDH and TfR were used as markers for cytosol and membrane fractions, respectively. (D) Densitometric quantification of lysates from control- or Arl8b-silenced cells subjected to western blot analysis using antibodies against endogenous HOPS subunit proteins from two independent experiments. (E) Control and Vps41-siRNA treated HeLa lysates were immunoblotted with anti-Vps41 and anti-actin antibodies. (F) An immunoblot of control or Vps41 shRNA-transduced HeLa cell lysates probed with Vps41 antisera or for tubulin (loading control).

Arl8b signal (membrane fraction) compared to control-siRNA (Fig. 3.15C). Importantly, western blotting with anti-hVps41 antibodies showed that there was a dramatic decreasein membrane-associated hVps41 levels in cells treated with Arl8b-siRNA compared to control-siRNA (Fig. 3.15C, compare lanes 2 and 4). A significant decrease in membrane association of other endogenous HOPS subunits, including hVps11 and hVps33a, was also observed upon depletion of Arl8b (Fig. 3.15C, compare lanes 2 and 4). In this assay, GAPDH and transferrin receptor (TfR) were used as markers for cytosol and membrane fractions, respectively. Notably, the relative expression of HOPS subunits (except Vps18) was less in total cell extracts from Arl8b-deleted cells with Vps11 and Vps41 levels dropping by 30-40% (Fig. 3.15D). These results strongly support our conclusion that Arl8b is required for recruitment of the human HOPS complex to lysosomes.

3.2.6 Depletion of hVps41 results in delayed trafficking and degradation of EGFR in lysosomes

A recent study by Pols et al. has shed light on the roles of hVps41 and hVps39 in regulating cargo traffic to lysosomes by mediating homotypic and heterotypic late endosome fusion (Pols, ten Brink et al. 2013). Our results from this study suggest that localization of hVps41 and other human HOPS subunit to lysosomes is mediated by the small GTPaseArl8b, implying Arl8b is an important regulator of human HOPS function in endocytic traffic. To address this, we first monitored the endoyctic degradation of EGFR as a model to study the role of hVps41 in this pathway. HeLa cells were treated with control siRNA or hVps41-siRNA or transduced with lentivirus particles containing scrambled shRNA (control) or shRNA against hVps41. The efficiency of hVps41 depletion using either siRNA or shRNA treatment was found to be >85% as determined by Western blotting (Fig. 3.15E,F). We monitored the

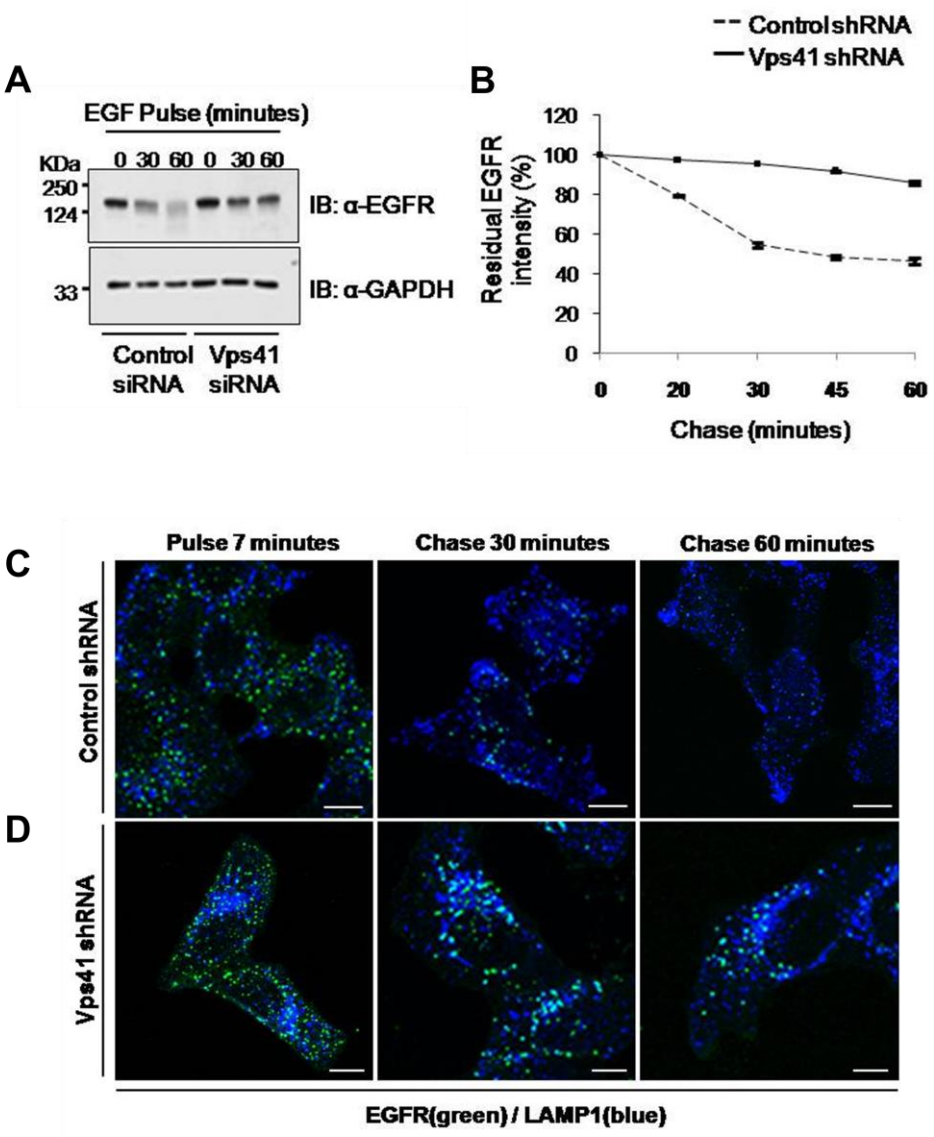

Figure 3.16. Arl8b regulates Vps41 function in endocytic degradation of EGFR. (A) Serum-starved control or Vps41-silenced HeLa cells were pulsed with unlabeled EGF (100 ng/ml) for the indicated time periods. The expression of EGFR and GAPDH (loading control) were detected by immunoblotting of whole-cell extracts. (B) Serum-starved control or Vps41-silenced HeLa cells were pulsed with Rhodamine–EGF (500 ng/ml) for 7 minutes and chased in complete medium for varying times. EGFR degradation was assessed by measuring residual EGFR intensity over these time periods in siRNA-treated cells. Values plotted are the mean±s.d. of three independent experiments (n=60 cells for each time point in every experiment). (C-D) EGFR levels remaining were also monitored over these time periods in control or Vps41 shRNA-treated cells by staining with anti-EGFR antibody and lysosomes were visualized by anti-LAMP1 staining. Merge images are depicted. Scale bars: 10 μM.

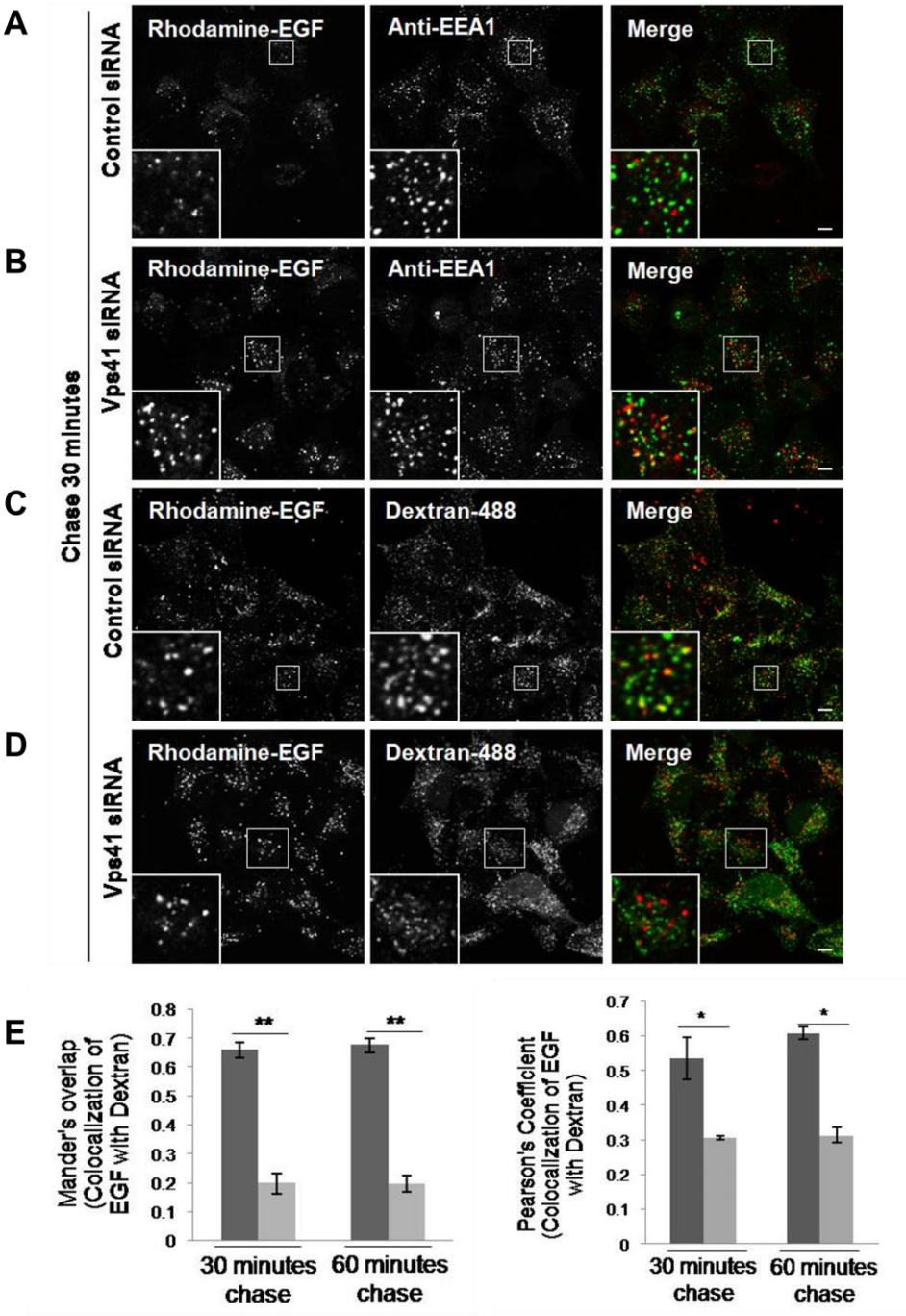

Figure 3.17. Delay in trafficking of EGFR to the dextran-labeled compartment in Vps41-silenced cells. (A-D) Control (A,C) or Vps41-silenced (B,D) HeLa cells were either left untreated or incubated overnight with Alexa-488 labeled dextran (250μg/ml). Cells were further serum starved, pulsed with Rhodamine-EGF (500ng/ml) for 7 minutes and chased in complete medium for varying times. After fixation, the untreated set was immunostained with EEA1. Shown are representative images of EGF compartmentalization to EEA1-or dextran-labeled endosomes after 30 minutes of chase. Co-localized pixels are visualized in the inset. (E) Mander's and Pearson's coefficients were determined for EGF signal coincident with dextran signal. Dark gray and light gray bars represent control-siRNA and Vps41-siRNA treated cells, respectively. Values plotted correspond to mean ± s.d. of three independent experiments (n=50 cells per experiment, * and ** indicate $p<0.05$ and $p<0.01$, respectively).Scale bar= 10 μM.

endocytic degradation of EGFR in hVps41-depleted cells by stimulating the cells with EGF and analyzing levels of EGFR in total cell lysates at different time points. In hVps41-siRNA treated cells, EGFR degradation was significantly delayed compared to control-siRNA treated cells (Fig. 3.16A). We also monitored the levels of EGFR remaining via immunofluorescence by incubating control- and hVps41-shRNA with rhodamine-labeled EGF followed by chase for various time points in complete medium (Fig. 3.16C,D). While in control-shRNA-transduced cells, EGFR signal was significantly reduced by 60 minutes of chase in complete medium (Fig. 3.16C), most of the EGFR signal in hVps41-shRNA transduced cells persisted during these time points, indicating that EGFR degradation is delayed in hVps41-depleted cells (Fig. 3.16D). Quantification of these images showed an approximately two-fold EGFR signal remaining in hVps41-depleted cells compared to control cells at 60 minutes of chase (Fig. 3.16B). Furthermore, colocalization of internalized EGF with early endosome marker EEA1 and with endocytosed Alexa-Fluor-488-labeled dextran (to label lysosomes) was also assessed to identify the compartment in which EGF signal was present in control versus Vps41-depleted cells (Fig 3.17). In comparison to control cells, in Vps41-depleted cells EGF failed to reach the dextrancompartment by 30 minutes of chase and several EGF-containing endosomes remained positive for EEA1, indicating that there is delay in EGFR trafficking to lysosomes upon depletion of Vps41 (Fig 3.17B,D, see the arrowheads, quantification of colocalization coefficients in Fig 3.17E).

3.2.7 Interaction of hVps41 with Arl8b is required for rescue of endocytic degradation of EGFR in hVps41-depleted cells

To elucidate if interaction of Vps41 with Arl8b is critical for its function in endocytic trafficking, we complemented control and hVps41-depleted cells with siRNA-

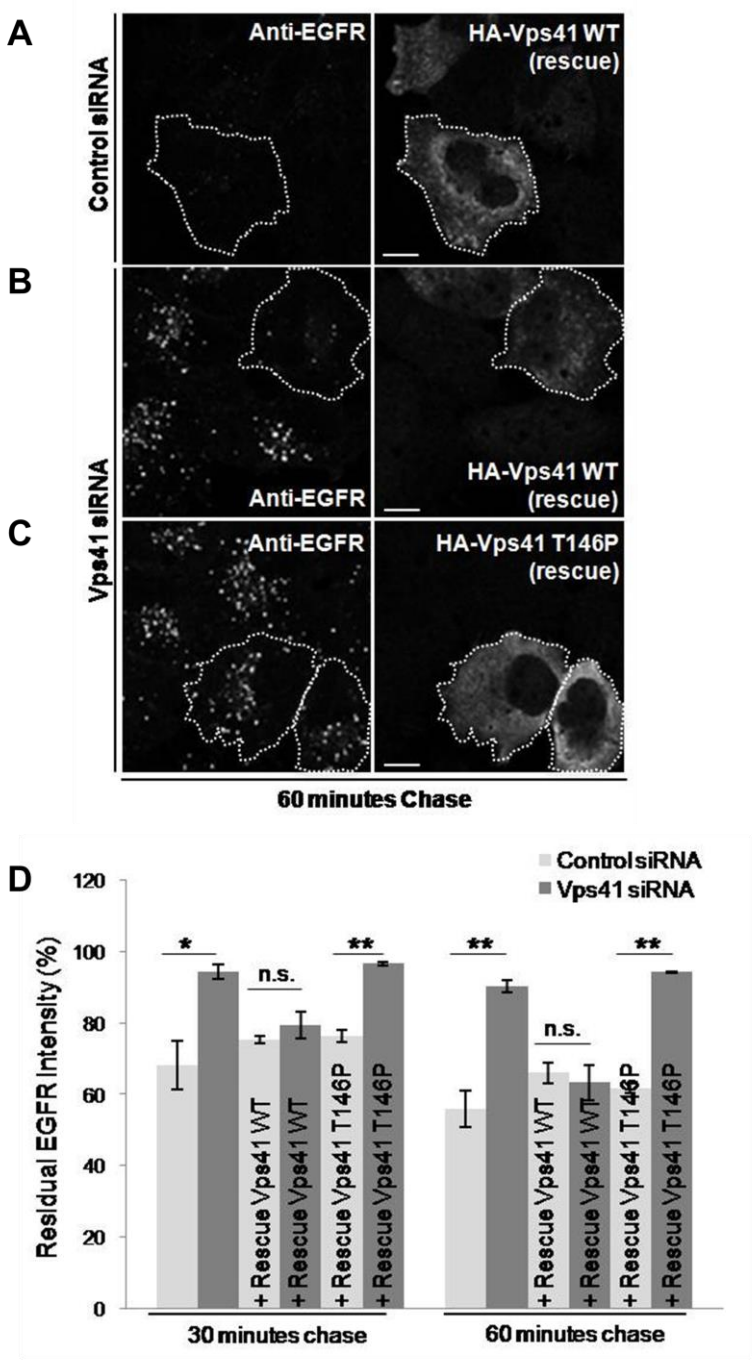

Figure 3.18. EGFR trafficking defects in Vps41-silenced cells can be rescued by expression of siRNA-resistant hVps41. (A-C) Control (A) or Vps41-silenced HeLa cells (B,C) were either transfected with siRNA-resistant wild-type (WT) HA–Vps41 (A,B), or with siRNA-resistant HA–Vps41-T146P (C), and EGFR degradation was monitored after 30 and 60 minutes of chase by staining with anti-EGFR antibody. Shown are representative images of EGFR trafficking to lysosomes after 60 minutes of chase. Scale bars: 10 µm. (D) Quantification of the percent residual EGFR fluorescence intensity in either case was performed for each indicated time point. Values plotted are the mean±s.d. of three independent experiments (n=100 cells for each time point per experiment); *P,0.05; **P,0.01; n.s., not significant.

resistant cDNAs for hVps41 WT or an Arl8b-binding defective mutant form (hVps41 T146P) and monitored EGFR signal remaining by pulse-chase experiment (Fig. 3.18A-C). While hVps41 WT was able to efficiently rescue the delay in EGFR degradation, the hVps41 T146P mutant that does not interact with Arl8b and therefore is not recruited to lysosome membranes failed to rescue effect of hVps41-depletion on EGFR degradation (Fig. 3.18B-C; quantification of images shown in Fig. 3.18D). These results clearly demonstrate that interaction of hVps41 with Arl8b is critical for function of hVps41 and probably of the human HOPS complex in the endocytic degradation pathway.

3.3 Discussion

The HOPS complex is a highly conserved multi-subunit tethering factor that regulates fusion of late endosomes-lysosomes and thereby cargo degradation in lysosomes. Despite several studies supporting the critical role of mammalian HOPS subunits in regulating the endocytic degradation pathway, little is known about how this complex is recruited to lysosomes and how its activity is regulated in mammalian cells.

In this study, we have demonstrated that the small GTPase Arl8b mediates recruitment of human HOPS complex to lysosome membranes. We noted that Arl8b, but not Rab7, directly interacts with the hVps41 subunit of the HOPS complex and regulates its association with lysosomes (Fig. 3.1). We also found that GTP-bound Arl8b was essential for localization of hVps41 to lysosomes, and that neither Rab7, nor its effector RILP which was previously shown to interact with, and recruit Vps41 to late endosomes, were able to rescue hVps41 membrane localization in Arl8b-depleted cells. (Fig. 3.2). It is interesting to note that in *S.cerevisiae* where there is no Arl8b homologue, the Rab7 homologue Ypt7 interacts with the Vps41p and Vps39p subunits of the HOPS complex and recruits the HOPS complex to vacuolar

membranes (Hickey, Stroupe et al. 2009). While previous studies had implicated that Rab7 regulates HOPS complex membrane localization in higher eukaryotes as well (Rink, Ghigo et al. 2005; Sun, Westphal et al. 2010), thus far, no evidence indicates a direct interaction of Rab7 with HOPS subunits or dependence of Rab7 for membrane recruitment of HOPS subunits in mammalian cells. Our findings suggest that at least in human cells; Rab7 does not directly bind to HOPS subunits or regulates the localization of the HOPS subunit hVps41 to lysosomes. Our results are further corroborated by evidence of a physical interaction of Arl8b and Vps41 in *C. elegans* and their coordinated function in phagolysosome formation (Sasaki, Nakae et al. 2013).

In a previous study by Harrington et al. two SNPs in the WD40 domain of hVps41 were reported to cause a loss-of-function phenotype in neuroprotection from α-synuclein-induced neurodegeneration in *C. elegans* and neuroglioma cells. However, the mechanisms by which these SNPs result in impaired Vps41 function have not been revealed. Our results indicate that one of these SNPs, which convert Threonine at position 146 to Proline (T146P), causes dramatic loss of binding to Arl8b and prevents hVps41 localization to lysosomes (Fig. 3.7). Moreover, this SNP in hVps41 also led to impairment in hVps41 function during endoyctic degradation of EGFR (Fig. 3.17). Collectively, our findings indicate that Arl8b-dependent localization of hVps41 to lysosomes is critical for its function in endocytic trafficking.

Since HOPS is a multi-subunit complex, it was important to understand how other subunits assemble on organelle membranes and form a functional complex. Thus far, no studies have reported how the mammalian HOPS complex assembles on lysosome membranes and what regulates membrane recruitment of this complex. We analyzed the recruitment of human HOPS subunits (hVps18, hVps11 and hVps16) to Arl8b and

Vps41 endosomes to understand the assembly and localization of the entire HOPS complex to lysosomes (Fig. 3.10). Our results indicate that direct interaction of hVps41 with hVps18 and similarly of hVps18, with hVps11 and hVps16, regulate their recruitment to Arl8b- and LAMP1-positive endosomes. Moreover, using knockdown approaches, we demonstrated that Arl8b is essential for membrane localization of the multiple subunits of human HOPS complex (Fig. 3.15). These results support a model whereby human HOPS subunits undergo hierarchical assembly to Arl8b-positive lysosomes guided by their subunit-subunit interactions.

Chapter IV

Arl8b effector SKIP interacts with Vps39 subunit of HOPS complex and regulates cargo trafficking to lysosomes

The following chapter has been published in Journal of Cell Science, 2015128:1746-61

Chapter 4

4.1 Introduction

While our findings that HOPS complex is recruited by Arl8b to lysosomes, explains the mode of action of Arl8b in regulating cargo traffic to lysosomes, another profound and probably better-understood function of Arl8b is in controlling microtubule-based lysosome motility. Arl8b in its GTP-bound form interacts with SKIP/PLEKHM2 that directly binds to kinesin light chain (KLC2) and recruits Kinsein-1 to drive plus-end-directed movement of lysosomes towards the cell periphery. Thus, Arl8b-SKIP-KLC2 complex functions in an opposing manner to Rab7-RILP-p150^{GLUED} complex, which promotes minus-end-directed transport of lysosomes towards the MTOC. Interestingly, recent studies have shown that Rab7-RILP complex associates with multiple HOPS subunits and this interaction is thought to couple minus-end transport and tethering steps during late endocytic trafficking. However, whether a similar cros-talk exists for the transport and vesicle tethering functions regulated by Arl8b and how this impacts association of HOPS complex with Rab7 and RILP is not understood. In this study we have identified a direct interaction between Arl8b effector SKIP and Vps39 subunit of the HOPS complex. SKIP promotes association of Vps39 with Arl8b, and Kinesin-1-positive peripheral lysosomes and possibly competes with RILP for association with the HOPS complex.

4.2 Results

4.2.1 Arl8b effector SKIP, directly interacts with Vps39 subunit of the HOPS complex and recruits it to Arl8b-positive lysosomes

To investigate the potential coordination of the transport and tethering events, we first tested if SKIP competes with or assists in the association of HOPS subunits with Arl8b. We found a strong colocalization of Arl8b-Vps41 and Arl8b-Vps41-Vps18

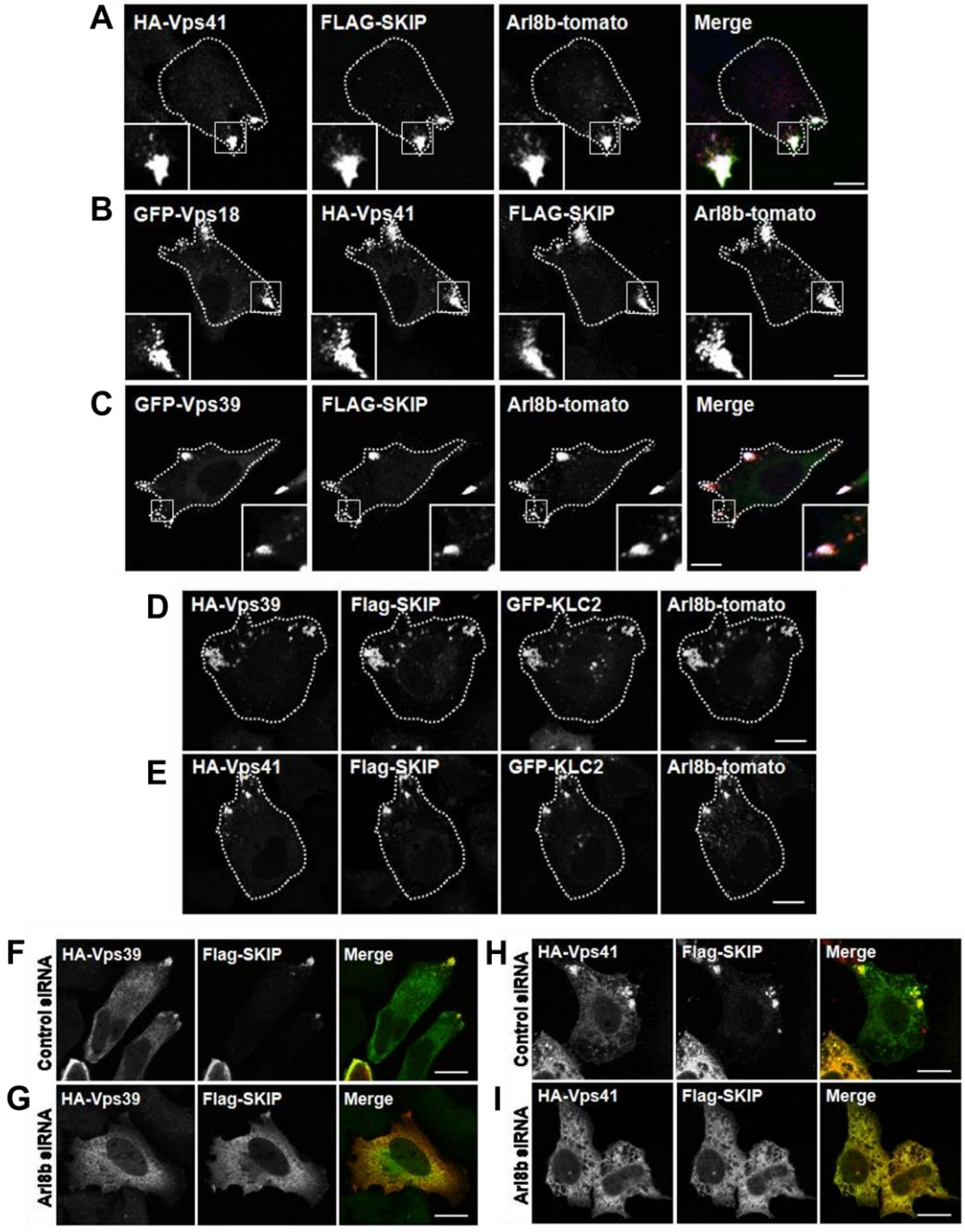

Figure 4.1. Arl8b and SKIP-expression induces peripheral distribution of Vps41 and Vps39 endosomes. (A–C) Representative confocal micrographs from HeLa cells transfected with the indicated constructs. The boundary of the transfected cell is highlighted to visualize the peripheral position of the colocalized endosomes. (D-E) Confocal micrographs depicting the localization of HOPS subunits Vps39 (D), and Vps41 (E), to SKIP, KLC2 and Arl8b-positive endosomes in transfected HeLa cells. (F-I) Control (F,H) or Arl8b-silenced (G,I) HeLa cells were transfected with Flag-SKIP and either HA-Vps39 (F,G) or HAVps41 (H,I), and visualized by immunofluorescence.

with SKIP on peripheral LAMP1-positive endosomes (Fig. 4.1A,B). A similar colocalization of Arl8b and SKIP was also observed with hVps39 subunit of the HOPS complex with all the three proteins present on the same peripheral lysosomes (Fig. 4.1C). Furthermore, co-expression of GFP-KLC2 showed that kinesin light chain was also entirely recruited to the HOPS-Arl8b-SKIP-positive lysosomes, which explains the spatial location of these compartments towards the cell periphery (Fig. 4.1D,E). Arl8b expression was found to be critical for association of HOPS subunits with SKIP-positive structures as siRNA against Arl8b led to a cytosolic redistribution of both Vps41 and Vps39 and their colocalization with SKIP was greatly reduced (Fig.4.1 G,I). Not surprisingly, SKIP also appeared more cytosolic in Arl8b-siRNA treated cells, as interaction with Arl8b has been previously implicated in recruitment of SKIP to lysosomes (Rosa-Ferreira and Munro 2011) .

Next, to investigate the role of SKIP in regulating HOPS complex, we treated cells with either control or SKIP-siRNA and scored their effect on the colocalization of Arl8b and HOPS subunits, Vps41 and Vps39. The efficiency of SKIP depletion was found to be >85% as measured by qRT-PCR (Fig. 4.2A). Interestingly, while no change was observed in the colocalization of Vps41 and Arl8b upon SKIP depletion (Fig. 4.2C,D), recruitment of Vps39 to Arl8b and LAMP1-positive compartment was strongly reduced in the SKIP-siRNA treated cells compared to control, indicating that SKIP is a critical linker that regulates Vps39 recruitment to Arl8b-positive lysosomes (Fig. 4.2E,F; see quantification graph, Fig. 4.2B).

This led us to test if there is a direct binding of SKIP with hVps39 subunit of the HOPS complex, which might lead to recruitment of Vps39 to Arl8b-positive lysosomes. To gain insight into this, we tested interaction of SKIP with the six subunits of the HOPS complex in a yeast two-hybrid assay. Interestingly, strong

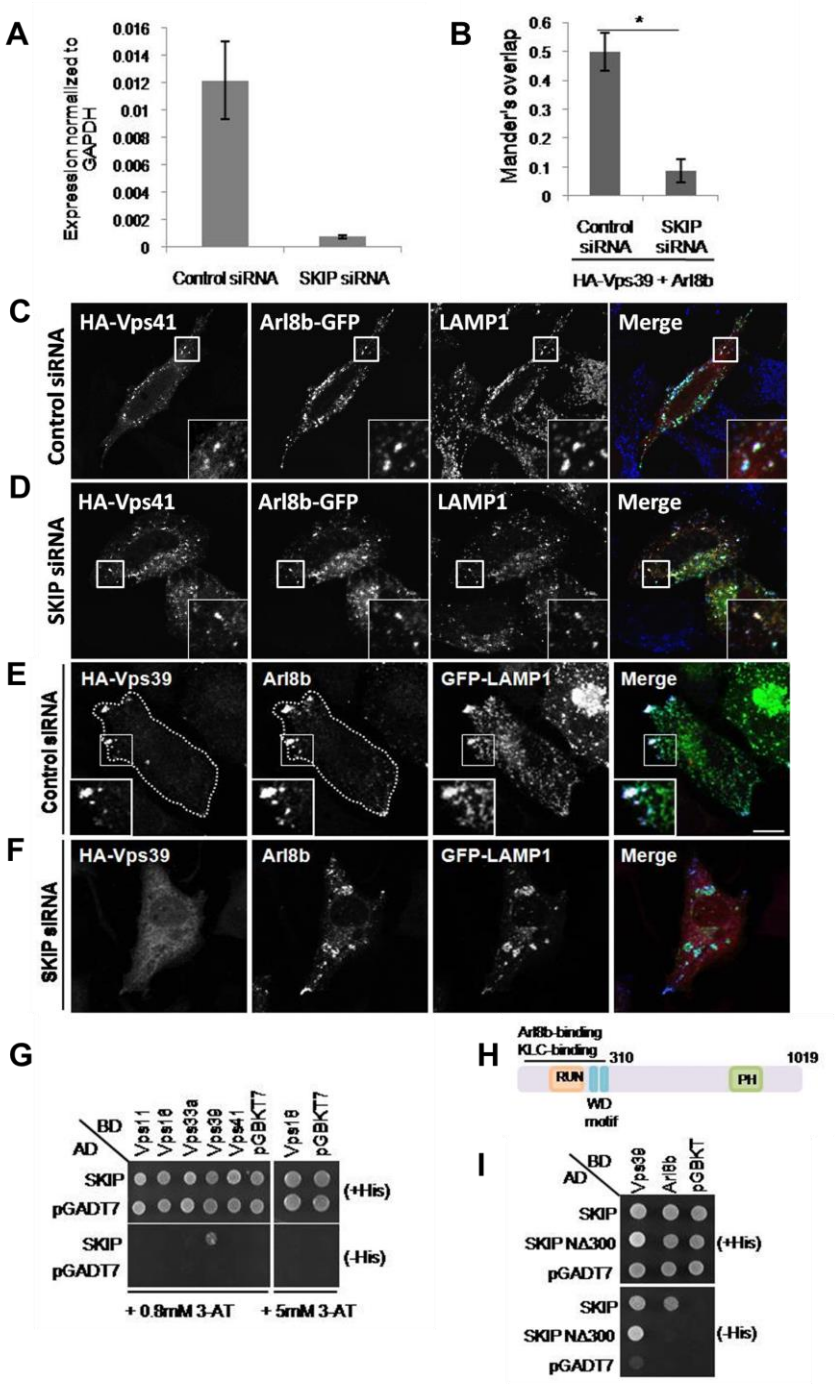

Figure 4.2. SKIP directly interacts with the Vps39 subunit of the HOPS complex and recruits it to Arl8b-positive lysosomes. (A) qRT-PCR analyses of SKIP levels in control and SKIP siRNA treated cells. (B-F) Immunofluorescence of control (C,E) or SKIP-silenced (D,F) HeLa cells cotransfected with either HA-Vps41 and Arl8b-GFP (C,D) or HA–Vps39 and Arl8b (E,F). Scale bars: 10 µm. Colocalization between Vps39 and Arl8b was analyzed using Mander's method over three independent experiments (n=30 cells per experiment); data show the mean±s.d.; *P,0.05 (B). (G) Interaction of human HOPS subunits with SKIP was tested in a yeast two-hybrid system by assaying for growth of co-transformants on non-selective medium, to confirm viability, and on selective medium, to detect interactions. AD, activating domain; BD, binding domain. (H) Domain architecture of SKIP. (I) Yeast two-hybrid interaction of Vps39 and Arl8b was tested with SKIP and SKIP ΔRUN by assaying for growth of co-transformants on non-selective medium to confirm viability and on selective medium to detect interactions.

binding of SKIP with only the hVps39, but not other HOPS subunits was observed, as revealed by yeast growth on the selection plate (Fig. 4.2G). Furthermore, we found that the binding of Vps39 to SKIP does not require the N-terminal RUN domain or "W-acidic" motifs of SKIP as interaction with Vps39 was also found with a domain deletion mutant of SKIP (Δ1-300) lacking these regions (Fig. 4.2H,I). As reported previously, interaction of Arl8b and KLC2 with SKIP is through the N-terminal RUN domain and W-acidic motifs of SKIP respectively (Rosa-Ferreira and Munro 2011; Pernigo, Lamprecht, Steiner and Dodding 2013), this indicates that Vps39 and Arl8b/KLC2 require different regions for binding to SKIP. These results were further corroborated by co-immunoprecipitation experiments, which clearly indicated a strong interaction of Vps39 with SKIP *in vivo* that is not dependent upon the RUN domain and W-acidic motifs of SKIP (Fig. 4.3A-C). More importantly, we also found that endogenous HOPS subunits Vps11 and Vps33a were also present in this complex of SKIP and Vps39 (Fig. 4.3A). Furthermore, HOPS subunits Vps39 and Vps41 were found colocalizing on Arl8b- and SKIP-positive endosomes indicating that they constitute a part of the same molecular complex (Fig. 4.3D).

RAB7 effector RILP has been previously shown to interact with multiple HOPS subunits including Vps41 and Vps39 and recruit them to late endosomes. The RILP and HOPS complex-positive late endosomes were shown to cluster at the microtubule organizing center (MTOC) as direct interaction of RILP with dynactin-dynein complex drives these endosomes towards the minus-end of microtubules (van der Kant, Fish et al. 2013). To understand how presence of SKIP would regulate association of HOPS with RILP, we compared colocalization between RILP and HOPS in cells with or without SKIP overexpression. A complete lack of colocalization between RILP and either Vps41 or Vps39 was observed in the presence

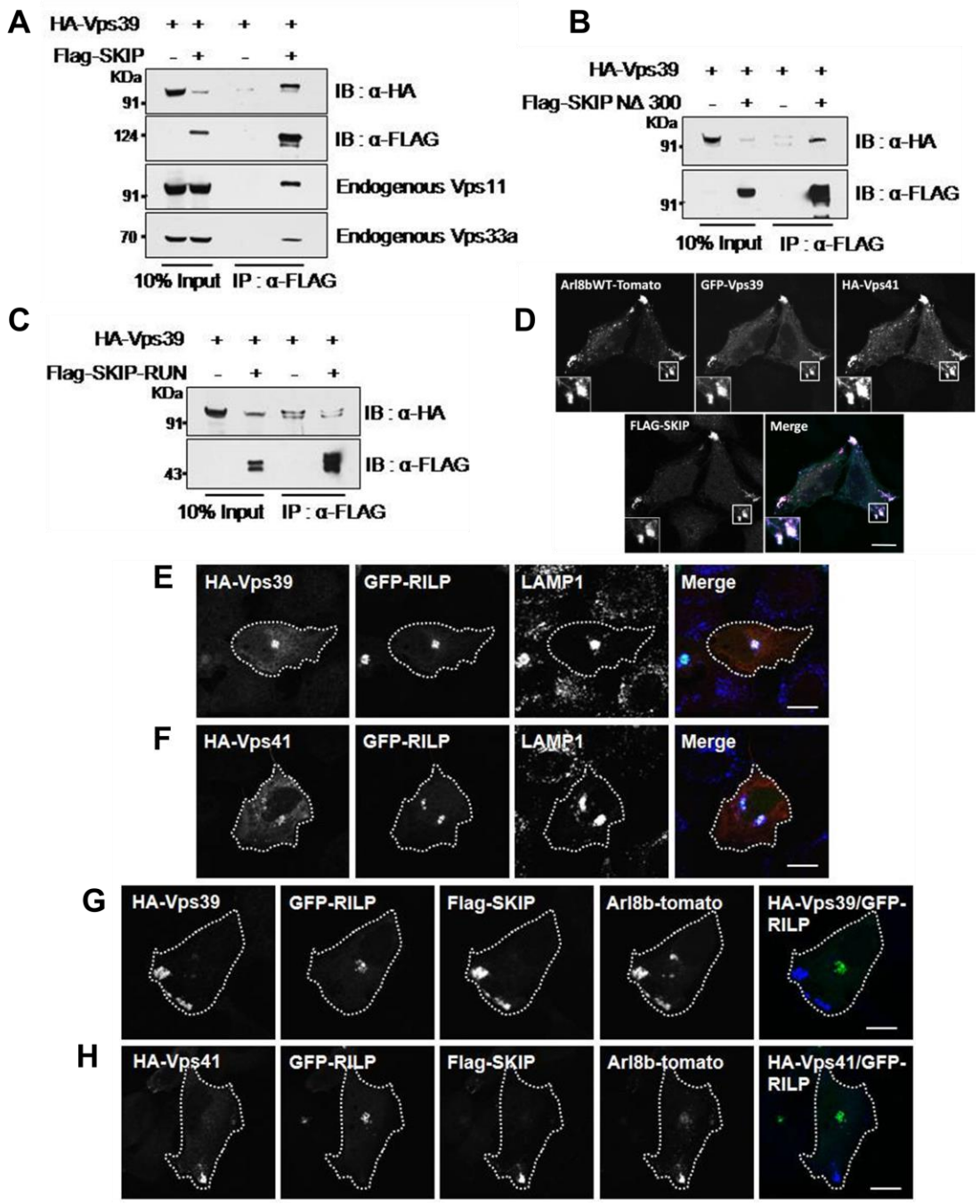

Figure 4.3. SKIP binds to multiple HOPS subunits and positions them away from the perinculear-clustered RILP compartment. (A-C) Lysates from HEK293T cells co-transfected with HA–Vps39 and Flag–SKIP wild-type or truncation mutants (as indicated), were subjected to immunoprecipitation (IP) with anti-Flag antibodies, and immunoblotted (IB) with HA antibodies to detect the interactions. After stripping, the membranes were re-probed with anti-Vps11 and anti-Vps33a antibodies (A). (D) Immunoflorescence depicting the colocalization of HOPS subunits with Arl8b and its effector SKIP. (E-H) HeLa cells were transfected with GFP-RILP and HA-Vps39 (E), or GFP-RILP and HA-Vps41 (F) or, these constructs additionally with Flag-SKIP and Arl8b-tomato (G,H), and analyzed for their localization by confocal microscopy. Merge images represent localization of RILP and HOPS subunits. Scale bar: 10μM.

of overexpressed Arl8b and SKIP as compared to without SKIP overexpression and HOPS subunits accumulated at the cell periphery with Arl8b and SKIP, while RILP retained its perinculear distribution (Fig. 4.3 E-H, see quantification in Fig. 4.4A). These results suggest that Arl8b-SKIP complex competes with Rab7-RILP complex for interaction with HOPS subunits and the two GTPases Arl8b and Rab7, drive motility of the HOPS-positive late endosomes and lysosomes in opposite directions.

4.2.2 SKIP/PLEKHM2 depletion results in delayed trafficking and degradation of EGFR in lysosomes

Our results shown here indicate that Arl8b effector SKIP plays a direct role in recruiting HOPS subunits to Arl8b-positive lysosomes. As Arl8b-association with HOPS is critical for endocytic degradation of EGFR, we tested if SKIP also regulates EGFR trafficking to lysosomes. Indeed, in SKIP-siRNA treated cells, EGFR degradation was significantly delayed compared to control-siRNA treated cells (Fig. 4.4B,C). We also monitored the levels of EGFR remaining via immunofluorescence by incubating control- and hVps41-shRNA with unlabeled EGF followed by chase for various time points in complete medium. While in control siRNA treated cells, EGFR signal was significantly reduced by 60 minutes of chase in complete medium, in SKIP siRNA treated cells, EGFR signal still persisted during this time point, indicating that EGFR degradation is delayed in SKIP-depleted cells (Fig. 4.4D,E).

In conclusion, these results show that Arl8b orchestrates membrane association and endocytic function of HOPS subunits by directly recruiting Vps41 to lysosomes and indirectly by association of its effector SKIP with Vps39.

4.3Discussion

The small GTPase Arl8b has been previously shown to mediate recruitment of molecular motor kinesin-1 via its effector SKIP on lysosome membranes (Rosa-

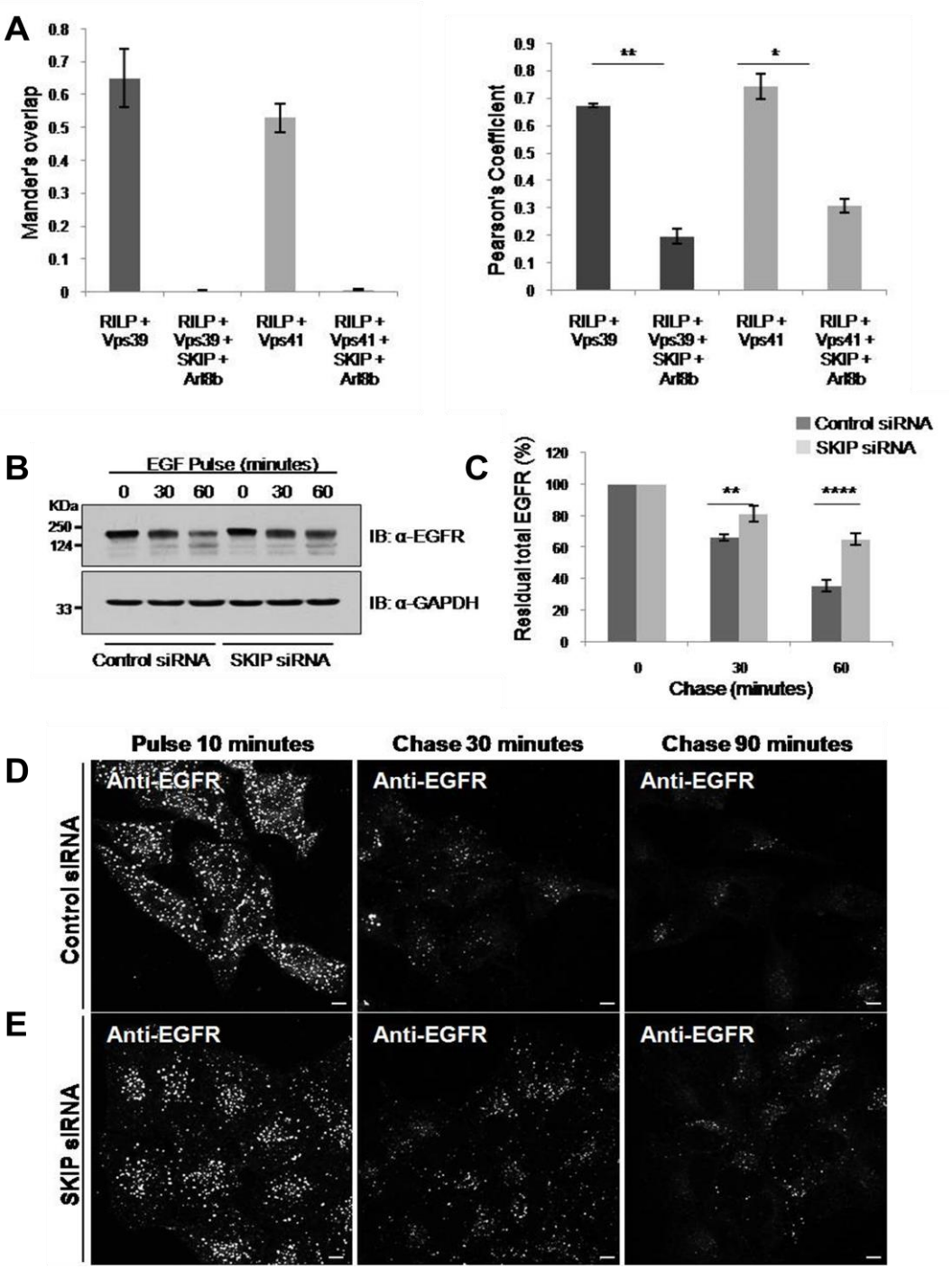

Figure 4.4. SKIP competes with RILP for association with HOPS subunits, and depletion of SKIP results in delayed EGFR degradation in lysosomes. (A) Colocalization of HA–Vps39 and HA–Vps41 with GFP–RILP in transfected HeLa cells was assessed by measuring the Mander's and Pearson's coefficients. Values plotted correspond to the mean±s.d. of three independent experiments (n=30 cells per experiment); *P,0.05; **P,0.01. (B) Control or SKIP-silenced HeLa cells were serum starved and pulsed with unlabeled EGF for the indicated time periods. The expression of EGFR and GAPDH (loading control) was detected by immunoblotting (IB) of whole-cell extracts. (C) Densitometric analysis of the residual total EGFR signal relative to GAPDH was performed for five independent experiments. Plotted values correspond to the mean6s.d. **P,0.01; ****P,0.0001. (D,E) Control (D) or SKIP-silenced (E) HeLa cells were serum starved, pulse-chased with unlabeled EGF for the indicated time periods, fixed for immunofluorescence and labeled with anti-EGFR. Scale bars: 10 μm.

Ferreira and Munro 2011). This tripartite complex of Arl8b-SKIP-KLC2 is implicated in plus-end movement of lysosomes towards the cell periphery. Interestingly, our results presented here indicate that HOPS subunits also associate with this Arl8b-SKIP-KLC2 complex and localize to cell periphery. Moreover, we found a novel interaction of SKIP with HOPS subunit Vps39 that regulates recruitment of Vps39 to Arl8b and LAMP1-positive endosomes (Fig. 4.2).

Since multi-subunit tethering factors are proposed to bridge the two membranes prior to vesicle fusion and predicted to have two membrane-binding sites, we propose a model whereby interaction of Arl8b on lysosome membranes and of Rab7-RILP on late endosomes with HOPS complex brings the two compartments in close proximity for fusion. Moreover, recruitment of molecular motors dynein and kinesin-1 by Rab7 and Arl8b effectors respectively to HOPS-containing endosomes drive motility of the HOPS-positive late endosomes and lysosomes in opposite directions. In agreement with this, we found that co-expression of SKIP and Arl8b prevents localization of HOPS subunits to RILP-positive compartment suggesting a competition between these two endocytic machineries.

In summary, our data clearly implicates the lysosome small GTPase Arl8b in regulating recruitment of human HOPS subunits to lysosomes via its direct interaction with hVps41 and via interaction of its effector SKIP with hVps39 subunit of the HOPS complex. Assembly of the core HOPS subunits to lysosomes is guided by their subunit-subunit interactions. We believe that findings presented in this manuscript will signify human HOPS complex as an Arl8b effector, a function that is performed by Rab7 homolog, Ypt7 in yeast and highlight this difference from yeast where no Arl8b homolog is present.

Chapter V

Characterization of Rabip4’ as a novel interaction partner of Arl8b

Chapter 5

5.1 Introduction

Small GTP-binding proteins of the Rab, Arfand Arf-like (Arl) families regulate intracellular traffic in various ways encompassing vesicular budding, transport and targeting to subcellular organelles. They alternate between an inactive GDP-bound state and an active GTP-bound conformation, that regulates GTPase localization and function in eukaryotic cells. Rab proteins have widespread functions ranging from vesicle budding to motility to fusion that are carried out by a diverse collection of effector molecules that bind to Rabs in their GTP-bound state. Recent advances have not only greatly extended the number of known Rab effectors, but have also begun to define the mechanisms underlying their distinct functions. Rab family of GTPases, particularly, has been linked to a class of effectors comprising RUN-domain containing proteins that act as regulators of membrane traffic, polarity, motility and signaling (Yoshida, Kitagishi, Okumura, Murakami, Nishimura et al. 2011). The RUN domains, named after RPIP8, UNC-14 and NESCA proteins, act as components of vesicle traffic and have been proposed to interact with a filamentous network linked to actin cytoskeleton or microtubules. For example, Rab8 associates with MyosinVI via RUN domain-containing protein Optineurin, and together they might be involved in presenting secretory vesicles from Golgi complex to plasma membrane before fusion (Chen and Wandinger-Ness 2001). A well-known regulator of endosome trafficking, Rab7, interacts with RUN domain-containing protein FYCO1 and regulatesthe anterograde movement of lysosomes and autophagosomes on microtubules and lysosometubulation in macrophages (Mrakovic, Kay et al. 2012). Sequence analysis has predicted that RUN domain is composed of six conserved blocks (A-F) which constitute the core of globular structure. The overall structure of

the RUN domain adopts a single globular fold consisting of eight α-helices. The conserved polar amino acids, particularly the basic ones in blocks A and D, might play a functional role in the interaction of RUN domains with small GTPases(Callebaut, de Gunzburg, Goud and Mornon 2001).

To date, atleast one member of Arf-like (Arl) family, Arl8b, has been shown to interact with multiple RUN-domain-containing proteins to regulate vesicular motility and trafficking. Early on after its discovery, Arl8b was recognized as a regulator of lysosome positioning in mammalian cells, thereby regulating nutrient sensing, cell migration, cancer cell metastasis, natural killer cell-mediated cytotoxicity, antigen presentation and the formation of tubular lysosomes in macrophages. While Arl8b depletion led to lysosome clustering at the microtubule-organizing center (MTOC), Arl8b overexpression increased the long-range, rapid movement of lysosomes on the microtubule track, resulting in some lysosomes dissociating from microtubules at the cell periphery and accumulating in the membrane projections. A search for Arl8b effectors using affinity chromatography revealed that Arl8b in its GTP-bound state interacts with the soluble RUN-domain containing protein SKIP (SifA and Kinesin-interacting Protein, also known as PLEKHM2), previously identified as a protein that binds to light chain of kinesin-1, and regulate anterograde motility of lysosomes in mammalian cells (Rosa-Ferreira and Munro 2011).

Another recently discovered effector of Arl8b and a well-known effector of Rab7, PLEKHM1, localizes to late endosomes/lysosomes, regulates lysosome motility and endocytic and autophagic cargo degradation in mammalian cells(McEwan, Popovic et al. 2015; Marwaha, Arya et al. 2017). PLEKHM1 binds to Arl8b via its RUN domain and shares ~40% similarity with SKIP over the length of RUN domain. While Rab7-RILP-PLEKHM1 complex promotes dynein-driven retrograde transport of late

endosomes/lysosomes, Arl8b-PLEKHM1 complex seems to act opposite to Arl8b-SKIP complex. In contrast to the perinuclear accumulation of lysosomes found upon Arl8b and SKIP knockdown, depletion of PLEKHM1 results in accumulation of lysosomes at the cell periphery. This study also provides an example that dual or shared effectors (in this case PLEKHM1) represent a point of convergence of Rab and Arl signals in membrane traffic (Marwaha, Arya et al. 2017).

Previous studies have shed light on RUN and FYVE domain-containing (RUFY) protein family that comprises four members RUFY1, RUFY2, RUFY3 and RUFY4. These proteins consisting of an amino-terminal RUN domain and a carboxyl-terminal FYVE domain have been reported to associate with phosphatidylinositol 3-phosphate in endosome membranes (Kitagishi and Matsuda 2013). Studies have implicated that RUFYs may act as docking proteins for multiple GTPases and their functions in the regulation of membrane trafficking and polarity have just begun to be understood. RUFY2, expressed in brain, lung and testis, interacts with Rab33 and Rab4 GTPases in yeast-two-hybrid and co-immunoprecipitation assays (Barbe, Lundberg, Oksvold, Stenius, Lewin et al. 2008), (Fukuda, Kobayashi, Ishibashi and Ohbayashi 2011). In a yeast-two-hybrid screen employing human fetal cDNA library, RUFY3 was found to be interacting with human Rab5A Q79L (constitutively GTP-bound) mutant used as bait, via its carboxyl-terminal region. GST-pulldown assays have suggested that the RUN domain of RUFY3 has an affinity for the GTPase Rap2. RUFY3 localizes in hippocampal neurons and accumulates in growth cones and axons, where it ensures the robustness of neuronal polarity by suppressing formation of surplus axons. Furthermore, RUFY3 overexpression leads to the formation of F-actin-enriched protrusive structures at the cell periphery and induces gastric cancer cell migration (Yoshida, Okumura, Kitagishi, Shirafuji and Matsuda 2010). RUFY4 has been shown

to be a positive regulator of macroautophagy in primary dendritic cells. Also, RUFY4 interacts with Rab7, and its ectopic expression induces aggregation of large LAMP1-positive membranous organelles in the perinuclear region of HeLa cells (Terawaki, Camosseto, Prete, Wenger, Papadopoulos et al. 2015).

RUFY1, the best-characterized member of the RUFY family of proteins, was discovered as an effector of small GTPase Rab4 and a regulator of early endosome dynamics (Cormont, Mari, Galmiche, Hofman and Le Marchand-Brustel 2001). Encoded by a single gene RUFY1, two isoforms of the protein are expressed as a result of transcription initiation at alternative promoters in RUFY1 gene: Rabip4' or the longer isoform comprising 708 amino acids and Rabip4 or the shorter isoform containing 600 amino acids. In addition to small GTPase-binding, both the N-terminal RUN domain and the C-terminal FYVE domain have been implicated in the early endosome localization of the two proteins (Ivan, Martinez-Sanchez, Sima, Oorschot, Klumperman et al. 2012). Moreover, tyrosine phosphorylation by Etk kinase at residues between the two coiled-coil domains is crucial for this localization (Yang, Kim, Wu and Qiu 2002). Rabip4 has been shown to be a dual effector of small GTPases Rab4 and Rab14 and together they mediate efficient recycling of endocytosed transferrin. Furthermore, reports have suggested an association of both isoforms of RUFY1 to a filamentous network with honeycomb appearance, possibly actin network, and their role in the migration of NIH 3T3 fibroblasts (Vukmirica, Monzo, Le Marchand-Brustel and Cormont 2006).

Interestingly, a recent study has unveiled the role of Rabip4' as a coordinator of lysosome positioning in mammalian cells. Rabip4' that was earlier functionally linked to early endosome small GTPases Rab4 and Rab5, was now found to interact with β3 subunit of Adaptor Protein 3 (AP-3). Silencing of Rabip4' promoted outgrowth of

plasma membrane protrusions, and polarized distribution and clustering of lysosomes at their tips. The most peripheral lysosomes were localized beyond microtubules, within the cortical actin network (Ivan, Martinez-Sanchez et al. 2012). However, since Rabip4' and AP-3 localize primarily to early/recycling endosomes, no mechanism implicating the direct involvement of the two proteins in regulating lysosome distribution was elucidated. Further, it was intriguing to note that RUN domain of Rabip4' shares 35% and 38% identity with those of Arl8b-interaction partners PLEKHM2/SKIP and PLEKHM1, respectively. Moreover, while Rab4 and Rab5 have an affinity for carboxy-terminal of Rabip4', no association of Rabip4' RUN-domain with any small GTPase has, thus far, been reported. These observations compelled us to probe if Rabip4' can interact with lysosome small GTPase Arl8b and regulate lysosome positioning and functions.

Here we report that Rabip4/Rabip4' (Rabip4s) interact with Arl8b via its RUN domain. Interaction of Rabip4swith Arl8b, possibly, drives their localization to late endosomes and lysosomes.

5.2 Results

5.2.1 Rabip4s interact with lysosomal small GTPase Arl8b via their N-terminal RUN domain

To investigate whether Rabip4s bind to Arl8b, we tested the interaction of Rabip4' with Arl8b in a co-immunoprecipitation assay (Fig. 5.1A). We observed haemagglutinin (HA)-tagged Arl8b could coimmunoprecipitate Flag-tagged Rabip4' from transfected HEK293T cell lysates (Fig. 5.1A). These results were further corroborated using a GST-pulldown assay in which either GST-tagged Rabip4 RUN (1-194) or PLEKHM1 RUN (1-198) (as a positive control) were used as bait to pull down Arl8b from transfected HEK293T cell lysates (Fig. 5.1B). We found that

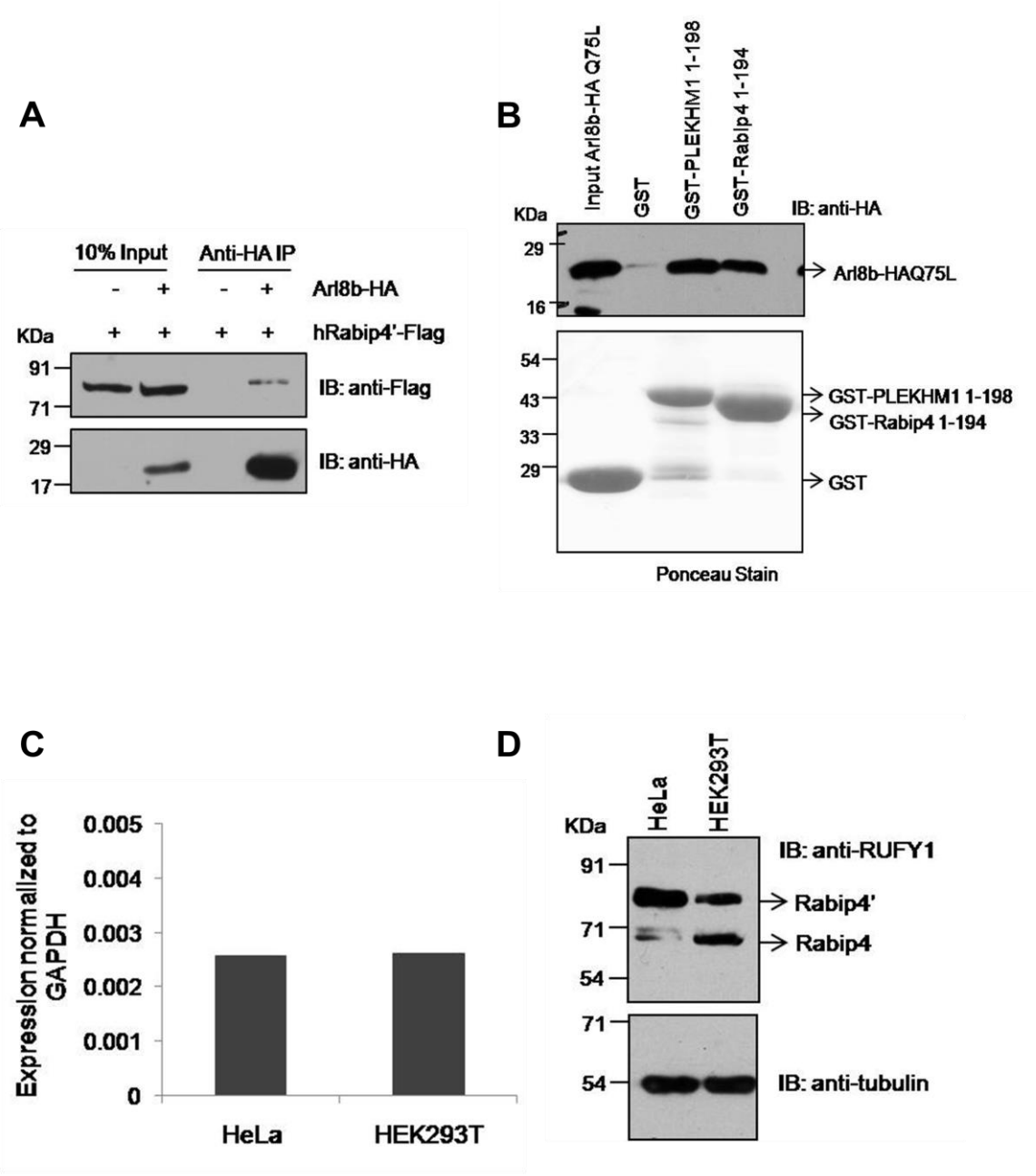

Figure 5.1. Rabip4s interact with small GTPase Arl8b. (A) A representative immunoblot (IB) of HEK293T cell lysates expressing either human Rabip4'-Flag alone or in combination with Arl8b-HA, immunoprecipitated (IP) with anti-HA-conjugated beads. (B) Western blot showing a GST pulldown assay using HEK293T cell lysates expressing Arl8b-HA incubated with glutathione-conjugated beads bound to the indicated GST proteins. GST-purified proteins were visualized with Ponceau staining. (C) qRT-PCR analyses of Rabip4s levels in HeLa and HEK293T cell lines (D) Western blot depicting the levels of Rabip4sin HeLa and HEK293T cell lines. Tubulin was used as the loading control.

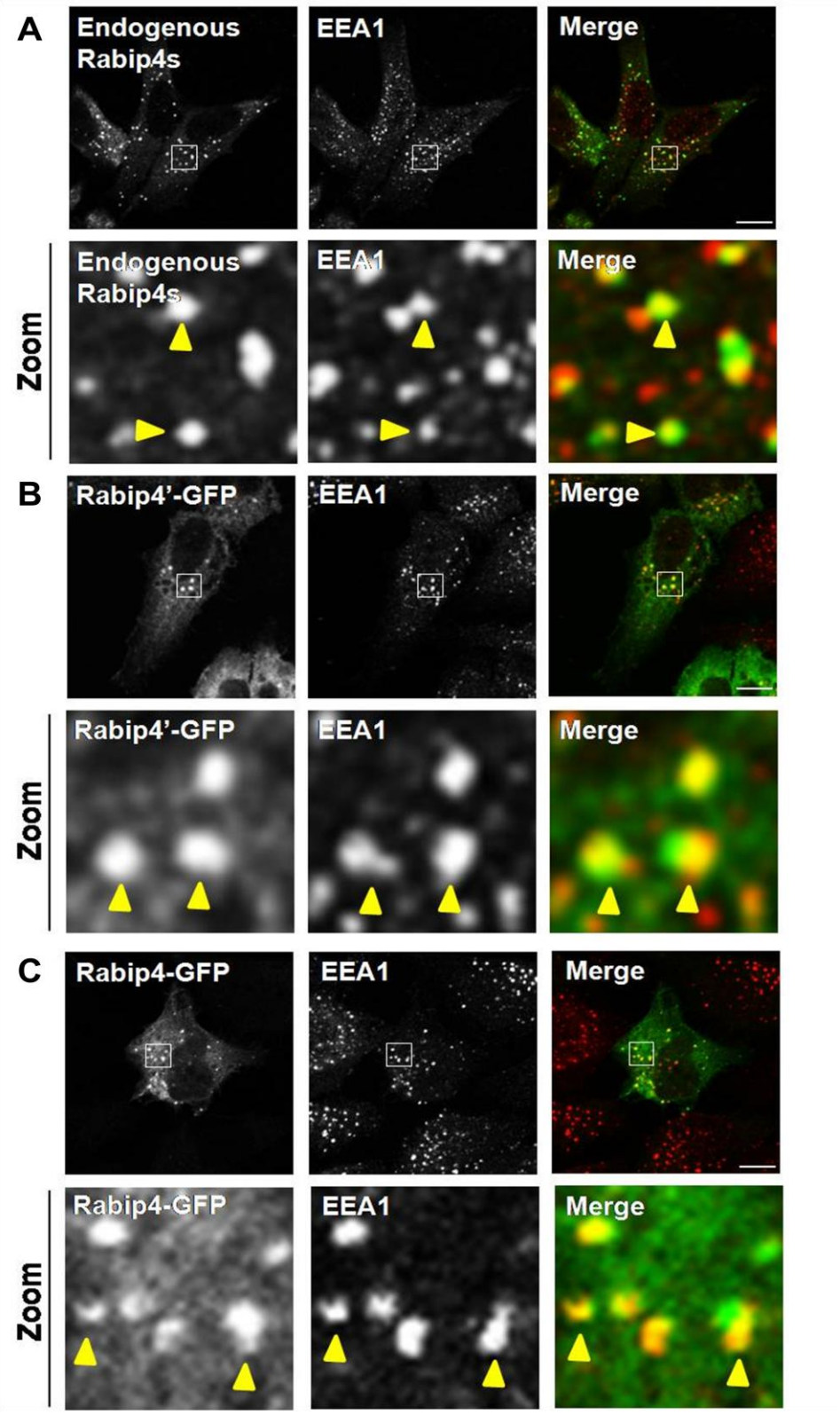

Figure 5.2. Rabip4s localize to early endosomes. (A-C) Immunofluorescence depicting HeLa cells either stained for endogenous Rabip4s (A) or transfected with either Rabip4'-GFP (B) or Rabip4-GFP (C) and co-stained with EEA1. Co-localized puncta are indicated in the zoomed in sections below the images, and marked by arrowheads. Scale bars: 10μm.

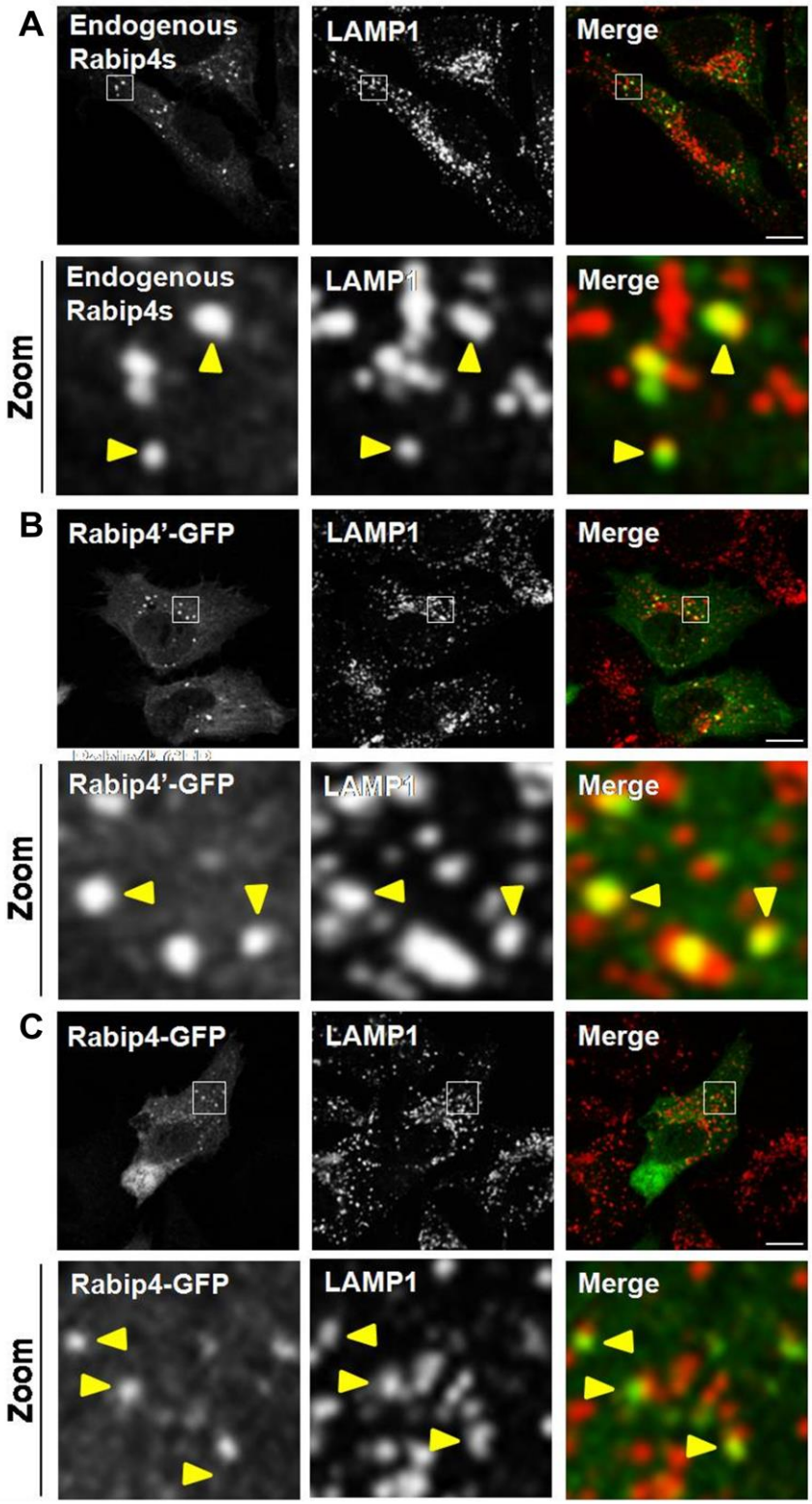

Figure 5.3. Rabip4s localize to lysosomes. (A-C) Confocal micrographs of HeLa cells either stained for endogenous Rabip4s (A) or transfected with either Rabip4'-GFP (B) or Rabip4-GFP (C) and co-stained with LAMP1. Co-localized puncta are indicated in the zoomed in sections below the images, and marked by arrowheads. Scale bars: 10μm.

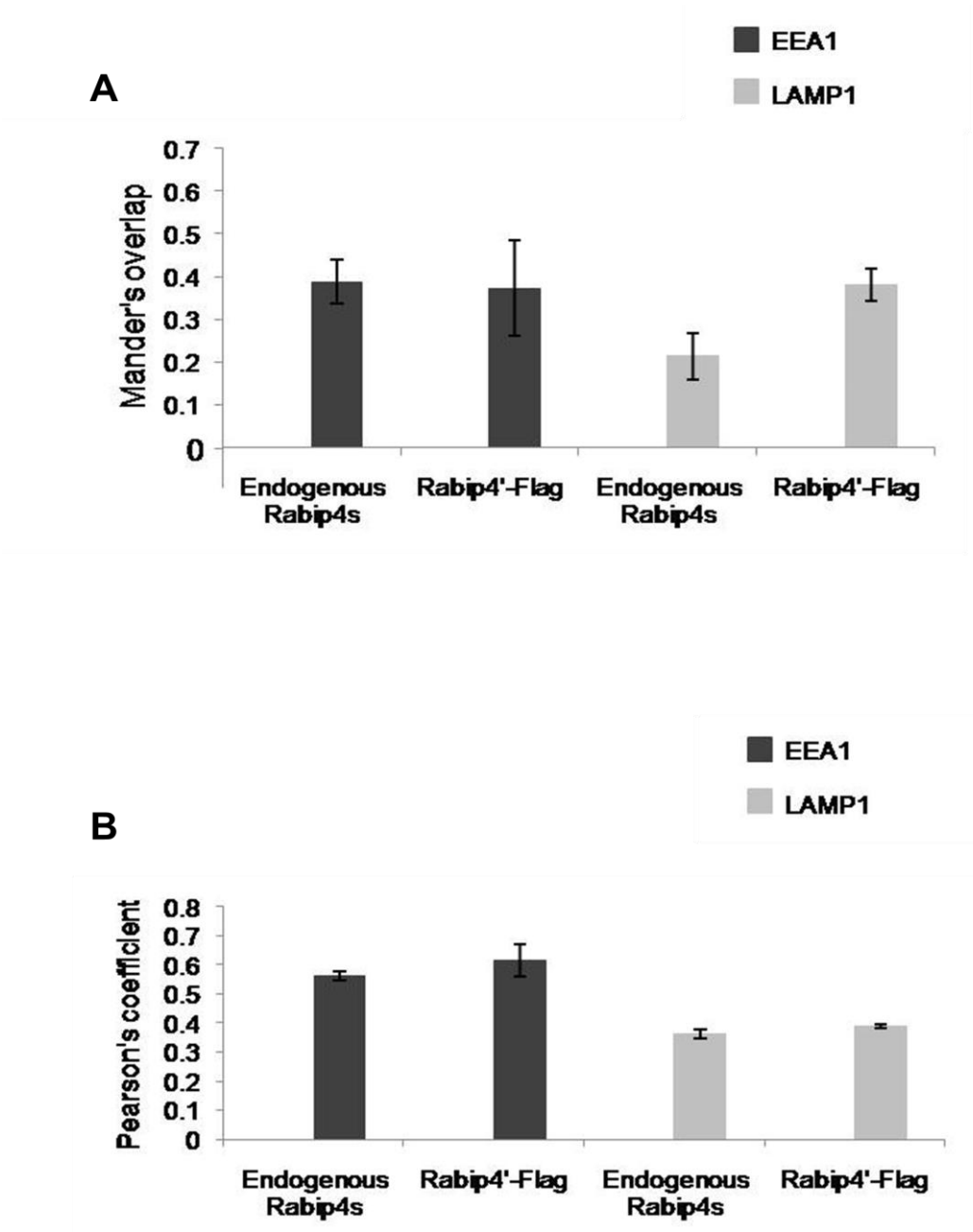

Figure 5.4. Rabip4s localize majorly to early endosomes and partially to lysosomes. (A) Mander's and Pearson's coefficients depicting the co-localization of endogenous Rabip4s and Rabip4'-Flag with EEA1 or LAMP1 endosomes were calculated, as indicated. Values plotted correspond to mean ± s.d. of three independent experiments (n=25 cells per experiment).

similar to PLEKHM1, interaction of Rabip4 with Arl8b is mediated via its RUN domain.

Rabip4s have been reported to localize to early endosomes and act as effectors of early/recycling endosome small GTPases. The mRNA expression of Rabip4s was confirmed in both HeLa and HEK293T cells by qRT-PCR (Fig. 5.1C). Immunoblotting indicated that the relative protein expression of the two isoforms is variable in these cell lines. While the longer isoform (Rabip4') predominates in HeLa cells, both the isoforms appear to have a similar expression in HEK293T cells (Fig. 5.1D). To understand the localization dynamics of Rabip4s in HeLa cells, we immunostained for endogenous Rabip4s either alone or in conjunction with Arl8b. As expected, when present alone both endogenous Rabip4s and overexpressed isoforms associate strongly with early endosomes (Fig. 5.2A-C, quantification shown in Fig. 5.4A,B). To our surprise, we found a small population of these proteins colocalizing with the late endosome and lysosome marker LAMP1 (Fig. 5.3A-C, quantification shown in Fig. 5.4A,B). When co-stained for Arl8b, we observed that endogenously Rabip4s and Arl8b colocalized on few puncta and in the cell periphery (Fig. 5.5A). Furthermore, there appeared a marked increase in the colocalization of two proteins when either Arl8b or both proteins were co-expressed in cells (Fig. 5.5B,C). In cells co-transfected with Arl8b and Rabip4', co-staining for EEA1 or LAMP1 yielded a significant population of Rabip4' and Arl8b-double positive endosomes that were colocalizing with EEA1 when compared to LAMP1 (Fig. 5.6A,D). Next, to determine whether the GTP-bound form of Arl8b promotes association of Rabip4s with lysosomes, we co-expressed putative GTP-locked (Q75L) and putative GDP-locked (T34N) mutants of Arl8b with Rabip4'. In contrast to the cells transfected with either wild-type or GTP-locked Arl8b, partial redistribution to the cytosol of Rabip4' was

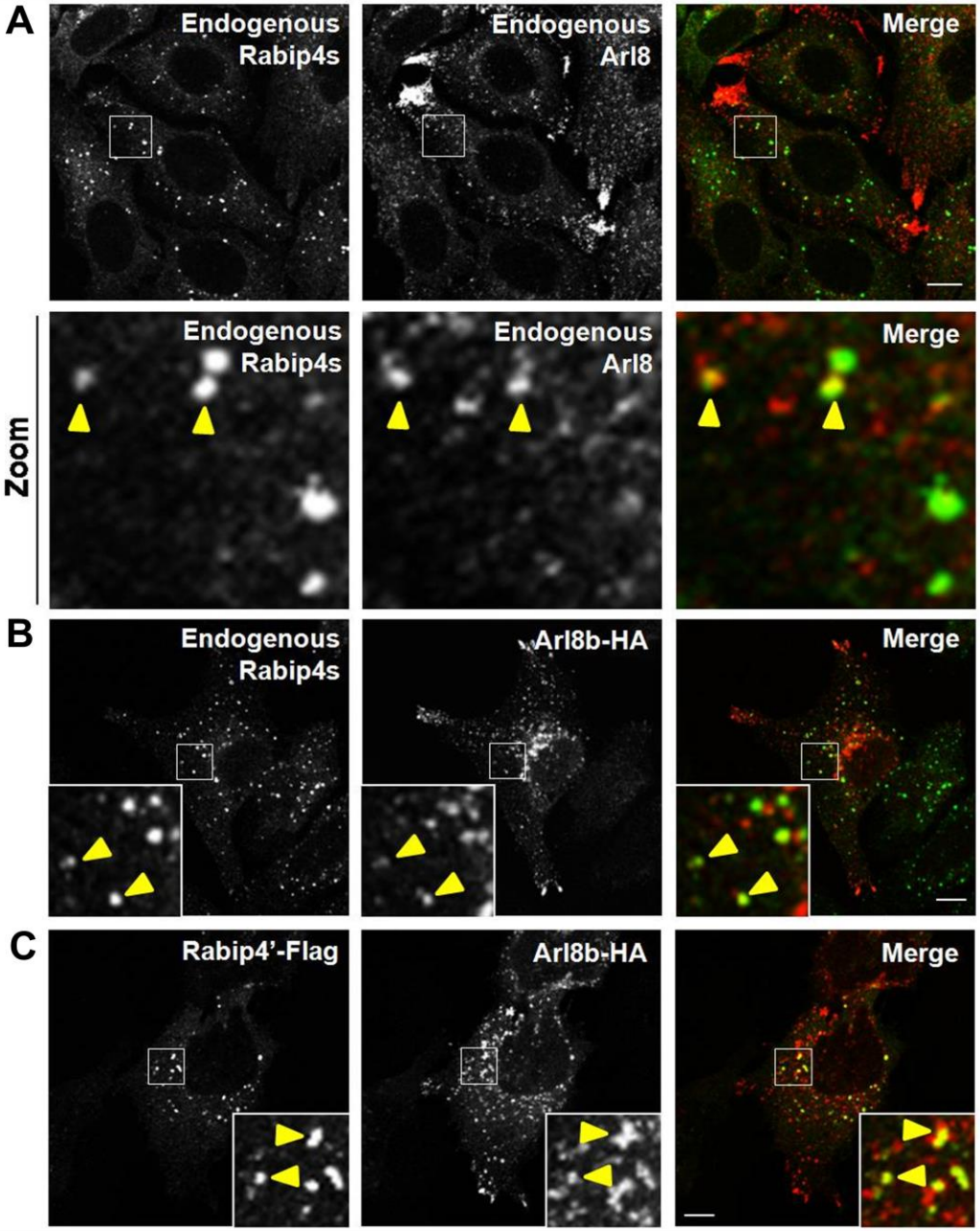

Figure 5.5. Rabip4s colocalizes with Arl8b-positive endosomes. (A) HeLa cells were co-stained for endogenous Rabip4s and Arl8 and their colocalization was analyzed by confocal microscopy. Co-localized puncta are indicated in the panel of zoomed in sections below. (B-C) Immunofluorescence depicting HeLa cells transfected with either Arl8b-HA WT alone and co-stained for endogenous Rabip4s (B) or co-transfected with Rabip4'-Flag (C). Co-localized puncta are indicated in the inset, and marked by arrowheads, Scale bars: 10μm.

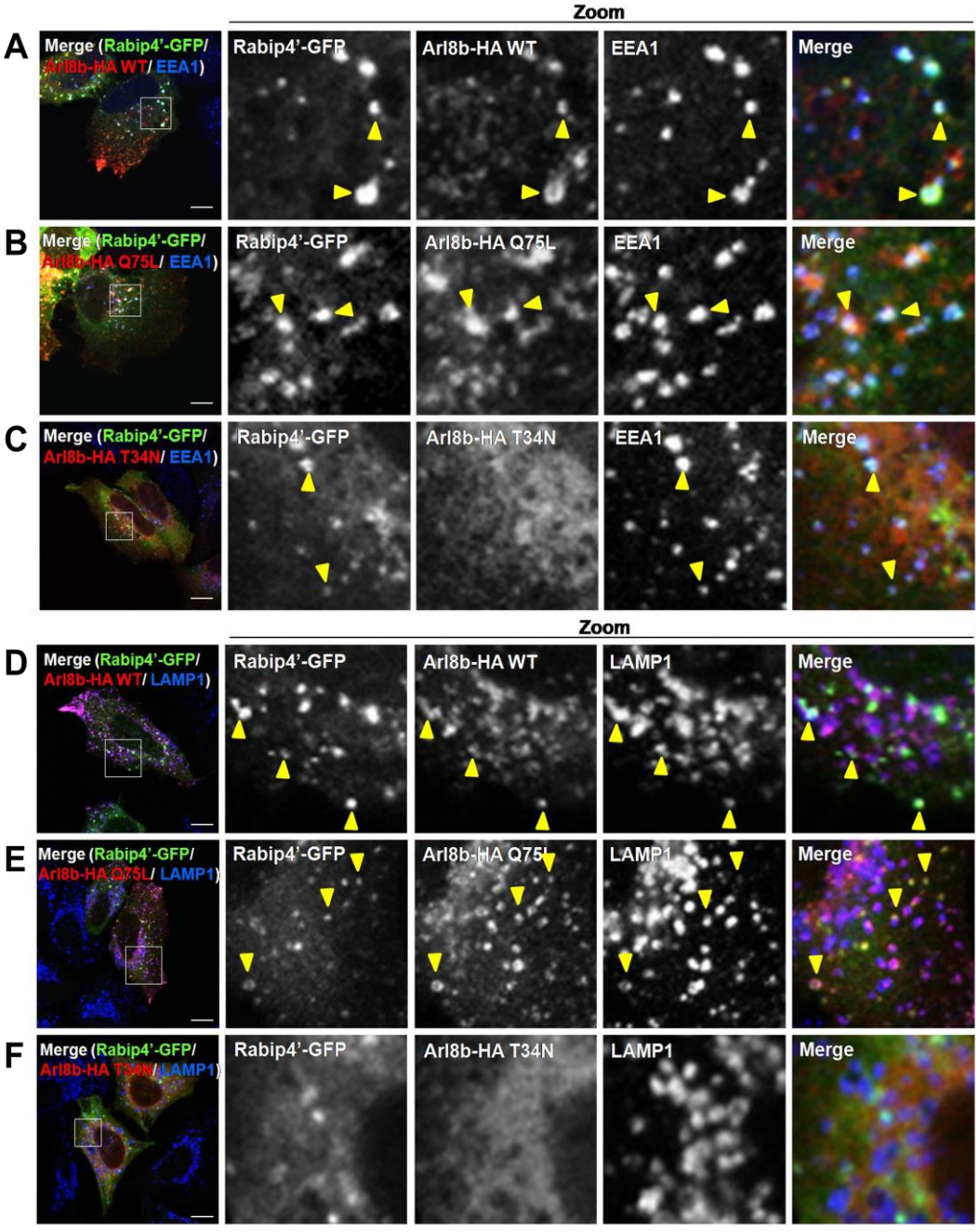

Figure 5.6. Rabip4' colocalizes with GTP-Arl8b on both EEA1- and LAMP1-positive puncta. (A-F) HeLa cells were co-transfected with Rabip4'-Flag and either Arl8b-tomato wild-type (WT) (A,D) or Arl8b-tomato Q75L (B,E) or Arl8b-tomato T34N (C,F) and co-stained with either EEA1 (A-C) or LAMP1 (D-F), and analyzed for early endosomal localization by confocal microscopy. Colocalized pixels are indicated in the inset. Scale bars: 10μm.

observed in cells co-expressing Arl8b-T34N (Fig. 5.6B-C, E-F). The remaining Rabip4' puncta in these cells were primarily EEA1-positive with a lack of LAMP1-positive ones, suggesting that GTP-bound state of Arl8b favors localization of Rabip4' to lysosomes (Fig. 5.6C,F).

5.2.2 Colocalization of Rabip4' with Arl8b is dependent upon conserved residues in RUN domain

Since we saw an *in-vitro* binding of Rabip4s RUN domain with Arl8b, we next analyzed whether RUN domain modulates the subcellular distribution of Rabip4s. Immunofluorescence data suggested that a mutant of Rabip4' lacking the RUN domain was completely cytosolic upon expression in HeLa cells, in contrast to wild-type Rabip4' that was found colocalizing with $LAMP1^{+}$ endosomes (Fig. 5.7A,B). This mutant failed to localize to any endosome structures even upon co-expression of Arl8b, indicating that binding of Rabip4s to Arl8b via RUN domain is essential for their membrane localization (Fig. 5.7D).

Previous studies have shed light on the presence of conserved basic residues in RUN domain that are implicated in interaction with small GTPases. Bioinformatic analyses depicted that, similar to PLEKHM1 and PLEKHM2, Rabip4s have conserved histidine and arginine residues in the RUN domain (Fig. 5.8A). Since deletion of RUN domain involves the loss of a large stretch of amino acids, we employed site-directed mutagenesis to mutate the arginine residues to alanine in Rabip4', and expressed them either alone or with Arl8b in HeLa cells. Similar to the RUN domain deleted protein, the arginine mutant was completely cytosolic (Fig. 5.9A,B)and continued to be so even upon co-transfection of Arl8b (Fig. 5.9C,D). Earlier reports have suggested that binding sites for small GTPases Rab4 and Rab14 lie in the C-terminal region of Rabip4s. Consistent with this, we observed a localization of

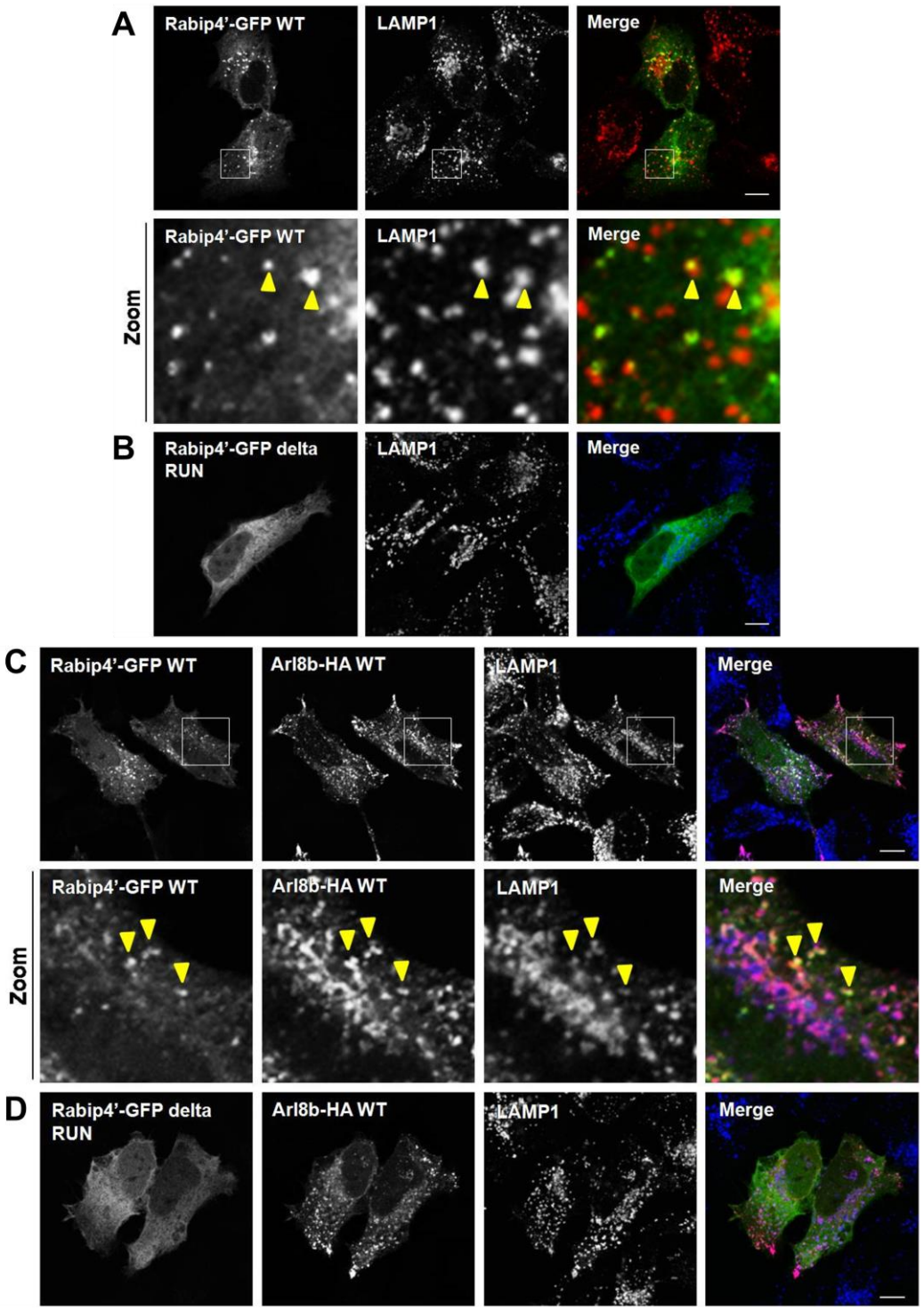

Figure 5.7. Rabip4' RUN domain is essential for its recruitment to Arl8b compartments. (A-B) Immunostaining with LAMP1 of HeLa cells transfected with either Rabip4'-GFP wild-type (WT) (A) or Rabip4'-GFP mutant lacking the RUN domain (delta RUN) (B). (C-D) Representative confocal micrographs depicting HeLa cells co-transfected with Arl8b-HA and either Rabip4'-GFP WT (C) or Rabip4'-GFP delta RUN (D), and co-stained with LAMP1. Co-localized puncta are indicated in the zoomed in images, and marked by arrowheads. Scale bars: 10μm.

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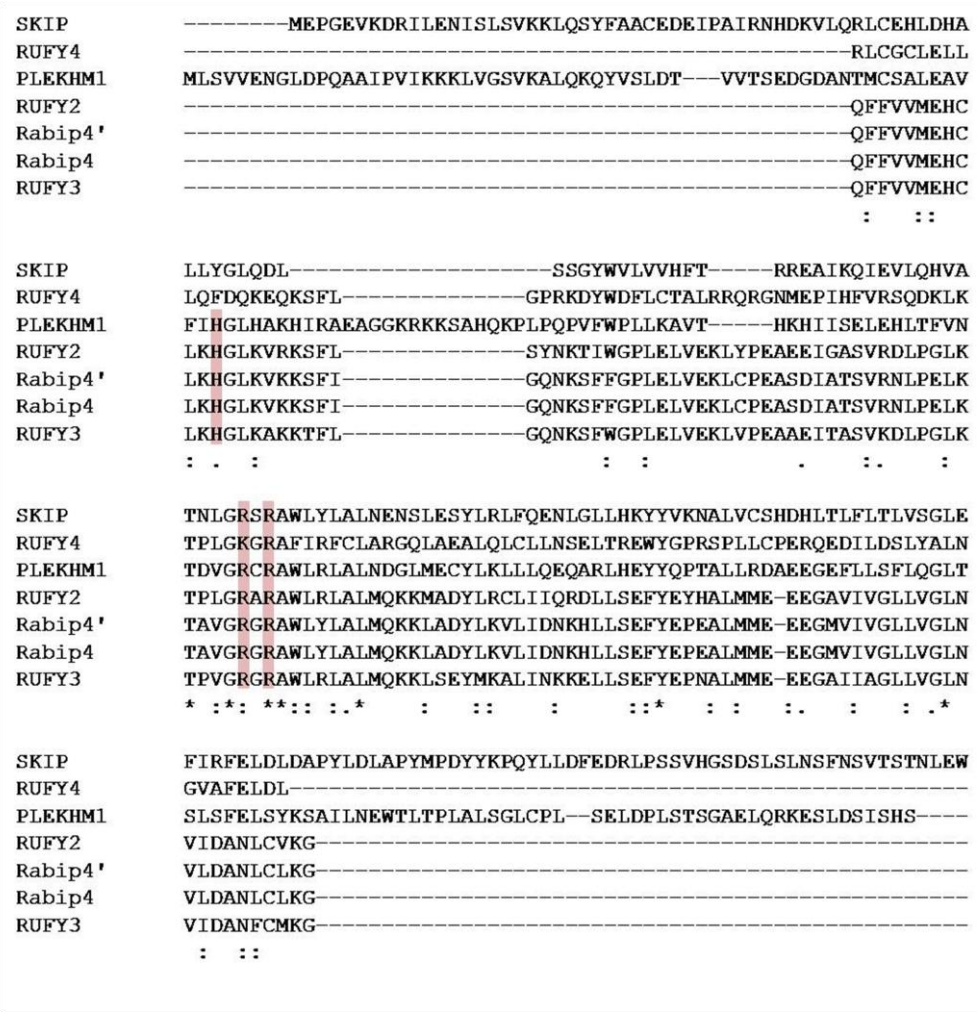

Figure 5.8. RUN domain-containing proteins depicting the conserved basic residues in RUN domain. (A) Multiple sequence alignment of RUN domains of indicated proteins highlighting the conserved basic (histidine and arginine) residues.

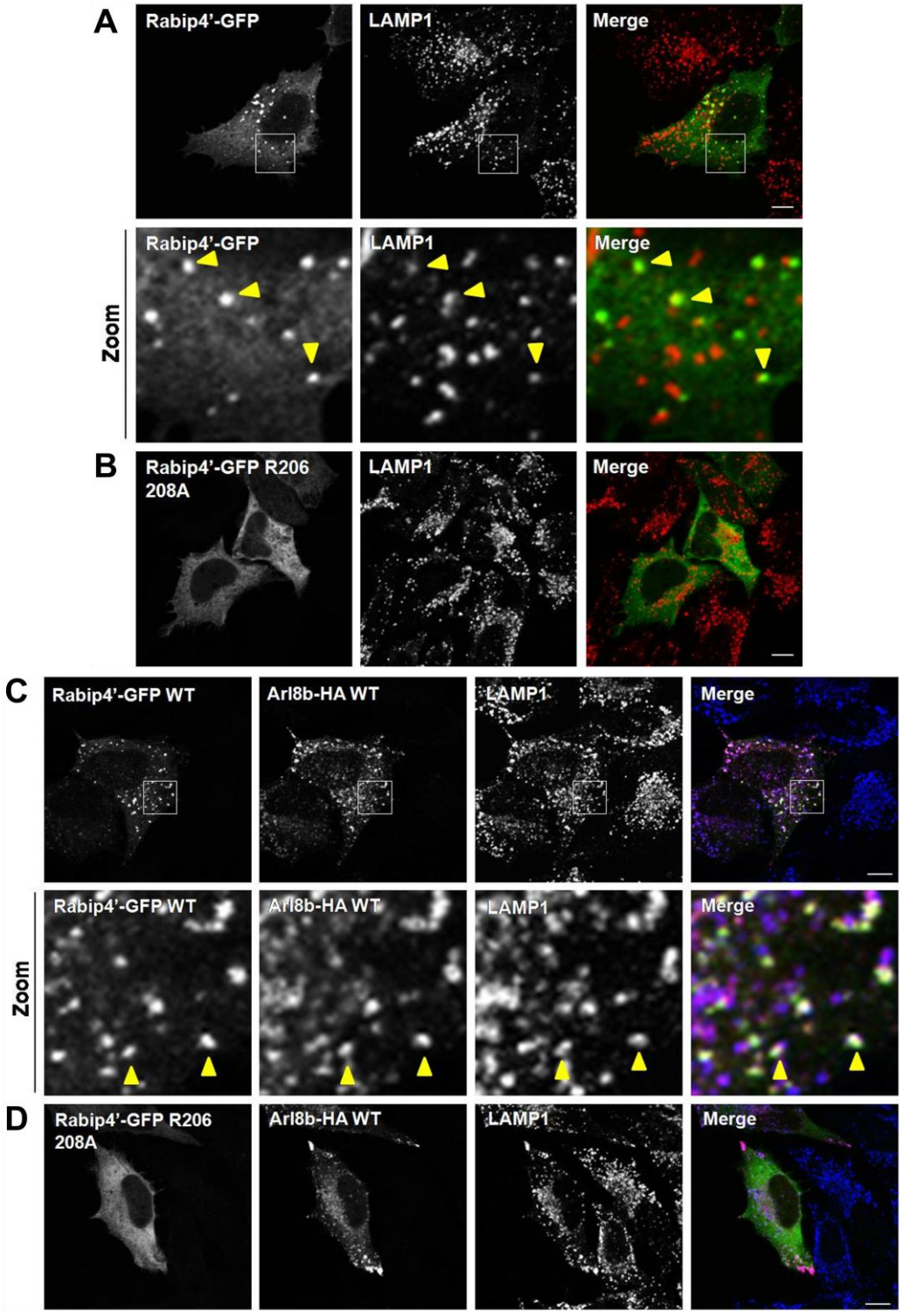

Figure 5.9. Conserved basic residues in Rabip4' RUN domain mediate its localization to Arl8b compartments. (A-B) HeLa cells transfected with either Rabip4'-GFP wild-type (WT) (A) or Rabip4'-GFP R206 208A (B) were analyzed for lysosomal localization of these proteins by confocal microscopy. (C-D) Immunofluorescence of HeLa cells co-transfected with Arl8b-HA and either Rabip4'-GFP WT (C) or Rabip4'-GFP R206 208A (D), and co-stained with LAMP1. Co-localized puncta are indicated in the zoomed in images, and marked by arrowheads. Scale bars: 10μm.

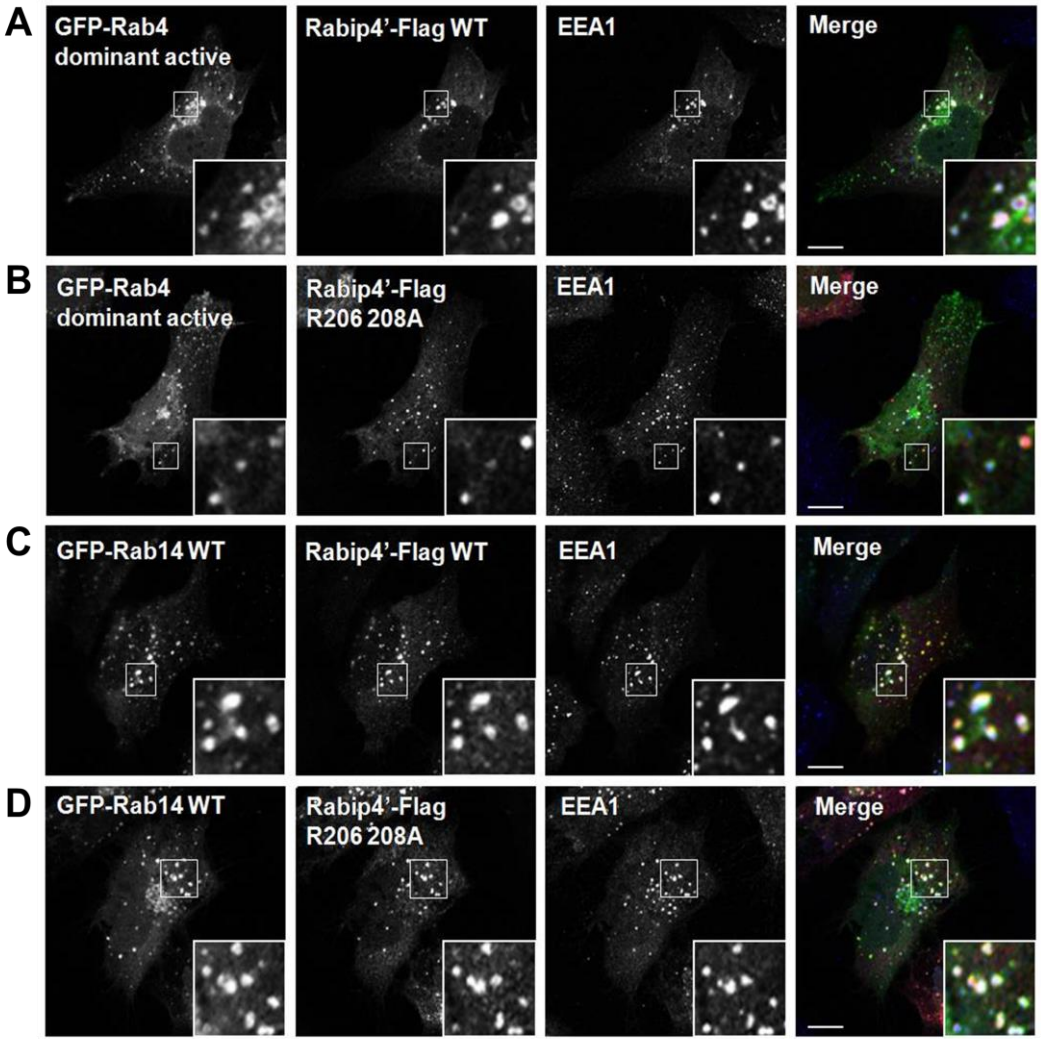

Figure 5.10. Conserved basic residues in Rabip4' RUN domain do not determine its localization to Rab4/Rab14 endosomes. (A-D) Colocalization of GFP-tagged small GTPases Rab4 (A,B) or Rab14 (C,D), and either Rabip4'-GFP wild-type (WT) (A,C) or Rabip4'-GFP R206 208A (B,D) was assessed in transfected HeLa cells by confocal microscopy. Co-localized pixels are indicated in the inset. Scale bars: 10μm.

Rabip4' arginine mutant to GFP-Rab4- and GFP-Rab14-positive endosomes (Fig. 5.10A-D).

5.2.3 Arl8b silencing disrupts association of Rabip4s with late endocytic membranes

To emphasize the significance of Arl8b in regulating Rabip4s membrane localization, we immunostained for endogenous Rabip4s in control and Arl8b siRNA-treated HeLa cells. The efficiency of Arl8b silencing using different oligonucleotides was confirmed by qRT-PCR and was found to be >80% (Fig. 5.12A). Unlike control siRNA-treated cells, where Rabip4s were present on endosome structures, they were redistributed to the cytoplasm of HeLa cells upon knockdown of Arl8b using multiple oligonucleotides (Fig. 5.11A-D). Importantly, while Rabip4s weremostly cytosolic in Arl8b-depleted cells, few endosomes were observed that were not LAMP1-positive but colocalized with EEA1 (Fig. 5.11E, quantification shown in Fig. 5.12B), indicating that Arl8b does not mediate association of Rabip4s with early endosomes but late endosomes/lysosomes. Next, to reaffirm the specificity of Arl8b depletion, we rescued its effect on Rabip4s localization in HeLa cells expressing siRNA-resistant Arl8b. As demonstrated, in siRNA-resistant Arl8b-transfected cells, Rabip4s were now present on many puncta (>5 endosomes per cell) that were also positive for Arl8b (Fig. 5.11F, quantification shown in Fig. 5.12B). It was noteworthy that, while Arl8b knockdown disrupted the membrane association of Rabip4s, it did not alter the localization of other early endosome proteins (Fig. 5.13A-D). Collectively, our findings suggest that Rabip4s interact with Arl8b via their RUN domain where the conserved basic residues within the RUN domain are required for this interaction.Furthermore, membrane localization of Rabip4s, especiallyto late endosomes and lysosomes, is regulated by interaction with Arl8b.

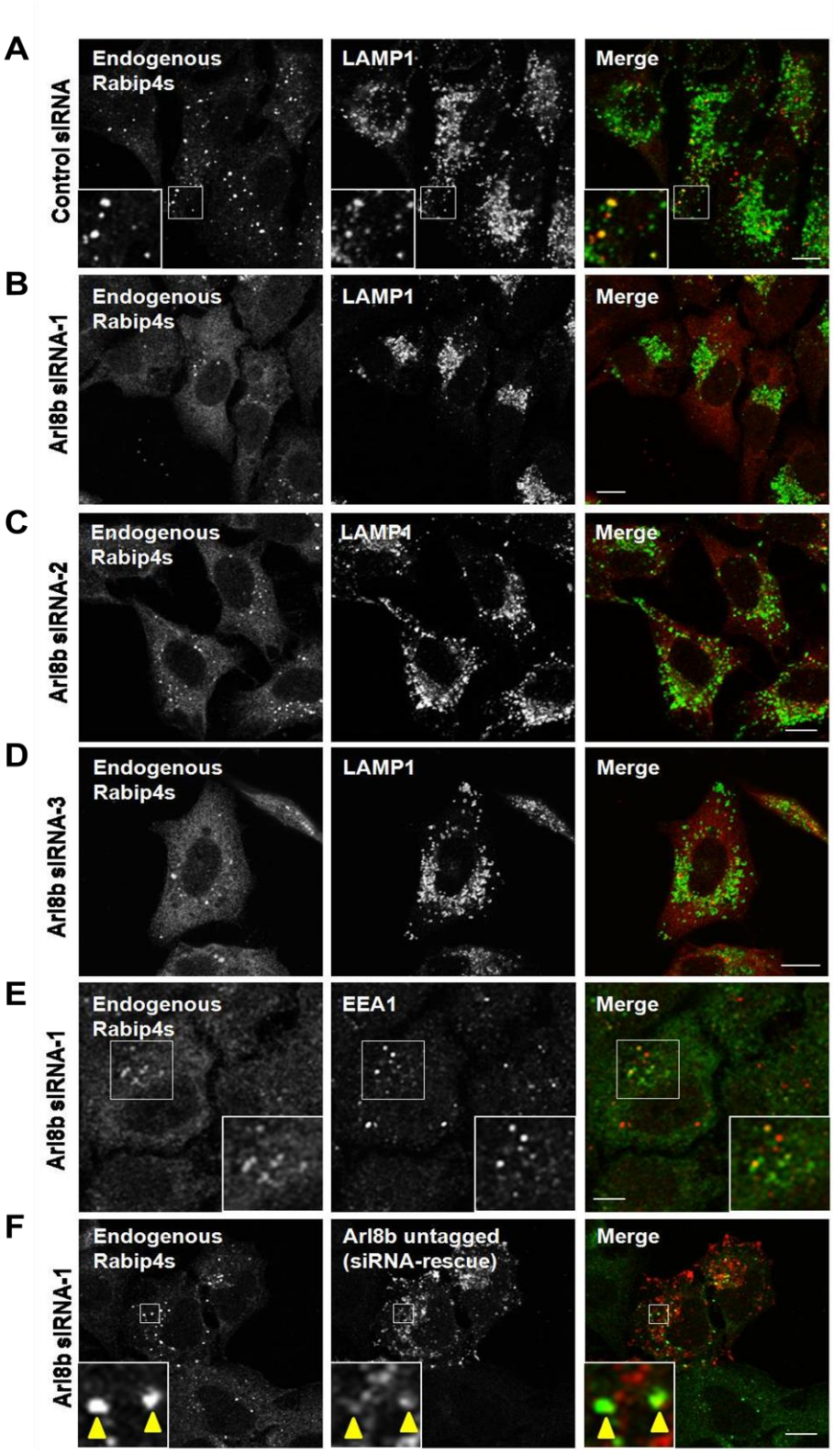

Figure 5.11. Arl8b silencing disrupts association of Rabip4s with late endocytic membranes. (A-F) Immunofluorescence depicting the localization of endogenous Rabip4s to either LAMP1^{+} (A-D) or EEA1^{+} (E) compartments in control (A) or Arl8b- (B-E) depleted cells, and Arl8b-silenced cells expressing siRNA-resistant Arl8b–tomato (F). Colocalized pixels are indicated in the inset, and indicated by arrowheads. Scale bar: 10μm.

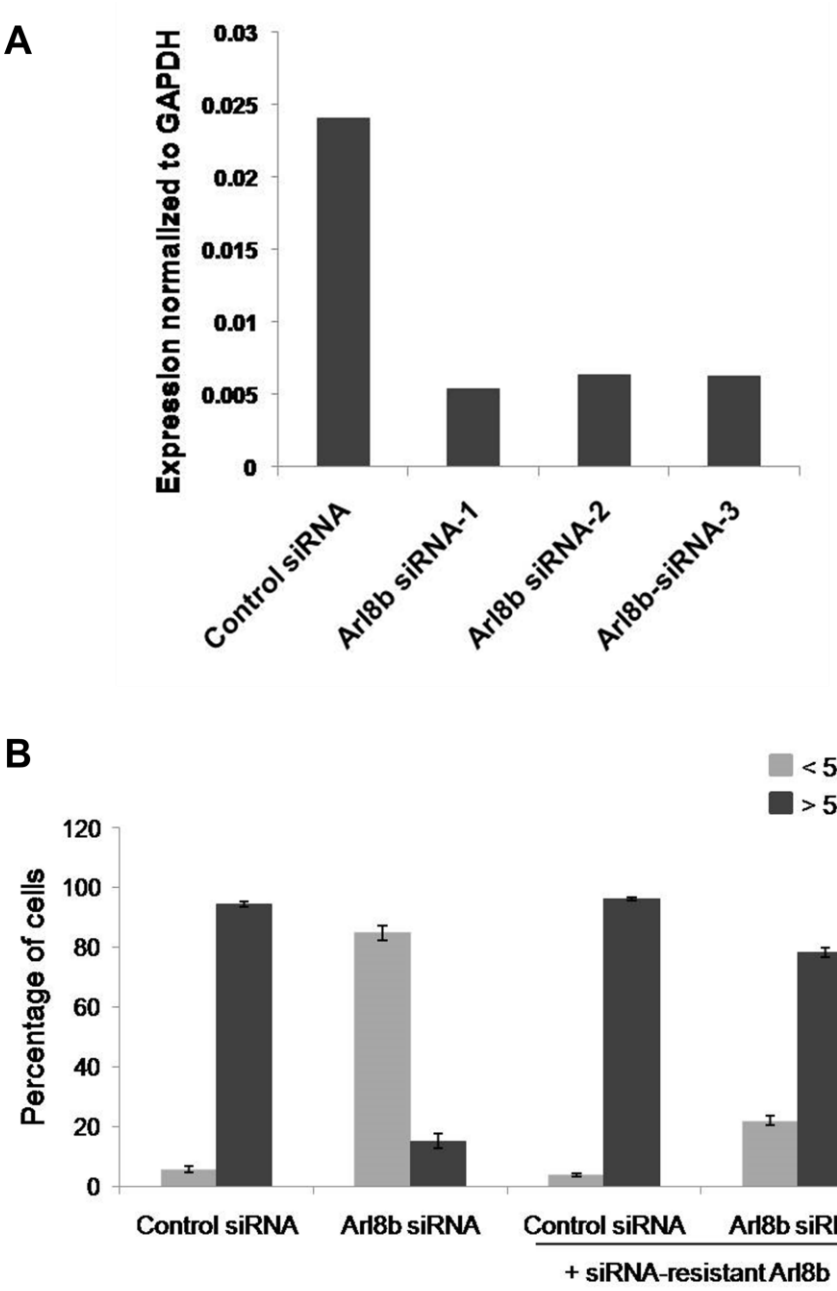

Figure 5.12. Arl8b expression is required for localization of Rabip4s to endosomal membranes. (A) qRT-PCR analyses of Arl8b levels in control and Arl8b siRNA treated cells. (B) Control or Arl8b-silenced cells were counted for the number of Rabip4s^{+} endosomes, and plotted as indicated. Values correspond to the mean ± s.d. of three independent experiments (n=35 cells per experiment).

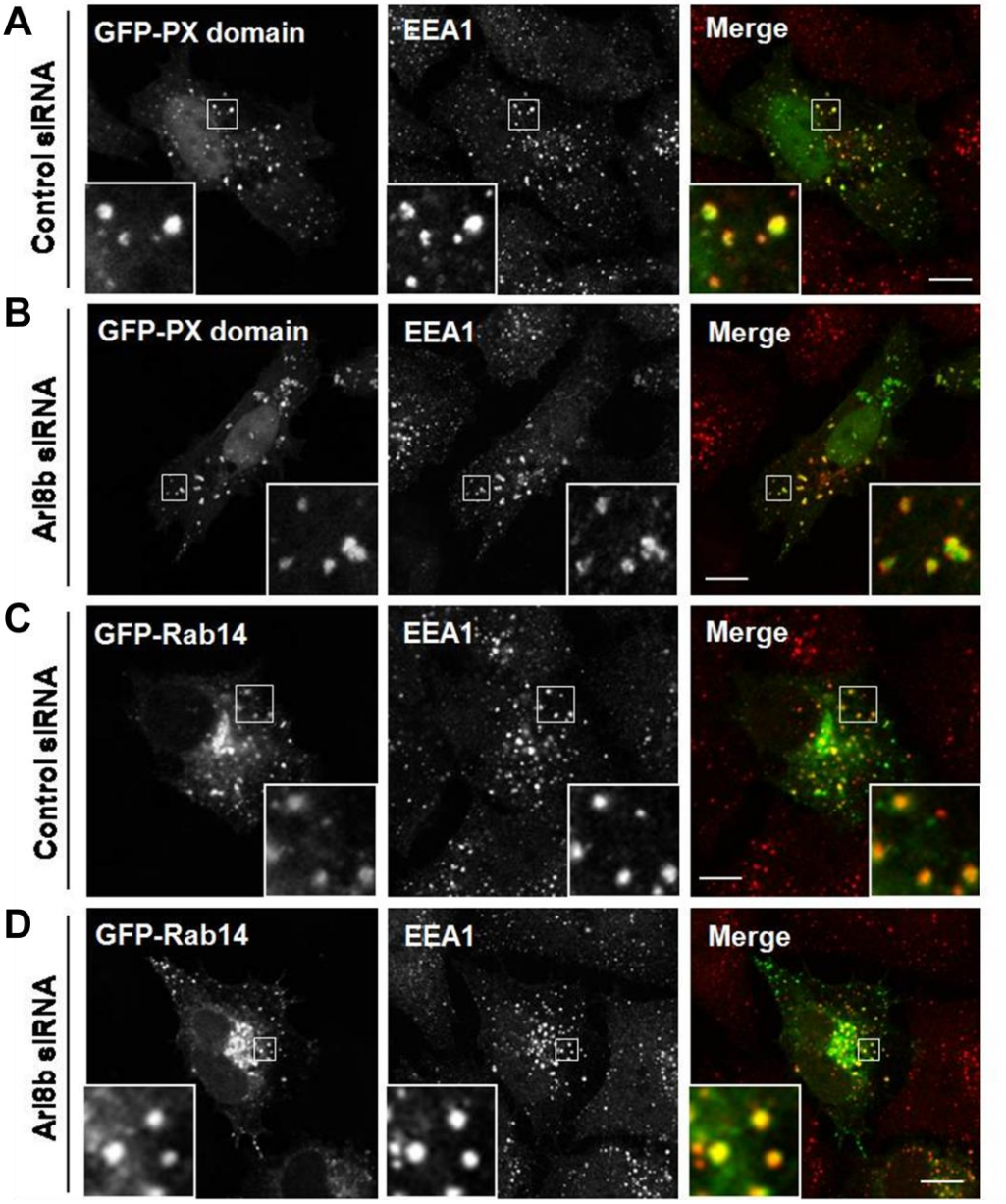

Figure 5.13. Arl8b silencing does not alter membrane association of other early endosomal proteins. (A-D) Control (A,C) and Arl8b siRNA (B,D) treated cells were transfected with either GFP-PX domain (A,B) or GFP-Rab14 (C,D), and immunostained for EEA1. Co-localized pixels are indicated in the inset. Scale bars: 10μm.

5.3 Discussion

RUN domain-containing proteins act as effectors of RabGTPasesand regulate membrane traffic, polarity and signaling. Earlier studies have determined the crystal structure of RUN domain protein, Rab6-interacting protein 1, R6IP1 in association with Rab6, which is also the largest Rab-effector complex structure solved to date. It was found that interaction of R6IP1 with Rab6 was mediated through the RUN domain(Fernandes, Franklin and Khan 2011). Interestingly, RUN domain proteins are increasingly being recognized as effectors of atleast one member of Arl family of GTPases, Arl8b. For example, RUN domain-containing proteins PLEKHM2 and PLEKHM1 bind to Arl8b and regulate lysosome motility and trafficking(Rosa-Ferreira and Munro 2011; Marwaha, Arya et al. 2017).

A previous study has brought to light the role of a member of RUN and FYVE domain-containing (RUFY) protein family, Rabip4', in regulating lysosome positioning in mammalian cells(Ivan, Martinez-Sanchez et al. 2012). It was intriguing that silencing of RUFY1, a protein reported to localize to early endosomesand mediate transferrin receptor recycling, could influence the localization of lysosomes, a hallmark function of Arl8b in mammalian cells. This prompted us to investigate if Rabip4s interact with Arl8b and together theycoordinate to perform this function.

Here we find that Rabip4s interact with Arl8b via their RUN domain. Both the isoforms are identical over the length of RUN domain but have variable expression levels in different cells. Consequently, it becomes important to study the relativecontribution of both proteins on lysosome motility and trafficking in different cell types. While GTP-Arl8b colocalized with Rabip4' on LAMP1 compartments, no colocalization of Rabip4' was found with the GDP-locked mutant of Arl8b, indicating that the binding of two proteins may be dependent on nucleotide state of Arl8b.

Surprisingly, the two proteins were also found colocalized on EEA1-positive endosomes, which could be an outcome of recruitment of Arl8b by Rabip4s due to overexpression. However, a potential role of Arl8b on early endosomes cannot be ruled out completely and needs to be explored.

We created a mutant of Rabip4'where conserved basic residues implicated in binding to small GTPaseswere mutated to alanine to disrupt binding with Arl8b putatively. The resulting mutant protein, Rabip4'R206/208A, localized completely to the cytoplasm of HeLa cells and was not recruited to Arl8b compartments even upon its co-expression. However, this mutant was present on Rab4/Rab14 and EEA1-positive endosomes indicating that binding of Rabip4' to these GTPases is independent of its binding to Arl8b.It also suggests that, in addition to Arl8b, there are other mechanisms that can regulate membrane binding of Rabip4', especially its localization to early endocytic compartments. Moreover, upon Arl8b depletion, while the majority of Rabip4s relocalize to cellular cytoplasm, a small population is retained on the early endosomes which maybe Rab4/Rab14 dependent. Previous studies have implicated Rab14 as a crucial factor for Rabip4 membrane localization(Yamamoto, Koga, Katoh, Takahashi, Nakayama et al. 2010), begging the question if Arl8b acts in conjunction with Rab14 to bring about this function. By and large, it is indicative of a model whereby Rabip4s may have a plausible role in the maturation of early to lateendosomesin the endocytic pathway, and the stability of its interaction with Rab4/Rab14 maybe dependent upon the availability of their downstream interaction partner Arl8b.According to earlier studies, Rab4/Rab14 bind to the C-terminus of Rabip4s(Yamamoto, Koga et al. 2010), while it is evident from our results that Arl8b-binding site lies in the N-terminal RUN domain. Thus it can be speculated that Rabip4s may act as shared effectors of Rab and ArlGTPases thatcoordinately regulate

traffic in the endocytic pathway. In this context, it will be important to delineate the consequences of Rabip4s knockdown in regulating cargo trafficking to lysosomes. In summary, our study expands the existing repertoire of Arl8b effectors in mammalian cells. Future experiments will be instrumental in providing interesting insights into the cooperative or competitive binding of Arl8b to multiple effectors and their role in the endocytic pathway.

Chapter 6

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