



Association of RABL6 with AP3 in trafficking membrane.

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Student: Moloud Farrokgi

Supervisor: Dr. Paul Manna

Examiner: Dr. Maria Algerin

Abstract

Via the membrane trafficking system, proteins and macromolecules dispense in different pathways into cells. Moreover, it transports proteins inside and outside of cells. AP3 and LAMP1 are crucial in the biogenesis of lysosomes and sorting the cargo proteins in the trafficking membrane. The result of immunoprecipitation and mass spectrometry proteomics that Paul Manna carried out showed that the RABL6 protein has a relation to AP3. RABL6 has been known as a proto-oncogene previously, and this is the first time that RABL6 may have a function in trafficking membrane. This study aimed to find the association of RABL6 with AP3 and the function of RABL6 via the distribution of LAMP1. HeLa cell line was transfected by a plasmid containing RABL6/GFP. The cells transfected by RABL6/GFP plasmids and wild-type cells were fixed and prepared for immunostaining. After immunostaining, confocal microscopy was used to show the interaction of AP3 and RABL6 and the distribution of LAMP1. In addition, HeLa cells were knockout for RABL6 to show the function of AP3 in RABL6 knockout cells. Optimizing immunostaining, the ratio of 1:100 for RABL6 antibody with PFA fixing shows the best result. The colocalization of RABL6 and AP3 was calculated in cells transfected by RABL6/GFP plasmid and wild-type cells and interpreted the association to each other. The knockout RABL6 cells were unsuccessful, although some differences were observed in the size of the cells. The distribution of LAMP1 in wild-type cells and RABL6 cells transfected by RABL6/GFP plasmid displayed statistical differences. In overexpressed cells, LAMP1 showed more intensity. In conclusion, the RABL6 is involved in trafficking membrane with AP3 and LAMP1.

Popular scientific summary

The trafficking membrane is an essential system for cells to be alive and preserve homeostasis. With this system, cells can move and transport molecules and proteins, lipids, and other molecules from the inner cells to outer or outer cell space to the internal area of cells. It includes vesicles, lysosomes, and membrane-bound organelles (endoplasmic reticulum, Golgi and Nucleus). Many proteins are involved in the system to regulate the trafficking membrane pathway. These proteins are involved in transferring vesicles and how they reach a specific destination. Traffick membrane has different pathways that maybe start from the outer membrane of cells and attach to the lysosome for perishing the unwanted particles that enter to cells such as antigen and viruses, or can start from the ER in the secretion and neurologic cells transfer protein and other cells to external space of cells. In addition, this system also works within a cell to move molecules from one organelle to other organelles. The lack of one particle of this system often causes many problems in the human body, such as many congenital syndromes such as HPS syndrome. Understanding this system's exact mechanism and definition is challenging, accordingly, scientists divided their research differently. The most critical part of this research is to find the correlation and function of the protein involved in the system.

Furthermore, according to the preliminary experiment by Dr Paul Manna, we decided to find the association of the molecules known as adaptors and assist the vesicle in sorting in the correct part with other protein RABL6. RABL6 belongs to the GTPase family that active some protein in regulating traffic membrane. In addition, RABL6 was known as a proto-oncogene in previous studies. Therefore, this project was done for the first time to show the function of RABL6 in the trafficking membrane.

This project optimized immunofluorescent staining approaches for detecting AP3 and RABL6 in HeLa cells. Furthermore, the clear colocalization of RABL6 and AP3 suggests they may function together.

Moreover, we evaluated the distribution of LAMP1 (lysosome marker) when RABL6/GFP plasmids transfected the cells. We show a different distribution of LAMP1 in cells transfected by RABL6/GFP plasmids and wild-type cells involved in the role of RABL6 in the lysosome. Finally, the generation of a RABL6 depleted HeLa cell line for functional studies was attempted. The CRISPR/Cas9 method depletes the RABL6 in HeLa cells. The generation of RABL6 depleted HeLa cell line for functional studies failed, and the RABL6 protein could not be removed entirely in cells. The knockout was assessed by immunofluorescence.

Abbreviations

AP	Adaptor protein
Con	concentration
COPI	coat protein complex
CRISPR	clustered regularly interspaced short palindromic repeats
<i>E. coli</i>	<i>Escherichia coli</i>
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
GFP	Green fluorescent protein
HPS2	Hermansky Pudlak syndrome type 2
LAMP	Lysosomal membrane glycoprotein
LB	Lysogeny broth
LRO	Lysosome related-organelles
NK cell	Natural killer cell
ON	overnight
PBS	Phosphate-buffered saline
RABL6	RAB, member Ras oncogene family like 6
RBEL1	Rab-like protein 1
RT	room temperature
sgRNA	Single-guide RNA
SNARE	SNAP stands for soluble NSF attachment protein
TGN	trans Golgi network
TM	temperature
WDR11	WD repeat containing protein

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Introduction

Membrane trafficking

To survive, cells require a permanent current of nutrients and the removal of unwanted or used materials. The formation, fusion and trafficking of membrane vesicles adjust vital cellular activities. Particular pathways exist in the cells called membrane trafficking pathways and aid in sustaining the transportation of proteins, lipids, and solutes between subcellular compartments (Tokarev et al., 2013). In eukaryotic cells, these subcellular compartments include the endoplasmic reticulum, the Golgi apparatus, endosomes, and lysosomes (Cheung & de Vries, 2008).

These subcellular compartments have a unique function reflected in different characteristics, such as the acidic environment within lysosomes. Different compartments contain a unique mixture of proteins to maintain these different characteristics. Different membrane trafficking pathways target the suitable protein in the right compartments (Canton & Battaglia, 2012).

The subcellular pH and changing the cell cycle factors adjusted the main characteristics of subcellular trafficking. In addition, membrane trafficking is a complex phenomenon that contains several trafficking vesicles which require endocytosis, exocytosis and cellular autophagy (Abdrabou & Wang, 2018).

Membrane trafficking is divided into two flow pathways endocytosis and exocytosis. These two pathways manage the cargo flow inside or outside the cells. As well as this, they work as delivery of newly synthesized proteins to their specific places inside the cells (Watson et al., 2005). Endocytic trafficking starts when the extracellular substance is uptake by creating a budding endocytic vesicle excised from the plasma membrane. There are several types of endocytosis: Clathrin-mediated, caveolar, phagocytosis, and micropinocytosis (Li et al., 2013). The exocytosis is initiated when vesicles brought from the trans-Golgi network fuse to the plasma membrane and are promoted to secrete their cargo to the extracellular space. There are two types of exocytosis, secretory and non-secretory. The exocytosis pathway is a complicated process, and the activation of receptors, channels, pumps and transporters is involved (D'Alessandro & Meldolesi, 2019).

The trafficking of proteins and lipids is retained by balanced targeting, maintenance, and detection mechanisms. Intracellular membrane transport systems make ready proteins for their journey by glycosylation and folding. Occasionally, it unfolds the folded protein in defined stress times and controls the cell's destiny. While the complex folding process is complemented, the protein is ready to transfer to its following location by leaving the endoplasmic reticulum as vesicles, which are either small or large, with the aid of the coat protein complex II (COPII). With the assistance of COPII, the cargo attains the Golgi complex and participates in glycosylation from the cis part of the Golgi to the trans part. Afterwards, the cargo will be packed based on their content and specific membranous carriers' similar lysosomes transferred to the plasma membrane (De Matteis & Luini, 2011).

Different moving pathway of protein

The protein synthesis starts from the ribosome, and the following destinations depend on their amino acid, which includes sorting signals. The decision about the next destination of protein is whether proteins remain in the compartment or move to the following places. Sorting signals assist protein in delivering to outside the cytosol. Proteins which do not have the sorting signals remain in the cytosol. Sorting signals lead to three pathways that protein transport from one compartment to another (Bonifacino & Glick, 2004).

These pathways contain gated transport (red), transmembrane transport (blue) and vesicular transport (black). see Figure 1. In the gated transport, protein vehicle from the cytosol and nucleus and nuclear pore complexes known as selective gates in this pathway. These permit the transport of macromolecules and micromolecules.

The transmembrane transport pathway is applied in cells, for instance, the transportation of proteins from the cytosol to ER lumen or from the cytosol into the mitochondria. In the pathway, the membrane- bound protein translocator assists in the moving of the proteins.

In the third pathway, vesicular transport, molecules cargos derived from the lumen of the first compartment separate from its membrane. Then they unloaded their cargo into the next compartment via fusing to the membrane of the next compartment. Sorting signals play a crucial role in the three pathways mentioned (Alberts et al., 2002).

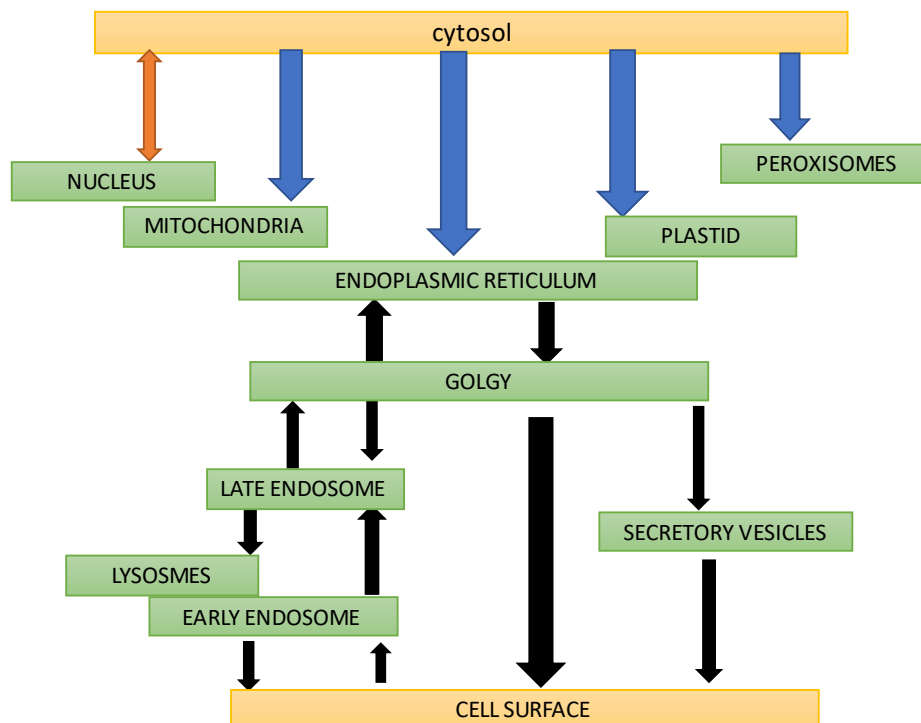


Figure1. subcellular transport pathways. The red arrow shows gated transport pathway, the blue arrows show transmembrane transport and black arrows show vesicular transport.

Control disruption of protein within cell

For the regulation of the mechanism of sorting, the intracellular membrane trafficking route is accompanied by multiple special parts of transport containing proteins like SNAREs, Rab GTPases, and Sec1 homologs, for the keeping of vesicle-docking and vascular-fusion reactions. The function of phosphatidylinositol and its phosphorylated derivatives, recognized as phosphoinositide, is introduced as second messengers in signal transduction pathways (Odorizzi et al., 2000). In addition, supramolecular complexes, known as protein coats, locate in the cytosolic part of the membrane and boost cargo sorting and formation of transport carriers in the trafficking membrane. Protein coats contain COPI, COPII, AP-1, AP-2, AP-3, AP-4, AP-5 and retromer. Depletion of these protein coats causes damage to a particular transport pathway (Dell'Angelica & Bonifacino, 2019; Robinson, 2015). The location of adaptors in transport pathway is shown in Figure 2.

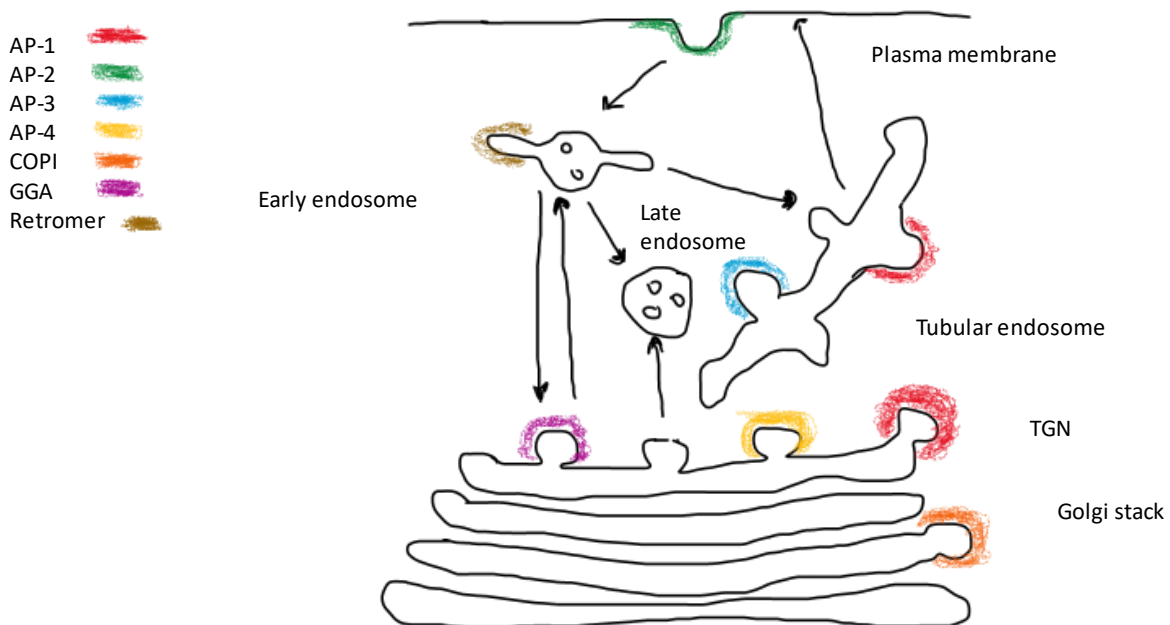


Figure 2. The location of each adaptor shown in the transport pathway. Each colour is related to a specific adaptor that displays on the left of the figure.

AP3 Trafficking, sorting signal

In transmembrane protein trafficking, AP complexes play a crucial role and carry out special sorting functions at distinct intracellular organelles. Cargo proteins in the Endocytic and secretory pathways are transported to distinct places through assembling in vesicular trafficking. In this process, AP (adaptor protein) attaches to sorting signals of cytoplasmic tails of cargo proteins, then takes on Clathrin and other accessory proteins, and collects cargo protein into vesicular carriers to carry from the donor membrane to the target organelle membrane (Park & Guo, 2014).

There are AP-1, AP-2, AP-3, AP-4 and AP-5. The AP3 complex is a hetero-tetrameric sorting adaptor containing two large subunits, called δ and $\beta 3$, a medium ($\mu 3$) and a small (σ) subunit. The medium and small subunits bind specific trans-membrane cargo proteins containing linear peptide motifs of the form Yxx Φ (Φ denotes bulky hydrophobic residues) or (D/E) xxxLL, respectively.

The structure of AP3 describes as a Micky mouse. COOH-terminal domains are similar to the head, and two large subunits protrude like ears (Heuser & Keen, 1988). AP-3 mediates cargo transport from tubular endosomes to late endosomes and is required for the biogenesis of LROs (lysosome-related organelles) (Blumstein et al., 2001). AP3 is absorbed into the endosome membrane by an interplay with the GTP-bound form of the small GTPase ARF1. AP3 acts as a sorting protein that assorts cargo proteins into forming membrane vesicles containing an AP3 coat (Ooi et al., 1997). The lack of AP complex plays a crucial role in the accuracy of transmembrane, broad cellular processes such as signal transduction, organelle dynamics, and tissue homeostasis. The physiological roles of AP3 complexes are explained through several inherited diseases and knockout mouse models. Molecular mutation of the $\beta 3A$ subunit in humans leads to HPS-2 (Hermansky-Pudlak syndrome type 2) (Dell'Angelica et al., 1999).

LROs

LROS have a variable content which contains lysosomal protein, and because of the acidic phase during their lifestyle, they need activation of ATPase in vacuolar (Dell'Angelica et al., 2000). The development of comprehension of the constitution and secretion of LROs has been greatly accelerated by the study of syndromic human genetic disorders like HPS. HPS is defined by different degrees of oculocutaneous albinism, with concomitant visual impairment and susceptibility to skin cancer, and by excessive bleeding and bruising (Wei, 2006). Mutation in 10 different genes in humans causes HPS. All of these genes encode subunits of four obligate multi-subunit protein complexes: AP-3 and the Biogenesis of Lysosome-related Organelles Complex (BLOC)-1, -2 and, -3. Disruption of the protein complexes in HPS takes part in a separate step of membrane trafficking which is required for LRO biogenesis or for additional functions in cell types that lack LROs (Wei, 2006).

RABL6

RABL6, also called RBEL1, is a conserved member of the Ras GTPase superfamily with little sequence similarity to other known Ras family members. The Ras superfamily proteins are small GTPases, and these include proteins involved in switching and controlling membrane trafficking (Smith et al., 2016). Interaction with a multiplex effector could control different levels of membrane trafficking (Jordens et al., 2005). A role for RABL6 in cell cycle progression and tumour cell proliferation has been suggested (especially in the interaction of p53) (Chen et al., 2018) though the protein is mainly uncharacterized (Montalbano et al., 2009). According to the unpublished data from a previous experiment that was carried out by Paul Manna, he identified robust association between RABL6 and AP3. The function of RABL6 in AP3 dependent sorting remains to be explored.

LAMP1

Lysosomal membrane glycoprotein (LAMP) involves the biogenesis of lysosome, autophagy and cholesterol homeostasis. There are two different Lysosomal membrane glycoproteins, LAMP1 and LAMP2. Moreover, it has a function in NK-cells cytotoxicity. It plays a role in transporting cytotoxic granules to the cell surface (Krzewski et al., 2013). It is known as the lysosome marker (Pugsley, 2017). Many diseases are due to lacking LAMP1, such as Mumps and Chediak Higashi Syndrome (Gil-Krzewska et al., 2016; Ueo et al., 2020). In previous studies, when the AP3 was depleted in mice, the LAMP1 was mis-sorted in cell surfaces. Hence, the LAMP1 up-tacking was utilized to find out the function of the AP3 pathway.

AIM

The thesis aims to find the association between RABL6 and AP3 via colocalization and LAMP1 up-taking. The aim was to categorize into five objectives.

1. Optimization of immunostaining (titration of RABL6 antibody and fixing cell solutions)
2. Assess colocalization of AP3 by RABL6 in HeLa cells transfected by RABL6/GFP plasmid
3. Assess colocalization of AP3 by RABL6 in wild type HeLa cells
4. Assess colocalization of AP3 by RABL6 in in CRISPR/Cas 9 knockout HeLa cells
5. Assess distribution of LAMP-1 in HeLa cells in cells transfected by RABL6/GFP plasmid and wild-type cells

Methods and material

Molecular and cell biological techniques were used to achieve the objectives outlines above. Below is a summary of the technical approaches to be employed for each objective.

Proteins that function together and physically interact likely share a similar subcellular distribution. In order to observe the subcellular distribution of RABL6 by light microscopy and compare it to that of AP3, it will be necessary to generate a RABL6 construct bearing a fluorescent protein fusion. This fluorescent

protein fusion construct was also used for colocalization of AP3 and LAMP1.

Fluorescent protein tagging via GFP/RABL6 plasmid

To generate the fluorescent protein fusion construct, GFP/RABL6 plasmid was constructed previously by cloning the cDNA sequence of human RABL6 into the pEGFP-C1 plasmid to create a RABL6 protein with GFP fused to its N-terminus. First, the RABL6 GFP plasmid was purified by E.Z.N.A Plasmid D.N.A. Mini kit I [omega BIO-TEK] and its concentration was measured by nanodrop. Then, the 2500 ng of RABL6/GFP plasmid was transformed into a 50 µl component of *Escherichia coli* (*E. coli* cells) (New England Biolabs) based on the manufacturer's instructions. The correct insertion of the RABL6 gene was assessed by digestion with BamHI & EcoRI endonuclease enzyme and selected bacteria via resistance to Kanamycin in L.B. agar. After transformation, *E. coli* was cultivated in L.B. agar with Kanamycin (concentration of 50 mg/mL) overnight at 37 °C and then purified by E.Z.N.A Plasmid D.N.A. Mini kit I [omega BIO-TEK]. Then the concentration of plasmid was measured by nanodrop. Finally, the 250 ng of plasmid was transfected by TransIT-HeLaMONSTER® Transfection [Mirus Bio] in HeLa cells 6-wells plate according to the manufacturer's instructions. The cells that absorbed plasmid were green under fluorescent microscopy due to GFP. These fluorescent-tagged RABL6 constructs allowed light microscopy-based localization of RABL6 and colocalization with AP3 and other endosomal marker proteins by either co-expression of further tagged constructs or antibody-based immunofluorescent approaches.

Cell culture

Due to their ease of experimental manipulation, the HeLa cell line was used. Cells was cultured in RPMI1640 (GIBCO), supplemented with 10% FBS (fetal bovine serum), 10 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Every 3 days the Hela cells was passaged to preserve cell. The protocol followed the protocol of subculturing adherent mammalian cells in Thermo Fischer website.

(Thermo Fisher Scientific-US. (n.d.). Retrieved June 16, 2022, from <https://www.thermofisher.com/se/en/home/references/gibco-cell-culture-basics/cell-culture-environment.html>)

Cells fixing

The cells that GFP/RABL6 plasmid inserted were seeded onto coverslips (10 mm No. 1.5). At first, cells from the 6-wells plate were lysed with 10% trypsin and diluted by RPMI1640, coverslips were placed in a new 6-wells plate, and lysis cells in media were added to coverslips and cultivated overnight at 37 °C in a humidified atmosphere with 5% CO₂. Finally, cells on coverslips were fixed using either 4% paraformaldehyde or methanol in ice for 10 minutes.

Immunofluorescent staining

After fixing, coverslips were rinsed twice with 1X PBS (Phosphate-buffered saline). Next, the triton x-100 20% solution in FBS (permeabilized solution) was added to coverslips and incubated at room temperature for 1 hour. Afterwards, a primary antibody with different concentrations according to the experiments was diluted by the triton x-100 20% solution in FBS and incubated at various times (one hour or overnight) at different temperatures and added to coverslips. Subsequently, coverslips were rinsed 2times by PBS. Then secondary antibodies diluted by the triton x-100 20% solution in FBS were added to coverslips and incubated for one hour at room temperature. Following fixation, coverslips were mounted in ProLong Gold mounting media containing DAPI (Molecular Probes, Invitrogen) for experiments solely involving ectopically expressed fluorescent protein fusion constructs. Imaging samples were carried out using laser scanning confocal microscopy (Zeiss LSM700). Moreover, subsequent data analysis was performed in FIJI software (NIH) with plugin JACOB. All the primary and secondary antibodies and their concentrations are mentioned in Tables 1 and 2.

Optimization of colocalization of RABL6 and AP3

Several experiments were designed to show the best result for colocalizing AP3 and RABL6 and repeat the experiment so that the results were not accidental. The data about the modification of experiments are mentioned in table 1. This information contained the purpose of experiments, primary antibodies concentration, time and temperature incubation, secondary antibodies, cell phenotype and fixing solution. The concentration, temperature, and time incubation for secondary antibodies in all experiments were the same. Other information on primary and secondary antibodies is written in tables 2 and 3.

Table 1. concentration and purpose of using each antibody

Experiment purpose	Fixing solution	Primary antibody for RABL6				Primary antibody for AP3, con, time, TM	Secondary antibodies, con, time, TM
		Name	con	Time	TM		
Optimization of RABL6 concentration	PFA	C9orf86	1:100	1(h)	RT	SA4/1:100/1(h)/RT	Alexa
			1:500				647/568/1:500/
			1:100				1(h)/RT
Checking other fixing solution (methanol)	Methanol	C9orf86	1:100	1(h)	RT	SA4/1:100/1(h)/RT	Alexa647/568/1:500/1(h)/RT

Colocalization of RABL6 with AP3 in wild type cells and transfected cells by RABL6/GFP plasmid	PFA	C9orf86	1:100	ON	F	SH2/1:100/ON/F	Alexa 647/568/1:500/ 1(h)/RT
Knockout cells for RABL6	PFA	C9orf86	1:100	ON	F	SH2/1:100/ON/F	Alexa 647/568/1:500/ 1(h)/RT

The meaning of abbreviations that were used in the table one was: ON: overnight, RT: room temperature, h: hour, Con: concentration, TM: temperature, F: fridge.

Table 2. characteristics of primary antibodies.

Target	Name /Company	Host species	Dilution of using
LAMP1	H4A3/ BD Biosciences	mouse	1:1000
AP3	SA4/DSHb	mouse	1:100
AP3	SH2/ Atlas Antibodies	Rabbit	1:100
RABL6	C9orf86 Polyclonal antibody/proteintech	Rabbit	1:50/1:100/1:200/1:500 1:1000

Table 3. Characteristics of secondary antibodies.

Conjugation	Target species	Host species	Company	Dilution of using
Alexa Fluor 568	Rabbit	Donkey	Thermo Fisher Scientific	1:500
Alexa Fluor 647	mouse	Donkey	Thermo Fisher Scientific	1:500
Alexa Fluor 488	mouse	Donkey	Thermo Fisher Scientific	1:500

CRISPR/Cas9 knockout

For CRISPR/Cas9 mediated knockout generation the PX330 system was used (Ran et al., 2013). Specific sgRNAs targeting RABL6 exons was selected by using CRISPick (Crispick. Retrieved June 16, 2022, from <https://portals.broadinstitute.org/gppx/crispick/public>).

These sgRNAs were incorporated into oligonucleotides bearing compatible overhangs for cloning into BbsI digested PX330. PX330 constructs harbouring sgRNAs targeting various exons of RABL6 were transfected into cells as described below alongside another plasmid encoding an antibiotic Puromycin resistance cassette (5:1 PX330: resistance cassette vector ratio). The method from the digest of PX330 until transfected HeLa cell was done according to the Zhang lab (Ran et al., 2013). The five times more PX330 plasmid than the Puromycin plasmid was utilized and transfected by Mirus TransIT-HeLaMONSTER Transfection kit [Mirus Bio] according to its protocols. For making a selective culture medium, the concentration of 10 U/ml of Puromycin was added to RPMI1640. The antibiotic Puromycin selected the Positive selective transfectants. The cells containing transfected Puromycin plasmid survived in media with Puromycin, although cells without Puromycin plasmid were eliminated in media. In this way, the cells containing Puromycin antibiotics were selected. Then, the selection of colonies transfers to the T25 flask to grow more for checking knockout.

Functional assays via up-tacking LAMP1

The effects of RABL6 on the subcellular distribution for LAMP1 were assessed by fluorescence-based microscopy. Furthermore, it is known that inhibition of AP3 function leads to a redistribution of the lysosomal protein LAMP1. Therefore, the function of the RABL6 pathway can be assessed using the uptake of an antibody targeting the luminal/extracellular domain of LAMP1 by live cells.

Assessment of LAMP1 distribution in transfected RABL6/GFP cells and non-transfected RABL6/GFP cells

At first, the HeLa cell was transfected by RABL6/GFP plasmid (that explained above the process of transfection) and treated by LAMP1 antibody with the ratio of 1:1000 (1 µl of Anti-LAMP-1 Antibody (H4A3) was diluted by 1 ml of Opti-MEM™ I Reduced Serum) for three hours at 37 °C. Then, fixing cells by PFA was done according to the protocol explained before and prepared for immune staining. Afterwards, the anti RABL6 (C9orf86 Polyclonal Antibody) was added in overnight incubation at room temperature. Finally, Donkey anti- Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor plus 647 as secondary antibody for LAMP1 and the Alexa Fluor™ 568 donkey anti-rabbit IgG (H+L) was added for RABL6 on coverslips for 1-hour incubation in room temperature. The T-test was utilized to confirm the distribution statistically different in transfected cells by RABL6/GFP plasmid and non-transfected cells by RABL6/GFP plasmid.

Result

Optimization of Anti RABL6

Due to the first immunostaining of RABL6, three different ratios of concentration of RABL6 antibody were selected, which contained: 1:100, 1:500 and 1:1000. The best ratio was 1:100 because the channel of green (RABL6) was more noticeable and brightest (Figure 3). Moreover, the red channel (AP3) was bright, and two channels of red and green were distinguished in the merge channel area in yellow (colocalization of AP3 and RABL6).

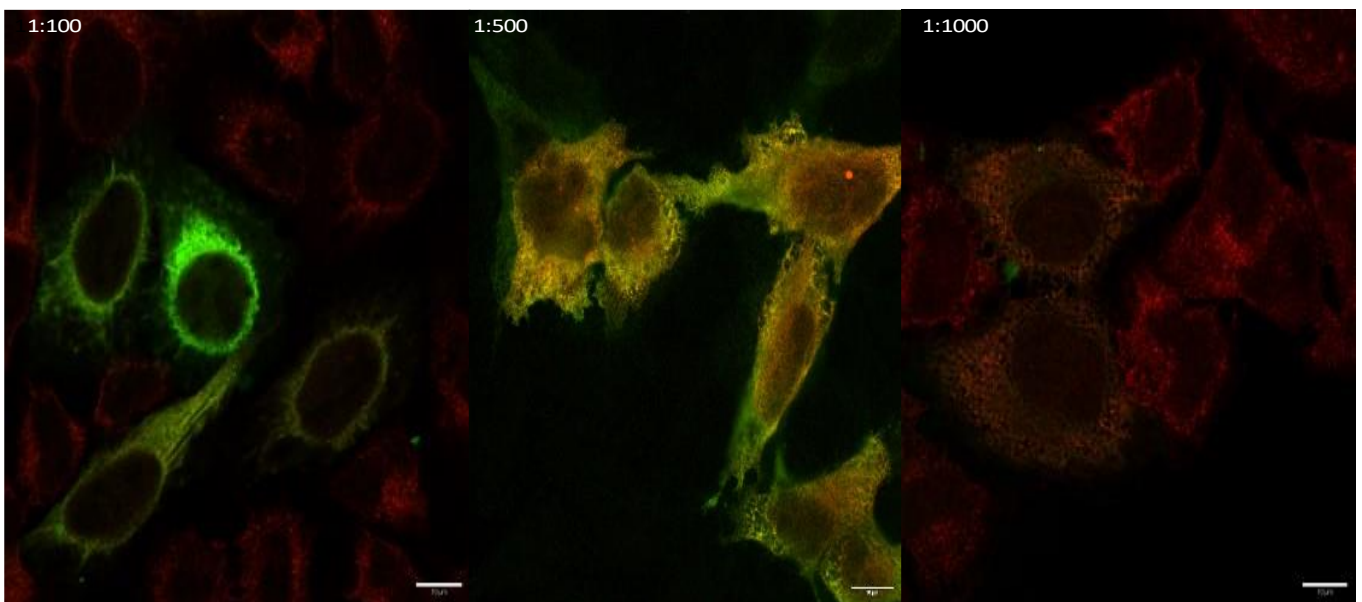


Figure 3. RABL6 antibody titration showing three different concentrations of antibody against RABL6. The green colour shows RABL6/GFP; the red colour shows AP3, and the area where two channels were mixed, shows colocalization of RABL6 and AP3 (yellow colour). The scale bar shows 10 μ m.

Checking other fixing solution (methanol)

Methanol was selected as the second candidate to find a better fixing solution. HeLa cells were transfected by RABL6/GFP plasmid, and prepared for immune staining via methanol. The optimal antibody ratio for RABL6 was added as in the previous experiment. In addition, the same ratio of antibodies for AP3 was also used. Image shows that the cells were fixed by methanol were not sharpest, brightness in channel red (AP3) and also channel green (RABL6) (Figure 4). In addition, it seemed the spots mixed, and it was hard to detect a particular spot.

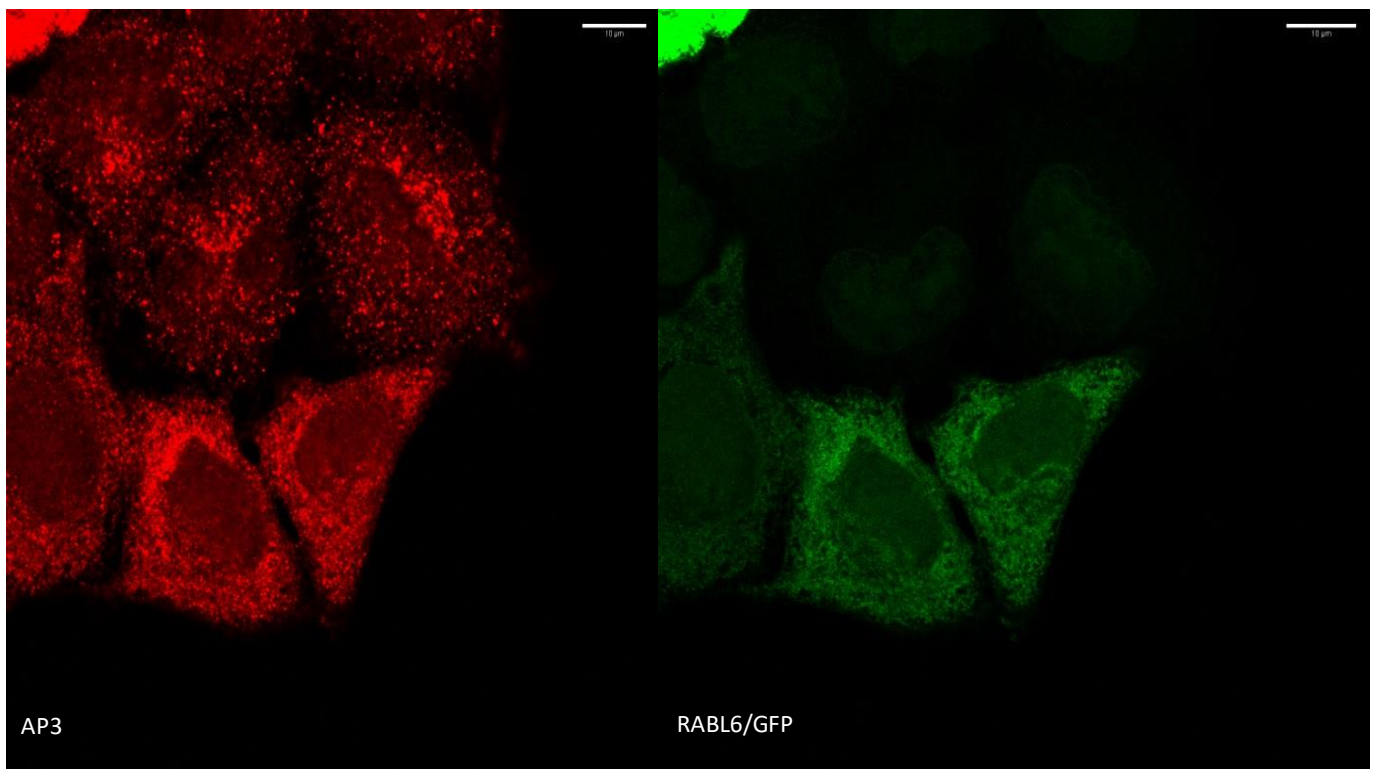
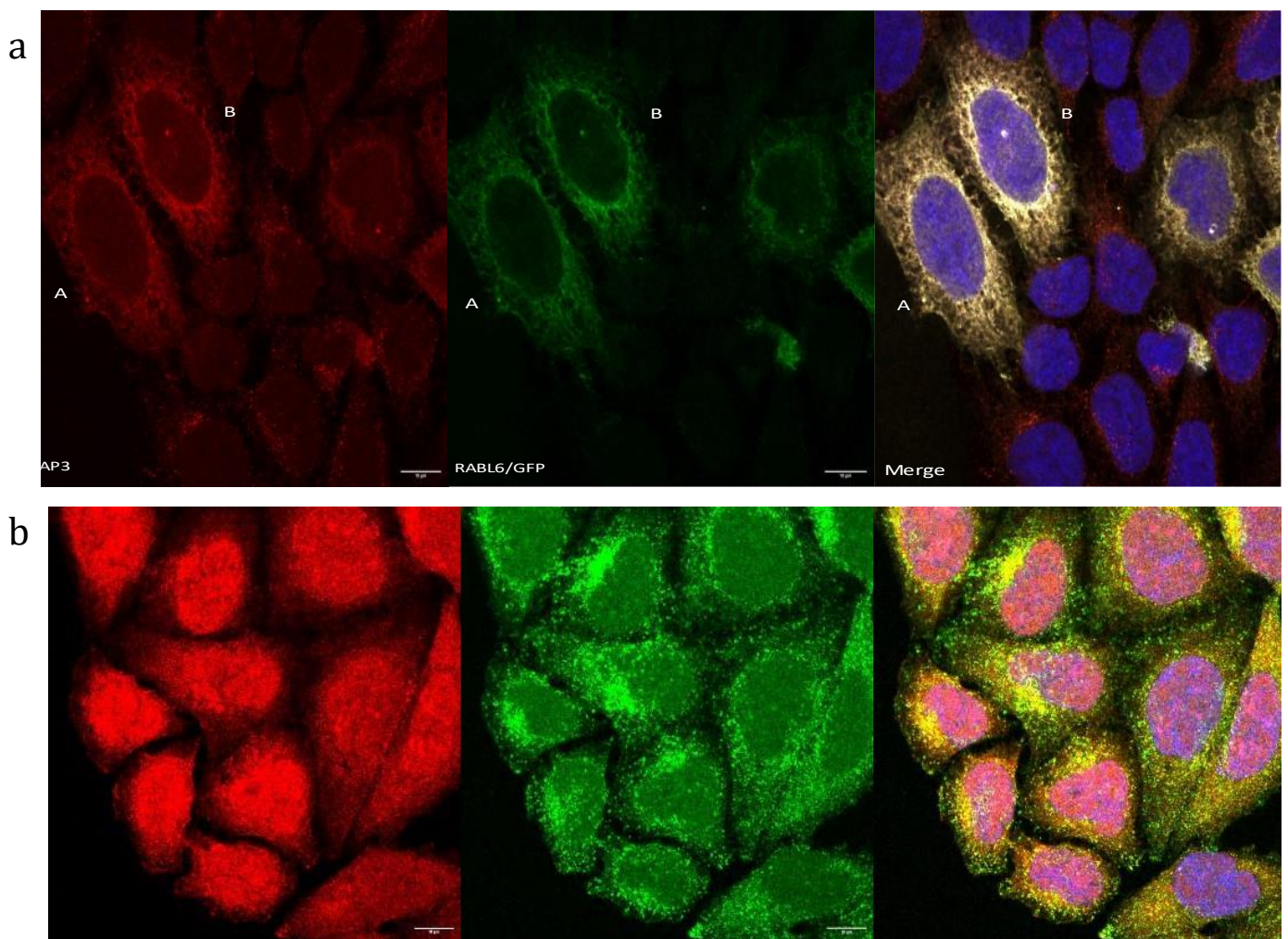


Figure 4. Methanol fixing. HeLa cells were transfected by RABL6/GFP plasmid, which were fixed by methanol. The AP3 showed in red, RABL6/GFP in green. The scale bar shows 10 μm .

Colocalization of RABL6 and AP3 in transfected cells by RABL6/GFP plasmid and wild type cells

The colocalizations between RABL6 and AP3 were calculated in transfected cells by RABL6/GFP plasmid. Colocalization assists in showing the possibility of the function of RABL6 and AP3 in the vesicles. The Pearson correlation coefficient was applied to show the colocalization between proteins. The α Pearson correlation coefficient was 0.810 in cells A and B (the cells transfected by RABL6) (Figure 5a). The mean value of the α Pearson coefficient was 0.600 in wild-type cells (Figure 5 B). Two-channels red (AP3) and green (RABL6), were shown separately. In the merged image, area of colocalization was yellow (Figures 5a and b). The two types of cells, wild-type and transfected by RABL6/GFP plasmid, showed the colocalization between RABL6 and AP3 (Figures 5 a and b).



Figures 5 a and b. (a)The colocalization of RABL6 with AP3 in the cells transfected by RABL6. A and B are the cells transfected by RABL6. AP3 was in red, RABL6/GFP was in green, and nuclei were blue. In the merged image, the colocalization shows by yellow light. (b) The colocalization of RABL6 and AP3 in wild type cells. The colours of AP3 and RABL6 were red and green, respectively. In the merged image, the colocalization shows by yellow light.

Knockout of RABL6 in HeLa cells

CRISPR-Cas9 plasmid (PX330) was transfected to HeLa cells to eliminate (knockout) RABL6 protein. If the RABL6 was knockout, the channel green should not exist in the image. The knockout of RABL6 was unsuccessful because the RABL6 antibody distinguished RABL6 (Figure 6). The colocalization was measured, and the α Pearson coefficient was equal to 0.08. The α Pearson coefficient was the same as wild-type cells. However, the size of the cell and pattern of distribution of AP3 and RABL6 was different in Knockout cells. The spindle shape of wild-type cells on the right of image changed to a round shape, and the knockout cells were smaller than wild-type cells (Figure 6). The blue circles show the difference around the selected cell (Figure 6).

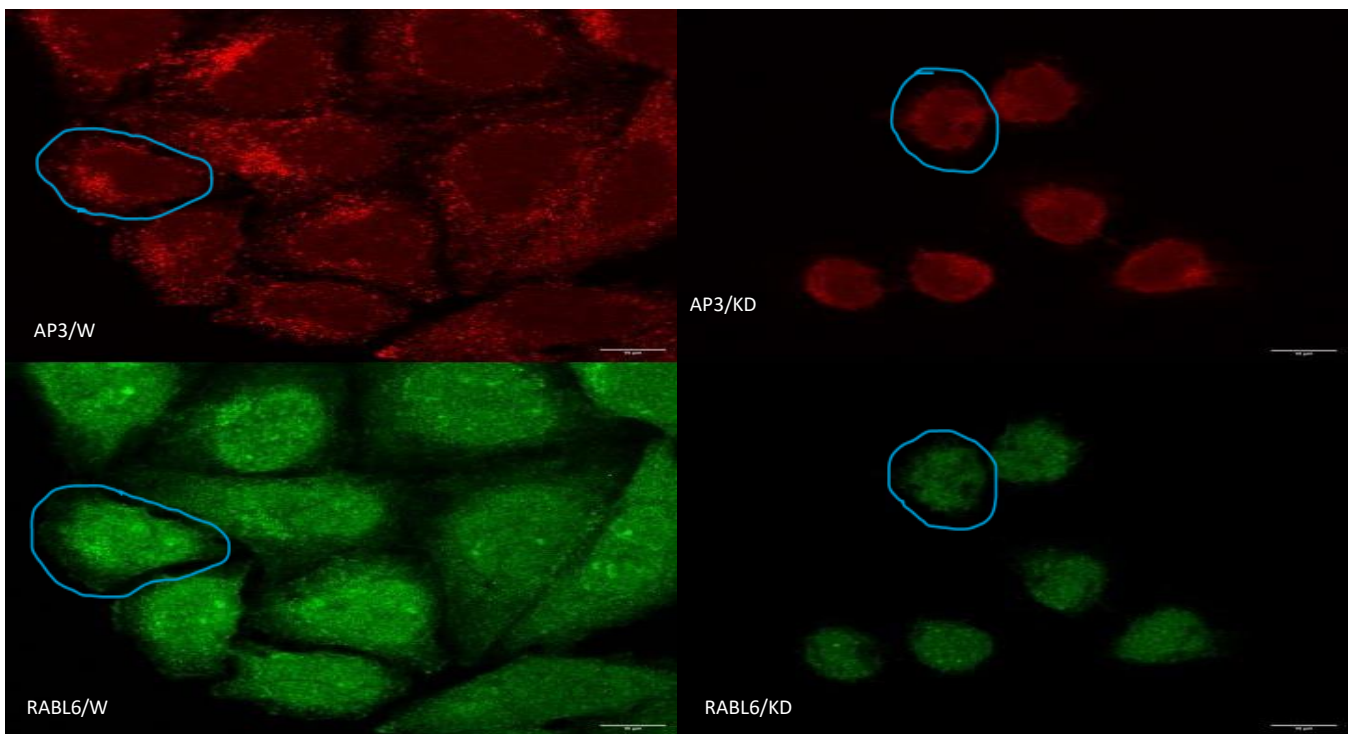


Figure 6. The difference of AP3 and RABL6 in wild type cells and CRISPR-Cas 9 knockout cells. W: wild type, KD: knockout. The scale bar shows 10 μ m. AP3 was in the red and RABL6/GFP was in green. The blue circles show around the cells.

LAMP1 distribution in transfected cells by RABL6/GFP plasmid and wild type cells

The difference in LAMP1 distribution in transfected cells by RABL6/GFP plasmid and wild-type cells was observed after treating cells with LAMP1 antibody (H4A3). The images showed the distribution of LAMP1 in transfected cells by RABL6/GFP plasmid and wild-type cells. Red particles (LAMP1) were brighter and denser in transfected cells by RABL6/GFP plasmid (Figure 7). The yellow arrows mentioned wild-type cells, and the blue arrows mentioned transfected cells by RABL6/GFP plasmid. Besides, the mean distribution value in transfected cells by RABL6/GFP was 466.9 with SD 298.10, while the non-overexpressed cells were 295.9 with SD 85.30. The t-test was done, and the p-value was 0.03. Hence there was a statistical significance between the distribution of LAMP-1 in transfected cells by RABL6/GFP plasmid and wild-type.

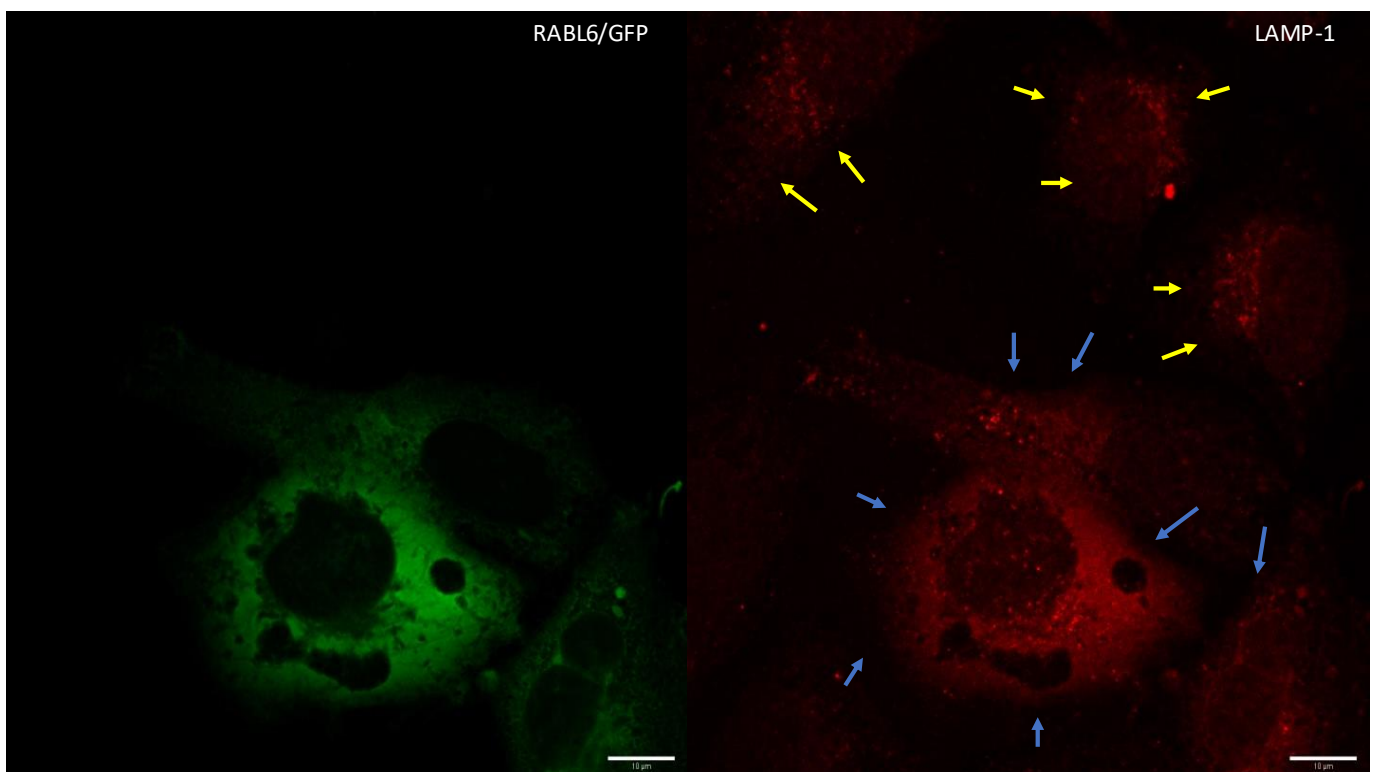


Figure 7. LAMP1 distribution. The LAMP1 distribution is in red, and RABL6/GFP is in green. Yellow arrows show the cells not transfected by RABL6 /GFP plasmid. The blue arrows show which transfected by RABL6 /GFP plasmid.

Discussion

Several studies use different optimization methods that contain maximum or minimum study parameters. The methods include minimizing cost and primary materials to obtain better results (Alonso et al., 2020). According to the recommendations of companies that produced the antibody for RABL6, the best ratio was 1:50. However, the mentioned ratio was general and different for the experiment. The antibodies have other applications, such as FACS and western blot; therefore, different proportions of antibodies are required for better results in each technique. In addition, because the transfected cells were used, the density of RABL6 increased in the cells that RABL6 transfected. When the concentration of protein increases, it causes to lack of recognition of protein by antibodies. The ratios less than 1:50 were used to optimize the concentration of the RABL6 antibody. Therefore, three ratios of 1:100, 1:500 and 1:1000 were selected. The result shows that the best concentration was 1:100. In this ratio, two channels of red (AP3) and green (RABL6) were completely obvious with different colours (Figure 3). In the ratio of 1:500, the channel of red was not prominent. In the ratio of 1:1000, the channel of green was not noticeable (Figure 3).

We expected that with the decrease of concentration of RABL6 antibody, the channel green showed less colour. Therefore, the results showed a declining trend. The protocol for titration of antibodies has followed the study of Penault-Llorca et al. (1994) used different ratios of antibodies to optimize the immunohistochemical for detection of ERBB2 in breast cancer (Penault-Llorca et al., 1994).

The second fixing solution, freezing methanol, was used in the experiment. Using methanol is the next step to optimizing immunostaining. The result shows that the channels of red (AP3) and green (RABL6/GFP) were visible (Figure 4). In addition, the AP3 (red) particles and RABL6 (green) spread out, and the distinction between particles seemed hard in two channels, red and green. It causes an overestimation of the calculation of colocalization of RABL6 and AP3. The colocalization was measured based on the exact localization of the protein in different colours. When the colour particles spread out, it is brought out overestimated colocalization. According to the study of Hagedorn et al. (2006), methanol demonstrated a better result than PFA since methanol amends the quality and construction of antibodies during fixing and immunostaining. In addition, methanol preserves the membrane from damage (Hagedorn et al., 2006). Our result was in agreement with the results shown by Hagedorn et al. (2006). The spreading colour channel of AP3 (red) and RABL6 (green) brings from that ultracold methanol caused hexagonal ice crystals in cells (Pegg, 2010).

The colocalization of RABL6 and AP3 cells transfected by RABL6/GFP plasmid was observed (Figure 5 a). The α Pearson coefficient was 0.810, which shows the association of RABL6 and AP3. However, the colocalization of RABL6 and AP3 in wild-type cells showed the fewer value of the Pearson coefficient (0.600) (Figure 5 b). The increase of α Pearson coefficients was due to an increase in RABL6 in cells transfected by RABL6/GFP plasmid.

The colocalization of AP3 and RABL6 transfected by RABL6/GFP plasmid and wild-type cells shows that these two proteins function in vesicles (Figures 5a and 5b). Since AP3 is a sorting protein involved in the biogenesis of lysosomes and carrier of vesicles, it showed that the RABL6 for transport in cells might require the

accompany of AP3. The study by Valencia et al. (2006); that was carried out about the role of adaptors in sorting Pmel17 in melanosomes displayed that AP3 did not involve in sorting Pmel17. However, other adaptors (AP1 and AP2) play a role in sorting (Valencia et al., 2006). To compare the study of Valencia, it seems each protein requires different adaptors. More experiments need to show that the other adaptors play a role in sorting RABL6.

In addition, the RABL6 has similarities to the sequence of the RAS GTPase family, the family involved in vesicle budding, vesicle delivery, vesicle binding, and fusion of the vesicle membrane. Therefore, the interaction of RABL6 and AP3 may prove the role of RABL6 in vesicle delivery like the RAS GTPase family (Jordens et al., 2005).

Furthermore, Kohlmeyer et al. (2021) showed that RABL6 works as a protooncogene that involves the proliferation of cancer cells (Kohlmeyer et al., 2021). Based on the result, via regulation of AP3 could make alternation expression of RABL6 in cells and observe the modification in cancer cells. However, more experiments were required to find out the exact role of AP3 against RABL6.

The association of RABL6 and AP3 may answer these questions about whether the AP3 might be involved in sorting RABL6 or the new role of RABL6 is recruitment in the trafficking membrane. At first, WDR11 (WD Repeat-Containing Protein), a tumour suppressor like RABL6, was known as a protooncogene. The function of WDR11 was unknown in vesicle transport until it was found that WDR11 was associated with adaptor AP1 in budding vesicles (Navarro et al., 2018). Therefore, RABL6 and AP3 may show the same function as each, such as WDR11 and AP1. Other studies find out the places of WDR11 in Nucleus autophagosome and TGN (trans-Golgi network) (Bassik et al., 2013; Kim et al., 2010; Taylor & Mossman, 2015). Our results showed that the RABL6 exist around the nuclei and transmembrane. Therefore, it may be such as AP3 involve in the movement of protein from Golgi to transmembrane or vice versa.

The next step was that when the RABL6 knockout occurred, what modification would happen for AP3. When RABL6 is knockout, if the distribution of AP3 changes, it will help to interpret the role of RABL6 in the trafficking pathway. We expected that in knockout cells, the RABL6 antibody could not recognize the RABL6. However, RABL6 was detected by the RABL6 antibody. Despite detecting RABL6, the shape and size of cells and nucleus were different in knockout cells (Figure 6). The size of the cells was more minor than wild-type cells. The sequencing also was demanded to show whether knockout occurred or not. It seemed semi knockout of RABL6 happened because the RABL6 antibody detected RABL6 (green), although the distribution of RABL6 was different from wild-type cells. The knockout of genes falls out in other genes because of an error in selecting sgRNA (Ran et al., 2013) and in general, CRISPR-Cas9 technology is prone to error (Gupta & Musunuru, 2014).

There are some rules for selected sgRNA: avoid select target site near the N-terminus of protein aiming to reduce the cell's capability to apply an alternative ATG downstream of the annotated start codon.

Moreover, target sites close to the C terminus of the protein were avoided to increase the chances of creating a non-functional allele. Selecting gRNAs for an experiment requires balancing reducing on-target activity while

reducing off-target activity. It is obvious, but it is often a difficult decision. For example, it is better to use a less-active sgRNA that targets an exactly unique site in the genome or a more-active sgRNA with one additional target site in a region of the genome with an unknown function (Ran et al., 2013).

Besides, the HeLa cells are cancer cells, and genes often duplicate in cancer cells (Glenfield & Innan, 2021). In addition, increasing the number of genes causes an increase in the amount of mRNA, so the sgRNA may not bind perfectly to its target mRNA.

In all previous experiments, the purposes were optimizing the ratio of antibodies and selecting the solution for fixing cells, colocalization and knockout. The following experiment investigated the distribution of LAMP1 in transfected by RABL6/GFP plasmid and wild-type cells. After the observation, the differences in the distribution were calculated by T-test. It shows the significance statistic differences between the two phenotypes. LAMP1 is a glycoprotein in Lysozyme and accompany by AP3 involved in the biogenesis of Lysozyme. The LAMP1 was used to show whether the proteins interact with LAMP1 in the Lysozyme. The increase in the distribution of LAMP1 in cells transfected by RABL6/GFP suggested that RABL6 is involved in the lysosomal pathway by AP3 and LAMP1.

Some studies show that with depletion of AP3, LAMP1 accumulate in cells. Because the AP3 possesses the sorting function and lysosomes in depletion, AP3 cells lose their next destination (Jones et al., 2013).

To figure out the complete function of RABL6 in the lysosomal pathway, the depletion of RABL6 cells was required. Because of the Unreliable RABL6 knockout in the previous experiment, the LAMP1 uptake was not performed in Knockout RABL6 cells.

Future prospective

In each step, some problems occurred that caused to make unpredictable results. Moreover, more methods were required to show more precise results. Therefore, to improve future experiments' results, we recommend some suggestions. The first one, using other adaptors like AP1,2,4 COPI and II, shows whether they are involved in RABL6 sorting. The second suggestion is that other cell lines can be used. Because to improve the result, whether the RABL6 function is the same in the HeLa cells. In addition, we estimate that since the HeLa cells are cancer cells, it causes the problem in the knockout of RABL6. If Non-cancerous cell lines were used, it would cause decreasing prone to knockout of RABL6. The third suggestion is that other techniques was used for knockout of RABL6, such as siRNA and CRISPR cas9 primer editing. Since these techniques are more reliable and precise than CRISPR-Cas9.

The final suggestion is that other imaging techniques such as immunogold will be used. Because the method is precise and it may help to show the exact location of RABL6. It results from the more reliable function of RABL6. In addition, where exactly is RABL6 involved in the trafficking membrane, exocytosis, lysozyme and the endosome. All the suggestions mentioned above can assist in showing the function of RABL6, although the factor of time and cost should not forget.

Ethical aspects

The proposed project does not require the use of animals or human subjects; therefore, no special ethical approvals are needed for this study. This project used the CRISPR system to detect an association between AP3 and RABL6. This study assesses what happens in the cell for these proteins when they would knockout out by CRISPR. Lack of AP3 causes the HPS syndrome. If the association between AP3 and RABL6 is found, it can help scientists determine whether RABL6 is involved in the cause of HPS syndrome. In addition, it would help the treatment of HPS syndrome in the future, and it may prevent the development of the syndrome via genetic modification by the CRISPR system in the embryo.

The frequency of this syndrome is 1 in 500,000 to 1,000,000 individuals worldwide; therefore, it is a rare syndrome. However, individuals affected by the syndrome suffer severe diseases such as albinism, skin damage, and scar tissue in the lung. The aim of the project is not the usage of CRISPR for the treatment of adults and embryos but if we think more deeply and assume that this interaction would be found. The gene-editing of embryos for HPS syndrome may be used. The use of CRISPR is a risky treatment; it may cause some problems in the future. The CRISPR technology does not have 100 per cent efficiency, so parents who probably have this gene must be informed about the risk of gene editing. In addition, one of the essential risks of CRISPR is causing off-target mutations; thus, the role of using sequencing is necessary to prevent occurring other mutations. Therefore, it is essential when we intend to use gene editing. We would use gene editing for purposes that have no other alternatives for treatment. For example, in some cases, such as preventing HIV infection in embryos, other ways can be used to hinder that instead of using gene editing. The HPS syndrome has an autosomal recessive pattern. Thus, there are other ways to prevent the birth of infants who carry the syndrome, such as genetic testing and counselling.

In conclusion, it is imperative to look at the cost of treatment and who is affected and compare it to the priority of other treatments with less risk and cost. Besides, our research can help to find out the function of RABL6 in the trafficking membrane system. Each problem in this system causes a problem in cells and the human body. This problem causes many human diseases, such as HPS, neurodegeneration disorder, and cancer. Therefore, by finding a new candidate (RABL6), the trafficking membrane understands more and can help treat diseases the trafficking membrane involves.

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