Amino acids stimulate the endosome-to-golgi trafficking through ragulator and small GTPase Arl5

Chen, Bing

2018

Chen, B. (2018). Amino acids stimulate the endosome-to-golgi trafficking through ragulator and small GTPase Arl5. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73545>

<https://doi.org/10.32657/10356/73545>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**AMINO ACIDS STIMULATE THE ENDOSOME-TO-
GOLGI TRAFFICKING THROUGH RAGULATOR AND
SMALL GTPASE ARL5**

CHEN BING

School of Biological Sciences

2017

**AMINO ACIDS STIMULATE THE ENDOSOME-TO-
GOLGI TRAFFICKING THROUGH RAGULATOR AND
SMALL GTPASE ARL5**

CHEN BING

School of Biological Sciences

**A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of
Doctor of Philosophy**

2017

Acknowledgement

First and foremost, my heartfelt thanks and sincere gratitude to my supervisor, Dr. Lu Lei for his valuable guidance, help, timely advice and continuous encouragement all these years. I appreciate the candor and the many sessions of in-depth discussions which have sharpened my thought process. His dedication and enthusiasm for scientific research, his knowledge which is both broad-based and focused, have always been a source of inspiration.

I would like to express my sincere thanks to my thesis advisory committee members, Dr. Koh Cheng Gee and Dr. Wong, Siew Peng Esther, for their valuable suggestions during my student annual academic meetings.

I would like to express my sincere gratitude to all my lab members, in particular, Dr. Boh Boon Kim, Dr. Shi Meng, Dr. Madugula Venkata Satya Uma Viswanadh, Dr. Divyanshu Mahajan, Tie Hieng Chiong and Sun Xiuping, for their encouragement and kindly help for my experiments. I thank them for providing a positive environment for research.

I am highly indebted to my mom and sisters for their continuous support and invariable love. Without their understanding and encouragement, this work cannot be done.

Last but not least, I'd like to thank all my friends for their constant support and encouragement.

The financial supports by the way of Research Scholarship from the Nanyang Technological University (NTU) are greatly acknowledged.

Table of contents

Acknowledgement	i
Table of contents.....	i
Abbreviations:.....	v
List of figures.....	viii
List of tables.....	xi
Abstract.....	1
1. Introduction.....	3
1.1 Nutrient-dependent signaling and pathways in eukaryotes	3
1.1.1 The mTORC1 signaling pathway	3
1.1.2 AA-regulated intracellular membrane trafficking	6
1.2 Intracellular membrane trafficking in eukaryotes.....	9
1.2.1 The endocytic pathways in mammalian cells	10
1.2.2 The endocytic retrograde pathways leading to the Golgi	10
1.2.3 The molecular machinery of endosome-to-Golgi trafficking pathway.....	14
1.2.4 Physiological importance of endosome-to-Golgi trafficking	18
1.3 Overview of Arl GTPases.....	19
1.3.1 Ras family small GTPases	19
1.3.2 Arf family GTPases	22
2. Objectives	28
3. Materials and Methods.....	29
3.1 Constructs	29
3.1.1 Arl5 constructs	29
3.1.2 Lamtor1 constructs.....	32
3.1.3 CD8a-reporter chimeras constructs.....	33

3.1.4 Lentivirus constructs	33
3.1.5 Other related constructs	35
3.2 Antibodies	35
3.2.1 Antibodies used in this study	35
3.2.2 Generation of polyclonal antibodies against human Arl5b.....	36
3.3 Main reagents.....	37
3.3.1 siRNA	37
3.3.2 Nutrient starvation and stimulation medium.....	38
3.3.3 Small molecules, drugs, antibiotics and chemicals.....	38
3.4 Cell culture, transfection, lentiviral production and transduction.....	39
3.4.1 Cell culture and transfection	39
3.4.2 Lentiviral production and transduction.....	39
3.5 Immunofluorescence.....	40
3.6 Fluorescence microscopy	40
3.7 Nutrient starvation and stimulation of cells	41
3.8 Internalization transport assay	41
3.8.1 CD8a-furin and CD8a-CI-M6PR internalization assay	41
3.8.2 Internalization transport assay under nutrient starvation and stimulation conditions.....	41
3.9 Western blot	42
3.10 Immunoprecipitation.....	42
3.11 Guanine nucleotide exchange of GST-Arl5b and GDT-Arl1	42
3.12 RT-qPCR.....	43
3.12.1 RNA extraction by Trizol	43
3.12.2 Reverse transcription	43

3.12.3 Quantitative reverse transcription PCR	44
3.13 Quantification method used for trafficking assay	45
4. Results.....	47
Chapter 1: AAs regulate the endosome-to-Golgi trafficking pathway	47
4.1 Characterization of CD8a-chimera reporters used in this study	47
4.2 Starvation reversibly induces the translocation of TGN membrane proteins to the endosomal pool	55
4.2.1 Nutrient starvation changes the subcellular distribution of TGN membrane proteins.....	55
4.2.2 Furin mainly localizes in the endosomal pool under nutrient starvation conditions	57
4.2.3 The effects of nutrient starvation on TGN membrane protein localization are reversible.....	61
4.3 Nutrients stimulate the endosome-to-Golgi trafficking	63
4.3.1 Nutrients stimulate the PM-to-Golgi trafficking.....	64
4.3.2 Nutrients stimulate the endosome-to-Golgi trafficking	69
4.4 AAs, especially glutamine, stimulate the endosome-to-Golgi trafficking.....	73
4.4.1 AAs but not glucose or growth factors, stimulate the endosome-to-Golgi trafficking.....	73
4.4.2 The effect of AAs on the endosome-to-Golgi trafficking is probably ubiquitous	74
4.4.3 Glutamine has the most acute effect in stimulating endosome-to-Golgi trafficking.....	76
4.4.4 The effect of AAs on stimulating the endosome-to-Golgi trafficking is not additive.....	79
Chapter 2: The AA-stimulated endosome-to-Golgi trafficking depends on v-ATPase, SLC38A9 and Ragulator but not Rag GTPases and mTORC1	82

4.5 v-ATPase is essential for AA-stimulated endosome-to-Golgi trafficking.....	82
4.6 SLC38A9 is required for the AA-stimulated endosome-to-Golgi trafficking	84
4.7 The Ragulator complex but not Rag GTPases depletion decreases the AA-stimulated endosome-to-Golgi trafficking pathway	87
4.8 AA-stimulated endosome-to-Golgi trafficking pathway is independent of mTORC1 activity.....	90
Chapter 3: Arl5b and its effector, GARP, are essential for the AA-stimulated endosome-to-Golgi trafficking	92
4.9 Arl5b interacts with Lamtor1	92
4.10 Arl5a and Arl5b are the two major paralogs of Arl5	94
4.11 Characterization of Arl5b antibody	96
4.12 Arl5a and Arl5b localize to the <i>trans</i> -Golgi	97
4.13 Arl5b colocalizes with Lamtor1 at the endosome and lysosome.....	100
4.14 Depletion of Arl5 decreases the endosome-to-Golgi trafficking of CD8a-furin .	102
4.15 Arl5b is essential for AA-stimulated endosome-to-Golgi trafficking	106
4.16 GARP is involved in the AA-stimulated endosome-to-Golgi trafficking pathway	109
5. Discussion	113
5.1 AAs stimulate the endosome-to-Golgi trafficking in mammalian cells	113
5.2 v-ATPase, SLC38A9 and Ragulator are required for AA-stimulated endosome-to-Golgi trafficking.....	115
5.3 AA-stimulated endosome-to-Golgi trafficking depends on Arl5b and its effector GARP	116
6. Bibliography	119

Abbreviations:

AAs	amino acids
AF	Alexa Fluor
AD	Alzheimer's disease
AP	adaptor proteins
APP	Amyloid precursor protein
Arf	ADP ribosylation factor
Arl	ADP ribosylation factor-like proteins
CD8a	cluster of differentiation 8a
CD-M6PR	cation dependent-Mannose 6 phosphate receptor
CI-M6PR	cation independent-Mannose 6 phosphate receptor
ConA	concanamycin A
DMEM	Dulbecco's modified Eagle medium
DMP	dimethyl pimelidate
DTT	dithiothreitol
EE	early endosome
EEA1	EE antigen 1
ER	endoplasmic reticulum
FBS	fetal bovine serum
GAP	GTPase activating protein
GARP	Golgi-associated retrograde protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GRIP	Golgin97, RanBP2 α , Imh1p and p230/Golgin245
GST	Glutathione S-transferase
GTP	guanosine triphosphate
HBSS	Hanks' balanced salt solution
HeLa	Henrietta Lacks
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP	horseradish peroxidase
IF	immunofluorescence
IgG	immunoglobulin G
IL-2	interleukin-2
IP	immunoprecipitation
IPTG	isopropyl β -D-thiogalactopyranoside
kd	kilo Dalton
Lamp1	lysosomal-associated membrane protein 1
LE	late endosome
M	molar
mTORC1	mechanistic target of rapamycin complex 1
mAb	monoclonal antibody
mg	milligram
mM	millimolar
Ni-NTA	nickel-nitrilotriacetic acid
nM	nanomolar
pAb	polyclonal antibody
PBS	phosphate buffered saline
PEI	polyethylenimine
PFA	paraformaldehyde
PM	plasma membrane
PVDF	polyvinylidene fluoride
Ran	Ras-related Nuclear protein
Ras	Rat sarcoma
RE	recycling endosome
RT-qPCR	quantitative reverse transcription PCR
RUFY1	RUN and FYVE domain-containing protein 1
Sar	Secretion associated and Ras related
SDsS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA

SLC38A9	solute carrier family 38 member 9
SNX	sorting-nexin
SNARE	soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor
STxB	Shiga toxin B fragment
TfR	transferrin receptor
TGN	<i>trans</i> -Golgi network
Tris	Tris (hydroxymethyl) aminomethane
v-ATPase	vacuolar H ⁺ -adenosine triphosphatase ATPase
Vps	vacuolar protein sorting
WB	western blot
μg	microgram
μl	microlitre
μm	micrometer
μM	micromolar

List of figures

Figure 1: Model for AA-induced mTORC1 activation.....	6
Figure 2: A model for the AAs-regulated Gap1p trafficking in the yeast.	8
Figure 3: Illustration of endocytic trafficking pathways in mammalian cells.	13
Figure 4: Major machinery components in endosome-to-Golgi trafficking.	17
Figure 5: Classification of human Ras superfamily small GTPases.	20
Figure 6: GDP-GTP exchange cycle of the Ras superfamily small GTPases.	22
Figure 7: Quantification of the fraction of Golgi-localized reporter of interest using <i>ImageJ</i>	46
Figure 8: Schematic representation of the reporters used for studying the endocytic trafficking assay.	48
Figure 9: CD8a-CI-M6PR is transported via EE and RE en route to the Golgi.	51
Figure 10: CD8a-furin is transported via LE en route to the Golgi.	54
Figure 11: Starvation causes changes in the subcellular distribution of endogenous furin and CI-M6PR.	56
Figure 12: Starvation causes changes in the subcellular distribution of CD8a-furin and CD8a-CI-M6PR.	57
Figure 13: CD8a-furin displays an EE and RE localization but not LE localization under nutrient starvation conditions.	58
Figure 14: Full-length furin displays endosomal localization under nutrient starvation conditions.	60
Figure 15: Full-length furin but not CD8a-furin localizes to the LE under nutrient starvation conditions.	61
Figure 16: Resupplying nutrients rapidly reverses the starvation-induced reduction of endogenous furin in the Golgi pool.	62
Figure 17: Resupplying nutrients rapidly reverses the starvation-induced reduction of furin-GFP in the Golgi pool.	63
Figure 18: Optimization of starvation time for the CD8a-furin endocytic trafficking assay.	64
Figure 19: Nutrients regulate the PM-to-Golgi trafficking of CD8a-furin.	66
Figure 20: Nutrients regulate the PM-to-Golgi trafficking of various CD8a-chimeras.	68

Figure 21: Nutrients do not significantly affect the endocytosis of CD8a-furin.	70
Figure 22: Nutrients stimulate the endosome-to-Golgi trafficking of CD8a-furin.....	71
Figure 23: Nutrients stimulate the endosome-to-Golgi trafficking of CD8a-CI-M6PR....	72
Figure 24: AAs but not growth factors or glucose, stimulate the endosome-to-Golgi trafficking of CD8a-furin.....	74
Figure 25: AAs stimulate endosome-to-Golgi trafficking in BSC-1 cells.....	75
Figure 26: AAs stimulate endosome-to-Golgi to the Golgi in HEK293T cells.....	76
Figure 27: Gln is one of the most acute AAs that stimulate effects on endosome-to-Golgi trafficking.....	77
Figure 28: Gln is essential for stimulating the endosome-to-Golgi trafficking of CD8a-furin.....	78
Figure 29: The effect of combining two AAs on endosome-to-Golgi trafficking is not additive.....	80
Figure 30: ConA treatment eliminates the effects of AAs on endosome-to-Golgi trafficking of CD8a-furin.....	83
Figure 31: Evaluation of shRNA-mediated knockdown of endogenous SLC38A9 levels in HeLa cells.	85
Figure 32: SLC38A9 is required for the AA-simulated endosome-to-Golgi trafficking of CD8a-furin.	86
Figure 33: Evaluation of shRNA-mediated knockdown of endogenous Lamtor1, Lamtor3 and Rag A/B GTPase levels in HeLa cells.	87
Figure 34: Lamtor1 and Lamtor3 but not Rag A/B are required for the AA-stimulated Golgi trafficking of CD8a-furin.....	89
Figure 35: mTORC1 is not required for the AA-stimulated endosome-to-Golgi trafficking of CD8a-furin.....	91
Figure 36: Arl5b, but not Arl1, specifically pulled down Lamtor1-GFP.	93
Figure 37: Arl5b-wt, -QL and -TN interact with Lamtor1-Myc.....	94
Figure 38: Arl5a, Arl5b are the two major paralogs of Arl5.	95
Figure 39: Characterization of anti-Arl5b rabbit pAb.	96
Figure 40: Arl5a and Arl5b localize to the Golgi.	97
Figure 41: Arl5a and Arl5b localize to the <i>trans</i> -Golgi.....	99

Figure 42: N-terminal myristoylation is probably required for the Golgi localization of Arl5b.	100
Figure 43: Arl5b-QL and -TN but not Arl5b-wt display endosomal localization, which colocalize with Lamtor1 under live cell conditions.	101
Figure 44: Lamtor1 localizes to the EE, LE and lysosome.....	102
Figure 45: siRNA mediated knockdown of endogenous Arl1 and Arl5.....	103
Figure 46: Endosome-to-Golgi trafficking of CD8a-furin is slowed in cells depleted of Arl1 or Arl5.....	105
Figure 47: Evaluation of siRNA-mediated knockdown of endogenous Arl5 levels in HeLa cells.	106
Figure 48: Arl5 is required for the AA-stimulated endosome-to-Golgi trafficking of CD8a-furin.	107
Figure 49: Evaluation of shRNA-mediated knockdown of endogenous Arl5a and Arl5b levels in HeLa cells.	108
Figure 50: Arl5a and Arl5b are essential for the AA-stimulated Golgi trafficking of CD8a-furin.....	109
Figure 51: Evaluation of shRNA-mediated knockdown of endogenous Arl5a and Arl5b levels in HeLa cells.....	111
Figure 52: GARP is required for the AA-stimulated Golgi trafficking of CD8a-furin. ..	112
Figure 53: Working model on how AAs stimulate the endosome-to-Golgi trafficking through Ragulator and Arl5b.	117

List of tables

Table 1: The mammalian Arl family G proteins.....	24
Table 2: List of antibodies used in this study.	35
Table 3: List of RT-qPCR primers used in this study.....	44

Abstract

The endosome-to-Golgi trafficking pathway is an important post-Golgi recycling route. However, there is a lack of knowledge about the regulatory mechanisms behind intracellular membrane trafficking processes in response to extracellular signals. In this study, we found that nutrient starvation reversibly caused the *trans*-Golgi network (TGN) membrane proteins, such as furin and CI-M6PR, translocate from the TGN to the endosomal pool. Using a series of CD8a tagged TGN membrane proteins as reporters, we demonstrated that nutrient could stimulate the endosome-to-Golgi trafficking. We found that amino acids (AAs), especially glutamine, but not growth factors or glucose, were the key factors regulating the endosome-to-Golgi trafficking in mammalian cells. Moreover, the stimulation effect of AAs on endosome-to-Golgi trafficking is probably ubiquitous, as it is observed in multiple cell lines. Thus, we made a novel discovery that the endosome-to-Golgi trafficking of cargos is inhibited and stimulated by the absence and presence, respectively, of AAs. Inspired by the mechanism of the AA-induced mTORC1 activation pathway, we hypothesized that the AA-stimulated endosome-to-Golgi trafficking pathway might share similar machinery. By selectively inhibiting or depleting each component of the AA-stimulated mTORC1 signaling pathway, it was revealed that SLC38A9, v-ATPase and Ragulator, but not Rag GTPases or mTORC1, are essential for AA-stimulated endosome-to-Golgi trafficking.

To accomplish the delivery of cargos from endosomes to the Golgi, various factors, including tethering factors, SNAREs and the small GTPases from the Rab and Arf-like family, are involved. Arl5, an Arf-like family small GTPases, has been found to regulate the membrane trafficking between the endosome and the Golgi. There are three closely related paralogs of Arl5 in vertebrates – Arl5a, b and c, where Arl5a, Arl5b are the dominant ones. Endogenously and exogenously expressed Arl5a and Arl5b were found to localize in the Golgi, while human Arl5c did not display a Golgi localization. Using yeast two-hybrid, pull-down and immunoprecipitation assays, we found that Arl5 interacts with Lamtor1. Live-cell imaging revealed that Arl5b colocalizes with Lamtor1 at the endosome and lysosome. Furthermore, both Arl5 and its effector, the Golgi-associated retrograde protein complex (GARP), are required for AA-stimulated trafficking. We have therefore

identified a mechanistic connection between nutrient signaling and the endosome-to-Golgi trafficking pathway, whereby SLC38A9 and v-ATPase sense AA-sufficiency. Moreover, the interaction between Lamtor1 and Arl5 might activate Arl5, which, together with its effector GARP, a tethering factor, likely facilitates the endosome-to-Golgi trafficking.

1. Introduction

1.1 Nutrient-dependent signaling and pathways in eukaryotes

For all living organisms, nutrients provide the bulk of energy and functions as essential building blocks to support the growth, proliferation and survival of a cell. Nutrient sufficiency stimulates anabolic metabolism such as protein translation and the biogenesis of organelles, whereas nutrient deficiency triggers catabolic pathways, like autophagy, to break down macromolecules in order to recycle much-needed materials for cell survival.

Cellular nutrients are simple organic compounds, such as glucose and related sugars, amino acids (AAs) and fatty acids. They serve not only as fundamental resources and substrates for the biosynthesis of macromolecules or the generation of high-energy molecules, but they are also important signaling molecules in diverse nutrient transduction signaling pathways ([Cooper, 2004](#); [Efeyan et al., 2015](#)). Intense research has been focused on how nutrients regulate cellular metabolism through transcription and translation. Among these, a particularly notable and crucial regulatory system is the mechanistic target of the rapamycin complex 1 (mTORC1) signaling pathway.

1.1.1 The mTORC1 signaling pathway

The mechanistic target of the rapamycin (mTOR) is a serine/threonine kinase, which belongs to the phosphatidylinositol 3-kinase-related kinase protein family ([Sengupta et al., 2010](#)) and is thought to be evolutionarily conserved ([Jewell and Guan, 2013](#)). In the past two decades, extensive research has established the critical role of mTOR in regulating cell growth and metabolism; furthermore, many studies have shown that the mTOR signaling pathway is deregulated in many human diseases, such as cancer, diabetes and Alzheimer's disease ([Saxton and Sabatini, 2017](#); [Zoncu et al., 2010](#)).

mTOR can form two structurally and functionally distinct protein complexes, known as mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2) ([Laplante and Sabatini, 2009](#)). In addition to the catalytic subunit mTOR, both complexes contain DEP domain containing mTOR-interacting protein (DEPTOR) and lethal with SEC13 protein 8 (mLST8/GβL). DEPTOR has been characterized as an inhibitor of both complexes ([Peterson et al., 2009](#)). Furthermore, mTORC1 has two other unique components:

regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT substrate 40 kDa (PRAS40). In contrast, mTORC2 contains rapamycin-insensitive companion of TOR (RICTOR), the mammalian stress-activated MAP kinase – interacting protein 1 (mSIN1) and protein observed with RICTOR (PROTOR) as signature components ([Costa-Mattioli and Monteggia, 2013](#)). Both RAPTOR and RICTOR function as scaffolding proteins that link mTOR kinase with other components. Similar to DEPTOR, PRAS40 acts as a negative regulator of mTORC1. Other components, such as mSIN1, can promote mTORC2 assembly and signaling, while the role of PROTOR remains incompletely defined ([Foster and Fingar, 2010](#)).

Whether the cell undergoes anabolic or catabolic processes is mainly determined by the mTORC1 signaling pathway, which senses and integrates multiple upstream signals from nutrients (e.g. glucose and AA), growth factors (e.g. insulin and epidermal growth factor), oxygen state and energy levels ([Efeyan et al., 2012](#); [Jewell and Guan, 2013](#); [Laplane and Sabatini, 2009](#); [Shimobayashi and Hall, 2014](#)). Most upstream signals, except AA ([Smith et al., 2005](#)), regulate mTORC1 signaling through the tuberous sclerosis heterodimer (TSC1–TSC2). The TSC1–TSC2 complex functions as a guanosine triphosphatase activating protein (GAP) for small Ras-related GTPase Rheb1 ([Inoki et al., 2003](#); [Tee et al., 2003](#)), which is a lysosome localization protein that can directly interact and stimulate the activity of mTORC1 in its GTP-bound state ([Long et al., 2005](#)). Unlike the TSC1–TSC2 complex, Rheb1 seems to be essential for all upstream signals, including AAs, to activate the mTORC1 signaling pathway ([Sancak et al., 2010](#)).

Among the mTORC1 upstream signals, AAs seem to be essential for mTORC1 activation, as growth factors are not fully able to activate mTORC1 when AAs are absent ([Hara et al., 1998](#); [Jewell et al., 2013](#); [Sancak et al., 2008](#)). Over the past few years, researchers have become increasingly interested in elucidating the mechanisms leading to activation of mTORC1 by AAs. It has been demonstrated that AA signaling initiates within the lysosomal lumen and ultimately causes the recruitment of mTORC1 to the lysosomal surface ([Sancak et al., 2010](#)), where it interacts with Rheb1 and is activated. This re-localization is determined by Rag GTPase, which belongs to the Ras family of GTP-binding proteins and exists as obligate heterodimers of RagA or RagB with RagC or

RagD ([Kim and Kim, 2016](#); [Sancak et al., 2008](#)). The GTP-bound RagA/B together with the GDP-bound RagC/D promotes the intracellular localization of mTORC1 to the lysosome and subsequent activation, whereas the GDP-bound RagA/B together with GTP-bound RagC/D inhibits mTORC1 activation by AAs ([Sancak et al., 2008](#)). Similar to other small GTPases, the activity of Rag GTPase is regulated by guanine nucleotide exchange factor (GEF) and GAPs. GATOR1, an octomeric complex comprising DEPDC5, Nprl2, and Nprl3, has been shown to have GAP activity toward RagA and RagB ([Bar-Peled et al., 2013](#)), while the tumor suppressor FLCN (folliculin) together with its binding partner FNIP1/2 functions as GAP for RagC/D GTPase ([Tsun et al., 2013](#)). The GEF for Rag GTPases, named Ragulator, a pentameric complex comprising Lamtor1-5 (also referred to as p18, p14, MP1, C7orf59 and HBXIP), was identified earlier than GAPs and has been found to play an important role in the AA-induced mTORC1 activation pathway ([Bar-Peled et al., 2012](#); [Sancak et al., 2010](#)). The N-terminal myristoylation and palmitoylation of Lamtor1 ([Nada et al., 2009](#)) are required for anchoring Ragulator and the Rag GTPase to the lysosomal surface to perform their functions ([Sancak et al., 2010](#)). The GEF activity of the Ragulator complex can be stimulated by v-ATPase, which is a proton pump responsible for the acidification of the lysosome and has been shown to be necessary for the activation of mTORC1 in response to lysosomal AAs. The interaction between v-ATPase and Ragulator is strengthened and weakened upon AA starvation and stimulation, respectively ([Zoncu et al., 2011](#)).

Despite extensive investigation into the AA-dependent mTORC1 signaling pathway over the past few years, little is known how AAs are sensed to control the mTORC1 pathway. Three studies published in 2015 ([Jung et al., 2015](#); [Rebsamen et al., 2015](#); [Wang et al., 2015](#)) collectively identified and demonstrated an uncharacterized lysosomal AA sensor, termed solute carrier family 38 member 9 SLC38A9 (SLC38A9), which acts upstream of the Rag heterodimers and binds to the Rag GTPase and Ragulator in an AA-dependent fashion. SLC38A9 belongs to the solute carrier 38 family (SLC38), which is Na⁺-dependent and functions as an AA transporter ([Bröer, 2014](#)). SLC38A9 is predicted to have 11 transmembrane domains and a long N-terminal cytosolic tail (119 AAs) ([Jung et al., 2015](#); [Wang et al., 2015](#)). When AAs accumulate in the lysosomal lumen, the cytosolic tail of SLC38A9 loosely interacts with the Rag–Ragulator complex and

eventually activates mTORC1. In contrast, the interaction between SLC38A9 and the Rag–Regulator complex is tightened in the absence of AAs, which causes the inactivation of Rag GTPase and induces mTORC1 to leave the lysosomal surface and become inactive ([Jung et al., 2015](#)). Thus, SLC38A9 is another key regulator in the AA-dependent mTORC1 signaling pathway. There are conflicting results regarding the preference of SLC38A9 for the transport of AAs: Wang et al showed that SLC38A9 is an excellent arginine transporter ([Wang et al., 2015](#)), while Rebsamen et al identified it as a glutamine transporter ([Rebsamen et al., 2015](#)). Additionally, the study from Jung et al’s lab proposed that SLC38A9 can act as a transporter for different AAs, such as arginine, glutamine and leucine ([Jung et al., 2015](#)).

Figure 1: Model for AA-induced mTORC1 activation.

In the absence of AAs, the v-ATPase, SLC38A9, Ragulator, and Rag small GTPases form a tightly bound complex, and mTORC1 is released throughout the cytoplasm and remains inactive. In the presence of AAs, AAs rapidly accumulate within the lumen of the lysosome. Luminal AAs trigger SLC38A9 and v-ATPase. The activated SLC38A9 and v-ATPase signal to Ragulator by rearranging their interaction with the latter. Following activation, Ragulator functions as the GEF for heterodimeric Rag GTPases. Finally, GTP-loaded Rag heterodimer recruits mTORC1 to the surface of the lysosome membrane, where the full kinase activity of mTORC1 is turned on by another small GTPase, Rheb1 (not shown). Modified from ([Bar-Peled et al., 2012](#)).

1.1.2 AA-regulated intracellular membrane trafficking

In contrast with numerous findings of nutrients regulating cellular metabolism via transcription and translation, little progress has been made in understanding how AAs regulate trafficking events in the cell.

In *Saccharomyces cerevisiae*, it is known that a number of AA permeases on the plasma membrane (PM) are regulated by AAs. The major role of AA-permeases is to transport AAs into the cell. AA-permeases can be classified into two classes: the constitutive permeases and the regulated permeases ([Roberg et al., 1997](#)). The general AA permease, Gap1p, is one of the most characterized regulated permeases, and the intracellular trafficking of Gap1p is strictly regulated in response to the availability of AAs, a general nitrogen source utilized by yeast ([Chen and Kaiser, 2002](#); [Godard et al., 2007](#); [Roberg et al., 1997](#)). During AA starvation or growth on nitrogen-poor sources like proline, the newly synthesized Gap1p rapidly traffics from the *trans*-Golgi network (TGN) to the cell surface and accumulates on the PM, where it is highly activated and helps to scavenge the extracellular nitrogen sources. However, under nitrogen sufficiency conditions or in the presence of AAs, especially Gln, the majority of Gap1p is directly transported from the TGN to the vacuolar degradation pathway without ever being delivered to the cell surface ([Chen and Kaiser, 2002](#); [Merhi and André, 2012](#); [O'Donnell et al., 2010](#)). Npr1p, a TORC1- as well as nutrient-regulated kinase, is a key factor in the AA-regulated Gap1p intracellular trafficking pathway. In the presence of AA, activated-TORC1 phosphorylates Npr1p kinase to inhibit its activity. Once it has been inactivated, Npr1p fails to phosphorylate the arrestin-like adaptors, Bul1p and Bul2p. As a result, the dephosphorylated Bul proteins interact with Gap1p and promote its polyubiquitylation by the ubiquitin ligase Rsp5p, which causes the sorting of Gap1p to vacuoles, and eventually Gap1p becomes degraded ([Bieschke et al., 2009](#); [Lauwers et al., 2009](#); [Merhi and André, 2012](#)). During AA starvation, the activated Npr1p phosphorylates the Bul adaptors, which are bound to the conserved eukaryotic proteins 14-3-3 and thus protects Gap1p against ubiquitylation ([Merhi and André, 2012](#)). Furthermore, the activated Npr1p also phosphorylates and positively regulates the activity of yeast α -arrestin Aly2p, a protein that directly interacts with the adaptor protein AP-1 and mediates the recycling of Gap1p from endosomes to the TGN and finally its trafficking to the PM ([O'Donnell et al., 2010](#)).

Figure 2: A model for the AAs-regulated Gap1p trafficking in the yeast.

Under AA-starvation conditions or growth on nitrogen-poor sources like proline (Pro), newly synthesized Gap1p transits from the late Golgi (TGN) to the PM (highlighted with red arrow), and the recycling of Gap1p from endosomes to the late-Golgi (TGN) is up-regulated. TORC1 fails to phosphorylate Npr1p, which remains in an active state. Active Npr1p phosphorylates the α -arrestin Aly2p, which may stimulate Gap1p incorporation into AP-1/clathrin-coated vesicles and traffic to the late Golgi (highlighted with red arrow). Furthermore, Npr1p activation also phosphorylates and negatively regulates the Bul1p and Bul2p adaptors, which are thus inhibited. Hence, Gap1p is not ubiquitinated by the Rsp5p and remains stable and active at the PM. In the presence of a sufficiency of AAs, or supply of a good nitrogen source such as glutamine (Gln) to cells, activated TORC1 phosphorylates Npr1p to inhibit its activity. Npr1 inactivation stimulates polyubiquitylation of Gap1p by Bul1p/Bul2p/Rsp5p. Therefore the majority of Gap1p is trafficked from the late Golgi (TGN) to the endosome/vacuole and eventually becomes degraded (highlighted with green arrow). Modified from ([O'Donnell et al., 2010](#)).

Besides Gap1p in yeast, the intracellular trafficking of an autophagy-related protein Atg9 has also been reported to be regulated by the nutrient availability in mammalian cells. In AA-rich medium, the mammalian Atg9 (mAtg9) is found in a juxta-nuclear region that colocalizes with the TGN marker TGN46 as well as in the peripheral pools that overlap with the late endosome (LE) markers, Rab7 and Rab9. After AA starvation, the juxta-nuclear pool of mAtg9 is rapidly lost, while an increase in the peripheral pool, which colocalizes with Rab7 and the autophagosome marker, GFP-LC3, is observed ([Webber and Tooze, 2010b](#); [Webber et al., 2007](#); [Young et al., 2006](#)). This redistribution of mAtg9 from the TGN to the peripheral punctuate structures is dependent on mAtg1 (also referred to as ULK1), as mAtg1 knockdown inhibits the starvation-induced redistribution of mAtg9 from the Golgi to the endosomes ([Young et al., 2006](#)). The dispersal of mAtg9 during starvation also requires the p38-interacting protein (p38IP), and the interaction between them is negatively regulated by the mitogen-activated protein kinase (MAPK) p38 α ([Webber and Tooze, 2010a](#)). Additionally, mAtg9 cycles between the TGN and endosomal pool under AA-sufficient conditions, which shows a similar trafficking pattern as CI-M6PR ([Young et al., 2006](#)). Thus, it has been proposed that the retrograde trafficking of mAtg9 could be regulated by the TIP47/Rab9, Retromer, or the mammalian Golgi-associated retrograde protein complex (GARP) ([Webber et al., 2007](#)), which needs to be further explored.

1.2 Intracellular membrane trafficking in eukaryotes

In eukaryotes, membrane trafficking plays a pivotal role in transporting cargos, such as the transport of proteins and lipids to different destinations inside and outside the cell. Various membrane-bound intracellular compartments, such as the endoplasmic reticulum (ER), Golgi apparatus and endosomes are connected by membrane trafficking pathways, which are crucial for them to communicate with the cellular environment and to maintain normal cellular functions. The intracellular membrane trafficking pathways can be classified into two major categories: 1) the exocytic pathway (also called biosynthetic-secretory pathway) that delivers newly synthesized proteins and lipids from the ER to the PM or the extracellular space; and 2) the endocytic pathway that functions in internalization of cargos from the PM or the cell milieu ([Grant and Donaldson, 2009](#); [Tokarev et al., 2009](#)).

1.2.1 The endocytic pathways in mammalian cells

There are two major groups of endocytic pathways: clathrin-dependent endocytosis (also known as clathrin-mediated endocytosis, CME) and clathrin-independent endocytosis (CIE). As one of the most studied and best-characterized endocytic pathways, CME proceeds through multiple stages including clathrin-coated pit (CCP) initiation, cargo selection, maturation of the CCP to a clathrin-coated vesicles (CCV), scission and uncoating ([Elkin et al., 2016](#); [McMahon and Boucrot, 2011](#)). A wide variety of proteins such as clathrin, adaptor proteins 2 (AP-2), dynamin and ATPase heat shock cognate 70 (HSC70) are involved in these stages ([McMahon and Boucrot, 2011](#)). Recycling of iron-bound transferrin and the uptake of low-density lipoprotein (LDL) are the two classical examples of CME ([Alberts, 2017](#)). Another endocytic pathway, CIE, including caveolin-dependent internalization, macropinocytosis, and phagocytosis, have gained a lot of interest in recent years ([Grant and Donaldson, 2009](#)). Cargos like the major histocompatibility complex class I proteins (MHCI), β -integrin and the ubiquitous glucose transporter Glut1 have been identified to travel in this pathway ([Grant and Donaldson, 2009](#)). Taken together, both pathways have important physiological roles, as mutations happened in the core components of endocytic pathways can cause numerous human diseases ([Elkin et al., 2016](#)).

Once internalized from the PM into cells through any of the above-mentioned endocytic pathways, cargos reach the first endocytic compartment – early endosome (EE), which serves as a common sorting station for cargos ([Jovic et al., 2010](#)). From there, the fates of the internalized cargos are decided: cargos can be 1) recycled back to the PM directly via the EE or indirectly via the recycling endosome (RE), 2) sorted to the lysosome via the LE/multivesicular body (MVB) for degradation, or 3) delivered to the Golgi via the endocytic retrograde trafficking pathway ([Elkin et al., 2016](#); [Jovic et al., 2010](#)).

1.2.2 The endocytic retrograde pathways leading to the Golgi

There are two retrograde pathways leading from different endosome compartments to the Golgi. Some cargos are directly transported from EE/RE to the Golgi without passing through the LE (EE/RE-to-Golgi), whereas others involve the late endocytic compartments (LE-to-Golgi) ([Lieu and Gleeson, 2011](#); [Sannerud et al., 2003](#)). A vast

range of cargos, including membrane and soluble proteins, lipids and some exogenous bacterial and plant toxins, have been reported to utilize these two retrograde pathways ([Maxfield and McGraw, 2004a](#)). Among these, most salient examples are acid-hydrolase receptors (e.g. mammalian mannose 6-phosphate receptors), TGN transmembrane enzymes (e.g. furin), Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNAREs, e.g. GS15), Shiga toxin, cholera toxin as well as proteins with undefined cellular functions (e.g. TGN38 in rat and TGN46 in human) ([Bonifacino and Rojas, 2006](#)).

One of the best-known proteins following the EE/RE-to-TGN recycling pathway is TGN38. By using a chimeric transmembrane protein Tac-TGN38, which consists of the luminal domain of the IL-2 receptor α chain (Tac) and the cytoplasmic and transmembrane domains of TGN38, the itinerary of TGN38 has been studied ([Ghosh et al., 1998](#)). Shortly after internalization, TGN38 is initially delivered to the EE, while a substantial amount of internalized TGN38 proteins rapidly exit the EE and then enter the RE. After passing through the RE, the majority of Tac-TGN38 returns to the PM, while the rest is delivered to the Golgi, without entering the LE ([Ghosh et al., 1998](#)). In addition to TGN38, cation-independent mannose-6-phosphate receptor (CI-M6PR) ([Lin et al., 2004](#)) and Shiga toxin B-fragment (STxB) ([Mallard et al., 1998](#); [McKenzie et al., 2012](#)) have also been reported to follow the similar pathway.

However, the itinerary of Tac-furin is different from the Tac-TGN38. Furin is a type-I integral endoprotease that is responsible for activating and catalyzing the maturation of various types of proproteins ([Thomas, 2002](#)). After internalization and transportation from the cell surface to the EE, Tac-furin is retained in the same compartment until the EE matures to become the LE. From the LE, Tac-furin reaches the Golgi ([Mallet and Maxfield, 1999](#); [Maxfield and McGraw, 2004b](#)). Interestingly, it has been shown that a fraction of endocytosed CI-M6PR can be detected at the LE in CHO cells ([Lin et al., 2004](#)), and this could be due to its enzyme delivery function. To transport the newly synthesized acid hydrolases from the TGN to lysosomes, CI-M6PR binds to the modified hydrolases and they transit to the LE together. Once in the LE, the acidic environment accelerates the release of the hydrolases from CI-M6PR, and the free CI-M6PR is then

recycled back to the Golgi via the LE-to-Golgi pathway to perform its normal function ([Ghosh et al., 2003](#)). Another explanation for detecting the CI-M6PR in the LE could be that the clearly defined boundaries among the EE, the RE and the LE do not exist. This is because they are undergoing continuous maturation and transformation ([Huotari and Helenius, 2011](#); [Lu and Hong, 2014](#)). Therefore, it is possible that the sorting of membrane-bound cargos in the endocytic pathway could take place continuously and simultaneously during endosome maturation, with some cargos sorted in early stages and others at late stages of maturation.

Figure 3: Illustration of endocytic trafficking pathways in mammalian cells.

In the endocytic trafficking pathway, cargos from the cell surface are internalized into vesicles that initially arrive at the EE (highlighted using black arrow). From there, cargos are sorted to the lysosome via the LE for degradation (highlighted using red arrows). Cargos could be selectively salvaged from the degradation pathway by returning directly or via RE back to the PM (highlighted using purple arrows). Alternatively, cargos can be transported to the Golgi either from EE/RE (e.g. TGN38, Shiga toxin and CI-M6PR) or from LE (e.g. furin and CI-M6PR) (highlighted using green arrows). Modified from ([Lu and Hong, 2014](#)).

1.2.3 The molecular machinery of endosome-to-Golgi trafficking pathway

In the endosome-to-Golgi trafficking pathway, the first step is budding of a vesicle that contains the soluble biomolecular cargos from the endosome membrane. Vesicles then be transported to and target the TGN membrane, followed by fusion with the membrane. Cargos will finally be delivered to the Golgi. A large number of trafficking factors, such as coat proteins, small GTPases, tethering factors and SNAREs, have been implicated in these steps ([Bonifacino and Rojas, 2006](#); [Cooper and Ganem, 1997](#); [Lu and Hong, 2014](#); [Mallet and Maxfield, 1999](#)). As described above, clathrin and its adaptors are essential for the formation of CCVs. The clathrin adaptor protein AP-1 was shown to be involved in the retrograde transport of several types of cargos, including M6PRs and furin, but it is not involved in the transport of STxB ([Hirst et al., 2012](#); [Meyer et al., 2000](#)). Another clathrin adaptor, EpsinR, which has been found to interact with AP-1 and clathrin ([Hirst et al., 2003](#)), is also required for efficient retrograde trafficking of STxB and M6PRs ([Saint-Pol et al., 2004](#)). The retromer complex is another important regulator of the retrograde pathway. It is a heteropentameric complex consisting of a sorting nexin (SNX) dimer and the cargo recognition trimer, Vps26-Vps29-Vps35, and has been shown to localize at the EE ([Johannes and Popoff, 2008](#); [Lu and Hong, 2014](#)). Retromer is critical for transiting cargos such as STxB, M6PRs and sortilin from endosomes to the Golgi ([Bonifacino and Hurley, 2008](#); [Bonifacino and Rojas, 2006](#)).

When transport vesicles arrive in the TGN, they tether to the TGN membrane by the TGN-localized tethering factors. There are two main types of tethering factors: the coiled-coil homodimer and the oligomeric multi-subunit complexes ([Lu and Hong, 2014](#); [Lupashin and Sztul, 2005](#)). The homodimeric coiled-coil tethers presented on the *cis*-face, rim or the *trans*-face of the Golgi apparatus are also referred to as Golgins ([Barr and Short, 2003](#); [Munro, 2011](#)). At least 15 different Golgins have now been identified. Among these, the TGN-localized GRIP (Golgin97-RanBP-Imh1p-p230) Golgins, which function in tethering the endosome-derived carriers to the TGN, have been well-characterized. The GRIP Golgins, including Golgin97, Golgin245/p230, Golgi localized coiled-coil protein (GCC) 185 and GCC88, contain a conserved GRIP domain at their C-termini. By interacting with the Arf-like small G protein Arl1, GRIP Golgins such as Golgin97 and Golgin245 can be recruited to the TGN membrane ([Goud and Gleeson, 2010](#); [Lu and](#)

[Hong, 2003](#)). Both Arl1 and Golgin97 are required for endosome-to-Golgi trafficking of STxB ([Lu et al., 2004](#)). In contrast to Golgin97 and Golgin245, the GRIP Golgins like GCC88 and GCC185 were identified to be Arl1-independent ([Luke et al., 2003](#)). They also play a critical role in the retrograde transport of cargos from endosomes to the Golgi. Depletion of GCC88 resulted in the retention of CI-M6PR and TGN38 at the EE but did not affect the retrograde transport of STxB ([Lieu et al., 2007](#)), which somewhat indicates that GCC88 specifically regulates transport from the EE to the Golgi. The GCC185 is required for efficient transport of STxB from the RE to the Golgi ([Derby et al., 2007](#)). Moreover, GCC185 is also involved in the tethering of M6PR- and furin-carrying cargos emanating from the LE, and this process is dependent on the small GTPase Rab9 ([Chia et al., 2011](#); [Reddy et al., 2006](#)). Apart from the homodimer complex, the oligomeric tethering complexes such as GARP, have gained increasing attention in recent years. It was initially identified in yeast, and the orthologs in humans have been found by sequence homology ([Bonifacino and Hierro, 2011](#)). The structure and function of GARP are highly conserved. It localizes to the TGN and endosomes, which comprises four subunits – vacuolar protein sorting 51 (Vps51), Vps52, Vps53 and Vps54. The primary role of GARP is to mediate tethering of the budded transport carriers containing cargos, such as the Golgi SNARE, TGN46 and CI-M6PR, from endosomes to the Golgi ([Conibear and Stevens, 2000](#); [Liewen et al., 2005](#); [Pérez-Victoria et al., 2010](#); [Siniossoglou and Pelham, 2002](#)). To exert its tethering function, GARP simultaneously binds to the retrograde carriers and acceptor membranes. This process is regulated by the Rab and Arf small G proteins. In yeast, GARP interacts with Arl1p and Ypt6p via its Vps52p and Vps53p subunits, respectively ([Bonifacino and Hierro, 2011](#); [Panic et al., 2003](#)). GARP has been found to lose its Golgi localization in Ypt6p-depleted cells ([Siniossoglou and Pelham, 2001](#)). Recently, it has been also reported that Arl5b, a member of the Arl family, mediates the recruitment of GARP to the Golgi in *Drosophila* as well as in mammalian cells ([Rosa-Ferreira et al., 2015](#)).

In addition to tethering factors, another group of membrane proteins, named SNARE, is also crucial for mediating transport vesicle docking and fusion to the target membrane. SNAREs can be divided into two types: v-SNARE (vesicle-SNARE) and t-SNARE (target-SNARE). Based on their highly conserved motifs, they have been also classified as

R-SNAREs (arginine containing SNARE) and Q-SNAREs (glutamine containing SNARE) ([Chen and Scheller, 2001](#); [Südhof and Rothman, 2009](#)). Most SNAREs anchor to the membrane using their C-terminus tail. A variety of SNAREs exist and localize in distinct subcellular compartments in eukaryotic cells ([Duman and Forte, 2003](#)). During membrane fusion, the v-SNAREs on the vesicle combine and interact with t-SNAREs on the target membrane to form a stable four-helical bundle complex ([Chen and Scheller, 2001](#); [Lu and Hong, 2014](#)). After fusion, the ATPase NSF (N-ethylmaleimide-sensitive factor) and α -SNAP (α -soluble NSF attachment protein) work together to release SNAREs for recycling ([Südhof and Rothman, 2009](#)). In endosome-to-Golgi trafficking, several Golgi-localized SNARE proteins, such as Syntaxin 5, Ykt6, GS28, and GS15, were shown to form a complex and are required for the retrograde transport of STxB ([Tai et al., 2004](#)). Others, like syntaxin16, syntaxin 6, Vtila and VAMP4 (or VAMP3), are also involved in endosome-to-Golgi transport ([Mallard et al., 2002](#)).

Figure 4: Major machinery components in endosome-to-Golgi trafficking.

Clathrin and accessory proteins, retromer and accessory proteins as well as Rab9 and accessory proteins are involved in the formation of transport carriers on the EE/LE; retrieval of transport carriers from endosomes to the Golgi also requires other regulatory factors such as small GTPases, tethering factors (highlighted using blue colors) and SNAREs (highlighted using purple colors). Modified from ([Lu and Hong, 2014](#)).

1.2.4 Physiological importance of endosome-to-Golgi trafficking

The endosome-to-Golgi trafficking pathway is one of the major intracellular vesicle recycling pathways, which is essential for the biogenesis and functional integrity of post-Golgi organelles, the secretion of cargos and maintenance of nutrient homeostasis ([Burd, 2011](#); [Chia et al., 2013](#)). Furthermore, it has also been reported to regulate signal transduction, like Wnt signaling ([Belenkaya et al., 2008](#); [Franch-Marro et al., 2008](#)) and contribute to the pathogenesis of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases ([Follett et al., 2014](#); [Johannes and Popoff, 2008](#); [Vilariño-Güell et al., 2011](#)). Wnt signaling pathway plays important roles in embryonic development. The Wnt family contains 19 secreted glycoproteins and the release of Wnt from the cell is controlled by an evolutionary conserved transmembrane protein Wntless ([Komiya and Habas, 2008](#)). After post-translational modifications in the Golgi, Wnt interacts with Wntless, which helps to send the Wnt from the Golgi to the PM for secretion. The PM-localized Wntless is then internalized and required to be recycled back to the Golgi to perform its normal function. This process is mediated by the retromer. When retromer activity is inhibited, Wntless becomes unstable and is delivered to the lysosome for degradation. Thus, Wnt proteins fail to be targeted to the PM and secreted from the cell ([Belenkaya et al., 2008](#); [Port et al., 2008](#); [Yang et al., 2008](#)).

The neurodegenerative Alzheimer's disease (AD) is one of the most common causes of dementia in older adults. A crucial step in the development of AD is production and accumulation of β -amyloid peptides ($A\beta$) within the brain ([O'Brien and Wong, 2011](#)). $A\beta$ is derived from the amyloid precursor protein (APP) through sequential cleavages by two proteases β - and γ -secretase. APP, β - and γ -secretase have been reported to cycle in the TGN, endosomes and PM ([Huse et al., 2000](#)). Thus, the production of $A\beta$ is determined by the localization of APP and proteases ([Burd, 2011](#)). The retrograde trafficking of APP has been found to be regulated by a Vps10 domain receptor SorLA, which interacts with the retromer subunit Vps26 to mediate sorting and transporting of APP ([Nielsen et al., 2007](#); [Rogaeva et al., 2007](#)). Several studies have reported that knockdown of retromer subunit (Vps35 or Vps26) or SorLA promote the production of $A\beta$ ([Small et al., 2005](#); [Vieira et al., 2010](#)). Since the endosome-to-Golgi trafficking is related to various

physiological and pathological processes such as metazoan development and neurological diseases, more research is needed to reveal how it modulate and affect these processes.

1.3 Overview of Arl GTPases

1.3.1 Ras family small GTPases

As described above, to accomplish the delivery of cargos to their final destination in vesicular trafficking, numerous factors, including tethering factors, SNAREs and the small GTPases from the Rab and Arl-family, are involved ([Itzen and Goody, 2011](#)). In this section, we will discuss small GTPases.

Small GTPases (also called small GTP-binding protein or G proteins) are a family of hydrolase enzymes that bind to and hydrolyze guanosine triphosphate (GTP), which act as regulators for a large number of cellular events, such as gene expression, cytoskeleton reorganization, microtubule organization, vesicular membrane trafficking, etc ([Takai et al., 2001](#)). There are more than a hundred small GTPases in eukaryotes ([Jékely, 2003](#)) and the most well-known members are the Ras superfamily. According to the sequence similarities and properties of function, the Ras superfamily can be classified into five main families: Ras, Rho, Rab, Ran and Arf ([Rojas et al., 2012](#); [Vigil et al., 2010](#)).

Ras superfamily small GTPases (human)

Ras	signal transduction, regulation of gene expression-regulation cell proliferation, survival,differentiation
Rho	signal transduction, regulation of actin organization-regulation cell shape and polarity, movement, cell-cell and cell-matrix interactions
Rab	vesicular trafficking-regulation of endocytosis and secretory processes
Arf	vesicular trafficking-regulation of endocytosis, secretory and microtubule dynamics
Ran	Nuclear-cytoplasmic transport,mitotic spindle organization

Figure 5: Classification of human Ras superfamily small GTPases.

Based on the sequence and functional similarities, the Ras superfamily GTPases can be divided into five major branches: Ras, Rho, Rab, Arf and Ran. They regulate a wide variety of cellular processes, including gene expression, cellular differentiation, cell movement, vesicular trafficking, etc. Modified from ([Vigil et al., 2010](#)).

Small GTPases share conserved AA sequence motifs (designated as the G1-G5 box), which are responsible for guanine nucleotide binding and hydrolysis ([Takai et al., 2001](#)). The G1 box, GXXXXGK(S/T) (X stands for any AA), contacts the α - and β -phosphates of the guanine nucleotide. The G2 box contains a conserved threonine (Thr), which is involved in coordination of Mg^{2+} . The G3 box with the consensus sequences, DXXXG(Q/H/T), provides the binding elements to the γ -phosphate of GTP. The conserved NKXD sequence in the G4 box is responsible for recognizing of the guanine nucleotide ring. The G5 box, (T/G)(C/S)A, primarily enforces the interaction with the guanine ring ([Colicelli, 2004](#); [Sprang, 1997](#)). Small GTPases generally exist in two exchangeable forms *in vivo*: the GTP-bound active and GDP-bound inactive forms. By

comparing the structure of active and inactive form of small GTPases, two flexible regions, defined as the switch I and switch II region, have been revealed. The G2 box resides in the switch I region and the G3 box is in the switch II region. Both switch I and switch II region are involved in the interaction between small GTPase and its effectors ([Vetter, 2014](#)).

The rate of conversion between active and inactive form of small GTPase is low, and this requires the help from its guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In general, small GTPase has similar affinity for GTP and GDP. Upon activation by its upstream signal, the GEF catalyzes the dissociation of the nucleotide from small GTPases, resulting increase GTP-bound form over GDP-bound form of small GTPase ([Bos et al., 2007](#); [Vigil et al., 2010](#)). This is due to that the cellular concentration of GTP is approximately ten times higher than the GDP ([Bos et al., 2007](#)). GTP-bound active small GTPase subsequently binds to its downstream effectors to perform their cellular function. To terminate the active state of small GTPase, a GAP is required, which accelerates the GTP hydrolysis and returns the small G protein to a GDP-bound inactive form ([Bos et al., 2007](#); [Cherfils and Zeghouf, 2013](#)). Besides, there exists a third class of crucial regulator, the guanine nucleotide dissociation inhibitors (GDIs), which act on small G proteins in the Rho and Rab families. GDIs bind to the GDP-bound form of small GTPases and maintain them in an inactive state in the cytosol ([Cherfils and Zeghouf, 2013](#)).

In addition, most small G proteins undergo lipid modification after being translated from mRNA. For example, the Ras members are farnesylated and palmitoylated at their C-terminus, whereas proteins from the Arf family are modified with myristic acid at their N-terminus (Gly residues) ([Castellano and Santos, 2011](#); [Takai et al., 2001](#)). Lipid modifications are generally necessary for the association of small G proteins with membranes and their interacting partners, and for activation of downstream effectors.

Figure 6: GDP-GTP exchange cycle of the Ras superfamily small GTPases.

The conversion of GTP-bound active form and GDP-bound inactive form of small GTPase are regulated by its GTPase activating protein (GAP) and guanine nucleotide exchange factor (GEF). For the Rho and Rab families of small GTPases, there exists a third class of regulators – the guanosine-nucleotide dissociation inhibitor (GDI). GDI binds to the farnesyl or geranylgeranyl group in C-terminal of the Rho and Rab GTPases, and helps to stabilize the inactive soluble GDP-bound forms of small GTPases. Modified from ([Cherfils and Zeghouf, 2013](#)).

1.3.2 Arf family GTPases

The small ADP ribosylation factor (Arf) GTP-binding proteins are known to play critical roles in intracellular membrane trafficking ([Pasqualato et al., 2002](#)). They are classified into three different major groups: the Arf, Arf-like (Arls) and remotely related secretion-associated and Ras-related (Sars) proteins ([Kahn et al., 2006](#)). In general, all Arf small G proteins have an N-terminal amphipathic helix containing a myristol or an acetyl group, which is critical for them binding to the membrane ([Donaldson and Jackson, 2011](#)).

1.3.2.1 Arf subfamily

In mammalian cells, there are 6 Arf proteins that can be categorized into three classes on the basis of their AA sequence identities: Class I, including Arf1, Arf2 and Arf3; Class II, including Arf4 and Arf5; and class III, including Arf6 ([Moss and Vaughan, 1998](#)). All of

them are ubiquitously expressed in humans with the exception of Arf2 ([Gillingham and Munro, 2007](#)). The Golgi-localized Arf1 and the PM-localized Arf6 are the most thoroughly studied mammalian Arf small G proteins. Arf1 has been reported to participate in multiple cellular events, such as regulation of membrane trafficking, stimulation the activity of enzymes that involved in phospholipid production and organization of the actin cytoskeleton ([Donaldson et al., 2005](#); [Donaldson and Jackson, 2011](#); [Takai et al., 2001](#)). The intracellular trafficking function of Arf1 is accomplished mainly through its recruitment of coat proteins, including coatamer complex I (COPI), the heterotetrameric AP-1/3/4 clathrin coats, and the three monomeric Golgi-localized, γ -ear-containing, Arf-binding proteins (GGA1/2/3), on to membranes ([Donaldson and Honda, 2005](#); [Donaldson et al., 2005](#); [Donaldson and Jackson, 2011](#); [Gillingham and Munro, 2007](#)). Arf3 were thought to function and localize identically as Arf1, since there are only 7 AA differences between them. However, it has been reported recently that the TGN localization of Arf3 is depends on 4 Arf3-specific N-terminal AAs, and this localization is temperature-sensitive ([Manolea et al., 2010](#)). Thus, it suggests that Arf3 might play a unique function at the TGN, which needs to be further explored. By contrast, little attention has been paid to the function of class II Arf proteins. Arf4 has been found to play a distinct role in generation of ciliary-targeted rhodopsin transport carriers ([Deretic et al., 2011](#); [Mazelova et al., 2009](#)). Moreover, Arf4 and Arf5 have been shown to interact with the Ca^{2+} -dependent activator protein for secretion, and regulate dense core vesicles trafficking in the TGN ([Sadakata et al., 2010](#)). The functions of the PM-localized Arf6 are multiple and complex. Arf6 participates in regulating vesicular trafficking, affecting the cortical actin cytoskeleton, remodelling of membrane lipids, etc ([Donaldson, 2003](#); [Donaldson and Jackson, 2011](#); [Gillingham and Munro, 2007](#)).

1.3.2.2 Arl subfamily

More than 20 Arl proteins have been identified in humans ([Gillingham and Munro, 2007](#)). Although members of Arl group are poorly studied at cellular and molecular levels, they are expected to have diverse functions on the localized intracellular compartments, such as ER, Golgi, lysosomes ([Hofmann and Munro, 2006](#)) and cilium ([Cevik et al., 2010](#); [Gillingham and Munro, 2007](#); [Houghton et al., 2012](#)). One of the widely conserved and best characterized Arls in eukaryotes is Arl1.

Arl1, a TGN-localized small G protein, is essential for Golgi structure maintenance and membrane trafficking between the endosome and the TGN ([Lu et al., 2001](#); [Lu et al., 2004](#); [Munro, 2005](#)). When Arl1 is activated with its GTP-bound form, it interacts with the GRIP domain of Golgin97 and Golgin245, and recruits them to the Golgi ([Lu and Hong, 2003](#)). In yeast, Arl1 has also been found to directly interact with Vps53p, one of the subunits of the tethering factor GARP, as mentioned above.

In addition to Arl1, another Arls member, Arl5, also localizes in the Golgi in mammalian cells ([Gillingham and Munro, 2007](#)). In vertebrates, Arl5 contains three closely related paralogs – Arl5a, Arl5b and Arl5c; the former two appear to be ubiquitously expressed ([Breiner et al., 1996](#); [Rosa-Ferreira et al., 2015](#)). It has been documented that Arl5a and Arl5b are localized to the *trans*-Golgi in HeLa cells, and Arl5b plays a role in the regulation of retrograde membrane transport from endosomes to the TGN ([Houghton et al., 2012](#)). More recently, two effectors of Arl5b have been identified by two different research groups: 1) Rosa-Ferreira et al found that Arl5b interacts with GARP and recruits it to the Golgi in both fly and human cells. Depletion of Arl5b decreased the GARP in *Drosophila* tissues as well as in HeLa cells ([Rosa-Ferreira et al., 2015](#)); and 2) Toh et al showed that Arl5b is associated with the adaptor protein AP-4, and contributes to the recruitment of AP-4 to the TGN membrane. The interaction between Arl5b and AP-4 is necessary for transporting the Amyloid precursor protein (APP) from the TGN to the endosomes ([Toh et al., 2017](#)). Despite these findings, the precise role of Arl5 and its downstream effectors are still not fully understood.

The subcellular localization, effectors and cellular functions of the mammalian Arl small G proteins are summarized and listed in Table 1.

Table 1: The mammalian Arl family G proteins.

Arl	subcellular localization	effectors	cellular functions
Arl1	TGN (Lu et al., 2001)	GRIP domain Golgins (Lu and Hong, 2003); Arfaptin-2 (Man et al., 2011 ; Nakamura et al., 2012)	Golgi structure (Lu et al., 2001); post-Golgi trafficking (Lu et al., 2004 ; Nishimoto-Morita et al., 2009 ; Yu and Lee, 2017)

Arl2	mitochondria (Newman et al., 2017 ; Sharer et al., 2002); centrosome (Zhou et al., 2006)	cofactor D (Bhamidipati et al., 2000); Bart (Sharer et al., 2002); PDEδ6 (Hanzal - Bayer et al., 2002); HRG4 (Kobayashi et al., 2003)	microtubule dynamics (Al-Bassam, 2017 ; Bhamidipati et al., 2000); mitochondrial transport of nucleotide (Sharer et al., 2002); mitochondrial fusion (Newman et al., 2017)
Arl3	cilium (Schwarz et al., 2012 ; Schwarz et al., 2017 ; Zhou et al., 2006); nucleus, Golgi, mitotic spindle (Zhou et al., 2006)	RP2 (Bartolini et al., 2002 ; Schwarz et al., 2012); Bart (Sharer and Kahn, 1999); UNC119 (Wright et al., 2011); PDEδ6 (Linari et al., 1999); Kif7 and Kif17 (Schwarz et al., 2017); PrBPδ (Wright et al., 2016); STAT3 (Togi et al., 2016)	microtubule dynamics, cytokinesis (Zhou et al., 2006); ciliary functions (Hanke-Gogokhia et al., 2016); prenylated protein trafficking (Wright et al., 2016); nuclear retention of STAT3 (Togi et al., 2016)
Arl4a,c,d	nucleus(Lin et al., 2000); PM (Hofmann et al., 2007)	cytohesin-2 (Hofmann et al., 2007)	spermatogenesis in mice (Arl4a) (Schürmann et al., 2002); tubulogenesis and tumourigenesis (Arl4c) (Matsumoto et al., 2016); actin cytoskeleton and cell migration (Hofmann et al., 2007)
Arl5a,b,c	Golgi (Arl5a, Arl5b) (Houghton et al., 2012)	GARP (Rosa-Ferreira et al., 2015); AP-4 (Toh et al., 2017)	retrograde trafficking (Arl5a, Arl5b) (Houghton et al., 2012 ; Rosa-Ferreira et al., 2015); anterograde transport of APP from the Golgi-to-EE (Toh et al., 2017)
Arl6	centrosome (Wiens et al., 2010); cilium (Fan et al., 2004)	BBSome (Jin et al., 2010)	ciliary protein targeting (Jin et al., 2010 ; Li et al., 2012a ; Wiens et al., 2010)
Arl8a,b	LE and lysosome (Hofmann and Munro, 2006);	SKIP (Arl8b) (Rosa-Ferreira and Munro, 2011); HOPS (Arl8b) (Khatter et al., 2015a)	lysosome trafficking and motility (Arl8b) (Hofmann and Munro, 2006 ; Khatter et al., 2015a ; Rosa-Ferreira and Munro, 2011); tubular lysosome biogenesis (Arl8b) (Mrakovic et al.,

			2012); phagosome-lysosome fusion (Khatter et al., 2015b)
Arl9	unknown	none known	unknown
Arl10	unknown	none known	unknown
Arl11	unknown	none known	tumor suppressor and apoptotic signaling (Jiang et al., 2017 ; Yendamuri et al., 2008 ; Yendamuri et al., 2007)
Arl13a	unknown	none known	unknown
Arl13b	cilium (Cantagrel et al., 2008); PM and tubular-vesicular structure (Barral et al., 2012)	exocyst complex (Seixas et al., 2016); Myh9 (Casalou et al., 2014); INPP5E (Humbert et al., 2012 ; Nozaki et al., 2017); UBC-9 (Li et al., 2012b)	cilium biogenesis and Shh signaling (Cantagrel et al., 2008 ; Larkins et al., 2011) ; ciliary retrograde protein trafficking (Humbert et al., 2012 ; Nozaki et al., 2017) endocytic recycling traffic, actin cytoskeleton (Barral et al., 2012 ; Casalou et al., 2014)
Arl14	lysosome (Paul et al., 2011)	ARF7EP (Paul et al., 2011)	MHCII trafficking (Paul et al., 2011)
Arl15	Golgi, cytoplasm (Zhao et al., 2017)	ASAP2 (Zhao et al., 2017)	insulin signaling pathway (Zhao et al., 2017)
Arl16	unknown	Rig-1 (Yang et al., 2011)	host response to viral infection (Yang et al., 2011)
ARFRP1	TGN	none known	activation of Arl1(Nishimoto-Morita et al., 2009 ; Zahn et al., 2006); post-Golgi membrane trafficking (Nishimoto-Morita et al., 2009 ; Shin et al., 2005 ; Zahn et al., 2006 ; Zahn et al., 2008); lipolysis (Hommel et al., 2010); lipidation of chylomicrons (Jaschke et al., 2012); normal growth and glycogen storage (Hesse et al., 2012)
TRIM23	Golgi and lysosome (Vitale et al., 2000)	cytohesin-1(Vitale et al., 2001); PPAR γ (Watanabe et al., 2015); UL144 (Poole et al., 2009)	adipocyte differentiation (Watanabe et al., 2015); NF- κ B signaling (Poole et al., 2009)

BART: binder of Arl Two; **PDE δ** : delta subunit of type 6 phosphodiesterase; **HRG4**: human retinal gene 4; **RP2**: retinitis pigmentosa 2; **Kif**: kinesin-like protein; **PrBP δ** : prenyl binding protein δ ; **STAT3**: signal transducer and activator of transcription 3; **APP**: amyloid precursor

protein; **BBSome**: Bardet-Biedl Syndrome protein complex; **SKIP**: SifA and kinesin-interacting protein; **HOPS**: homotypic fusion and protein sorting complex; **Shh**: sonic hedgehog; **Myh9**: non-muscle myosin heavy chain IIA; **INPP5E**: phosphoinositide 5-phosphatase; **UBC-9**: the sole E2 small ubiquitin-like modifier (SUMO)-conjugating enzyme; **ARF7EP**: ARF7 effector protein; **ASAP2**: ARF-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2; **Rig-1**: retinoic acid-inducible gene 1; **PPAR γ** : peroxisome proliferator-activated receptor γ ; **NF- κ B**: nuclear factor- κ B.

1.3.2.3 Sar subfamily

The Sar subfamily comprises only one member – Sar1. Sar1 presents in all eukaryotes examined so far. Active Sar1 embeds in the ER membranes through its N-terminal amphipathic helix, which functions in initiation of membrane curvature and recruitment of COPII coat for subsequent ER-to-Golgi transport ([Bielli et al., 2005](#); [Lee et al., 2005](#)). Sar1 has been reported to regulate the ER-mitochondria contact sites through its effects on membrane curvature ([Ackema et al., 2016](#)). The ER-localized Sec12 and the COPII-vesicle-localized Sec23 act as the GEF and GAP for Sar1, respectively ([Barlowe and Schekman, 1993](#); [Bielli et al., 2005](#)).

2. Objectives

Cellular nutrients, including glucose and related sugars, AAs and other carbon sources, not only function as most fundamental resources for the growth and proliferation of cells, but also serve as important signaling molecules to regulate cellular metabolism through transcription and translation. However, whether & how nutrients regulate the intracellular membrane trafficking, especially the endosome-to-Golgi pathway is still unknown. In this study we attempt to investigate the role of nutrients on the endosome-to-Golgi trafficking.

3. Materials and Methods

The techniques and reagents used for this study were described in detail in this section.

3.1 Constructs

Various DNA plasmids including bacterial and mammalian expression plasmids, gene knock-down plasmids as well as viral plasmids were either constructed or acquired for this study.

3.1.1 Arl5 constructs

1) Arl5a-wt-GFP: The coding sequence (CDS) of human Arl5a was PCR amplified from a cDNA clone (GenBank Accession No.: NM_012097) using a pair of oligonucleotides 5'-CCG GAA TTC GCC ACC ATG GGA ATT CTC TTC ACT AGA ATA-3' and 5'-CGC GGA TCC CGT CTA ATC TTA AGT CGT GAC ATC-3' as primers. The resulting PCR product was purified and double digested by EcoRI/BamHI sites, which was further ligated into EcoRI/BamHI digested pEGFP-N1 (Clontech) vector.

2) Arl5a-QL-GFP: To introduce point mutation into Arl5a at the position 70, two PCR amplifications were performed by using Arl5a-wt-GFP as the template and primer pairs (5'-CCG GAA TTC GCC ACC ATG GGA ATT CTC TTC ACT AGA ATA-3' and 5'-AGA ACG AAG AGA TTC AAG GCC ACC AAT ATC CCA-3') and (5'-TGG GAT ATT GGT GGC CTT GAA TCT CTT CGT TCT-3' and 5'-CGC GGA TCC CGT CTA ATC TTA AGT CGT GAC ATC-3'). The two PCR fragments were mixed as template and subjected to second round of PCR amplification using the first and the fourth primer. The resulting product was purified and double digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-N1 vector.

3) Arl5a-TN-GFP: To introduce point mutation into Arl5a at the position 30, two PCR amplifications were performed by using Arl5a-wt-GFP as the template and primer pairs (5'-CCG GAA TTC GCC ACC ATG GGA ATT CTC TTC ACT AGA ATA-3' and 5'-TTG GTA AAG AAT GGT AGT TTT CCC TGC ATT ATC-3') and (5'-GAT AAT GCA GGG AAA ACT ACC ATT CTT TAC CAA-3' and 5'-CGC GGA TCC CGT CTA ATC TTA AGT CGT GAC ATC-3') The two PCR fragments were mixed as template and subjected to second round of PCR using the first and the fourth primer. The resulting

product was double digested by EcoRI/BamHI and ligated into pEGFP-N1 vector digesting using the same sites.

4) Arl5b-wt-GFP: The CDS of human Arl5b was PCR amplified from a cDNA clone (GenBank Accession No.: BQ270027) using a pair of oligonucleotides 5'-CCG GAA TTC GCC ACC ATG GGG CTG ATC TTC GCC AAA CTG TG-3' and 5'-CTA GCT GGA TCC CGT CTC ACA CCA ATC CGG GAG-3' as primers and ligated into EcoRI/BamHI digested pEGFP-N1 vector using the same sites.

5) Arl5b-QL-GFP: To introduce point mutation into Arl5b at the position 70, two PCR amplifications were performed by using Arl5b-wt-GFP as the template and primer pairs (5'-CCG GAA TTC GCC ACC ATG GGG CTG ATC TTC GCC AAA CTG TG-3' and 5'-GAT CGC AGA GAC TCA AGA CCA CCA ATA TCC CAC-3') and (5'-GTG GGA TAT TGG TGG TCT TGA GTC TCT GCG ATC-3' and 5'-CTA GCT GGA TCC CGT CTC ACA CCA ATC CGG GAG-3'). The two PCR fragments were mixed and subjected to second round of PCR using the first and the fourth primer. The resulting product was purified and double digested by EcoRI/BamHI and ligated into digested pEGFP-N1 vector using the same sites.

6) Arl5b-QL-mCherry: To construct the Arl5b-QL-mCherry plasmid, the fragment of Arl5b-QL was released from Arl5b-QL-GFP with EcoRI/BamHI and ligated into EcoRI/BamHI digested pmCherry-N1 (Takara Bio) vector.

7) Arl5b-TN-GFP: To introduce point mutation into Arl5b at the position 30, two PCR amplifications were performed by using Arl5b-wt-GFP as the template and primer pairs (5'-CCG GAA TTC GCC ACC ATG GGG CTG ATC TTC GCC AAA CTG TG-3' and 5'-GAT AAT GCA GGG AAA AAT ACC ATT CTT TAC C-3') and (5'-GGT AAA GAA TGG TAT TTT TCC CTG CAT TAT C-3' and 5'-CTA GCT GGA TCC CGT CTC ACA CCA ATC CGG GAG-3'). The two PCR fragments were mixed and subjected to second round of PCR using the first and the fourth primer. The resulting product was purified and double digested by EcoRI/BamHI and ligated into pEGFP-N1 vector using the same sites.

8) Arl5b-TN-mCherry: To construct the Arl5b-TN-mCherry plasmid, the fragment of Arl5b-TN was released from Arl5b-TN-GFP with EcoRI/BamHI and ligated into EcoRI/BamHI digested pmCherry-N1 vector.

9) Arl5b-wt-His: The coding region of human Arl5b was PCR amplified using a pair of oligonucleotides (5'-ACG ATA AGA TCT GCC ACC ATG GGG CTG ATC TTC GCC AAA C-3' and 5'-AGT TCA AAG CTT TCT CAC ACC AAT CCG GGA GGT CAT CCA C-3') as primers. The resulting PCR product was digested by BglII/HindIII and inserted into pET-30a vector (Novagen) using the same sites.

10) Arl5b-wt-GST: To construct the Arl5b-wt-GST plasmid, the fragment of Arl5b-wt was released from Arl5b-wt-GFP with EcoRI/BamHI and ligated into EcoRI/BamHI digested pGEB vector (a modified pGEX-KG vector from GE Healthcare).

11) Arl5b-QL-G2A-GFP: To substitute of glycine (G) residue by alanine (A) at the Arl5b-QL-GFP position 2, PCR amplification was performed using Arl5b-QL-GFP as the template and a pair of oligonucleotides (5'-ACC GCA GAA TTC GCC ACC ATG GCG CTG ATC TTC GCC AAA CTG-3' and 5'-BamHI-CAT GAC GGA TCC CGT CTC ACA CCA ATC CGG GAG GTC ATC-3' as primers. The resulting PCR product was digested by EcoRI/BamHI and ligated into pEGFP-N1 vector using the same sites.

12) Arl5c-wt-GFP: To construct human and mouse Arl5c-wt-GFP, the CDS of human and mouse Arl5c were amplified from two cDNA clones (GenBank Accession No.: NM_001143968 and BC065791.1, respectively) by using primer pairs (5'-GCA CCG GAA TTC GCC ACC ATG GGA CAG CTG ATC GCC-3' and 5'-CTA GCT GGA TCC CGG TTA GCA GCG GCC TGA G-3') and (5'-GCG ATC GAA TTC GCC ACC ATG GGA CAG CTG ATA GCC AAG-3' and 5'-CAC TAC GGA TCC CCG TTG GCG GTG GCC TGA GCT TGC AT-3'), respectively. The resulting PCR products were digested with EcoRI/BamHI and inserted into same enzymes digested pEGFP-N1 vector.

13) mArl5c-QL-GFP: To introduce point mutation into mouse Arl5c at the position 70, two PCR amplifications were performed by using mouse mArl5c-wt-GFP as the template and primer pairs (5'-GCG ATC GAA TTC GCC ACC ATG GGA CAG CTG ATA GCC AAG-3' and 5'-GCC TCC AGG CCC CCT AGG TCC CAC ATG-3') and (5'-CAT GTG

GGA CCT AGG GGG CCT GGA GGC-3' and 5'-CAC TAC GGA TCC CCG TTG GCG GTG GCC TGA GCT TGC AT-3'). The two PCR fragments were mixed and subjected to second round of PCR using the first and the fourth primer. The resulting product was purified and double digested by EcoRI/BamHI and ligated into EcoRI/BamHI digested pEGFP-N1 vector.

14) mArl5c-TN-GFP: To introduce point mutation into mouse Arl5c at the position 30, two PCR amplifications were performed by using mouse mArl5c-wt-GFP as the template and primer pairs (5'-GCG ATC GAA TTC GCC ACC ATG GGA CAG CTG ATA GCC AAG-3' and 5'-GAG AAT GGT GTT CTT CCC TGC-3') and (5'-GCA GGG AAG AAC ACC ATT CTC-3' and 5'-CAC TAC GGA TCC CCG TTG GCG GTG GCC TGA GCT TGC AT-3'). The two PCR fragments were mixed as template and subjected to PCR using the first and the fourth primer. The resulting product was digested by EcoRI/BamHI and ligated into pEGFP-N1 vector using the same sites.

3.1.2 Lamtor1 constructs

1) Lamtor1-GFP: The CDS of Lamtor1 was PCR amplified using a full length clone, which was recovered from our yeast two-hybrid screening, as the template and a pair of oligonucleotides 5'-GAC TAG CTC GAG ATG GGG TGC TGC TAC AGC AGC-3' and 5'-GAA CTC GAA TTC GTG GGA TCC CAA ACT GTA CAA CCA G-3' as the primer pair. The resulting PCR product was digested by XhoI/EcoRI and ligated into pEGFP-N1 vector using the same sites.

2) Lamtor1-mCherry: Lamtor1-GFP was digested by XhoI/EcoRI and the insert released was ligated into pmCherry-N1 using the same sites.

3) Lamtor1-Myc: Oligonucleotides 5'-AAT TCA GTA CTC AGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GTA AAG C-3' and 5'-GGC CGC TTT ACA GAT CCT CTT CTG AGA TGA GTT TTT GTT CTG AGT ACT G-3' were annealed to generate a fragment encoding Myc-tag and ligated into EcoRI/NotI digested Lamtor1-GFP using the same sites.

3.1.3 CD8a-reporter chimeras constructs

CD8a-fused furin, CI-M6PR, CD-M6PR and sortilin in pCI-neo vector (Promega) were previously described ([Mahajan et al., 2013](#)).

3.1.4 Lentivirus constructs

1) pLVX-CD8a-furin: The fragment encoding CD8a-furin was PCR amplified from CD8a-furin in pCI-neo using oligonucleotides 5'-GTC TAG AAT TCA GCC ACC ATG GCC TTA CCA GTG ACC GCC TTG C-3' and 5'-GAC CTG TCT AGA TTA GAG GGC GCT CTG GTC TTT GAT AAA GGC G-3' as primers. The resulting fragment was digested by EcoRI/XbaI and ligated into pLVX-Puro vector (Clontech) using the same sites.

2) pLKO.1-GL2 shRNA: The firefly luciferase (GL2) shRNA was used as a control in shRNA knockdown experiment. The two following oligonucleotides: 5'-CCG GAA CGT ACG CGG AAT ACT TCG ACT CGA GTC GAA GTA TTC CGC GTA CGT TTT TTT G-3' and 5'-AAT TCA AAA AAA CGT ACG CGG AAT ACT TCG ACT CGA GTC GAA GTA TTC CGC GTA CGT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector (Addgene # 10878; a gift from D. Root).

3) pLKO.1-Arl5a shRNA: The two following oligonucleotides 5'-CCG GAA TGA TCT CTA CTG ACC TCT TCT CGA GAA GAG GTC AGT AGA GAT CAT TTT TTT G-3' and 5'-AAT TCA AAA AAA TGA TCT CTA CTG ACC TCT TCT CGA GAA GAG GTC AGT AGA GAT CAT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

4) pLKO.1-Arl5b shRNA: The two following oligonucleotides 5'-CCG GAA TAC CTC ACC CTT AGT TCA ACT CGA GTT GAA CTA AGG GTG AGG TAT TTT TTT G-3' and 5'-AAT TCA AAA AAA TAC CTC ACC CTT AGT TCA ACT CGA GTT GAA CTA AGG GTG AGG TAT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

5) pLKO.1-Vps51 shRNA #1: Two synthetic oligonucleotides 5'-CCG GAA CCT CTT GAG CAA TAT CCA GCT CGA GCT GGA TAT TGC TCA AGA GGT TTT TTT G-3' and 5'-AAT TCA AAA AAA CCT CTT GAG CAA TAT CCA GCT CGA GCT GGA

TAT TGC TCA AGA GGT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

6) pLKO.1-Vps51 shRNA #2: Two synthetic oligonucleotides 5'-CCG GAA CGT ATT GAT GTG TTC AGC CCT CGA GGG CTG AAC ACA TCA ATA CGT TTT TTT G-3' and 5'-AAT TCA AAA AAA CGT ATT GAT GTG TTC AGC CCT CGA GGG CTG AAC ACA TCA ATA CGT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector to obtain the desire clone.

7) pLKO.1-Vps54 shRNA #1: The two following oligonucleotides 5'-CCG GAA CAT TGC TCA CCA GAT CTC TCT CGA GAG AGA TCT GGT GAG CAA TGT TTT TTT G-3' and 5'-AAT TCA AAA AAA CAT TGC TCA CCA GAT CTC TCT CGA GAG AGA TCT GGT GAG CAA TGT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

8) pLKO.1-Vps54 shRNA #2: The two following oligonucleotides 5'-CCG GAA CCA GCT GAA GTT CTT ATT GCT CGA GCA ATA AGA ACT TCA GCT GGT TTT TTT G-3' and 5'-AAT TCA AAA AAA CCA GCT GAA GTT CTT ATT GCT CGA GCA ATA AGA ACT TCA GCT GGT T-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector to obtain the desire clone.

9) pLKO.1-SLC38A9 shRNA #1: The two following oligonucleotides 5'-CCG GGC CTT GAC AAC AGT TCT ATA TCT CGA GAT ATA GAA CTG TTG TCA AGG CTT TTT G-3' and 5'-AAT TCA AAA AGC CTT GAC AAC AGT TCT ATA TCT CGA GAT ATA GAA CTG TTG TCA AGG C-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

10) pLKO.1-SLC38A9 shRNA #2: Two synthetic oligonucleotides 5'-CCG GCC TCT ACT GTT TGG GAC AGT ACT CGA GTA CTG TCC CAA ACA GTA GAG GTT TTT G-3' and 5'-AAT TCA AAA ACC TCT ACT GTT TGG GAC AGT ACT CGA GTA CTG TCC CAA ACA GTA GAG G-3' were annealed and ligated into AgeI/EcoRI digested pLKO.1 vector.

3.1.5 Other related constructs

1) furin-GFP or mCherry: The full length CDS of furin was PCR amplified by using a cDNA clone (GenBank Accession No.: BC012181.1) as the template and the following oligonucleotides 5'-CAG ATC TCG AGC TCA AGC TTC GAA TTC GCC ACC ATG GAG CTG AGG CCC TGG-3' and 5'-GAT CCC GGG CCC GCG GTA CCG TCG ACC CGA GGG CGC TCT GGT CTT TG-3' as primers. The PCR fragment was digested by EcoRI/SalI and ligated into pEGFP-N1 or pmCherry-N1 vector, respectively, using the same sites.

2) GalT-mCherry: the fragment of β 1, 4-galactosyltransferase (GalT) was released from GalT-tdTomato ([Lu et al., 2013](#)) with NheI/BamHI and subsequently ligated into NheI/BamHI digested pmCherry-N1 vector (Takara Bio, Shiga, Japan).

3) Gift and purchased plasmids: To produce the lentivirus, three packaging plasmids pLP1, pLP2 and pLP/VSVG were purchased from Invitrogen. Plasmids pLKO.1-Lamtor1 shRNA (#26631), pLKO.1-RagA shRNA #1 (#30319), pLKO.1-RagB shRNA #1 (#26627) and pLKO.1-Lamtor3 shRNA (#26632) were purchased from Addgene. Plasmids like GFP-Rab7-wt, TfR-GFP and mCherry-Rab5 were gifts from T. Kirchhausen.

3.2 Antibodies

The antibodies used in this study were either made in this lab or obtained commercially.

3.2.1 Antibodies used in this study

The source, dilution and application of the antibodies used are described in detail below (Table 2).

Table 2: List of antibodies used in this study.

Antibody against	Host Species	Source	Dilution
Giantin	Rabbit polyclonal	Biolegend	1:2000 (IF)
Golgin245 (p230)	Mouse monoclonal	BD Bioscience	1:100 (IF)
CD8a (OKT8)	Mouse monoclonal	DHSB	1:500 (IF)
Transferrin receptor (OKT9)	Mouse monoclonal	DHSB	1:250 (IF)
Lamp1 (H4A3)	Mouse monoclonal	DHSB	1:500 (IF)

EEA1	Mouse monoclonal	BD Bioscience	1:1000 (IF)
RUFY1	Rabbit polyclonal	Proteintech	1:200 (IF)
GM130	Mouse monoclonal	BD Bioscience	1:100 (IF)
Furin	Rabbit polyclonal	Thermo Scientific	1:100 (IF)
M6PR	Mouse monoclonal	Thermo Scientific	1:200 (IF)
c-Myc	Mouse monoclonal	Santa Cruz	1:200 (IF)
Mouse (AF488 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
Mouse (AF594 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
Mouse (AF647 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
Rabbit (AF488 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
Rabbit (AF594 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
Rabbit (AF647 conjugated)	Goat polyclonal	Thermo Scientific	1:500 (IF)
GFP	Mouse monoclonal	Santa Cruz	1:1000 (WB)
Phospho-p70S6 Kinase (Thr389)	Mouse monoclonal	Cell Signaling	1:1000 (WB)
GAPDH	Rabbit polyclonal	Santa Cruz	1:1000 (WB)
α -tubulin	Rabbit polyclonal	Abcam	1:1000 (WB)
β -tubulin	Mouse monoclonal	Sigma Aldrich	1:1000 (WB)
RagA	Rabbit monoclonal	Cell Signaling	1:750 (WB)
Mouse IgG-HRP	Goat polyclonal	Bio-Rad	1:10000 (WB)
Rabbit IgG-HRP	Goat polyclonal	Bio-Rad	1:10000 (WB)
Protein A-HRP	-	Abcam	1:3000 (WB)
IgG	Rabbit polyclonal	Lu Lei lab	1 μ g/ml (IP)
GFP	Rabbit polyclonal	Lu Lei lab	1 μ g/ml (IP)
Lamtor1	Rabbit monoclonal	Cell Signaling	1:250 (IF), 1:1000 (WB)
Arl5b	Rabbit polyclonal	This study	1 μ g/ml (IP), 1:750 (WB)
Depleted Arl5b	Rabbit polyclonal	This study	1 μ g/ml (IP), 1:750 (WB)

3.2.2 Generation of polyclonal antibodies against human Arl5b

1) Preparation of the antigen: The bacterial expression plasmid Arl5b-wt-His was transformed into BL21 (DE3) competent *E.coli*. To induce protein expression, 0.5 mM Isopropyl β -D-1-thiogalactopy (IPTG) was added and cultured overnight at room

temperature. The collected bacteria pellet was re-suspended in 8 M urea in PBS and subjected to sonication. Cell lysate was incubated for 2 h at room temperature to completely lyse the cell. After spinning down, the supernatant was incubated with Ni-NTA beads at room temperature for 2 h. The beads were then washed with 25 mM imidazole in urea/PBS followed by elution in 250 mM imidazole in urea/PBS. The eluted protein was dialyzed against PBS. After concentration, protein was sent for antibody production (Genemed Synthesis, Inc).

2) Purification of the antibody: The purified Arl5b-wt-GST protein on Glutathione Sepharose 4B (GST) Agarose Beads were washed with cold PBS followed by 0.2 M sodium borate buffer (pH 9.0) for 2 times. The beads were incubated overnight with 50 mM dimethyl pimelimidate (DMP, Sigma) in 0.2 M sodium borate buffer at 4°C. After cross linking, beads were washed with sodium borate buffer and incubated with 0.2 M ethanolamine (pH 8.0) for 2 h at room temperature. The beads were then washed with PBS and incubated with 6 ml of PBS diluted Arl5b-wt-His generated rabbit serum for 1 h at room temperature. After washing thoroughly with PBS, bound antibody was eluted in IgG elution buffer (100 mM glycine, pH 2.8) and neutralized with 1 M Tris buffer (pH 8.0). Finally, the collected elute was dialyzed in ice cold PBS overnight and concentrated using Amicon Ultra-15 Centrifugal Filter Units. The purified antibody was stored at -20°C in 50% glycerol.

For purifying the depleted-Arl5b antibody, the serum was collected after two times binding with Arl5b-wt-GST beads and then incubated with Protein A beads (Sigma-Aldrich). Similarly, bound antibody on the beads were eluted and neutralized, followed by dialysis and concentration as mentioned above.

3.3 Main reagents

3.3.1 siRNA

In this study, all siRNA oligos were synthesized and purchased from GE Dharmacon: the firefly luciferase GL2 (#D-001100-01-20) and siRNA SMART pools for human Lamtor1 (#L-020916-02-0005), human Arl5a (#L-012408-00-0005), Arl5b (#L-017861-02-0005) and Arl5c (#L-030887-02-0005).

GL2: 5'-CGU ACG CGG AAU ACU UCG A-3'

Lamtor1 (SMART pool): 5'-UCU CCA GGA UAG CUG CUU A-3'; 5'-GGC UUA UAC AGU ACC CUA A-3'; 5'-AAG UGA GGG UAG AAC CUU U-3'; 5'-GUU UGU CAC CCU CGA UAA A-3'.

3.3.2 Nutrient starvation and stimulation medium

1) Hank's Balanced Salt Solution (HBSS): One of the AA and serum starvation medium used in this study is HBSS. The components of HBSS (pH ~7.2) were: 137 mM NaCl, 5.36 mM KCl, 1.26 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 1000 mg/L Glucose.

2) DMEM-base: It was modified from the Life Technologies Dulbecco's Modified Eagle Medium (DMEM), high glucose (Thermofisher Scientific, #11965). The DMEM-base contains inorganic salts (1.8 mM CaCl₂, 2.5×10^{-4} mM Fe(NO₃)₃·9H₂O, 0.8 mM MgSO₄, 5.3 mM KCl, 44 mM NaHCO₃, 110 mM NaCl and 0.91 mM NaH₂PO₄·H₂O), 110 mg/L C₃H₃NaO₃ and vitamins (life technologies, #11120052). It was also used as the AA and serum starvation medium in this study.

3) DMEM/-AAs: 4.5g/ L glucose was added to the DMEM-base to make the DMEM/-AAs.

4) Nutrient stimulation medium: DMEM (GE Healthcare Life Science, #SH30002.03); complete medium [DMEM plus 10% fetal bovine serum (FBS) (Gibico, #10270-106)] were used as nutrient stimulation medium in this study.

5) Others: Selective AA(s) was(were) added to DMEM/-AAs to make corresponding media containing defined AAs; DMEM/-Gln and DMEM/-Leu were prepared by supplying Leu and Gln, respectively, to DMEM/-Gln/-Leu (MP Biomedicals, #1642149).

3.3.3 Small molecules, drugs, antibiotics and chemicals

G418 (also known as Geneticin, Invitrogen); Polybrene (Sigma-Aldrich); Puromycin dihydrochloride (Sigma-Aldrich); concanamycin A (conA, Abcam); Torin1 (Tocris Bioscience); Rapamycin (InvivoGen); Nocodazole (Sigma-Aldrich); GMPPNP (Sigma-Aldrich) and GDP (Sigma-Aldrich) are all commercially available.

Except Gln (Invitrogen) and His (Fluka), all AAs were from Sigma-Aldrich.

Dialyzed-serum was prepared by dialyzing the serum in 3.5 kDa molecular weight cut-off dialysis tubing (Thermo Fisher, #68035) against PBS followed by passing through a syringe-driven 0.22 μ m filter unit (Sartorius).

3.4 Cell culture, transfection, lentiviral production and transduction

3.4.1 Cell culture and transfection

HeLa, HEK293T and BSC-1 cells were cultured in DMEM High glucose medium supplemented with 3.7 g/L NaHCO₃ and 10% FBS at 37°C in 5% CO₂ humidified atmosphere. HEK293FT cells which are used for lentiviral production were maintained in the same medium containing 500 μ g/ml G418.

Polyethylenimine (PEI) (Polysciences, Inc.) was used for transfecting plasmid DNA and Lipofectamine2000 (Invitrogen) was used to perform siRNA transfection. Cells were seeded at a confluency of ~85-90% and transfected according to the manufacturer's protocol.

3.4.2 Lentiviral production and transduction

1) Lentivirus Production: 293FT cells seeded in the 6-well-plate (about 90% confluent) were transfected with pLenti expression vector (containing the gene of interest) and the three packaging plasmids pLP1, pLP2 and pLP/VSVG in 4:2:1:1 ratio. Cells were incubated at 37°C for 18 h and replaced with fresh medium to incubate another 24-48 h to harvest virus-containing supernatants. The viral supernatants were then filtered through the Millipore 0.45 μ m filter unit (Sartorius) to remove any debris of cells. The collected viral supernatants could be used for transduction immediately or store at -80°C.

2) Lentivirus Transduction: HeLa cells were seeded in the 6-well-plate till 60-70% confluent. Before transduction, lentiviral stock was diluted into completed cell culture medium containing 8 μ g/ml polybrene in 1:1 ratio and added into each well of the cells. To increase the transduction efficiency, another time of transduction was performed. After 48-72 h of transduction, the infected cells can be used for the specific cell assays or processed for 1 μ g/ml puromycin selection for generating the stable cell lines.

3.5 Immunofluorescence

After washing with PBS to remove any trace amounts of medium, cells on coverslips (ø 12 mm) were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature or 4°C (for trafficking assay). Cells were then washed two times with 100 mM NH₄Cl to quench any reactive aldehyde groups, followed by washing two more times with PBS. The fixed cells were then incubated with the diluted primary antibody for 1 h at room temperature, followed by PBS washing and incubation with secondary antibody for 1 h at room temperature. Both primary and secondary antibodies were diluted in Fluorescence Dilution Buffer (FDB, PBS containing 5% FBS and 2% Bovine serum albumin (BSA)) supplemented with 0.1% Saponin. After washing three times with PBS, cells were then mounted in a 100 mM Tris (pH 8.5) made up of 12% Mowiol 4-88 (EMD Millipore, Billerica, MA, United States) and 30% glycerol.

For live cell imaging, cells grown on the ø 25 mm coverslips in 6-well plate were transfected with respective plasmids and cultured for 24 h. On the day, the culture chamber (SKE) containing 1 ml of CO₂ independent medium (Invitrogen) supplemented with 10% FBS and 4 mM Glutamine was used to hold the coverslip, and cells were viewed on the microscope. Temperature was maintained in the microscope incubator chamber.

3.6 Fluorescence microscopy

High resolution images were acquired under an inverted wide-field microscope system, comprising Olympus IX83 equipped with a Plan Apo oil objective lens (40X, NA1.30; 63X NA1.42; or 100X, NA1.40), a motorized stage, motorized filter cubes, a scientific complementary metal-oxide semiconductor camera (Neo; Andor) and a 200 W metal-halide excitation light source (Lumen Pro 200; Prior Scientific). Dichroic mirrors and filters in filter turrets were optimized for GFP/Alexa Fluor 488, mCherry/Alexa Fluor 594 and Alexa Fluor 647. The microscope system was controlled by MetaMorph software (Molecular Devices). The pixel size was 64 nm as measured by the micro-ruler (Geller MicroAnalytical Laboratory, Topsfield, MA, United States).

3.7 Nutrient starvation and stimulation of cells

To investigate the nutrient effects on the steady state of endogenous or overexpressed furin/CI-M6PR, HeLa cells grown on coverslips were cultured till 90% confluent or transfected with CD8a-furin/CI-M6PR or furin-GFP/mCherry for 24 h before the assay. Cells were rinsed with and incubated in complete medium, DMEM or HBSS for 2 h. For nutrient stimulation, cells were firstly rinsed with and starved in HBSS for 2 h, and stimulated with DMEM for different time. Cells were then fixed and processed for IF as described above.

3.8 Internalization transport assay

3.8.1 CD8a-furin and CD8a-CI-M6PR internalization assay

HeLa cells grown on the coverslips were transfected with CD8a-furin or CD8a-CI-M6PR, together with TfR-GFP or GFP-Rab7 respectively. After 24 h incubation, cells were cooled down on ice for 10 min to stop all cellular trafficking processes, followed by incubation with anti-CD8a antibody for 60 min on ice for surface-labeling. After washing away the unbound CD8a antibody, coverslips were incubated with fresh medium at 37°C for different time (0 min, 6 min, 12 min, 24 min, 48 min and 96 min). Cells were then PFA-fixed and processed for IF.

3.8.2 Internalization transport assay under nutrient starvation and stimulation conditions

In this study, CD8a-chimeras, especially CD8a-furin, were used as reporters for studying the internalization transport assay under nutrient starvation and stimulation conditions.

CD8a-furin stable cells or the cells after transfection, siRNA knockdown or shRNA knockdown were cultured in 24-well plate with coverslips for 24 h. Cells were then rinsed with and starved for 2 h (or different periods) using HBSS or DMEM/-AAs solution at 37°C incubator. After surface-labeling, cells on the coverslips were washed three times with ice-cold PBS, followed by addition of 400 µl starvation medium (continuously starved) or stimulation medium (stimulated) as described in section 3.3.2 for 20 min (or different periods) at 37°C. For studying the endocytosis assay, HeLa cells expressing CD8a-furin were chased at 37°C for different time after surface-labeling. Cells were then cooled on ice and washed with acetic acid buffer (PBS containing 0.2 M glacial acetic

acid and 0.5 M NaCl, pH 2.0) to remove any surface-bound antibody. For studying endosome-to-Golgi trafficking assay, cells after anti-CD8a antibody surface-labeling were firstly incubated at 18°C for 2 h to allow the CD8a-furin to accumulate in the EE/RE, followed by 37°C incubation for different time. After the assay, cells were fixed and subjected to IF.

3.9 Western blot

Cells lysates were prepared using 1×SDsS sample buffer and boiled for 10 min. The protein samples were resolved on SDsS-PAGE and transferred to PVDF membrane (Bio-Rad). The membrane was then blocked with 5% non-fat milk in PBST (1× PBS containing 0.05% Tween 20) for 1 h at room temperature, followed by incubation with diluted primary antibody for overnight at 4°C or 1 h at room temperature. After washing three times with PBST, the membrane was incubated with diluted HRP conjugated secondary antibody for 1 h at room temperature. The membrane was then washed with PBST and incubated with Luminol Enhancer and Hydrogen Peroxide solution (Advansta) in 1:1 ratio for 2 min. Subsequently, ImageQuant LAS-4000 (GE healthcare life sciences) was used to detect the signal.

3.10 Immunoprecipitation

293T cells were either seeded in the dishes or transfected with plasmids. After culturing for 24 h, cells were then lysed in 40 mM HEPES (pH 7.4) with 100 mM NaCl, 1 mM DTT and 0.1% Triton X-100. The lysate was allowed to rotate on a shaker for 30 min in cold room (4°C) followed by centrifugation at 17000×g for 30 min to remove membrane debris. After centrifugation, the supernatant was collected and incubated with respective antibodies at 4°C for overnight, followed by incubation with 20 µl pre-washed Protein A/G beads (Pierce) at 4°C for 4 h. The beads were extensively washed 5 times with lysis buffer and boiled in 2×SDS sample buffer to elute the proteins. WB was then performed to analyze the IP result.

3.11 Guanine nucleotide exchange of GST-Arl5b and GDT-Arl1

Glutathione bead-immobilized GST-Arl5b or GST-Arl1 was washed twice with the exchange buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM EDTA, 5 mM MgCl₂

and 1 mM DTT) and incubated with the buffer supplemented with 10 unit/ml calf intestinal alkaline phosphatase (New England Biolab) at room temperature for 2 h. Next, beads were washed by the exchange buffer and incubated with the same buffer supplemented with 0.5 mM GMPPNP or GDP (final concentration) for 1 h at the room temperature. 5 mM (final concentration) MgCl_2 was subsequently added to the system and beads were further incubated for 1 h at the room temperature. The exchanged GST-Arl5b on beads was stored at 4°C until use.

3.12 RT-qPCR

3.12.1 RNA extraction by Trizol

HeLa cells in 6-well plate were rinsed with ice-cold PBS followed by addition of 1 ml Trizol (Invitrogen, #15596-018) into each well to lyse the cells. The lysate was transferred into 1.5 ml falcon tubes and incubated at room temperature for 5 min to permit completely dissociation of nucleoprotein complexes. After adding 0.2 ml chloroform (1/5 volume), samples were vortexed for 15 sec and incubated at room temperature for 5 min, followed by centrifugation at 12,000 g for 15 min at 4°C. After centrifugation, the upper phase was transferred into fresh tubes. 0.5 ml isopropanol was added to precipitate the RNA at room temperature for 10 min, followed by centrifugation at 12000 g for 10 min at 4°C. The supernatant was removed and pellet was washed with 1 ml 75% ethanol. After centrifugation at 7500 g for 5 min at 4°C, supernatant was discarded completely and pellet was allowed to dry at room temperature for few minutes. 15-50 μl of DEPC water was added to dissolve the RNA pellet.

3.12.2 Reverse transcription

The reverse transcription was conducted by using the nanoScript 2 Reverse Transcription kits from Primerdesign. Firstly, the random reverse transcription primer was annealed to the denatured RNA at 65°C for 5 min, followed by addition of appropriate reaction buffer and dNTP mix to enable the reverse transcriptase to reverse transcribe the RNA into cDNA at 42°C for 20 min. The enzyme was then inactivated at 75°C for 10 min. The synthesized cDNA can be used as template for RT-qPCR or stored at -20°C until use.

3.12.3 Quantitative reverse transcription PCR

The Quantitative reverse transcription PCR (RT-qPCR) was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System with the PrecisionFAST qPCR MasterMix-Low Rox-SYBR kit (Primerdesign). The RT-qPCR program is as follows:

95°C 10 min
95°C 3 sec
60°C 1 min } 50 cycles
Melt curve

The primers used for RT-qPCR in this study were summarized below (Table 3). To ensure the efficient amplification and avoid amplification of contaminating genomic DNA, we followed these two guidelines to design the RT-qPCR primer: 1) the size of PCR amplicon should be smaller than 200 bp; and 2) the designed primer must span an exon-exon junction (one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon). To check the specificity of RT-qPCR primers and to ensure the RT-qPCR reaction specificity, the post-amplification melting-curve analysis was done.

Table 3: List of RT-qPCR primers used in this study.

Target protein	RT-qPCR primer
Arl5a	Forward primer: 5'-GTT AGC GCA TGA GGA CCT AAG-3' Reverse primer: 5'-CTT GGC ACA ATC CCT CGC CAG TTA C-3'
Arl5b	Forward primer: 5'-TGG CTC ATG AGG ATT TAC GGA AG-3' Reverse primer: 5'-CCT TGG CAT AAC CCT TCT CCT GTG-3'
Arl5c	Forward primer: 5'-TGG CCC ATG AGG CTC TAC AGG ATG-3' Reverse primer: 5'-TCC ATC CAC TGA AGT CTG GCA G-3'
Lamtor3	Forward primer: 5'-CCT GTT ATT AAA GTG GCA AAT GAC AAT GC-3' Reverse primer: 5'-TTG AAC CAC CTG GTA GGT GTT ATA G-3'
Vps51	Forward primer: 5'-CTC AGC CAC AGA CAC CAT CCG G-3' Reverse primer: 5'-GCG AGC GCT GAA GTC GGT GAT C-3'
Vps54	Forward primer: 5'-GTT GTT GTG AAG CTT GCA GAT CAG-3'

	Reverse primer: 5'-TGT TGC CTT CAC TCT CTG TAG G-3'
SLC38A9	Forward primer: 5'-CCT AGC ATT TTC CAT GTG CTG-3'
	Reverse primer: 5'-GCT CCT GAA TAT CTT ATG ATC CCT CC-3'

3.13 Quantification method used for trafficking assay

For imaging analysis, random fields of view were imaged. Image analysis was performed in *ImageJ* (<http://imagej.nih.gov/ij/>). In transient transfection, cells have various levels of expression of the reporter. Therefore, different cells should have distinct background fluorescence intensities. In order to make the images smooth for quantification, sliding paraboloid background subtraction (300 pixels) was first performed to all images. The region of interest (ROI) of the cell was manually drawn by tracing the cell's contour (Figure 7a). The ROI of the Golgi was generated by intensity thresholding using the co-stained endogenous Golgi marker (such as giantin or Golgin245) signal (Figure 7b). The image was background-subtracted by using ROIs outside cells. In the channel of the reporter fluorescence, A_{cell} and A_{Golgi} are the area (in pixels) of the cell and the Golgi ROI respectively, while I_{cell} and I_{Golgi} are the mean intensity of the cell and the Golgi ROI respectively. f is a constant value between 0 and 1. $f=0.5$ was used for all image quantification with either transfected or endogenous reporters. In each image to be quantified, border cells were excluded and all the rest cells positive for the reporter of interest were analyzed. In short, the fraction of Golgi-localized reporter of interest was measured by the following formula:

$$\text{Fraction of Golgi-localized reporter of interest} = \frac{(I_{Golgi} - f \times I_{cell}) \times A_{Golgi}}{(1 - f) \times I_{cell} \times A_{cell}}$$

To measure the fold of AAs effect, the AA stimulated Golgi trafficking of CD8a-furin was calculated as follows:

$$\text{AA stimulated Golgi trafficking of CD8a-furin} = \frac{\text{Fraction value in AA stimulation medium}}{\text{Fraction value in AA starvation medium}}$$

Figure 7: Quantification of the fraction of Golgi-localized reporter of interest using ImageJ.

(a) The channel of the reporter fluorescence. After sliding paraboloid background subtraction, the region of interest (ROI) of the cell was drawn manually by tracing the cell's contour. The image was then background-subtracted by using ROIs outside cells (highlighted using green box). **(b)** The channel of the Golgi marker (giantin or Golgin245) fluorescence. Similarly, sliding paraboloid background subtraction was performed to make the image smooth for quantification. The threshold value for the giantin or Golgin245 labeled Golgi was determined and applied to create a binary mask. A Golgi ROI was generated by the segmentation of giantin or Golgin245 signal within the binary mask. The fraction of Golgi-localized reporter of interest was determined by dividing the Golgi intensity of the test protein by the total cell intensity.

The analysis of data and student's t-test (P -values) were performed and determined in Microsoft Excel (two-tailed distribution and two-sample unequal variance). The graphs were plotted using Origin 8.5 (OriginLab).

4. Results

Chapter 1: AAs regulate the endosome-to-Golgi trafficking pathway

4.1 Characterization of CD8a-chimera reporters used in this study

In mammalian cells, the imaging based assay is one widely used approach to study the endosome-to-Golgi trafficking pathway ([Lu and Hong, 2014](#)). It depends on high resolution microscopy and reporters. As discussed in the introduction, most known *trans*-Golgi network (TGN)-localized transmembrane proteins, such as furin, CI-M6PR and TGN38 are commonly used reporters for endosome-to-Golgi trafficking ([Ghosh et al., 1998](#); [Mallet and Maxfield, 1999](#); [Seaman, 2004](#)). They cycle between the plasma membrane (PM) and Golgi through endosomes ([Lu and Hong, 2014](#)). Their relative distribution between the Golgi and endosomal pool shifts as a result of the change in endocytic trafficking.

In this study, the reporters used for studying endocytic trafficking are summarized below (Figure 8). Among these, we first examined the itinerary of CD8a-furin and CD8a-CI-M6PR.

The Rab small GTPases family has been shown to function as regulators of distinct steps in membrane trafficking pathways. Different Rab GTPases are localized to the surface of specific intracellular membrane-bound organelles ([Stenmark and Olkkonen, 2001](#)). Hence, they can be used as markers for particular organelles. Among these, Rab7 was first found to be localized within the late endosome (LE) ([Chavrier et al., 1990](#); [Pereira-Leal and Seabra, 2001](#)). Another reference marker used in this study is transferrin receptor (TfR), which has been well known to cycle between the PM, early endosome (EE) and recycling endosome (RE) ([Gruenberg and Maxfield, 1995](#); [Mayle et al., 2012](#)).

Figure 8: Schematic representation of the reporters used for studying the endocytic trafficking assay.

From (a) to (d) are the CD8a-chimeras, in which the cytoplasmic domain of CD8a was replaced by that of furin, CI-M6PR, CD-M6PR or sortilin. The C-terminal of the furin protein fused with (e) GFP or (f) mCherry were also constructed for the endocytic trafficking assay.

To determine the endocytic itinerary of CD8a chimeras, HeLa cells were transfected with CD8a-furin/CI-M6PR together with TfR-GFP or GFP-Rab7. After surface-labeling with anti-CD8a antibody, cells were chased at 37°C for various lengths of time to allow endocytic trafficking.

As expected, the antibody–CI-M6PR complexes were restricted to the cell surface at 0°C (Figure 9a and 9b, 0 minutes). After 6 min of chase, the surface-labeled antibody–CI-M6PR complexes had been internalized and were found to localize in endosomal structures distributed in the cell periphery and throughout the cytoplasm. By 12 min, most of the CI-M6PR-positive endosomal structures were TfR positive. After 24 min of internalization, CD8a-CI-M6PR overlapped extensively with the EE/RE marker TfR-GFP. At this time point, some colocalization of CI-M6PR with giantin labeled Golgi was also

detected. These results suggest that the majority of the internalized CI-M6PR first entered the TfR-positive EE/RE before being delivered to the Golgi. By 48 min, a substantial amount of the internalized CI-M6PR colocalized with the Golgi marker, while the endosomal localization also remained positive. As the chase proceeded to 96 min, CD8a-CI-M6PR achieved its steady state distribution, which was colocalized well with the Golgi marker, giantin. However, no obvious colocalization between CD8a-CI-M6PR and the LE marker GFP-Rab7 was observed throughout the period of internalization (Figure 9b), which indicated that the CD8a-CI-M6PR does not pass through the LE before reaching the Golgi. Thus, our data is consistent with previous reports that the majority of the PM CI-M6PR traffics to the Golgi by passing through the EE and the RE ([Lin et al., 2004](#); [McKenzie et al., 2012](#)).

Figure 9: CD8a-CI-M6PR is transported via EE and RE en route to the Golgi.

Time course images show the endocytic trafficking of CD8a-CI-M6PR to the Golgi. HeLa cells were transfected with CD8a-CI-M6PR together with (a) TfR-GFP or (b) GFP-Rab7 for 24 h and surface-labeled with anti-CD8a antibody for 60 min on ice. Cells were chased at 37°C for various lengths of time before immunolabeling of CD8a and giantin. TfR-GFP was used as the EE/RE marker, while GFP-Rab7 was used as the LE marker. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. Bars, 10 μ m.

On the contrary, CD8a-furin followed a different trafficking pathway. As shown in Figure 10, the CD8a-furin signal accumulated at the periphery of PM at 0 min. Similar to CD8a-CI-M6PR, the antibody–CD8a-furin complexes were efficiently internalized and arrived at the TfR-positive structures after 6 min of chase. Unlike CD8a-CI-M6PR, the localization of CD8a-furin with the LE marker GFP-Rab7 was detected at 12 min and showed an increase over 24 min. However, only a low level of overlap was observed between CD8a-furin and TfR-GFP after 24 min of internalization. By 48 minutes, the majority of internalized CD8a-furin complexes were concentrated in the perinuclear region, which colocalized well with the Golgi marker giantin. At this time point, there is almost no colocalization of CD8a-furin with TfR-GFP. After 96 min of internalization, a substantial amount of the internalized furin colocalized with the giantin. Thus, the itinerary of CD8a-furin was different from CD8a-CI-M6PR, which was transported via LE and eventually accumulated in the Golgi.

Figure 10: CD8a-furin is transported via LE en route to the Golgi.

Time course images show the endocytic trafficking of CD8a-furin to the Golgi. HeLa cells were transfected with CD8a-furin together with (a) TfR-GFP or (b) GFP-Rab7 for 24 h and surface-labeled with anti-CD8a antibody for 60 min on ice. Cells were chased at 37°C for various lengths of time before immunolabeling of CD8a and giantin. TfR-GFP was used as the EE/RE marker, while GFP-Rab7 was used as the LE marker. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. Bars, 10 μ m.

4.2 Starvation reversibly induces the translocation of TGN membrane proteins to the endosomal pool

To determine whether nutrients play any roles in endocytic membrane trafficking, we firstly compared the subcellular distribution of a few TGN resident transmembrane proteins, such as furin and CI-M6PR in different nutrient treatment conditions.

4.2.1 Nutrient starvation changes the subcellular distribution of TGN membrane proteins

HeLa cells incubated in the complete medium (DMEM supplemented with 10% FBS), DMEM or HBSS for 1 h at 37°C were stained with antibodies against either furin or CI-M6PR with Golgin245 or giantin as the Golgi marker, respectively. As expected, endogenous furin mainly colocalized with Golgin245 at the TGN in the complete medium (Figure 11a, c). When serum or growth factor was withdrawn by incubation in DMEM for 1 h, no noticeable change in furin was observed in cells (Figure 11a, c). However, when cells were starved of both AAs and growth factors by incubating in HBSS for 1 h, furin appeared diffused throughout the cytosol and no Golgi localization was observed (Figure 11a, c). Similarly, the localization of endogenous CI-M6PR was also changed under HBSS starvation (Figure 11b, d), though the effect seemed not as obvious as endogenous furin.

Figure 11: Starvation causes changes in the subcellular distribution of endogenous furin and CI-M6PR.

HeLa cells seeded on coverslips were incubated with complete, DMEM or HBSS starvation medium for 1 h. Cells were stained with (a) anti-furin and anti-Golgin245 antibody or (b) anti-CI-M6PR and anti-giantin antibody. Quantification results of the fraction of Golgi-localized (c) furin or (d) CI-M6PR in different media. Error bars indicate SEMs and *P*-values were determined by student's t-test. Bars, 10 μ m.

To further confirm this observation, HeLa cells expressing CD8a-furin or CD8a-CI-M6PR were subjected to the same assay. The chimeric proteins were observed at the perinuclear region that colocalize well with the Golgi marker, giantin in the complete medium, as well as the medium without serum or growth factor (DMEM) (Figure 12a, b). In contrast, after 1 h of HBSS starvation, the CD8a-chimeric proteins were distributed more extensively throughout the cytoplasm in punctuate structures (Figure 12a, b). The fraction of Golgi-localized CD8a-chimeras quantified further validated these observations (Figure 12c, d).

Figure 12: Starvation causes changes in the subcellular distribution of CD8a-furin and CD8a-CI-M6PR.

HeLa cells transfected with CD8a-furin or CD8a-CI-M6PR were incubated with complete, DMEM or HBSS for 1 h. **(a) & (b)** Representative images show the distribution of CD8a-furin or CD8a-CI-M6PR in different media. Quantification results of the fraction of Golgi-localized **(c)** CD8a-furin or **(d)** CD8a-CI-M6PR in different media. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

4.2.2 Furin mainly localizes in the endosomal pool under nutrient starvation conditions

Since both endogenous furin and the ectopically expressed CD8a-furin lost its Golgi pool and was spread throughout the cytosol in HBSS-treated cells, it was unclear where the exact endosomal localization of this protein was. A series of co-localization experiments were then performed.

Figure 13: CD8a-furin displays an EE and RE localization but not LE localization under nutrient starvation conditions.

HeLa expressing CD8a-furin was either stained with anti-RUFY1 antibody or co-expressed with TfR-GFP or GFP-Rab7 and subjected to HBSS starvation for 1 h, followed by immunofluorescence labeling. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. Bars, 10 μ m.

After 1 h of HBSS starvation treatment, CD8a-furin proteins expressed in the HeLa cells were distributed in the cell periphery and throughout the cytoplasm, which were found to clearly overlap with the EE marker (RUN and FYVE domain-containing protein 1, RUFY1). Interestingly, the colocalization between CD8a-furin and TfR-GFP was also observed in peripheral EE as well as perinuclear RE, suggesting that CD8a-furin traffics to the RE in starvation conditions. Surprisingly, CD8a-furin and the LE marker GFP-Rab7 showed almost no colocalization under HBSS treatment conditions (Figure 13).

As the CD8a-furin only contains the cytosolic domain of furin (Figure 8a), we tested whether the full-length furin has the same endosomal localization as CD8a-furin under starvation conditions. Since the furin antibody commercially available gave poor staining of the endosomal pool (Figure 11a), two full-length furin proteins fused to GFP or mCherry (Figure 8e, f) were constructed and used for the assay. After incubation in the

DMEM for 1 h, the majority of full-length furin fusion proteins remained concentrated in the perinuclear region together with a small fraction residing in the EE (which are colocalized with RUFY1) and LE (which are colocalized with GFP-Rab7) as expected (Figure 14a-c). In addition, a trace amount of furin was also observed to overlap with TfR-GFP under DMEM conditions (Figure 14b). By contrast, the localization of the full-length furin with the EE marker RUFY1 (Figure 14a) and the EE/RE marker TfR-GFP (Figure 13b) was substantially increased after 1 h starvation with HBSS, which is consistent with the finding for the CD8a-furin (Figure 13). However, unlike the CD8a-furin, the LE localization of furin-mCherry was detected under starvation conditions; they overlapped well with the LE marker GFP-Rab7 (Figure 14c).

Figure 14: Full-length furin displays endosomal localization under nutrient starvation conditions.

(a) HeLa cells expressing furin-GFP were incubated with DMEM or HBSS medium for 1 h. Cells were stained with anti-RUFY1 antibody. (b) HeLa cells were co-transfected with furin-mCherry and TfR-GFP and subjected to incubation with DMEM or HBSS for 1 h, followed by immunofluorescence labeling. (c) HeLa cells were transfected to express furin-mCherry and GFP-Rab7 and subjected to incubation with DMEM or HBSS for 1 h, followed by immunofluorescence labeling. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. Bars, 10 μ m.

We then triple co-expressed furin-mCherry, GFP-Rab7 and CD8a-furin in the HeLa cells and evaluated their colocalization. Similarly, full-length furin-mCherry proteins were found to extensively overlap with GFP-Rab7 (Figure 15), while no colocalization was observed between CD8a-furin and the LE marker GFP-Rab7. Furthermore, there was a fraction of furin-mCherry that colocalized with the CD8a-furin in the endosomal structures, which could be the EE/RE. Collectively, these data indicated that both the transmembrane and cytosolic domain of furin, which are missing in CD8a-furin, are important for its endosomal sorting. This has been previously suggested ([Chia et al., 2011](#)). Nevertheless, both the CD8a-furin and the full-length furin localization data support the conclusion that HBSS starvation changes the subcellular distribution of TGN membrane proteins from the Golgi to the endosome pool.

Figure 15: Full-length furin but not CD8a-furin localizes to the LE under nutrient starvation conditions.

HeLa cells triple co-expressed furin-mCherry, GFP-Rab7 and CD8a-furin were incubated with HBSS medium for 1 h. The colocalization between furin-mCherry and GFP-Rab7 were enlarged in the upper right corner (box1). The colocalization between furin-mCherry and CD8a-furin were enlarged in the lower right corner (box2). Arrows indicate colocalization. Bars, 10 μ m.

4.2.3 The effects of nutrient starvation on TGN membrane protein localization are reversible

To determine whether the starvation-induced reduction of TGN membrane proteins in the Golgi pool and the corresponding increase in the peripheral endosomal pool were reversible, nutrients were supplied to the nutrient-starved cells by incubating with DMEM. Endogenous furin reappeared in the Golgi after 30 min of nutrient (DMEM) stimulation. Subsequently, the colocalization between furin and Golgin245 increased rapidly after 1 h

of DMEM incubation, in which the protein had already recovered to its steady-state status (Figure 16).

Figure 16: Resupplying nutrients rapidly reverses the starvation-induced reduction of endogenous furin in the Golgi pool.

HBSS-treated HeLa cells were supplied with DMEM at 37°C for different time and were stained with antibodies against furin and Golgin245. Bars, 10 μ m.

It was difficult to quantify the fraction of Golgi-localized endogenous furin due to the high level of background nuclear staining signal (Figure 16) caused by the commercial furin antibody. Thus, we performed a similar experiment using furin-GFP as the reporter. Furin-GFP expressing cells were either incubated in the HBSS for different time (Figure 17b) or were directly starved for 2 h and resupplied with DMEM and incubated for various lengths of time. Before HBSS starvation, furin-GFP was found to localize to the Golgi, which was colocalized well with the TGN marker Golgin245 (Figure 17a). As expected, the extent of colocalization declined dramatically with increasing incubation time in HBSS (Figure 17b). By 120 min of HBSS starvation, substantial amounts of furin-GFP was detected in the endosomal pool (Figure 17a). By contrast, the amount of furin-GFP in the Golgi showed an increase after 20 min of DMEM stimulation. Eventually, the

majority of furin-GFP was redistributed to the Golgi and recovered to its pre-starvation state (Figure 17a). These observations have further been validated by quantifying the fraction of Golgi-localized furin-GFP for each time point (Figure 17b). Therefore, the effects of nutrient starvation on furin localization are reversible.

Figure 17: Resupplying nutrients rapidly reverses the starvation-induced reduction of furin-GFP in the Golgi pool.

(a) HeLa cells expressing furin-GFP were either starved for different time or were resupplied with DMEM and incubated for various lengths of time after 2 h of HBSS starvation. Cells were stained with antibodies against Golgin245. (b) Quantification results of the fraction of Golgi-localized CD8a-furin under different treatment. Error bars indicate SEMs. Bars, 10 μ m.

4.3 Nutrients stimulate the endosome-to-Golgi trafficking

The reduction of the Golgi pool and the concomitant increase in the endosomal pool of the TGN membrane proteins under the HBSS treatment indicates that DMEM or complete medium may stimulate endosome-to-Golgi trafficking. Thus, we first tested the effects of nutrients on PM-to-Golgi trafficking.

4.3.1 Nutrients stimulate the PM-to-Golgi trafficking

Figure 18: Optimization of starvation time for the CD8a-furin endocytic trafficking assay.

HeLa cells transfected with CD8a-furin were starved (**a**) 0 h, (**b**) 2 h or (**c**) 4 h followed by surface-labeling of anti-CD8a antibody for 1 h on ice. Cells were then incubated in DMEM or HBSS and chased at 37°C for 20 min. (**d**) Quantification results of the fraction of Golgi-localized CD8a-furin under different HBSS starvation times and 20 min trafficking conditions. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μm.

HeLa cells expressing CD8a-furin were surface-labeled with anti-CD8a antibody for 1 h on ice. After washing away the unbound antibody, cells were incubated with DMEM or HBSS and chased at 37°C for 20 min to allow endocytic trafficking. The amount of Golgi-localized CD8a-furin was determined by dividing CD8a-furin fluorescence intensity located in the giantin-defined Golgi region to the total intensity of cellular fluorescence. The localization of CD8a-furin was detected in the Golgi after 20 min of internalization (Figure 18a) and we found that the CD8a-furin localized more in the Golgi under DMEM stimulation compared with HBSS treatment, although the difference was not statistically significant (*p*=0.08) (Figure 18d). This is suggestive of a role of nutrients in the endocytic trafficking pathway.

Based on this finding, we proceeded to try different starvation times before the 20 min endocytic trafficking assay. Surprisingly, we observed that the amount of CD8a-furin reaching the Golgi under 20 min of DMEM stimulation was significantly higher after 2 h of HBSS starvation ($p=4\times 10^{-03}$) (Figure 18b, d). After 4 h of starvation, the fraction of Golgi-localized CD8a-furin was decreased in both nutrient treatment, compared with the 0 h or 2 h of HBSS starvation conditions (Figure 18c, d). Although the amount of CD8a-furin localized to the Golgi under DMEM stimulation was still higher than HBSS starvation, there was no statistically significant difference between them ($p=0.2$) (Figure 18d). Thus, 2 h of HBSS starvation before the endocytic trafficking assay was chosen for the following experiments.

We then tested different trafficking time from PM-to-Golgi under different nutrient treatment conditions. HeLa cells transiently transfected with CD8a-furin were starved for 2 h before the internalization assay. After surface-labeling, the antibody-bound complexes were internalized for 20, 40, 80 and 160 min at 37°C under DMEM or HBSS treatment conditions. As illustrated in Figure 18, regardless of different nutrient treatment, a continuous increase of CD8a-furin in the Golgi pool from 20 min to 160 min was observed. After 2 h of HBSS starvation and 20 min of chase, the amount of Golgi-localized CD8a-furin was significantly higher in DMEM-treated cells than in HBSS-treated cells ($p=9\times 10^{-03}$) (Figure 19a, e). This observation is in agreement with the results shown above (Figure 18b, d). By 40 min of internalization, a small amount of surface-labeled CD8a-furin protein reached the Golgi, while the majority was still located in the endosomes under HBSS treatment as compared with DMEM stimulation (Figure 19b). The Golgi-localized CD8a-furin remained much higher in DMEM stimulation than HBSS treatment after 80 min of chase ($p=0.04$) (Figure 19c, e). At 160 min, majority of internalized CD8a-furin was accumulated in the perinuclear region, which overlapped with the Golgi marker giantin (Figure 19d), and no statistically significant difference ($p=0.8$) was observed between HBSS starvation and DMEM stimulation (Figure 19e).

Figure 19: Nutrients regulate the PM-to-Golgi trafficking of CD8a-furin.

HeLa cells expressing CD8a-furin were starved for 2 h followed by surface-labeling using anti-CD8a antibody. Cells were then internalized for (a) 20, (b) 40, (c) 80 and (d) 160 min at 37°C under DMEM or HBSS treatment. CD8a and endogenous giantin were stained. (e) Quantification results of the fraction of CD8a-furin in the Golgi at different trafficking time points. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

To further confirm the role of nutrients in the PM-to-Golgi trafficking, other CD8a-chimera reporters, such as CD8a-CI-M6PR, CD8a-CD-M6PR and CD8a-sortilin (Figure 20a-d), were also tested.

After 2 h of HBSS starvation followed by 20 min of internalization, the amount of CD8a-chimeras transported to the Golgi in DMEM treatment were significantly higher than in HBSS starvation (CD8a-CD-M6PR, $p=3\times 10^{-03}$; CD8a-CI-M6PR, $p=2\times 10^{-08}$; CD8a-furin, $p=2\times 10^{-04}$; CD8a-sortilin, $p=5\times 10^{-04}$) (Figure 20). In conclusion, all tested CD8a-chimeras showed a similar trend in response to the nutrient stimulation during the PM-to-Golgi trafficking assay, though CD8a-CI-M6PR demonstrated the greatest effect. Considering that endogenous CI-M6PR was less sensitive than furin in response to the nutrient changes (Figure 11b, d) and both CD8a-CI-M6PR and CD8a-furin responded similar (Figure 12), we therefore chose the CD8a-furin as the major reporter to study the endocytic trafficking assay in the following experiments. Altogether, it could be concluded that nutrients play an important role in stimulating the PM-to-Golgi trafficking pathway.

Figure 20: Nutrients regulate the PM-to-Golgi trafficking of various CD8a-chimeras. HeLa cells expressing CD8a-chimeras were starved for 2 h followed by surface-labeling. CD8a antibody-bound complexes were internalized for 20 min at 37°C under nutrient starvation or stimulation. CD8a and endogenous giantin were stained. **(a–d)** Representative images for endocytic trafficking of CD8a-chimeras are shown. **(e)** Quantification results of the fraction of CD8a-chimeras in the Golgi after 2 h of starvation and 20 min of internalization under nutrient starvation or stimulation. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

4.3.2 Nutrients stimulate the endosome-to-Golgi trafficking

The endocytic trafficking of the TGN membrane proteins can be divided into two consecutive steps: clathrin-dependent endocytosis from the PM to the endosome and the endosome-to-Golgi trafficking. Since nutrients affect the PM-to-Golgi trafficking, we wanted to further explore in which step nutrients play a role.

HeLa cells expressing CD8a-furin were starved for 2 h, followed by surface-labeling with anti-CD8a antibody. The antibody–CD8a-furin complex was then internalized at 37°C for different time (Figure 21a). After chase, cells were cooled on ice and washed with acetic acid to remove the surface-bound antibody. The relative total intensity per cell was determined by dividing the total intensity of background-subtracted image by the number of cells in the image. As shown in Figure 21a, the total CD8a-furin fluorescence intensity of the cell increased along with the chase time and the internalized CD8a-furin distributed extensively throughout the cytoplasm regardless of whether it was under DMEM or HBSS treatment. Furthermore, quantitative analysis indicated that the internalized CD8a-furin within 6 min of chase did not display a statistically significant difference between DMEM stimulation and HBSS treatment ($p=0.06$) (Figure 21b), therefore suggesting that endocytosis was not the target of nutrient stimulation.

Figure 21: Nutrients do not significantly affect the endocytosis of CD8a-furin.

HeLa cells transiently expressing CD8a-furin were incubated in HBSS for 2 h followed by surface-labeling by anti-CD8a antibody. Next, the labeled CD8a-furin was chased at 37°C for the indicated time under DMEM or HBSS treatment before being subjected to acid wash on ice to remove the surface-bound antibody. **(a)** Total cellular CD8a-furin was stained. **(b)** The relative total intensity per cell was quantified as (total intensity of background-subtracted image)/(number of cells in the image). n=9 images were used for each time point. Error bars indicate SDs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

Hence, we focused on testing the endosome-to-Golgi trafficking. Antibody-labeled CD8a-furin or CI-M6PR was first allowed to accumulate and synchronize at the EE/RE by incubating at 18°C in HBSS for 2 h ([Mallard et al., 1998](#); [Tai et al., 2004](#)). The Golgi localization was subsequently quantified after a chase at 37°C for different time.

Figure 22: Nutrients stimulate the endosome-to-Golgi trafficking of CD8a-furin.

HeLa cells transiently expressing CD8a-furin were surface-labeled with anti-CD8a antibody and synchronized at endosomes at 18°C in HBSS for 2 h before being chased at 37°C in HBSS or DMEM for various lengths of time. **(a)** Time course images showing the endocytic trafficking of CD8a-furin to the Golgi. CD8a and endogenous giantin were stained. **(b)** The fraction of Golgi-localized CD8a-furin was quantified. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

At 0 min of chase, the CD8a-furin accumulated at peripheral endosomes (Figure 22a). After 6 min of chase, the amount of endocytic trafficking of CD8a-furin to the Golgi in both DMEM and HBSS treatment was still low, and there was no statistically significant difference between them ($p=0.1$). However, in comparison with the HBSS treatment, CD8a-furin showed a significant increase in the perinuclear region, which overlapped with the Golgi marker giantin after 12 min of internalization under DMEM stimulation

($p=3\times 10^{-4}$) (Figure 22a, b). At 18 min, the amount of CD8a-furin in the Golgi was still significantly higher in DMEM than in the HBSS treatment ($p=4\times 10^{-4}$). A similar result was also obtained for CD8a-CI-M6PR (Figure 23a, b). Collectively, these data demonstrate that nutrients are capable of stimulating the endosome-to-Golgi trafficking of furin as well as other TGN membrane residents. Upon nutrient-deficiency, endosome-to-Golgi trafficking is compromised, thus tipping the balance of the distribution of a TGN membrane protein by elevating its endosomal pool.

Figure 23: Nutrients stimulate the endosome-to-Golgi trafficking of CD8a-CI-M6PR. HeLa cells were transiently transfected to express CD8a-CI-M6PR. Cells were surface-labeled with anti-CD8a antibody and synchronized at endosomes at 18°C in HBSS for 2 h before being chased at 37°C in HBSS or DMEM for various lengths of time. (a) Time course images showing the endocytic trafficking of CD8a-CI-M6PR to the Golgi. CD8a and endogenous giantin were stained. (b) The fraction of Golgi-localized CD8a-CI-M6PR was quantified. Error bars indicate SEMs and *P*-values were determined by student's t-test. Bars, 10 μ m.

4.4 AAs, especially glutamine, stimulate the endosome-to-Golgi trafficking

The data obtained thus far strongly indicate that nutrients can stimulate the endosome-to-Golgi trafficking. The nutrient stimulation medium we mainly used in this study was DMEM, which consists of DMEM-base (inorganic salts and vitamins), AAs (15 AAs including Gln) and glucose. In addition, the complete medium was also used as the stimulation medium, which contains serum as the source of growth factors. Thus, the next objective of this study was to identify which component(s) was (were) the key factor(s) for stimulating the endosome-to-Golgi trafficking pathway.

4.4.1 AAs but not glucose or growth factors, stimulate the endosome-to-Golgi trafficking

The endosome-to-Golgi trafficking assay was conducted in the testing medium comprising DMEM-base supplemented with combinations of dialyzed-serum, AAs and glucose. To that end, CD8a-furin expressing cells were first starved in DMEM-base for 2 h, followed by surface-labeling with anti-CD8a antibody. The antibody-CD8a-furin complex was monitored along the endocytic trafficking en route to the Golgi.

We found that dialyzed-serum was unable to stimulate the endocytic trafficking of CD8a-furin to the Golgi (Figure 24a, b). Similarly, the addition of neither high (4.5 g/L) nor low (1.0 g/L) concentrations of glucose caused a statistically significant difference in the internalized CD8a-furin in the Golgi ($p=0.4$ and $p=0.5$, respectively) (Figure 24a, b). However, to our surprise, we observed that AAs alone were sufficient to stimulate the endosome-to-Golgi trafficking of CD8a-furin (Figure 24a). After incubating the cells in the AA-rich medium for 20 min, the amount of CD8a-furin in the Golgi was significantly higher than any other treatments ($p=3\times 10^{-10}$) (Figure 24b). Moreover, the supplementation of AAs, dialyzed-serum and glucose in DMEM-base failed to produce more stimulation effect than AAs alone, though it was able to significantly stimulate the endocytic trafficking of CD8a-furin to the Golgi ($p=3\times 10^{-05}$) (Figure 24a, b). Thus, AAs are necessary and sufficient for stimulating the endosome-to-Golgi trafficking.

Figure 24: AAs but not growth factors or glucose, stimulate the endosome-to-Golgi trafficking of CD8a-furin.

(a) Cells stably expressing CD8a-furin were treated with DMEM-base for 2 h. The surface-exposed CD8a-furin was labeled with anti-CD8a antibody and chased for 20 min in the testing medium consisting of DMEM-base supplemented with the indicated components. Cells were stained with anti-giantin antibodies. dial. serum, 10% dialyzed-serum. (b) Quantification results of the fraction of Golgi-localized furin under different nutrient treatment conditions. Error bars indicate SEMs and *P*-values were determined by student's t-test. Bars, 10 μ m.

4.4.2 The effect of AAs on the endosome-to-Golgi trafficking is probably ubiquitous

Apart from HeLa cells, other mammalian cells such as BSC-1 and HEK293T have also been used to test the role of AAs in stimulating endosome-to-Golgi trafficking. In BSC-1

cells, after 2 h of HBSS starvation and 20 min of chase, the amount of Golgi-localized CD8a-furin under DMEM stimulation was significantly higher than DMEM/-AAs or HBSS starvation ($p=2\times 10^{-03}$) (Figure 25a, b).

Figure 25: AAs stimulate endosome-to-Golgi trafficking in BSC-1 cells.

(a) BSC-1 cells transiently expressing CD8a-furin were treated with HBSS for 2 h. The surface-exposed CD8a-furin was labeled with anti-CD8a antibody and chased for 20 min under DMEM, DMEM/-AA or HBSS treatment. CD8a and endogenous giantin were stained. (b) Quantification results of the fraction of Golgi-localized furin. Error bars indicate SEMs and *P*-values were determined by student t-test. Bars, 10 μ m.

Similarly, in comparison with the HBSS starvation, CD8a-furin was observed to localize more in the Golgi during 20 min of AA stimulation in HEK293T cells ($p=0.01$) (Figure 26a, b). Taken together, these results demonstrate that the effect of AAs on endosome-to-Golgi trafficking is probably ubiquitous.

Figure 26: AAs stimulate endosome-to-Golgi to the Golgi in HEK293T cells.

(a) HEK293T cells transiently expressing CD8a-furin were starved for 2 h. The surface-exposed CD8a-furin was labeled with anti-CD8a antibody and chased for 20 min under DMEM or HBSS treatment. CD8a and endogenous giantin were stained. (b) Quantification results of the fraction of Golgi-localized furin. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

4.4.3 Glutamine has the most acute effect in stimulating endosome-to-Golgi trafficking

After confirming the effects of AAs in the endosome-to-Golgi trafficking, we wanted to further examine which AA(s) is (are) responsible for stimulating this trafficking pathway. Thus, we individually tested 20 AAs at the same concentration (0.8 mM) for their effect on the CD8a-furin endosome-to-Golgi trafficking. HeLa cells stably expressing CD8a-furin were starved with HBSS for 2 h, followed by surface-labeling with anti-CD8a antibody. After washing away the unbound antibody, cells were chased in HBSS, DMEM or DMEM/-AAs supplemented with the indicated AA at 0.8 mM for 20 min to allow internalization. As observed, the majority of AAs stimulated trafficking to various degrees. In addition, among the tested 20 AAs, one of a non-essential AA, Gln, displayed the strongest stimulation effect (Figure 27a, green dashed line). By contrast, Leu, which is an essential AA and the most acute stimulator for mTORC1 signaling ([Lynch, 2001](#)), showed the weakness effect at 0.8 mM (Figure 27a, blue dashed line).

Figure 27: Gln is one of the most acute AAs that stimulate effects on endosome-to-Golgi trafficking.

(a) HeLa stably expressing CD8a-furin was starved with HBSS for 2 h followed by surface-labeling. Cells were then chased in HBSS, DMEM or DMEM/-AAs supplemented with the indicated AA at 0.8 mM for 20 min. After staining with CD8a and endogenous giantin, the fraction of Golgi-localized CD8a-furin was quantified. Error bars indicate SEMs. (b) HeLa cells stably expressing CD8a-furin were treated with DMEM/-AAs for 2 h followed by surface-labeling. The antibody-labeled CD8a-furin was subsequently chased at 37°C for 20 min in DMEM-base supplemented by the indicated AA at different concentrations, ranging from 0.01 mM to 5.12 mM. After staining with CD8a and giantin, cells were imaged and the Golgi-localized (I_{Golgi}) and total CD8a-furin intensities (I_{total}) within each field of view were acquired. The fraction of Golgi-localized CD8a-furin was calculated as $I_{\text{Golgi}}/I_{\text{total}}$. The plot incorporates n=4 (Ala and Gln) or 5 (Leu) fields of views with each field having more than 60 cells. Error bars indicate SDs.

To further explore whether the AA effects on stimulating the endosome-to-Golgi trafficking is concentration dependent, we chose three AAs for the tests: Gln (the strongest effect), Leu (the weakness effect) and Ala. HeLa cells stably expressing CD8a-furin were starved with DMEM/-AAs for 2 h. After surface-labeling, the antibody-labeled CD8a-furin was subsequently chased at 37°C for 20 min in DMEM-base supplemented with respective AA at different concentrations (0.01 mM, 0.02 mM, 0.04 mM, 0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, 2.56 mM and 5.12 mM). We found that the stimulating effect of Gln was concentration dependent and peaked at ~0.64 mM (Figure 27b, green line). The highest stimulation effect for Ala was observed 2.56 mM (Figure 27b, blue line). Conversely, Leu always showed the lowest value throughout the test

(Figure 27b, red line). Moreover, when >0.64 mM Leu was used, there was a sharp decline in the fraction of Golgi-localized CD8a-furin.

Figure 28: Gln is essential for stimulating the endosome-to-Golgi trafficking of CD8a-furin.

(a) HeLa stably expressing CD8a-furin was starved with HBSS for 2 h followed by surface-labeling using anti-CD8a antibody. Cells were then chased in DMEM or DMEM selectively leaving out the indicated AAs for 20 min. CD8a and endogenous giantin were stained. (b) Gln is essential for Leu to stimulate mTORC1. HeLa cells were starved with HBSS for 2 h before treatment with indicated medium or Torin1 (in the complete medium) for 20 min. Endogenous phospho-T389-S6K1 (p-S6K1) and GAPDH were blotted. (c) The fraction of Golgi-localized CD8a-furin was quantified. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m.

Accordingly, to further test whether Gln plays an essential role in the endosome-to-Golgi trafficking in a parallel comparison with Leu, the subcellular distribution of CD8a-furin in DMEM selectively leaving out Gln (DMEM/-Gln), Leu (DMEM/-Leu) or both (DMEM/-

Leu/-Gln) was investigated. To ensure these media work well, we first tested mTORC1 activity by detecting the phosphorylation state of T389 of S6K1 (Figure 28b). We observed that Gln was able to stimulate mTORC1 activity in the absence of Leu (DMEM/-Leu) (Figure 28b). However, the activity of mTORC1 was severely depressed when HeLa cells were incubated in medium without Gln, regardless of whether Leu was present (DMEM/-Gln) or absent (DMEM/-Leu/Gln) (Figure 28b). This observation was consistent with previous findings ([Chiu et al., 2012](#); [Durán et al., 2012](#); [Nicklin et al., 2009](#)) that Gln is essential for Leu to stimulate mTORC1.

As shown in Figure 28a, the majority of CD8a-furin was distributed throughout the cytosol and almost no Golgi localization was observed in media without Gln (DMEM/-Leu/-Gln and DMEM/-Gln), indicating an essential role of Gln in the endosome-to-Golgi trafficking; in the presence of Gln (DMEM/-Leu and DMEM), CD8a-furin prominently accumulated in the perinuclear region, which overlapped well with the Golgi marker giantin (Figure 28a). In contrast, the Golgi localization of CD8a-furin was not affected by the availability of Leu, since CD8a-furin mainly localized to the Golgi when cells were incubated in DMEM/-Leu (Figure 28a, c). This further solidifies the finding that Gln, but not Leu, plays the most acute stimulation effect on endosome-to-Golgi trafficking.

4.4.4 The effect of AAs on stimulating the endosome-to-Golgi trafficking is not additive

From the data of the 20 individual AAs (Figure 27a), we hypothesized that the effects of AAs on stimulating the endosome-to-Golgi trafficking is not additive, since DMEM, which contains 15 AAs, displayed less activity than DMEM/-AAs supplemented with Gln. To test this hypothesis, we randomly selected two groups of AAs (Ala plus Trp, Gln plus Leu) and examined the effect of combining two different AAs on the endosome-to-Golgi trafficking pathway.

Figure 29: The effect of combining two AAs on endosome-to-Golgi trafficking is not additive.

HeLa cells stably expressing CD8a-furin were treated with DMEM/-AAs for 2 h. The surface-exposed CD8a-furin was labeled with anti-CD8a antibody and subsequently chased for 20 min in DMEM/-AAs supplemented by the indicated AA at 0.8 mM before immunolabeling of CD8a and giantin. **(a)** The fraction of CD8a-furin localized to the Golgi was quantified. Error bars indicate SEMs and *P*-values were determined by student's *t*-test. Bars, 10 μ m. **(b)** The endosome-to-Golgi trafficking of CD8a-furin under DMEM/-AAs or DMEM. **(c)** The endosome-to-Golgi trafficking of CD8a-furin under DMEM/-AAs supplemented with Gln, Leu or Gln plus Leu. **(d)** The endosome-to-Golgi trafficking of CD8a-furin under DMEM/-AAs supplemented with Ala, Trp or Ala plus Trp.

As shown in Figure 29, HeLa cells stably expressing CD8a-furin were starved for 2 h, followed by surface-labeling. The antibody-labeled CD8a-furin was subsequently chased at 37°C for 20 min in DMEM-base supplemented by 0.8 mM individual AA or the mixture of the two AAs. In agreement with our earlier observation (Figure 27a), the CD8a-furin prominently accumulate in the Golgi after 20 min of chase in the medium containing Gln, Ala and Trp, while the majority of CD8-furin showed punctate structures in the DMEM/-AAs plus Leu treatment (Figure 29c, d). However, the fraction of Golgi-localized CD8a-furin did not significantly increase under DMEM/-AAs plus Ala and Trp or Gln and Leu conditions compared with DMEM/-AAs plus Ala ($p=0.1$), Trp ($p=0.5$), or Gln alone ($p=0.6$), respectively (Figure 29a), indicating that the effect of AA on the endosome-to-Golgi trafficking might not be simply additive. The mechanism behind this is currently unknown and awaits future exploration. It is possible that AAs can positively or negatively modulate each other's activities, by acting as exchangers factor ([Krall et al., 2016](#)).

Chapter 2: The AA-stimulated endosome-to-Golgi trafficking depends on v-ATPase, SLC38A9 and Ragulator but not Rag GTPases and mTORC1

The next objective of this study was to identify the mechanism behind nutrients, especially AAs, stimulate the endosome-to-Golgi trafficking pathway. It is known that AA signaling culminates in the activation of mTORC1 through SLC38A9, v-ATPase, Ragulator and heterodimeric Rag GTPases ([Bar-Peled et al., 2012](#); [Jung et al., 2015](#); [Rebsamen et al., 2015](#); [Sancak et al., 2008](#); [Wang et al., 2015](#)). To test whether AA-stimulated endosome-to-Golgi trafficking utilizes a similar pathway, we selectively inhibited or depleted each component through small molecule inhibitors or RNAi-mediated knockdowns, respectively, and explore the resulting effect on the endosome-to-Golgi trafficking.

Moreover, to compare and contrast the nutrient stimulatory effect, the fraction of Golgi-localized CD8a-furin under AA-stimulation was normalized by that under AA-starvation to yield a relative quantity referred to as “AA-stimulated Golgi trafficking”.

4.5 v-ATPase is essential for AA-stimulated endosome-to-Golgi trafficking

In the AA-stimulated mTOR signaling pathway, the signal begins within the lumen of the lysosome and subsequently relays to the nucleotide loading of Rag GTPase, which ultimately causes the recruitment of mTORC1 to the lysosomal surface to be activated by Rheb1. v-ATPase is required for this process that acts downstream of AAs and upstream of the Rags ([Zoncu et al., 2011](#)).

Figure 30: ConA treatment eliminates the effects of AAs on endosome-to-Golgi trafficking of CD8a-furin.

HeLa stably expressing CD8a-furin was starved with HBSS for 2 h. After surface-labeling, cells were chased in DMEM or HBSS for 20 min. During the 2 h starvation period and 20 min trafficking time, cells were treated with (a) DMSO (an equal amount to conA) or (b) 2.5 μ M conA. Cells were subsequently stained with anti-CD8a and anti-giantin. (c) The AA-stimulated Golgi trafficking was quantified by imaging. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 90 cells. Error bars indicate SDs and P -values were determined by student's t -test. Bars, 10 μ m.

To test whether the AA-stimulated endosome-to-Golgi trafficking requires v-ATPase, HeLa cells stably expressing CD8a-furin were treated with concanamycin A (conA), an inhibitor of v-ATPase ([Bowman et al., 2006](#)), before the trafficking assay. There was a marked reduction in the endosome-to-Golgi transport of internalized CD8a-furin in cells treated with conA compared with control-treated cells (Figure 30a, b). The majority of CD8a-furin was arrested in endosomal punctate structures in conA-treated cells regardless of whether it is under AA stimulation (DMEM) or starvation (HBSS) conditions (Figure 30b). As expected, after treatment with conA, the AA-stimulated Golgi trafficking of CD8a-furin decreased significantly compared with DMSO-treated control cells ($p=9 \times 10^{-3}$)

(Figure 30c). This is a direct evidence for the role of v-ATPase in regulating not only the mTORC1 signaling pathway but also the AA-stimulated endosome-to-Golgi trafficking pathway.

4.6 SLC38A9 is required for the AA-stimulated endosome-to-Golgi trafficking

As SLC38A9 has been claimed as a high-affinity transporter for Gln ([Rebsamen et al., 2015](#)), and from Figure 27a, Gln had the most acute effect in stimulating endosome-to-Golgi trafficking, we next sought to investigate if SLC38A9 was also involved in AA-stimulated endosome-to-Golgi trafficking pathway.

As shown in the Figure 31a, both SLC38A9 shRNAs were able to knock down the target gene by 50% to 70%. Furthermore, in agreement with previous findings ([Jung et al., 2015](#); [Rebsamen et al., 2015](#); [Wang et al., 2015](#)), depletion of SLC38A9 reduced the AA-stimulated mTORC1 activity by detecting the phosphorylation level of S6K1 (Figure 31c).

After verifying the knockdown efficiency of SLC38A9 shRNA, we proceeded to perform the trafficking assay. As shown in Figure 32a, CD8a-furin altered its cellular localization from the cell periphery to the perinuclear region in response to the AA stimulation in firefly luciferase GL2 control-depleted cells. However, when endogenous SLC38A9 was depleted by the shRNAs, the majority of CD8a-furin was distributed at periphery endosomes regardless of whether it was in AA starvation or stimulation conditions (Figure 32b, c). The value of AA-stimulated Golgi trafficking of CD8a-furin further validated these observations (Figure 32d). Thus, it seems that SLC38A9 is involved in regulating the AA-stimulated endosome-to-Golgi trafficking.

Figure 31: Evaluation of shRNA-mediated knockdown of endogenous SLC38A9 levels in HeLa cells.

(a) Endogenous SLC38A9 was knocked down by lentivirus-transduced shRNA #1 and #2 as assessed by RT-qPCR. (b) Melting curve analysis for RT-qPCR of SLC38A9 and GAPDH in control GL2- and SLC38A9-depleted templates. (c) The knockdown of endogenous SLC38A9 attenuated the AA-stimulated mTORC1 activity. SLC38A9 knockdown cells were incubated with DMEM/-AAs for 50 min followed by incubation with DMEM for 20 min. Cell lysates were immunoblotted for p-S6K1 and GAPDH.

Figure 32: SLC38A9 is required for the AA-simulated endosome-to-Golgi trafficking of CD8a-furin.

(a–c) HeLa cells knocked down by control GL2 or SLC38A9 shRNAs were transfected to express CD8a-furin and subjected to treatment with HBSS for 2 h followed by surface-labeling. Cells were then chased in DMEM or HBSS for 20 min, and subsequently stained with anti-CD8a and anti-giantin. (d) The AA-stimulated endosome-to-Golgi trafficking was quantified by imaging. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 21 cells. Error bars indicate SDs and P -values were determined by student's t -test. Bars, 10 μm .

4.7 The Ragulator complex but not Rag GTPases depletion decreases the AA-stimulated endosome-to-Golgi trafficking pathway

Figure 33: Evaluation of shRNA-mediated knockdown of endogenous Lamtor1, Lamtor3 and Rag A/B GTPase levels in HeLa cells.

HeLa cells were subjected to lentivirus-transduced control GL2 shRNA or shRNA targeting the indicated proteins. **(a)** The expression level of Lamtor1, RagA and RagB (RagA/B) were assessed by WB. α -tubulin was used as a loading control. **(b)** Depletion of Lamtor1 or Rag A/B attenuated the AA-stimulated mTORC1 activity. Lamtor3 or RagA/B knockdown cells were incubated with DMEM/-AAs for 50 min followed by incubation with DMEM for 20 min. Cell lysates were immunoblotted for p-S6K1 and GAPDH. **(c)** The expression level of Lamtor3 was assessed by RT-qPCR. **(d)** Melting curve analysis for RT-qPCR of Lamtor3 and GAPDH in control GL2- and Lamtor3-depleted templates.

Since the Ragulator complex acts downstream of v-ATPase and SLC38A9 and upstream of Rag GTPases in the AA-regulated mTORC1 signaling pathway ([Bar-Peled et al., 2012](#); [Sancak et al., 2010](#); [Wang et al., 2015](#); [Zoncu et al., 2011](#)), we subsequently explored whether Ragulator and Rag GTPases are also necessary for AA-stimulated endosome-to-Golgi trafficking.

Lamtor1 and Lamtor3, two subunits of the Ragulator complex, together with RagA and RagB (RagA/B) were chosen to perform the experiment. The shRNA-mediated Lamtor1, Lamtor3 as well as RagA/B knockdown levels were evaluated by WB or RT-qPCR (Figure 33a, c). The AA-stimulated mTORC1 activity was strongly reduced in the Lamtor3 or RagA/B GTPases-depleted cells (Figure 33b) as previously reported ([Sancak et al., 2010](#); [Sancak et al., 2008](#)).

When Lamtor1 and Lamtor3 were individually knocked down by corresponding shRNAs, the internalized CD8a-furin was distributed more extensively throughout the cytoplasm in punctate structures (Figure 34b, c). Consistently, the value of AA-stimulated Golgi trafficking of CD8a-furin in Lamtor1- or Lamtor3-depleted cells decreased significantly in comparison with the control ($p=0.02$) (Figure 34e), which suggested that the intact Ragulator complex is required for the AA-stimulated endosome-to-Golgi trafficking.

We then tested whether Rag GTPases can also regulate the AA-stimulated endosome-to-Golgi trafficking pathway. In contrast, a substantial amount of CD8a-furin still localized in the Golgi under DMEM stimulation conditions in the RagA and RagB simultaneous knockdown cells (Figure 34d). Depletion of RagA/B did not significantly affect the AA-stimulated Golgi trafficking ($p=0.4$) (Figure 34e). Therefore, Rag GTPases are not involved in the AA-stimulated endosome-to-Golgi trafficking pathway, suggesting that AA-stimulated Golgi trafficking could be independent of mTORC1.

Figure 34: Lamtor1 and Lamtor3 but not Rag A/B are required for the AA-stimulated Golgi trafficking of CD8a-furin.

(a–d) Control GL2, Lamtor1, Lamtor3 and RagA/RagB shRNAs-mediated knockdown cells expressing CD8a-furin are subjected to treatment with HBSS for 2 h followed by surface-labeling using anti-CD8a antibody. Cells were chased in DMEM or HBSS for 20 min before stained with CD8a and giantin. (e) The AA-stimulated Golgi trafficking was quantified. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 61 cells. Error bars indicate SDs and P -values were determined by student's t -test. Bars, 10 μ m.

4.8 AA-stimulated endosome-to-Golgi trafficking pathway is independent of mTORC1 activity

The findings we have presented so far for the AA-stimulated endosome-to-Golgi trafficking pathway seem consistent with the AA-stimulated mTORC1 signaling pathway, except for the Rag GTPases. Since Rag GTPases are necessary and sufficient for AAs to activate mTORC1, we hypothesized that mTORC1 is not required for the AA-stimulated endosome-to-Golgi trafficking pathway.

To verify this hypothesis, we inhibited the activity of mTORC1 using rapamycin or Torin1 ([Ballou and Lin, 2008](#); [Thoreen et al., 2009](#)) and then tested ensuing effects on the AA-stimulated endosome-to-Golgi trafficking pathway. As expected, there was no visible difference in the Golgi localization of CD8a-furin under DMEM stimulation between control and rapamycin treated cells (Figure 35a, b), although the activity of mTORC1 was strongly inhibited (Figure 35d). Moreover, the value of AA-stimulated Golgi trafficking of CD8a-furin was also not significantly change ($p=0.6$) (Figure 35e). A similar result was observed using another highly potent and selective ATP-competitive mTOR inhibitor, Torin1 (Figure 35c, e) ([Thoreen et al., 2009](#)). Altogether, these data demonstrated that SLC38A9, v-ATPase and Ragulator, but not Rag GTPases or mTORC1, are probably involved in AA-stimulated endosome-to-Golgi trafficking.

Figure 35: mTORC1 is not required for the AA-stimulated endosome-to-Golgi trafficking of CD8a-furin.

(a–c) HeLa stably expressing CD8a-furin was starved with HBSS for 2 h followed by surface-labeling. Cells were chased in DMEM or HBSS for 20 min. During the 2 h starvation period and 20 min of chase, cells were treated with 100 nM rapamycin or 250 nM Torin1. DMSO was used as a control treatment. Cells were stained with anti-CD8a and anti-giantin. (d) Rapamycin and Torin1 inhibited the AA-stimulated mTORC1 activity. The experiment was conducted similarly to that shown in (a–c) and cell lysates were subjected to blot with p-S6K1 and GAPDH. (e) AA-stimulated Golgi trafficking was quantified. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 51 cells. Error bars indicate SDs and P -values were determined by student's t -test. Bars, 10 μm .

Chapter 3: Arl5b and its effector, GARP, are essential for the AA-stimulated endosome-to-Golgi trafficking

As discussed in section 1.3, small GTPases from the Rab and Arl-family have been known to play important roles in regulating endosome-to-Golgi trafficking ([Bonifacino and Rojas, 2006](#); [Lu and Hong, 2014](#); [Pfeffer, 2009](#)). Arl1 is one of the best characterized small GTPases involved in maintenance of the Golgi structure as well as regulating the membrane trafficking between the endosome and TGN ([Lu et al., 2004](#); [Trull, 2012](#)). In addition to Arl1, Arl5b is another small GTPase, which has been reported to reside on the Golgi and whose knockdown greatly inhibits the endosome-to-Golgi trafficking ([Houghton et al., 2012](#); [Rosa-Ferreira et al., 2015](#)). Despite those findings, the precise role of Arl5b in the endosome-to-Golgi trafficking pathway is still not fully understood. Our lab mainly focuses on studying the Arls small GTPases, thus we performed a yeast two-hybrid screen of the human kidney cDNA library, using the Arl5b GTP-bound mutant form as the bait to gain further insight into the upstream regulators and downstream effectors of Arl5b. Interestingly, one of the strongest hits identified was full-length Lamtor1, which has been demonstrated to be involved in the AA-stimulated endosome-to-Golgi trafficking pathway in this study (section 4.7).

4.9 Arl5b interacts with Lamtor1

To verify the interaction between Arl5b and Lamtor1, a GST pull-down assay was performed. Bead-immobilized GST-Arl5b and control Arl, GST-Arl1, were loaded in vitro with GMPPNP (a non-hydrolyzable GTP analog) and GDP. The beads were then incubated with HEK293T cell lysate expressing Lamtor1-GFP. As expected, both GMPPNP and GDP-loaded Arl5b could pull down Lamtor1-GFP (Figure 36, lane IV and V), whereas neither GMPPNP nor GDP-loaded control GST-Arl1 could pull down the Lamtor1-GFP (Figure 36, lane VI and VII), demonstrating that the interaction between Arl5b and Lamtor1 is specific.

Figure 36: Arl5b, but not Arl1, specifically pulled down Lamtor1-GFP.

Bead-immobilized GST-Arl5b or Arl1 (used as the bait control) was loaded in vitro with GDP or GMPPNP and subsequently incubated with HEK293T cell lysates expressing GFP (used as the prey control) or Lamtor1-GFP. Pull-down samples were blot with antibody against GFP. Loading of GST fusion proteins was shown by Coomassie blue staining. Number 1 and 2 refer to the Lamtor1-GFP and GFP bands, respectively.

The interaction between Arl5b and Lamtor1 was further confirmed by forward co-IP. The assay was performed by co-expressing C-terminally GFP-tagged Arl5b-wt, QL (QL, the constitutively active GTP-bound mutant form) or TN (TN, the constitutively inactive GDP-bound form) together with Lamtor1-Myc in HEK293T cells. GFP was used as a negative control and showed no interaction with Lamtor1 (Figure 37, lane IV). Although Arl5b-wt-GFP, Arl5b-QL-GFP and Arl5b-TN-GFP were all found to interact with Lamtor1-Myc (Figure 37, lane I, II and III), GDP-mutant form of Arl5b (Arl5b-TN-GFP) appeared to interact more strongly. Similar results were obtained by reverse co-IP, which were performed in our lab by Dr. Shi Meng and Dr. Boh Boon Kim. In addition, they also characterized the interaction between Arl5b and individual subunits of Ragulator complex. During the GST pull-down experiments, they found that immobilized GST-Arl5b was able to pull down Lamtor2-Lamtor3 and Lamtor4-Lamtor5 sub-complexes only when Lamtor1 are co-expressed (data not shown). Moreover, immobilized GST-Arl5b can also pull down endogenous Lamtor1 (data not shown). In summary, we conclude that Arl5b interacts with Ragulator through Lamtor1.

Figure 37: Arl5b-wt, -QL and -TN interact with Lamtor1-Myc.

HEK293T cells co-transfected with Lamtor1-Myc and Arl5b-wt-GFP or Arl5b-QL-GFP or Arl5b-TN-GFP or pEGFP-N1 were subjected to IP using anti-GFP antibody and blotted with antibodies against Lamtor1 and GFP. GFP served as a negative control. Number 1 and 2 refer to the Arl5b-(wt, QL or TN)-GFP and GFP band, respectively.

4.10 Arl5a and Arl5b are the two major paralogs of Arl5

Arl5b, together with Arl5a and Arl5c were shown to be three closely related paralogs of Arl5. We have comparatively analyzed these three protein coding sequences from human and mouse. They shared more than 64% identity with each other (Figure 38a). Surprisingly, we found that human Arl5c does not have a typical G3 box (Figure 38a), which is different from human Arl5a and Arl5b.

We then used RT-qPCR to perform the absolute quantification of the human Arl5a, Arl5b and Arl5c cDNA levels in HeLa cells (Figure 38b-d). Using the purified Arl5a-GFP, Arl5b-GFP and hArl5c-GFP cDNA plasmids as calibration standards, RT-qPCR result revealed that transcripts levels of Arl5a and b were roughly equal (Figure 38b, c), while

that of Arl5c (Figure 38d) was ~30-folds lower than Arl5a and b, implying that Arl5c protein is probably present in minor amounts.

Figure 38: Arl5a, Arl5b are the two major paralogs of Arl5.

(a) Alignment of AA sequences of human and mouse Arl5a, b and c was conducted in Vector NTI (Invitrogen). The GenBank Accession number of each protein sequence is indicated. The five highly conserved guanine nucleotide binding motifs, G1–5 boxes, are underlined. RT-qPCR was used to perform the absolute quantification of the Arl5a, Arl5b and Arl5c cDNA levels in HeLa cells. For generating the calibration standard curve, (b) Arl5a-GFP; (c) Arl5b-GFP; (d) Arl5c-GFP cDNA plasmids were used to prepare a 10-fold dilution series in the range of $1000-1 \times 10^{-3}$ pg. To quantify the cDNA level of Arl5a, b, c, HeLa cells seeded in one well of a 6-well plate were harvested and used to extract RNA. After reverse transcription, 25 ng of cDNA was used as the template for each reaction in RT-qPCR. The C_t value of Arl5a, b and c were shown in the plot.

4.11 Characterization of Arl5b antibody

To further study the role of Arl5b in endosome-to-Golgi trafficking and test whether the interaction between Arl5b and Lamtor1 is important for AA-stimulated endosome-to-Golgi trafficking, we raised a rabbit polyclonal antibody against human Arl5b. The antibody was affinity-purified and characterized by WB, IP as well as IF.

As shown in the Figure 39a, the anti-Arl5b antibody preferentially recognizes Arl5b. In HeLa cells, the anti-Arl5b antibody stained the perinuclear region, which colocalizes well with the Golgi marker GS28 (Figure 39b). Additionally, the Arl5b but not the Arl5b-depleted antibody could efficiently and specifically perform IP of endogenous Arl5b (Figure 39c) as well as the overexpressed Arl5b-GFP in HeLa cells (Figure 39d).

Figure 39: Characterization of anti-Arl5b rabbit pAb.

(a) HeLa cells, either un-transfected or transiently transfected with GFP-tagged Arl5a, Arl5b and mArf5c were subjected to immunoblot with antibodies against GFP and Arl5b. (b) HeLa cells were co-stained with anti-Arl5b and anti-GS28 antibody. (c) HeLa cell lysate was subjected to IP by the indicated antibodies, and IPs were immunoblotted by anti-Arl5b antibody. (d) HeLa cell lysate transiently expressing Arl5b-GFP were subjected to IP with the indicated antibodies, and IPs were analyzed by immunoblotting using antibody against GFP. Bars, 10 μ m.

4.12 Arl5a and Arl5b localize to the *trans*-Golgi

Figure 40: Arl5a and Arl5b localize to the Golgi.

HeLa cells transiently expressing (a) Arl5a-GFP, (b) Arl5b-GFP or (c) mouse Arl5c (mAr15c) in QL, TN or wt form were fixed, and endogenous Golgin245 was stained. (d) HeLa cells transiently transfected with C-terminally GFP-tagged human Arl5c (hArl5c) were subjected to stain with anti-giantin. Bars, 10 μm.

It has been reported that Arl5a and Arl5b are Golgi-localized proteins ([Houghton et al., 2012](#)), and the anti-Arl5b pAb we raised also detected their Golgi localization (Figure 39b). To further confirm this localization and define their sub-Golgi localization, we generated several mutants and transfected into HeLa cells to compare their localization. The constitutively active mutants Arl5a-QL and Arl5b-QL showed stronger Golgi

localization (Figure 40a, b), compared with the wild type (Arl5a-wt and Arl5b-wt) proteins. However, the constitutively inactive forms Arl5a-TN and Arl5b-TN had reduced Golgi localization with concomitantly increased cytosolic pool (Figure 40a, b). Unlike Arl5a-QL and Arl5b-QL, mouse mArl5c-QL was found to localize more weakly to the Golgi (Figure 40c). In contrast to the mouse mArl5c-wt, the human hArl5c-wt, which lacks a typical G3 box (Figure 38a), diffused in the cytosol and showed no colocalization with the Golgi marker giantin (Figure 40d).

In addition, taking advantage of Golgi protein localization by imaging centers of mass (GLIM), the newly quantitative localization method for Golgi proteins developed in our lab ([Tie et al., 2016](#)), we were able to define the sub-Golgi localization of the Arl5. It is based on centers of fluorescence masses (hereafter center) of Golgi ministacks induced by the nocodazole treatment. The sub-Golgi localization of a test protein can be quantitatively expressed as the localization quotient (LQs) ([Tie et al., 2016](#)). Since the Arl5a- and Arl5b-TN forms (Figure 40a, b), mArl5c (Figure 40c) and hArl5c (Figure 40d) showed weak Golgi or no Golgi localization, we selected the wt and QL mutant forms of Arl5a and Arl5b to do the quantification. HeLa cells co-expressing the tested GFP tagged Arl5 protein and GalT-mCherry (used to mark the *trans*-Golgi) were subjected to treatment with nocodazole for 3 h, followed by immunofluorescence labeling with GFP and GM130 (used to mark the *cis*-Golgi). Cells were then imaged on a wide-field microscope. Golgi ministacks were selected to calculate the LQ. LQ is defined as d_x/d_1 , where d_x is the distance from the center of a tested protein (x) to that of GM130 and d_1 is the distance from the center of GalT-mCherry to that of GM130 ([Tie et al., 2017](#); [Tie et al., 2016](#)). Thus, the LQs of GM130 and GalT-mCherry are 0.00 and 1.00, respectively. As illustrated in Figure 41b, regions of the Golgi were operationally defined according to the LQ. The LQs of GFP-tagged Arl5a and b were measured (Figure 41a) and plotted on the localization map (Figure 41b). The result indicates that both Arl5a and Arl5b are mainly localized in the *trans*-Golgi.

Figure 41: Arl5a and Arl5b localize to the *trans*-Golgi.

Nocodazole-treated HeLa cells expressing indicated Arl5 proteins and GalT-mCherry were stained for endogenous GM130. Cells were imaged on a wide-field microscope, and the LQs of tested Arl5 proteins were measured according to (Tie et al., 2016) (a) LQs of Arl5a-wt-GFP, Arl5a-QL-GFP, Arl5b-wt-GFP and Arl5b-QL-GFP. (b) A localization map of tested Arl5 proteins in HeLa cells. Region including the ER Export Sites/ER-Golgi intermediate compartment (ERES/ERGIC), *cis*-, medial-, and *trans*-Golgi, and TGN are quantitatively and operationally defined (highlighted in blue), GalT-mCherry, GFP-Rab6, Arl1, Golgin97, Golgin245, CI-M6PR and furin were calculated by (Tie et al., 2016) and used as reference markers.

It is well known that the myristoylation site is needed for the GTP-bound form of Arf small GTPases to attach to the membrane (Cherfils and Zeghouf, 2013; Takai et al., 2001). Thus, we mutated the Gly at position 2, which could be a potential myristoylation site, and tested the localization (Figure 42). Similar to Arl1 (Lu et al., 2001), the C-terminally

GFP-tagged Arl5b-QL-G2A failed to localize in the Golgi, indicating that the N-terminal myristoylation of Arl5b is essential for its Golgi localization.

Figure 42: N-terminal myristoylation is probably required for the Golgi localization of Arl5b.

HeLa cells transiently expressing Arl5b-QL-GFP or Arl5b-QL-GFP harboring the G2A mutation were fixed and endogenous Golgin245 was stained. Bars, 10 μ m.

4.13 Arl5b colocalizes with Lamtor1 at the endosome and lysosome

We found that Arl5b interacts with Lamtor1, a component of the Ragulator complex that is predominantly distributed in the LE ([Nada et al., 2009](#)), while Arl5b was observed to localize in the Golgi in fixed cells. Thus, we tried to verify the localization in live cells. Interestingly, live-cell imaging revealed that the C-terminally mCherry or GFP-tagged QL and TN form of Arl5b colocalized to peripheral punctate structures labeled by the EE marker mCherry-Rab5 as well as the the LE or lysosome marker Lamp1-GFP (Figure 43a, b), demonstrating that Arl5b located in the endosomes and lysosome under live-cell conditions. Moreover, the peripheral punctate structures of GFP-tagged Arl5b-QL or TN were found to extensively overlap with Lamtor1-mCherry (Figure 43a, b). In contrast, we failed to detect any peripheral puncta in Arl5b-wt-GFP expressing cells under live cell imaging (Figure 43c).

Figure 43: Arl5b-QL and -TN but not Arl5b-wt display endosomal localization, which colocalize with Lamtor1 under live cell conditions.

(a) Live cell imaging of GFP/mCherry tagged Arl5b-QL with respective endosome markers (mCherry-Rab5 or Lamp1-GFP) and Lamtor1-mCherry. (b) Live cell imaging of GFP/mCherry tagged Arl5b-TN with respective endosome markers (mCherry-Rab5 or Lamp1-GFP) and Lamtor1-mCherry. (c) Live cell imaging of GFP tagged Arl5b-wt with endosome markers mCherry-Rab5 and Lamtor1-mCherry. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. Bars, 10 μm.

We also tested the endosomal localization of Lamtor1. As shown in Figure 44a, a substantial amount of Lamtor1 localized in the punctate structure that overlapped well with the LE (GFP-Rab7) and lysosomal (Lamp1) markers. Most importantly, we also observed that a large amount of Lamtor1 localizes to the EE (Figure 44a), while no colocalization between Lamtor1 and the Golgi marker Golgin245 was detected (Figure

44b). Therefore, it could be concluded that the interaction between Arl5b and Lamtor1 may take place on the surface of the endosome and lysosome.

Figure 44: Lamtor1 localizes to the EE, LE and lysosome.

(a) HeLa cells were transfected with GFP-Rab7 and stained with Lamtor1; HeLa cells were co-stained for Lamtor1 and EEA1 or Lamp1, respectively. Boxed regions are enlarged in the upper right corner. Arrows indicate colocalization. (b) HeLa cells were stained for endogenous Lamtor1 and Golgin245. Bars, 10 μ m.

4.14 Depletion of Arl5 decreases the endosome-to-Golgi trafficking of CD8a-furin

As described in the introduction, Arl5b plays an important role in regulating transport along the endosome-to-Golgi pathway ([Houghton et al., 2012](#)). The trafficking cargos they used were TGN38 and STxB. In this study, we wanted to further confirm this result by using CD8a-furin as the trafficking reporter. Since Arl1 has also been reported to regulate the endosome-to-Golgi trafficking pathway ([Lu et al., 2004](#)), we selected it as the positive control in this experiment.

Figure 45: siRNA mediated knockdown of endogenous Arl1 and Arl5.

HeLa cells were transfected with Arl1, Arl5 siRNA (Arl5a, b and c siRNA mixture) or control GL2 siRNA for 72 h. **(a)** The decrease in endogenous levels of Arl1 was observed by WB. GAPDH was used as a loading control. **(b)** The decrease in endogenous levels of Arl5b was observed by WB. GAPDH was used as a loading control. # refers to the non-specific band.

HeLa cells were first subjected to siRNA knockdown (Figure 45), followed by transfection with CD8a-furin. Due to the potential redundancy of three Arl5 paralogs, endogenous Arl5 a, b and c were simultaneously knocked down by a mixture of three siRNAs targeting the three paralogs, respectively (Figure 45b). The Arl1 siRNA was able to efficiently knock down the endogenous Arl1, which was demonstrated by WB (Figure 45a). The knockdown efficiency of Arl5a, b and c siRNA could reach to 60%. Cells were then surface-labeled with anti-CD8a antibody and chased for different time at 37°C.

At 0 min of chase, CD8a-furin was restricted to the cell surface as fine puncta, which did not colocalize with the Golgi marker giantin (Figure 46a–c, 0 min). After 20 min of chase, CD8a-furin was internalized in control GL2-, Arl1- and Arl5-depleted cells. In control GL2-depleted cells, a perinuclear localization of CD8a-furin which colocalized with the Golgi marker giantin was observed, whereas the majority of furin accumulated at peripheral endosomes in both Arl5- and Arl1-depleted cells, and the amount of CD8a-furin transported to the Golgi was significantly lower than control (Arl5a,b and c, $p=0.04$; Arl1, $p=0.01$) (Figure 46d). At 40 min, although CD8a-furin was found to present in the Golgi region in Arl5- and Arl1-depleted cells, the fraction of Golgi-localized CD8a-furin was still significantly lower than control-depleted cells (Arl5a,b and c, $p=0.03$; Arl1,

$p=2\times 10^{-05}$) . After 80 min of chase, majority of CD8a-furin reached the Golgi, while the punctate localization was still observed in the Arl1- and Arl5-depleted cells (Figure 46b, c). As the chase proceeded to 160 min, the CD8a-furin complexes achieved their steady-state distributions, where they overlapped well with the Golgi marker giantin. Additionally, although the amount of Golgi-localized CD8a-furin in Arl5-depleted cells was lower than the control-depleted cells after 80 min and 160 min of chase, the difference was not statistically significant (80 min, $p=0.2$; 160 min, $p=0.3$). This is inconsistent with previous reports that the majority of internalized STxB was still located in punctate structures after 90 min of incubation in Arl5b-depleted cells ([Houghton et al., 2012](#)). This might due to the different features of the trafficking reporters. Another possible reason could be that the Arl5b knockdown efficiency had not reached 100% (Figure 45b). Nevertheless, these data further support the conclusion that depletion of Arl5 perturbed the endosome-to-Golgi trafficking of CD8a-furin.

Figure 46: Endosome-to-Golgi trafficking of CD8a-furin is slowed in cells depleted of Arl1 or Arl5.

HeLa cells were transfected with control GL2 siRNA, Arl1 siRNA or Arl5 siRNA for 48 h and transfected with CD8a-furin for an additional 24 h. Monolayers were then incubated with anti-CD8a antibody for 60 min on ice and incubated in complete media for different time at 37°C, before immunolabeling of CD8a and giantin. **(a–c)** Time course images showing the endocytic trafficking of CD8a-furin in control-, Arl1- or Arl5- depleted cells. **(d)** Quantification results of the fraction of Golgi-localized furin in siRNA treated cells after different time of internalization at 37°C. Error bars indicate SEMs. The *P*-values (by student's t-test) of selected pairs of data, blue for Arl5a, b and c siRNA mixture and red for Arl1 siRNA with respect to control siRNA, respectively. Bars, 10 µm.

4.15 Arl5b is essential for AA-stimulated endosome-to-Golgi trafficking

Our findings prompted us to test the hypothesis that Arl5b participates in AA-stimulated endosome-to-Golgi trafficking. As shown in Figure 47, the Arl5a, b and c siRNAs were able to efficiently knock down endogenous Arl5a and Arl5b.

Figure 47: Evaluation of siRNA-mediated knockdown of endogenous Arl5 levels in HeLa cells.

(a) HeLa cells after GL2, Lamtor1 and Arl5abc siRNAs-mediated knockdown were subjected to immunoblot with antibodies against Arl5b and β -tubulin. (b–c) Endogenous Arl5a and Arl5b were knocked down by siRNA as assessed by RT-qPCR. (d) Melting curve analysis for RT-qPCR of Arl5a, Arl5b and GAPDH in control GL2- and Arl5a, b and c-depleted templates.

Figure 48: Arl5 is required for the AA-stimulated endosome-to-Golgi trafficking of CD8a-furin.

(a) Control GL2 and (b) Arl5 siRNA-treated HeLa cells were transiently transfected to express CD8a-furin and subjected to treatment with HBSS for 2 h, followed by surface-labeling using anti-CD8a antibody. Cells were then chased in DMEM or HBSS for 20 min and subsequently stained with anti-CD8a and anti-giantin. (c) The AA-stimulated Golgi trafficking was quantified by imaging. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 51 cells. Error bars indicate SDs and P -values were determined by student's t-test. Bars, 10 μm .

After the siRNA-mediated knockdown, HeLa cells were transiently transfected with CD8a-furin, followed by incubation with HBSS for 2 h and surface-labeled with anti-CD8a antibody. The CD8a antibody-bound complexes were then internalized at 37°C for 20 min. Under AA-stimulation conditions, there was a marked reduction in the endocytic trafficking of CD8a-furin to the Golgi after Arl5a and b was simultaneous knockdown (Figure 48a, b). The value of AA-stimulated Golgi trafficking of CD8a-furin (Figure 48c) also reflected this pattern ($p=0.03$). This is suggestive of a role of Arl5 in regulating AA-stimulated endosome-to-Golgi trafficking pathway.

To further confirm the effect of silencing Arl5 on the AA-stimulated endosome-to-Golgi trafficking pathway, we knocked down Arl5a and Arl5b separately by using their respective shRNAs. Both Arl5a and Arl5b shRNAs were able to efficiently knock down the endogenous Arl5a and Arl5b (Figure 49). Similarly, knockdown of Arl5b inhibited AA stimulation effects on the endosome-to-Golgi trafficking (Figure 50c). Moreover, we also observed that knockdown of Arl5a also affected the AA-stimulated endosome-to-Golgi trafficking (Figure 50b). In conclusion, Arl5 is required for the AA-stimulated endosome-to-Golgi trafficking.

Figure 49: Evaluation of shRNA-mediated knockdown of endogenous Arl5a and Arl5b levels in HeLa cells.

(a) HeLa cells were subjected to knockdown of endogenous Arl5a or Arl5b by lentivirus-mediated transduction of the corresponding shRNA. The knockdown efficiency was assessed by RT-PCR. (b) Melting curve analysis for RT-qPCR of Arl5a, Arl5b and GAPDH in control GL2-, Arl5a- and Arl5b-depleted templates.

Figure 50: Arl5a and Arl5b are essential for the AA-stimulated Golgi trafficking of CD8a-furin.

(a–c) Control GL2-, Arl5a- or Arl5b-depleted HeLa cells were transiently transfected to express CD8a-furin and subjected to treatment with HBSS for 2 h, followed by surface-labeling using anti-CD8a antibody. Cells were then chased in DMEM or HBSS for 20 min before immunolabeling of CD8a and giantin. (d) The AA-stimulated Golgi trafficking was quantified by imaging. The displayed value is the mean of n=3 independent experiments with each experiment analyzing ≥17 cells. Error bars indicate SDs and *P*-values were determined by student's *t*-test. Bars, 10 μm.

4.16 GARP is involved in the AA-stimulated endosome-to-Golgi trafficking pathway

Arl5b has been shown to regulate endosome-to-Golgi trafficking ([Houghton et al., 2012](#)). However, only a few effectors have been reported to interact with Arl5b. The tethering factor GARP has recently been identified to interact with Arl5b in *Drosophila* ([Rosa-Ferreira et al., 2015](#)). As mentioned in section 1.2.3, there are four subunits in GARP:

Vps51–54 ([Bonifacino and Hierro, 2011](#)). Thus, we tested whether GARP is also involved in this AA-stimulated endosome-to-Golgi trafficking. We chose two subunits of GARP, Vps51 and Vps54, to perform the knockdown assay. For each protein, shRNA targeting two different sequences were designed and knockdown efficiency was assessed by RT-qPCR (Figure 51).

Control GL2-, Vps51- or Vps54-depleted HeLa cells were transiently transfected with CD8a-furin. Cells were then incubated in HBSS for 2 h. After surface-labeling, cells were chased at 37°C for 20 min under DMEM stimulation or continuous HBSS starvation conditions. Upon depleting endogenous Vps51 or Vps54, the internalized CD8a-furin was found to localize in punctate structures regardless of the AA-stimulation or starvation conditions (Figure 52b–e). Furthermore, the AA-stimulated Golgi trafficking in the Vps51- or Vps54-depleted cells was found to be substantially attenuated in comparison with control knockdown cells (Vps51 #1: $p=0.02$; Vps51 #2: $p=0.02$; Vps54 #1: $p=0.06$; Vps54 #2: $p=0.03$) (Figure 52f). Together, our data demonstrate that GARP, the effector of Arl5, is essential for AA-stimulated endosome-to-Golgi trafficking.

Figure 51: Evaluation of shRNA-mediated knockdown of endogenous Arl5a and Arl5b levels in HeLa cells.

(a) HeLa cells were subjected to knockdown of endogenous Vps51 or Vps54 by lentivirus-mediated transduction of the corresponding shRNA. The knockdown efficiency was assessed by RT-PCR. (b) & (c) Melting curve analysis for RT-qPCR of Vps51, Vps54 and GAPDH in control GL2-, Vps51- and Vps54-depleted templates.

Figure 52: GARP is required for the AA-stimulated Golgi trafficking of CD8a-furin.

(a–e) Control GL2-, Vps51- or Vps54-depleted HeLa cells were transiently transfected to express CD8a-furin and subjected to treatment with HBSS for 2 h followed by surface-labeling using anti-CD8a antibody. Cells were then chased in DMEM or HBSS for 20 min before stained with CD8a and giantin. (f) AA-stimulated Golgi trafficking was quantified by imaging. The displayed value is the mean of $n=3$ independent experiments with each experiment analyzing ≥ 31 cells. Error bars indicate SDs and P -values were determined by student's t -test. Bars, 10 μ m.

5. Discussion

5.1 AAs stimulate the endosome-to-Golgi trafficking in mammalian cells

Membrane trafficking is important for cells of all organisms to connect and exchange materials with the surrounding environment, as well as to maintain cellular homeostasis. Defects in membrane trafficking have been revealed to cause a series of human disease ([Howell et al., 2006](#)). Despite extensive research in intracellular membrane trafficking pathway, there is limit knowledge on how this pathway is regulated by extracellular signals. In yeast, the localization of the general AA permease Gap1p has been demonstrated to be regulated by the nitrogen source such as AAs in the growth medium ([Chen and Kaiser, 2002](#); [Roberg et al., 1997](#)). In addition, the intracellular membrane trafficking of an autophagy-related protein, Atg9, has also been reported to be affected by the nutrient conditions in mammalian cells ([Webber et al., 2007](#); [Young et al., 2006](#)). Except for these two proteins, it is not yet fully known if and how nutrients regulate intracellular membrane trafficking in mammalian cells, especially the endosome-to-Golgi trafficking pathway. In this study, we made a novel discovery that extracellular AAs, but not growth factors or glucose, are essential and sufficient to regulate the endosome-to-Golgi trafficking in mammalian cells.

Firstly, we found that nutrient starvation changes the subcellular localization of TGN-localized transmembrane proteins, such as furin and CI-M6PR. In both complete medium (DMEM plus 10% FBS) and DMEM, endogenous furin and CI-M6PR are located in the juxta-nuclear region, which is positive for the Golgi marker Golgin245 or giantin, as previously reported ([Ghosh et al., 2003](#); [Molloy et al., 1994](#)). However, after 1 h of HBSS starvation, the majority of furin and CI-M6PR showed endosomal localization, although the effect differs between these two proteins. The effect of nutrient starvation on the steady-state localization of TGN membrane proteins was further confirmed by using the CD8a-furin and CD8a-CI-M6PR reporters. The CD8a-furin during starvation was found to colocalize well with the EE marker RUFY1 and the RE marker TfR-GFP, whereas no colocalization between CD8a-furin and the LE marker GFP-Rab7 was detected. Our data is different from previous observations that the majority of furin traffics to the Golgi is from the LE instead of passing through the RE ([Bosshart et al., 1994](#); [Chia et al., 2011](#);

[Mallet and Maxfield, 1999](#)). The colocalization study using full-length GFP-tagged furin protein showed similar results, except that the LE localization was also observed. This difference between full-length furin and CD8a-furin might be due to the contribution of the native transmembrane domain, as reported by Chia et.al ([Chia et al., 2011](#)). The starvation-induced translocation of TGN membrane proteins to the endosomes is reversible. When HBSS-treated cells were subsequently supplied with nutrients by incubating with DMEM, furin rapidly re-appeared in the Golgi and recovered to the pre-starvation state after 2 h, demonstrating that furin was probably not degraded under starvation conditions.

Based on these findings, we next examined the role of nutrients in endocytic trafficking. Interestingly, we found that nutrients can stimulate the PM-to-Golgi trafficking of CD8a-tagged reporter proteins. We then further demonstrated that the target of nutrient stimulation is the endosome-to-Golgi pathway, but not endocytosis. Since the nutrient stimulation medium used was DMEM or the complete medium, which contains more than one component, we tried to reveal the key factor(s) controlling the nutrient-dependent endocytic pathway. Surprisingly, we found that AAs, but not FBS or glucose, were sufficient to stimulate endosome-to-Golgi trafficking of cargos in HeLa cells. Moreover, a similar nutrient-stimulation effect was also observed in BSC-1 and HEK293T cells, which indicate that the effect of AAs on the endosome-to-Golgi trafficking is probably ubiquitous. Furthermore, we observed that Gln, one of the most effective and preferred nitrogen sources for yeast ([Chen and Kaiser, 2002](#); [Crespo et al., 2002](#)), stood out as the most acute stimulator for the endosome-to-Golgi trafficking pathway. Although Gln is a non-essential AA, it is the most abundant AA in blood plasma and has the highest concentration in cell culture media (2–4 mM). Gln plays diverse roles in the cell, such as acting as a nitrogen donor, serving as a carbon source and modulating cell signaling pathways ([Daye and Wellen, 2012](#)). For example, the oxidation of Gln maintains the tricarboxylic acid cycle and contributes to the synthesis of protein and lipid, which, together with glucose, serve as the major energy source for animal cells in the cell culture medium ([Hassell et al., 1991](#)). In addition to its direct function in cell metabolism, it also participates in promoting the activation of mTORC1 ([Durán et al., 2012](#)). Indeed, many cancer cells rely heavily on Gln for their continuous growth and proliferation ([Jin et al.,](#)

[2015](#); [Wise and Thompson, 2010](#)), the complete mechanism of which remains to be elucidated. It is possible that the maintenance of the endocytic retrograde trafficking contributes to the cellular demand for Gln.

The finding of AA-stimulated endosome-to-Golgi trafficking might affect cellular metabolism in at least two-ways. First, the endosome-to-Golgi trafficking has been known to retrieve the post-Golgi cycling cargos such as proteins and lipids from the degradation pathway, which prolong the half-lives of them ([Bonifacino and Rojas, 2006](#); [Lu and Hong, 2014](#)); and second cells are able to adjust the PM-localized transporters and receptors via AA-stimulated endosome-to-Golgi trafficking to ensure an optimal uptake of nutrients and engagement with their environment.

5.2 v-ATPase, SLC38A9 and Ragulator are required for AA-stimulated endosome-to-Golgi trafficking

We elucidated a signaling pathway from the sensing of AAs to the trafficking of membrane carriers from the endosome to the Golgi. We next sought to explore how AAs stimulate the endosome-to-Golgi trafficking pathway. Interestingly, we found that some of the components involved in the AA-sensing module of the mTORC1 pathway also play a similar role in the AA-stimulated endosome-to-Golgi trafficking. In the AA-induced mTORC1 signaling pathway, AAs accumulation in the lysosomal pool triggers the GEF activity of Ragulator complex toward Rag GTPase in a v-ATPase- and SLC38A9-dependent manner ([Bar-Peled et al., 2012](#); [Rebsamen et al., 2015](#); [Sancak et al., 2008](#); [Zoncu et al., 2011](#)). In our study, we observed that the majority of CD8a-furin was arrested in the endosomal pool and failed to transport to the Golgi even under AA-stimulation in cells treated with the v-ATPase inhibitor conA. Consistently, when SLC38A9 was depleted by its shRNAs, AAs failed to stimulate the endosome-to-Golgi trafficking. Similarly, when Lamtor1 and Lamtor3, two subunits of Ragulator, were individually depleted by the corresponding shRNAs, the AA-stimulated Golgi trafficking of CD8a-furin decreased significantly in comparison with the control treatment. However, neither simultaneous depletion of GTPase RagA and RagB nor inhibition of mTORC1 activity significantly affected AA-stimulated Golgi trafficking. Hence AA-regulated endosome-to-Golgi trafficking and mTORC1 signaling share common components,

including SLC38A9, v-ATPase and Ragulator, while the activation of mTORC1 occurs via Rag GTPases, and the promotion of the endosome-to-Golgi trafficking requires the Arl family small GTPase – Arl5b, which is discussed in the next section.

5.3 AA-stimulated endosome-to-Golgi trafficking depends on Arl5b and its effector GARP

The Golgi-localized Arl5b has been previously reported to regulate the endosome-to-Golgi trafficking ([Houghton et al., 2012](#); [Rosa-Ferreira et al., 2015](#)). There are two known downstream effectors of Arl5b – GARP and AP-4 ([Rosa-Ferreira et al., 2015](#); [Toh et al., 2017](#)). In this study, we further discovered that Arl5b and GARP are also required for the AA-stimulated endosome-to-Golgi trafficking. In Arl5b-depleted cells, the AA-stimulated endosome-to-Golgi trafficking was significantly decreased. A similar effect can also be achieved when the GARP subunits Vps51 or Vps54 were depleted. In addition, using yeast two-hybrid screening and in vitro protein-interaction assays, we found that Arl5b interacted with Ragulator through Lamtor1. We then explored the sub-Golgi localization of Arl5b, which was found to localize in the *trans*-Golgi. In addition to the Golgi localization in the fixed cell, the constitutively active (-QL) or inactive (-TN) form of Arl5b was also found to localize in the punctate structures that colocalized with the EE, LE and lysosomal markers as well as Lamtor1, under live cell imaging. These data, taken together, indicate that the interaction between Arl5b and Ragulator occurs on the endolysosome via its scaffolding subunit, Lamtor1. The biochemical assay performed in our lab also demonstrated that AAs stimulate the guanine nucleotide exchange of Arl5b from GDP to GTP in a Ragulator-, v-ATPase- and SLC38A9-dependent manner. Most importantly, Ragulator might function as a GEF for the activation of Arl5b,

Figure 53: Working model on how AAs stimulate the endosome-to-Golgi trafficking through Ragulator and Arl5b.

In AA-sufficient conditions, accumulation of AAs within the lumen of the endolysosome generates an activating signal to activate the GEF activity of Ragulator toward Arl5b. This activation process is dependent on SLC38A9 and v-ATPase; the activated Arl5b recruits the GARP to the transport carrier, which functions in tethering and fusion of the budded transport carriers with the TGN membrane.

From the observations thus far, we propose here a working model to summarize the AA-regulated signaling pathway that leads to the endosome-to-Golgi trafficking (Figure 53): when extracellular AAs are abundant, it leads to the rapid accumulation of AAs in the endolysosomal lumen; luminal AAs generate an activating signal to activate Ragulator in a SLC38A9- and v-ATPase-dependent fashion; following the activation, Ragulator functions as a GEF of Arl5b GTPase to activate the Arl5b by guanine nucleotide exchange; GTP-loaded Arl5b recruits the tethering factor GARP to the endosome-derived transport carrier, which finally facilitates the tethering and fusion of the transport carrier with the TGN membrane.

The endosome-to-Golgi trafficking has been reported to control a variety of physiological and pathological processes, including the early metazoan development, nutrient homeostasis, Alzheimer's and other neurological diseases ([Burd, 2011](#); [Lu and Hong, 2014](#)). Our discovery provides a possible mechanistic connection between the endosome-to-Golgi trafficking and the nutrient signaling pathway, which implies that nutrients might play roles in modulating these physiological and pathological processes and requires further study.

6. Bibliography

- Ackema, K.B., C. Prescianotto-Baschong, J. Hench, S.C. Wang, Z.H. Chia, H. Mergentaler, F. Bard, S. Frank, and A. Spang. 2016. Sar1, a Novel Regulator of ER-Mitochondrial Contact Sites. *PLoS one*. 11:e0154280.
- Al-Bassam, J. 2017. Revisiting the tubulin cofactors and Arl2 in the regulation of soluble $\alpha\beta$ -tubulin pools and their effect on microtubule dynamics. *Molecular biology of the cell*. 28:359-363.
- Alberts, B. 2017. Molecular biology of the cell. Garland science.
- Ballou, L.M., and R.Z. Lin. 2008. Rapamycin and mTOR kinase inhibitors. *Journal of chemical biology*. 1:27-36.
- Bar-Peled, L., L. Chantranupong, A.D. Cherniack, W.W. Chen, K.A. Ottina, B.C. Grabiner, E.D. Spear, S.L. Carter, M. Meyerson, and D.M. Sabatini. 2013. A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science*. 340:1100-1106.
- Bar-Peled, L., L.D. Schweitzer, R. Zoncu, and D.M. Sabatini. 2012. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell*. 150:1196-1208.
- Barlowe, C., and R. Schekman. 1993. SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature*. 365:347-349.
- Barr, F.A., and B. Short. 2003. Golgins in the structure and dynamics of the Golgi apparatus. *Current opinion in cell biology*. 15:405-413.
- Barral, D.C., S. Garg, C. Casalou, G.F. Watts, J.L. Sandoval, J.S. Ramalho, V.W. Hsu, and M.B. Brenner. 2012. Arl13b regulates endocytic recycling traffic. *Proceedings of the National Academy of Sciences*. 109:21354-21359.
- Bartolini, F., A. Bhamidipati, S. Thomas, U. Schwahn, S.A. Lewis, and N.J. Cowan. 2002. Functional overlap between retinitis pigmentosa 2 protein and the tubulin-specific chaperone cofactor C. *Journal of Biological Chemistry*. 277:14629-14634.
- Belenkaya, T.Y., Y. Wu, X. Tang, B. Zhou, L. Cheng, Y.V. Sharma, D. Yan, E.M. Selva, and X. Lin. 2008. The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. *Developmental cell*. 14:120-131.
- Bhamidipati, A., S.A. Lewis, and N.J. Cowan. 2000. ADP ribosylation factor-like protein 2 (Arl2) regulates the interaction of tubulin-folding cofactor D with native tubulin. *The Journal of cell biology*. 149:1087-1096.
- Bielli, A., C.J. Haney, G. Gabreski, S.C. Watkins, S.I. Bannykh, and M. Aridor. 2005. Regulation of Sar1 NH2 terminus by GTP binding and hydrolysis promotes membrane deformation to control COPII vesicle fission. *The Journal of cell biology*. 171:919-924.
- Bieschke, K.J., A. Abels, E. Adams, M. Miville, B. Schreier, Council Counseling Psychology Training Programs, Association of Counseling Center Training Agencies, and Society of Counseling Psychology. 2009. Counseling Psychology Model Training Values Statement Addressing Diversity. *Couns Psychol*. 37:641-643.
- Bonifacino, J.S., and A. Hierro. 2011. Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. *Trends in cell biology*. 21:159-167.

- Bonifacino, J.S., and J.H. Hurley. 2008. Retromer. *Current opinion in cell biology*. 20:427-436.
- Bonifacino, J.S., and R. Rojas. 2006. Retrograde transport from endosomes to the trans-Golgi network. *Nature reviews Molecular cell biology*. 7:568-579.
- Bos, J.L., H. Rehmann, and A. Wittinghofer. 2007. GEFs and GAPs: critical elements in the control of small G proteins. *Cell*. 129:865-877.
- Bosshart, H., J. Humphrey, E. Deignan, J. Davidson, J. Drazba, L. Yuan, V. Oorschot, P.J. Peters, and J.S. Bonifacino. 1994. The cytoplasmic domain mediates localization of furin to the trans-Golgi network en route to the endosomal/lysosomal system. *Journal of Cell Biology*. 126:1157-1172.
- Bowman, B.J., M.E. McCall, R. Baertsch, and E.J. Bowman. 2006. A model for the proteolipid ring and bafilomycin/concanamycin-binding site in the vacuolar ATPase of *Neurospora crassa*. *Journal of Biological Chemistry*. 281:31885-31893.
- Breiner, M., A. Schürmann, W. Becker, and H.-G. Joost. 1996. Cloning of a novel member (ARL5) of the ARF-family of Ras-related GTPases. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. 1308:1-6.
- Bröer, S. 2014. The SLC38 family of sodium–amino acid co-transporters. *Pflügers Archiv-European Journal of Physiology*. 466:155-172.
- Burd, C.G. 2011. Physiology and Pathology of Endosome - to - Golgi Retrograde Sorting. *Traffic*. 12:948-955.
- Cantagrel, V., J.L. Silhavy, S.L. Bielas, D. Swistun, S.E. Marsh, J.Y. Bertrand, S. Audollent, T. Attié-Bitach, K.R. Holden, and W.B. Dobyns. 2008. Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. *The American Journal of Human Genetics*. 83:170-179.
- Casalou, C., C. Seixas, A. Portelinha, P. Pintado, M. Barros, J.S. Ramalho, S.S. Lopes, and D.C. Barral. 2014. Arl13b and the non-muscle myosin heavy chain IIA are required for circular dorsal ruffle formation and cell migration. *J Cell Sci*. 127:2709-2722.
- Castellano, E., and E. Santos. 2011. Functional specificity of Ras isoforms so similar but so different. *Genes & cancer*. 2:216-231.
- Cevik, S., Y. Hori, O.I. Kaplan, K. Kida, T. Toivenon, C. Foley-Fisher, D. Cottell, T. Katada, K. Kontani, and O.E. Blacque. 2010. Joubert syndrome Arl13b functions at ciliary membranes and stabilizes protein transport in *Caenorhabditis elegans*. *The Journal of cell biology*. 188:953-969.
- Chavrier, P., R.G. Parton, H.P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell*. 62:317-329.
- Chen, E.J., and C.A. Kaiser. 2002. Amino acids regulate the intracellular trafficking of the general amino acid permease of *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*. 99:14837-14842.
- Chen, Y.A., and R.H. Scheller. 2001. SNARE-mediated membrane fusion. *Nature reviews Molecular cell biology*. 2:98-106.
- Cherfils, J., and M. Zeghouf. 2013. Regulation of small gtpases by gefs, gaps, and gdis. *Physiological reviews*. 93:269-309.

- Chia, P.Z.C., I. Gasnereau, Z.Z. Lieu, and P.A. Gleeson. 2011. Rab9-dependent retrograde transport and endosomal sorting of the endopeptidase furin. *J Cell Sci.* 124:2401-2413.
- Chia, P.Z.C., P. Gunn, and P.A. Gleeson. 2013. Cargo trafficking between endosomes and the trans-Golgi network. *Histochemistry and cell biology.* 140:307-315.
- Chiu, M., S. Tardito, A. Barilli, M.G. Bianchi, V. Dall'Asta, and O. Bussolati. 2012. Glutamine stimulates mTORC1 independent of the cell content of essential amino acids. *Amino acids.* 43:2561-2567.
- Colicelli, J. 2004. Human RAS superfamily proteins and related GTPases. *Science's STKE: signal transduction knowledge environment.* 2004:RE13.
- Conibear, E., and T.H. Stevens. 2000. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Molecular biology of the cell.* 11:305-323.
- Cooper, G.M., and D. Ganem. 1997. The Cell: A Molecular Approach. *Nature Medicine.* 3:1042-1042.
- Cooper, T. 2004. Nutrient-Induced Responses in Eukaryotic Cells: Topics in Current Genetics.
- Costa-Mattioli, M., and L.M. Monteggia. 2013. mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nature neuroscience.* 16:1537-1543.
- Crespo, J.L., T. Powers, B. Fowler, and M.N. Hall. 2002. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. *Proceedings of the National Academy of Sciences.* 99:6784-6789.
- Daye, D., and K.E. Wellen. 2012. Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. In *Seminars in cell & developmental biology.* Vol. 23. Elsevier. 362-369.
- Derby, M.C., Z.Z. Lieu, D. Brown, J.L. Stow, B. Goud, and P.A. Gleeson. 2007. The trans - Golgi Network Golgin, GCC185, is Required for Endosome - to - Golgi Transport and Maintenance of Golgi Structure. *Traffic.* 8:758-773.
- Deretic, D., J. Wang, and Y. Morita. 2011. Arf4GTP-ASAP1 Trafficking Module Coordinates VxPx Motif-dependent Ciliary Targeting Of Rhodopsin With Rab11a-Rabin8-Rab8 Ciliogenesis Cascade. *Investigative Ophthalmology & Visual Science.* 52:4406-4406.
- Donaldson, J., and A. Honda. 2005. Localization and function of Arf family GTPases. Portland Press Limited.
- Donaldson, J.G. 2003. Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *Journal of Biological Chemistry.* 278:41573-41576.
- Donaldson, J.G., A. Honda, and R. Weigert. 2005. Multiple activities for Arf1 at the Golgi complex. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research.* 1744:364-373.
- Donaldson, J.G., and C.L. Jackson. 2011. ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nature reviews. Molecular cell biology.* 12:362.
- Duman, J.G., and J.G. Forte. 2003. What is the role of SNARE proteins in membrane fusion? *American Journal of Physiology-Cell Physiology.* 285:C237-C249.

- Durán, R.V., W. Oppliger, A.M. Robitaille, L. Heiserich, R. Skendaj, E. Gottlieb, and M.N. Hall. 2012. Glutaminolysis activates Rag-mTORC1 signaling. *Mol. Cell.* 47:349-358.
- Efeyan, A., W.C. Comb, and D.M. Sabatini. 2015. Nutrient sensing mechanisms and pathways. *Nature.* 517:302.
- Efeyan, A., R. Zoncu, and D.M. Sabatini. 2012. Amino acids and mTORC1: from lysosomes to disease. *Trends in molecular medicine.* 18:524-533.
- Elkin, S.R., A.M. Lakoduk, and S.L. Schmid. 2016. Endocytic pathways and endosomal trafficking: a primer. *Wiener Medizinische Wochenschrift.* 166:196-204.
- Fan, Y., M.A. Esmail, S.J. Ansley, O.E. Blacque, K. Boroevich, A.J. Ross, S.J. Moore, J.L. Badano, H. May-Simera, and D.S. Compton. 2004. Mutations in a member of the Ras superfamily of small GTP-binding proteins causes Bardet-Biedl syndrome. *Nature genetics.* 36:989.
- Follett, J., S.J. Norwood, N.A. Hamilton, M. Mohan, O. Kovtun, S. Tay, Y. Zhe, S.A. Wood, G.D. Mellick, and P.A. Silburn. 2014. The Vps35 D620N mutation linked to Parkinson's disease disrupts the cargo sorting function of retromer. *Traffic.* 15:230-244.
- Foster, K.G., and D.C. Fingar. 2010. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *Journal of Biological Chemistry.* 285:14071-14077.
- Franch-Marro, X., F. Wendler, S. Guidato, J. Griffith, A. Baena-Lopez, N. Itasaki, M.M. Maurice, and J.-P. Vincent. 2008. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nature cell biology.* 10:170-177.
- Ghosh, P., N.M. Dahms, and S. Kornfeld. 2003. Mannose 6-phosphate receptors: new twists in the tale. *Nature reviews Molecular cell biology.* 4:202-213.
- Ghosh, R.N., W.G. Mallet, T.T. Soe, T.E. McGraw, and F.R. Maxfield. 1998. An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. *The Journal of cell biology.* 142:923-936.
- Gillingham, A.K., and S. Munro. 2007. The small G proteins of the Arf family and their regulators. *Annu. Rev. Cell Dev. Biol.* 23:579-611.
- Godard, P., A. Urrestarazu, S. Vissers, K. Kontos, G. Bontempi, J. van Helden, and B. André. 2007. Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Molecular and cellular biology.* 27:3065-3086.
- Goud, B., and P.A. Gleeson. 2010. TGN golgins, Rabs and cytoskeleton: regulating the Golgi trafficking highways. *Trends in cell biology.* 20:329-336.
- Grant, B.D., and J.G. Donaldson. 2009. Pathways and mechanisms of endocytic recycling. *Nature reviews. Molecular cell biology.* 10:597.
- Gruenberg, J., and F.R. Maxfield. 1995. Membrane transport in the endocytic pathway. *Current opinion in cell biology.* 7:552-563.
- Hanke-Gogokhia, C., Z. Wu, C.D. Gerstner, J.M. Frederick, H. Zhang, and W. Baehr. 2016. Arf-like protein 3 (ARL3) regulates protein trafficking and ciliogenesis in mouse photoreceptors. *Journal of Biological Chemistry.* 291:7142-7155.

- Hanzal - Bayer, M., L. Renault, P. Roversi, A. Wittinghofer, and R.C. Hillig. 2002. The complex of Arl2 - GTP and PDE δ : from structure to function. *The EMBO journal*. 21:2095-2106.
- Hara, K., K. Yonezawa, Q.-P. Weng, M.T. Kozlowski, C. Belham, and J. Avruch. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *Journal of Biological Chemistry*. 273:14484-14494.
- Hassell, T., S. Gleave, and M. Butler. 1991. Growth inhibition in animal cell culture. *Appl. Biochem. Biotechnol.* 30:29-41.
- Hesse, D., A. Jaschke, T. Kanzleiter, N. Witte, R. Augustin, A. Hommel, G.P. Püschel, K.-J. Petzke, H.-G. Joost, and M. Schupp. 2012. GTPase ARFRP1 is essential for normal hepatic glycogen storage and insulin-like growth factor 1 secretion. *Molecular and cellular biology*. 32:4363-4374.
- Hirst, J., G.H. Borner, R. Antrobus, A.A. Peden, N.A. Hodson, D.A. Sahlender, and M.S. Robinson. 2012. Distinct and overlapping roles for AP-1 and GGAs revealed by the “knocksideways” system. *Current Biology*. 22:1711-1716.
- Hirst, J., A. Motley, K. Harasaki, S.Y.P. Chew, and M.S. Robinson. 2003. EpsinR: an ENTH domain-containing protein that interacts with AP-1. *Molecular biology of the cell*. 14:625-641.
- Hofmann, I., and S. Munro. 2006. An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility. *Journal of cell science*. 119:1494-1503.
- Hofmann, I., A. Thompson, C.M. Sanderson, and S. Munro. 2007. The Arl4 family of small G proteins can recruit the cytohesin Arf6 exchange factors to the plasma membrane. *Current biology*. 17:711-716.
- Hommel, A., D. Hesse, W. Völker, A. Jaschke, M. Moser, T. Engel, M. Blüher, C. Zahn, A. Chadt, and K. Ruschke. 2010. The ARF-like GTPase ARFRP1 is essential for lipid droplet growth and is involved in the regulation of lipolysis. *Molecular and cellular biology*. 30:1231-1242.
- Houghton, F.J., S.A. Bellingham, A.F. Hill, D. Bourges, D.K. Ang, T. Gemetzi, I. Gasnereau, and P.A. Gleeson. 2012. Arl5b is a Golgi-localised small G protein involved in the regulation of retrograde transport. *Experimental cell research*. 318:464-477.
- Howell, G.J., Z.G. Holloway, C. Cobbold, A.P. Monaco, and S. Ponnambalam. 2006. Cell biology of membrane trafficking in human disease. *International review of cytology*. 252:1-69.
- Humbert, M.C., K. Weihbrecht, C.C. Searby, Y. Li, R.M. Pope, V.C. Sheffield, and S. Seo. 2012. ARL13B, PDE6D, and CEP164 form a functional network for INPP5E ciliary targeting. *Proceedings of the National Academy of Sciences*. 109:19691-19696.
- Huotari, J., and A. Helenius. 2011. Endosome maturation. *The EMBO journal*. 30:3481-3500.
- Huse, J.T., D.S. Pijak, G.J. Leslie, V.M.-Y. Lee, and R.W. Doms. 2000. Maturation and endosomal targeting of β -site amyloid precursor protein-cleaving enzyme The Alzheimer's disease β -secretase. *Journal of Biological Chemistry*. 275:33729-33737.

- Inoki, K., Y. Li, T. Xu, and K.-L. Guan. 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes & development*. 17:1829-1834.
- Itzen, A., and R.S. Goody. 2011. GTPases involved in vesicular trafficking: structures and mechanisms. In *Seminars in cell & developmental biology*. Vol. 22. Elsevier. 48-56.
- Jaschke, A., B. Chung, D. Hesse, R. Kluge, C. Zahn, M. Moser, K.-J. Petzke, R. Brigelius-Flohé, D. Puchkov, and H. Koepsell. 2012. The GTPase ARFRP1 controls the lipidation of chylomicrons in the Golgi of the intestinal epithelium. *Human molecular genetics*. 21:3128-3142.
- Jékely, G. 2003. Small GTPases and the evolution of the eukaryotic cell. *Bioessays*. 25:1129-1138.
- Jewell, J.L., and K.-L. Guan. 2013. Nutrient signaling to mTOR and cell growth. *Trends in biochemical sciences*. 38:233-242.
- Jewell, J.L., R.C. Russell, and K.-L. Guan. 2013. Amino acid signalling upstream of mTOR. *Nature reviews Molecular cell biology*. 14:133-139.
- Jiang, Y., C.-Y. Zhao, L.-C. Cheng, B. Xu, and H.-Y. Lv. 2017. ARLTS1 polymorphism is associated with an increased risk of familial cancer: evidence from a meta-analysis. *Hereditary Cancer in Clinical Practice*. 15:8.
- Jin, H., S.R. White, T. Shida, S. Schulz, M. Aguiar, S.P. Gygi, J.F. Bazan, and M.V. Nachury. 2010. The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia. *Cell*. 141:1208-1219.
- Jin, L., G. Alesi, and S. Kang. 2015. Glutaminolysis as a target for cancer therapy. *Oncogene*.
- Johannes, L., and V. Popoff. 2008. Tracing the retrograde route in protein trafficking. *Cell*. 135:1175-1187.
- Jovic, M., M. Sharma, J. Rahajeng, and S. Caplan. 2010. The early endosome: a busy sorting station for proteins at the crossroads. *Histology and histopathology*. 25:99.
- Jung, J., H.M. Genau, and C. Behrends. 2015. Amino acid-dependent mTORC1 regulation by the lysosomal membrane protein SLC38A9. *Molecular and cellular biology*. 35:2479-2494.
- Kahn, R.A., J. Cherfils, M. Elias, R.C. Lovering, S. Munro, and A. Schurmann. 2006. Nomenclature for the human Arf family of GTP-binding proteins: ARF, ARL, and SAR proteins. *The Journal of cell biology*. 172:645-650.
- Khatter, D., V.B. Raina, D. Dwivedi, A. Sindhwani, S. Bahl, and M. Sharma. 2015a. The small GTPase Arl8b regulates assembly of the mammalian HOPS complex on lysosomes. *J Cell Sci*. 128:1746-1761.
- Khatter, D., A. Sindhwani, and M. Sharma. 2015b. Arf-like GTPase Arl8: Moving from the periphery to the center of lysosomal biology. *Cellular logistics*. 5:e1086501.
- Kim, J., and E. Kim. 2016. Rag GTPase in amino acid signaling. *Amino acids*. 48:915-928.
- Kobayashi, A., S. Kubota, N. Mori, M.J. McLaren, and G. Inana. 2003. Photoreceptor synaptic protein HRG4 (UNC119) interacts with ARL2 via a putative conserved domain. *FEBS letters*. 534:26-32.
- Komiya, Y., and R. Habas. 2008. Wnt signal transduction pathways. *Organogenesis*. 4:68-75.

- Krall, A.S., S. Xu, T.G. Graeber, D. Braas, and H.R. Christofk. 2016. Asparagine promotes cancer cell proliferation through use as an amino acid exchange factor. *Nature communications*. 7.
- Laplante, M., and D.M. Sabatini. 2009. mTOR signaling at a glance. *Journal of cell science*. 122:3589-3594.
- Larkins, C.E., G.D.G. Aviles, M.P. East, R.A. Kahn, and T. Caspary. 2011. Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins. *Molecular biology of the cell*. 22:4694-4703.
- Lauwers, E., C. Jacob, and B. André. 2009. K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. *The Journal of cell biology*. 185:493-502.
- Lee, M.C., L. Orci, S. Hamamoto, E. Futai, M. Ravazzola, and R. Schekman. 2005. Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle. *Cell*. 122:605-617.
- Li, Y., K. Ling, and J. Hu. 2012a. The emerging role of Arf/Arl small GTPases in cilia and ciliopathies. *Journal of cellular biochemistry*. 113:2201-2207.
- Li, Y., Q. Zhang, Q. Wei, Y. Zhang, K. Ling, and J. Hu. 2012b. SUMOylation of the small GTPase ARL-13 promotes ciliary targeting of sensory receptors. *J Cell Biol:jcb*. 201203150.
- Lieu, Z.Z., M.C. Derby, R.D. Teasdale, C. Hart, P. Gunn, and P.A. Gleeson. 2007. The golgin GCC88 is required for efficient retrograde transport of cargo from the early endosomes to the trans-Golgi network. *Molecular biology of the cell*. 18:4979-4991.
- Lieu, Z.Z., and P.A. Gleeson. 2011. Endosome-to-Golgi transport pathways in physiological processes. *Histology and histopathology*. 26:395-408.
- Liewen, H., I. Meinhold-Heerlein, V. Oliveira, R. Schwarzenbacher, G. Luo, A. Wadle, M. Jung, M. Pfreundschuh, and F. Stenner-Liewen. 2005. Characterization of the human GARP (Golgi associated retrograde protein) complex. *Experimental cell research*. 306:24-34.
- Lin, C.-Y., P.-H. Huang, W.-L. Liao, H.-J. Cheng, C.-F. Huang, J.-C. Kuo, W.A. Patton, D. Massenburg, J. Moss, and F.-J.S. Lee. 2000. ARL4, an ARF-like protein that is developmentally regulated and localized to nuclei and nucleoli. *Journal of Biological Chemistry*. 275:37815-37823.
- Lin, S.X., W.G. Mallet, A.Y. Huang, and F.R. Maxfield. 2004. Endocytosed cation-independent mannose 6-phosphate receptor traffics via the endocytic recycling compartment en route to the trans-Golgi network and a subpopulation of late endosomes. *Molecular biology of the cell*. 15:721-733.
- Linari, M., M. Hanzal-Bayer, and J. Becker. 1999. The delta subunit of rod specific cyclic GMP phosphodiesterase, PDE δ , interacts with the Arf-like protein Arl3 in a GTP specific manner. *FEBS letters*. 458:55-59.
- Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa, and J. Avruch. 2005. Rheb binds and regulates the mTOR kinase. *Current Biology*. 15:702-713.
- Lu, L., R.N. Hannoush, B.C. Goess, S. Varadarajan, M.D. Shair, and T. Kirchhausen. 2013. The small molecule dispergo tubulates the endoplasmic reticulum and inhibits export. *Molecular biology of the cell*. 24:1020-1029.

- Lu, L., and W. Hong. 2003. Interaction of Arl1-GTP with GRIP domains recruits autoantigens Golgin-97 and Golgin-245/p230 onto the Golgi. *Molecular biology of the cell*. 14:3767-3781.
- Lu, L., and W. Hong. 2014. From endosomes to the trans-Golgi network. In *Seminars in cell & developmental biology*. Vol. 31. Elsevier. 30-39.
- Lu, L., H. Horstmann, C. Ng, and W. Hong. 2001. Regulation of Golgi structure and function by ARF-like protein 1 (Arl1). *Journal of cell science*. 114:4543-4555.
- Lu, L., G. Tai, and W. Hong. 2004. Autoantigen Golgin-97, an effector of Arl1 GTPase, participates in traffic from the endosome to the trans-golgi network. *Molecular biology of the cell*. 15:4426-4443.
- Luke, M.R., L. Kjer-Nielsen, D.L. Brown, J.L. Stow, and P.A. Gleeson. 2003. GRIP domain-mediated targeting of two new coiled-coil proteins, GCC88 and GCC185, to subcompartments of the trans-Golgi network. *Journal of Biological Chemistry*. 278:4216-4226.
- Lupashin, V., and E. Sztul. 2005. Golgi tethering factors. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 1744:325-339.
- Lynch, C.J. 2001. Role of leucine in the regulation of mTOR by amino acids: revelations from structure–activity studies. *The Journal of Nutrition*. 131:861S-865S.
- Mahajan, D., B.K. Boh, Y. Zhou, L. Chen, T.C. Cornvik, W. Hong, and L. Lu. 2013. Mammalian Mon2/Ysl2 regulates endosome-to-Golgi trafficking but possesses no guanine nucleotide exchange activity toward Arl1 GTPase. *Scientific reports*. 3:3362.
- Mallard, F., C. Antony, D. Tenza, J. Salamero, B. Goud, and L. Johannes. 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. *The Journal of cell biology*. 143:973-990.
- Mallard, F., B.L. Tang, T. Galli, D. Tenza, A. Saint-Pol, X. Yue, C. Antony, W. Hong, B. Goud, and L. Johannes. 2002. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *The Journal of cell biology*. 156:653-664.
- Mallet, W.G., and F.R. Maxfield. 1999. Chimeric forms of furin and TGN38 are transported from the plasma membrane to the trans-Golgi network via distinct endosomal pathways. *The Journal of cell biology*. 146:345-360.
- Man, Z., Y. Kondo, H. Koga, H. Umino, K. Nakayama, and H.-W. Shin. 2011. Arfaptins are localized to the trans-Golgi by interaction with Arl1, but not Arfs. *Journal of Biological Chemistry*. 286:11569-11578.
- Manolea, F., J. Chun, D.W. Chen, I. Clarke, N. Summerfeldt, J.B. Dacks, and P. Melançon. 2010. Arf3 is activated uniquely at the trans-Golgi network by brefeldin A-inhibited guanine nucleotide exchange factors. *Molecular biology of the cell*. 21:1836-1849.
- Matsumoto, S., S. Fujii, and A. Kikuchi. 2016. Arl4c is a key regulator of tubulogenesis and tumorigenesis as a target gene of Wnt– β -catenin and growth factor–Ras signalling. *The Journal of Biochemistry*. 161:27-35.
- Maxfield, F.R., and T.E. McGraw. 2004a. Endocytic recycling. *Nature reviews. Molecular cell biology*. 5:121.

- Maxfield, F.R., and T.E. McGraw. 2004b. Endocytic recycling. *Nature reviews Molecular cell biology*. 5:121-132.
- Mayle, K.M., A.M. Le, and D.T. Kamei. 2012. The intracellular trafficking pathway of transferrin. *Biochimica et Biophysica Acta (BBA)-General Subjects*. 1820:264-281.
- Mazelova, J., L. Astuto - Gribble, H. Inoue, B.M. Tam, E. Schonteich, R. Prekeris, O.L. Moritz, P.A. Randazzo, and D. Deretic. 2009. Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4. *The EMBO journal*. 28:183-192.
- McKenzie, J.E., B. Raisley, X. Zhou, N. Naslavsky, T. Taguchi, S. Caplan, and D. Sheff. 2012. Retromer Guides STxB and CD8 - M6PR from Early to Recycling Endosomes, EHD1 Guides STxB from Recycling Endosome to Golgi. *Traffic*. 13:1140-1159.
- McMahon, H.T., and E. Boucrot. 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nature reviews. Molecular cell biology*. 12:517.
- Merhi, A., and B. André. 2012. Internal amino acids promote Gap1 permease ubiquitylation via TORC1/Npr1/14-3-3-dependent control of the Bul arrestin-like adaptors. *Molecular and cellular biology*. 32:4510-4522.
- Meyer, C., D. Zizioli, S. Lausmann, E.L. Eskelinen, J. Hamann, P. Saftig, K. von Figura, and P. Schu. 2000. μ 1A - adaptin - deficient mice: lethality, loss of AP - 1 binding and rerouting of mannose 6 - phosphate receptors. *The EMBO journal*. 19:2193-2203.
- Molloy, S., L. Thomas, J. VanSlyke, P. Stenberg, and G. Thomas. 1994. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *The EMBO Journal*. 13:18.
- Moss, J., and M. Vaughan. 1998. Molecules in the ARF orbit. *Journal of Biological Chemistry*. 273:21431-21434.
- Mrakovic, A., J.G. Kay, W. Furuya, J.H. Brumell, and R.J. Botelho. 2012. Rab7 and Arl8 GTPases are necessary for lysosome tubulation in macrophages. *Traffic*. 13:1667-1679.
- Munro, S. 2005. The Arf-like GTPase Arl1 and its role in membrane traffic. Portland Press Limited.
- Munro, S. 2011. The golgin coiled-coil proteins of the Golgi apparatus. *Cold Spring Harbor perspectives in biology*. 3:a005256.
- Nada, S., A. Hondo, A. Kasai, M. Koike, K. Saito, Y. Uchiyama, and M. Okada. 2009. The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes. *The EMBO journal*. 28:477-489.
- Nakamura, K., Z. Man, Y. Xie, A. Hanai, H. Makyio, M. Kawasaki, R. Kato, H.-W. Shin, K. Nakayama, and S. Wakatsuki. 2012. Structural basis for membrane binding specificity of the Bin/Amphiphysin/Rvs (BAR) domain of Arfaptin-2 determined by Arl1 GTPase. *Journal of Biological Chemistry*. 287:25478-25489.
- Newman, L.E., C.R. Schiavon, R.E. Turn, and R.A. Kahn. 2017. The ARL2 GTPase regulates mitochondrial fusion from the intermembrane space. *Cellular Logistics*. 7:e1340104.
- Nicklin, P., P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, H. Yang, M. Hild, C. Kung, and C. Wilson. 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell*. 136:521-534.

- Nielsen, M.S., C. Gustafsen, P. Madsen, J.R. Nyengaard, G. Hermey, O. Bakke, M. Mari, P. Schu, R. Pohlmann, and A. Dennes. 2007. Sorting by the cytoplasmic domain of the amyloid precursor protein binding receptor SorLA. *Molecular and cellular biology*. 27:6842-6851.
- Nishimoto-Morita, K., H.-W. Shin, H. Mitsuhashi, M. Kitamura, Q. Zhang, L. Johannes, and K. Nakayama. 2009. Differential effects of depletion of ARL1 and ARFRP1 on membrane trafficking between the trans-Golgi network and endosomes. *Journal of Biological Chemistry*. 284:10583-10592.
- Nozaki, S., Y. Katoh, M. Terada, S. Michisaka, T. Funabashi, S. Takahashi, K. Kontani, and K. Nakayama. 2017. Regulation of ciliary retrograde protein trafficking by the Joubert syndrome proteins ARL13B and INPP5E. *J Cell Sci*. 130:563-576.
- O'Brien, R.J., and P.C. Wong. 2011. Amyloid precursor protein processing and Alzheimer's disease. *Annual review of neuroscience*. 34:185-204.
- O'Donnell, A.F., A. Apffel, R.G. Gardner, and M.S. Cyert. 2010. α -Arrestins Aly1 and Aly2 regulate intracellular trafficking in response to nutrient signaling. *Molecular biology of the cell*. 21:3552-3566.
- Panic, B., J.R. Whyte, and S. Munro. 2003. The ARF-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Current biology*. 13:405-410.
- Pasqualato, S., L. Renault, and J. Cherfils. 2002. Arf, Arl, Arp and Sar proteins: a family of GTP - binding proteins with a structural device for 'front - back' communication. *EMBO reports*. 3:1035-1041.
- Paul, P., T. van den Hoorn, M.L. Jongsma, M.J. Bakker, R. Hengeveld, L. Janssen, P. Cresswell, D.A. Egan, M. van Ham, and A. ten Brinke. 2011. A Genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell*. 145:268-283.
- Pereira-Leal, J.B., and M.C. Seabra. 2001. Evolution of the Rab family of small GTP-binding proteins. *Journal of molecular biology*. 313:889-901.
- Pérez-Victoria, F.J., C. Schindler, J.G. Magadán, G.A. Mardones, C. Delevoye, M. Romao, G. Raposo, and J.S. Bonifacino. 2010. Ang2/fat-free is a conserved subunit of the Golgi-associated retrograde protein complex. *Molecular biology of the cell*. 21:3386-3395.
- Peterson, T.R., M. Laplante, C.C. Thoreen, Y. Sancak, S.A. Kang, W.M. Kuehl, N.S. Gray, and D.M. Sabatini. 2009. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell*. 137:873-886.
- Pfeffer, S.R. 2009. Multiple routes of protein transport from endosomes to the trans Golgi network. *FEBS letters*. 583:3811-3816.
- Poole, E., I. Groves, A. Macdonald, Y. Pang, A. Alcami, and J. Sinclair. 2009. Identification of TRIM23 as a cofactor involved in the regulation of NF- κ B by human cytomegalovirus. *Journal of virology*. 83:3581-3590.
- Port, F., M. Kuster, P. Herr, E. Furger, C. Bänziger, G. Hausmann, and K. Basler. 2008. Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nature cell biology*. 10:178.
- Rebsamen, M., L. Pochini, T. Stasyk, M.E. de Araújo, M. Galluccio, R.K. Kandasamy, B. Snijder, A. Fauster, E.L. Rudashevskaya, and M. Bruckner. 2015. SLC38A9 is a

- component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature*. 519:477-481.
- Reddy, J.V., A.S. Burguete, K. Sridevi, I.G. Ganley, R.M. Nottingham, and S.R. Pfeffer. 2006. A functional role for the GCC185 golgin in mannose 6-phosphate receptor recycling. *Molecular biology of the cell*. 17:4353-4363.
- Roberg, K.J., N. Rowley, and C.A. Kaiser. 1997. Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*. *The Journal of cell biology*. 137:1469-1482.
- Rogaeva, E., Y. Meng, J.H. Lee, Y. Gu, T. Kawarai, F. Zou, T. Katayama, C.T. Baldwin, R. Cheng, and H. Hasegawa. 2007. The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. *Nature genetics*. 39:168.
- Rojas, A.M., G. Fuentes, A. Rausell, and A. Valencia. 2012. The Ras protein superfamily: evolutionary tree and role of conserved amino acids. *J Cell Biol*. 196:189-201.
- Rosa-Ferreira, C., C. Christis, I.L. Torres, and S. Munro. 2015. The small G protein Arl5 contributes to endosome-to-Golgi traffic by aiding the recruitment of the GARP complex to the Golgi. *Biology open*. 4:474-481.
- Rosa-Ferreira, C., and S. Munro. 2011. Arl8 and SKIP act together to link lysosomes to kinesin-1. *Developmental cell*. 21:1171-1178.
- Sadakata, T., Y. Shinoda, Y. Sekine, C. Saruta, M. Itakura, M. Takahashi, and T. Furuichi. 2010. Interaction of calcium-dependent activator protein for secretion 1 (CAPS1) with the class II ADP-ribosylation factor small GTPases is required for dense-core vesicle trafficking in the trans-Golgi network. *Journal of Biological Chemistry*. 285:38710-38719.
- Saint-Pol, A., B. Yélamos, M. Amessou, I.G. Mills, M. Dugast, D. Tenza, P. Schu, C. Antony, H.T. McMahon, and C. Lamaze. 2004. Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Developmental cell*. 6:525-538.
- Sancak, Y., L. Bar-Peled, R. Zoncu, A.L. Markhard, S. Nada, and D.M. Sabatini. 2010. Regulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*. 141:290-303.
- Sancak, Y., T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 320:1496-1501.
- Sannerud, R., J. Saraste, and B. Goud. 2003. Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. *Current opinion in cell biology*. 15:438-445.
- Saxton, R.A., and D.M. Sabatini. 2017. mTOR signaling in growth, metabolism, and disease. *Cell*. 168:960-976.
- Schürmann, A., S. Koling, S. Jacobs, P. Saftig, S. Krauß, G. Wennemuth, R. Kluge, and H.-G. Joost. 2002. Reduced sperm count and normal fertility in male mice with targeted disruption of the ADP-ribosylation factor-like 4 (Arl4) gene. *Molecular and cellular biology*. 22:2761-2768.
- Schwarz, N., A.J. Hardcastle, and M.E. Cheetham. 2012. Arl3 and RP2 mediated assembly and traffic of membrane associated cilia proteins. *Vision research*. 75:2-4.

- Schwarz, N., A. Lane, K. Jovanovic, D.A. Parfitt, M. Aguila, C.L. Thompson, L. da Cruz, P.J. Coffey, J.P. Chapple, and A.J. Hardcastle. 2017. Arl3 and RP2 regulate the trafficking of ciliary tip kinesins. *Human Molecular Genetics*. 26:2480-2492.
- Seaman, M.N. 2004. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. *J Cell Biol.* 165:111-122.
- Seixas, C., S.Y. Choi, N. Polgar, N.L. Umberger, M.P. East, X. Zuo, H. Moreiras, R. Ghossoub, A. Benmerah, and R.A. Kahn. 2016. Arl13b and the exocyst interact synergistically in ciliogenesis. *Molecular biology of the cell*. 27:308-320.
- Sengupta, S., T.R. Peterson, and D.M. Sabatini. 2010. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Molecular cell*. 40:310-322.
- Sharer, J.D., and R.A. Kahn. 1999. The ARF-like 2 (ARL2)-binding Protein, BART Purification, cloning, and initial characterization. *Journal of Biological Chemistry*. 274:27553-27561.
- Sharer, J.D., J.F. Shern, H. Van Valkenburgh, D.C. Wallace, and R.A. Kahn. 2002. ARL2 and BART enter mitochondria and bind the adenine nucleotide transporter. *Molecular biology of the cell*. 13:71-83.
- Shimobayashi, M., and M.N. Hall. 2014. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nature reviews Molecular cell biology*. 15:155-162.
- Shin, H.-W., H. Kobayashi, M. Kitamura, S. Waguri, T. Suganuma, Y. Uchiyama, and K. Nakayama. 2005. Roles of ARFRP1 (ADP-ribosylation factor-related protein 1) in post-Golgi membrane trafficking. *Journal of Cell Science*. 118:4039-4048.
- Siniosoglou, S., and H.R. Pelham. 2001. An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. *The EMBO Journal*. 20:5991-5998.
- Siniosoglou, S., and H.R. Pelham. 2002. Vps51p links the VFT complex to the SNARE Tlg1p. *Journal of Biological Chemistry*. 277:48318-48324.
- Small, S.A., K. Kent, A. Pierce, C. Leung, M.S. Kang, H. Okada, L. Honig, J.P. Vonsattel, and T.W. Kim. 2005. Model - guided microarray implicates the retromer complex in Alzheimer's disease. *Annals of neurology*. 58:909-919.
- Smith, E.M., S.G. Finn, A.R. Tee, G.J. Browne, and C.G. Proud. 2005. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *Journal of Biological Chemistry*. 280:18717-18727.
- Sprang, S.R. 1997. G protein mechanisms: insights from structural analysis. *Annual review of biochemistry*. 66:639-678.
- Stenmark, H., and V.M. Olkkonen. 2001. The rab gtpase family. *Genome biology*. 2:reviews3007. 3001.
- Südhof, T.C., and J.E. Rothman. 2009. Membrane fusion: grappling with SNARE and SM proteins. *Science*. 323:474-477.
- Tai, G., L. Lu, T.L. Wang, B.L. Tang, B. Goud, L. Johannes, and W. Hong. 2004. Participation of the syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the trans-Golgi network. *Molecular biology of the cell*. 15:4011-4022.
- Takai, Y., T. Sasaki, and T. Matozaki. 2001. Small GTP-binding proteins. *Physiological reviews*. 81:153-208.

- Tee, A.R., B.D. Manning, P.P. Roux, L.C. Cantley, and J. Blenis. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Current Biology*. 13:1259-1268.
- Thomas, G. 2002. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nature reviews Molecular cell biology*. 3:753-766.
- Thoreen, C.C., S.A. Kang, J.W. Chang, Q. Liu, J. Zhang, Y. Gao, L.J. Reichling, T. Sim, D.M. Sabatini, and N.S. Gray. 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *Journal of Biological Chemistry*. 284:8023-8032.
- Tie, H.C., B. Chen, X. Sun, L. Cheng, and L. Lu. 2017. Quantitative Localization of a Golgi Protein by Imaging Its Center of Fluorescence Mass. *Journal of Visualized Experiments*.
- Tie, H.C., D. Mahajan, B. Chen, L. Cheng, A.M. VanDongen, and L. Lu. 2016. A novel imaging method for quantitative Golgi localization reveals differential intra-Golgi trafficking of secretory cargoes. *Molecular biology of the cell*. 27:848-861.
- Togi, S., R. Muromoto, K. Hirashima, Y. Kitai, T. Okayama, O. Ikeda, N. Matsumoto, S. Kon, Y. Sekine, and K. Oritani. 2016. A new STAT3-binding partner, ARL3, enhances the phosphorylation and nuclear accumulation of STAT3. *Journal of Biological Chemistry*. 291:11161-11171.
- Toh, W.H., J.Z.A. Tan, K.L. Zulkefli, F.J. Houghton, and P.A. Gleeson. 2017. Amyloid precursor protein traffics from the Golgi directly to early endosomes in an Arl5b - and AP4 - dependent pathway. *Traffic*. 18:159-175.
- Tokarev, A.A., A. Alfonso, and N. Segev. 2009. Overview of intracellular compartments and trafficking pathways. In *Trafficking Inside Cells*. Springer. 3-14.
- Trull, T. 2012. Clinical Psychology. In *Kindle*.
- Tsun, Z.-Y., L. Bar-Peled, L. Chantranupong, R. Zoncu, T. Wang, C. Kim, E. Spooner, and D.M. Sabatini. 2013. The folliculin tumor suppressor is a GAP for the RagC/D GTPases that signal amino acid levels to mTORC1. *Molecular cell*. 52:495-505.
- Vetter, I.R. 2014. The structure of the G domain of the ras superfamily. In *Ras Superfamily Small G Proteins: Biology and Mechanisms* 1. Springer. 25-50.
- Vieira, S.I., S. Rebelo, H. Esselmann, J. Wiltfang, J. Lah, R. Lane, S.A. Small, S. Gandy, E.F.d.C. e Silva, and O.A.d.C. e Silva. 2010. Retrieval of the Alzheimer's amyloid precursor protein from the endosome to the TGN is S655 phosphorylation state-dependent and retromer-mediated. *Molecular neurodegeneration*. 5:40.
- Vigil, D., J. Cherfils, K.L. Rossman, and C.J. Der. 2010. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nature reviews. Cancer*. 10:842.
- Vilariño-Güell, C., C. Wider, O.A. Ross, J.C. Dachsel, J.M. Kachergus, S.J. Lincoln, A.I. Soto-Ortolaza, S.A. Cobb, G.J. Wilhoite, and J.A. Bacon. 2011. VPS35 mutations in Parkinson disease. *The American Journal of Human Genetics*. 89:162-167.
- Vitale, N., V.J. Ferrans, J. Moss, and M. Vaughan. 2000. Identification of lysosomal and Golgi localization signals in GAP and ARF domains of ARF domain protein 1. *Molecular and cellular biology*. 20:7342-7352.

- Vitale, N., J. Moss, and M. Vaughan. 2001. Purification and properties of ARD1, an ADP-ribosylation factor (ARF)-related protein with GTPase-activating domain. *Methods in enzymology*. 329:324.
- Wang, S., Z.-Y. Tsun, R.L. Wolfson, K. Shen, G.A. Wyant, M.E. Plovanich, E.D. Yuan, T.D. Jones, L. Chantranupong, and W. Comb. 2015. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science*. 347:188-194.
- Watanabe, M., H. Takahashi, Y. Saeki, T. Ozaki, S. Itoh, M. Suzuki, W. Mizushima, K. Tanaka, and S. Hatakeyama. 2015. The E3 ubiquitin ligase TRIM23 regulates adipocyte differentiation via stabilization of the adipogenic activator PPAR γ . *Elife*. 4:e05615.
- Webber, J.L., and S.A. Tooze. 2010a. Coordinated regulation of autophagy by p38 α MAPK through mAtg9 and p38IP. *The EMBO journal*. 29:27-40.
- Webber, J.L., and S.A. Tooze. 2010b. New insights into the function of Atg9. *FEBS letters*. 584:1319-1326.
- Webber, J.L., A.R. Young, and S.A. Tooze. 2007. Atg9 trafficking in mammalian cells. *Autophagy*. 3:54-56.
- Wiens, C.J., Y. Tong, M.A. Esmail, E. Oh, J.M. Gerdes, J. Wang, W. Tempel, J.B. Rattner, N. Katsanis, and H.-W. Park. 2010. Bardet-Biedl syndrome-associated small GTPase ARL6 (BBS3) functions at or near the ciliary gate and modulates Wnt signaling. *Journal of Biological Chemistry*. 285:16218-16230.
- Wise, D.R., and C.B. Thompson. 2010. Glutamine addiction: a new therapeutic target in cancer. *Trends in biochemical sciences*. 35:427-433.
- Wright, K.J., L.M. Baye, A. Olivier-Mason, S. Mukhopadhyay, L. Sang, M. Kwong, W. Wang, P.R. Pretorius, V.C. Sheffield, and P. Sengupta. 2011. An ARL3–UNC119–RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. *Genes & development*. 25:2347-2360.
- Wright, Z.C., R.K. Singh, R. Alpino, A.F. Goldberg, M. Sokolov, and V. Ramamurthy. 2016. ARL3 regulates trafficking of prenylated phototransduction proteins to the rod outer segment. *Human molecular genetics*. 25:2031-2044.
- Yang, P.-T., M.J. Lorenowicz, M. Silhankova, D.Y. Coudreuse, M.C. Betist, and H.C. Korswagen. 2008. Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells. *Developmental cell*. 14:140-147.
- Yang, Y.-K., H. Qu, D. Gao, W. Di, H.-W. Chen, X. Guo, Z.-H. Zhai, and D.-Y. Chen. 2011. ARF-like protein 16 (ARL16) inhibits RIG-I by binding with its C-terminal domain in a GTP-dependent manner. *Journal of Biological Chemistry*. 286:10568-10580.
- Yendamuri, S., F. Trapasso, and G.A. Calin. 2008. ARLTS1—a novel tumor suppressor gene. *Cancer letters*. 264:11-20.
- Yendamuri, S., F. Trapasso, M. Ferracin, R. Cesari, C. Seignani, M. Shimizu, S. Rattan, T. Kuroki, K.R. Dumon, and F. Bullrich. 2007. Tumor suppressor functions of ARLTS1 in lung cancers. *Cancer research*. 67:7738-7745.
- Young, A.R., E.Y. Chan, X.W. Hu, R. Köchl, S.G. Crawshaw, S. High, D.W. Hailey, J. Lippincott-Schwartz, and S.A. Tooze. 2006. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *Journal of cell science*. 119:3888-3900.

- Yu, C.-J., and F.-J.S. Lee. 2017. Multiple activities of Arl1 GTPase in the trans-Golgi network. *J Cell Sci.* 130:1691-1699.
- Zahn, C., A. Hommel, L. Lu, W. Hong, D.J. Walther, S. Florian, H.-G. Joost, and A. Schürmann. 2006. Knockout of Arfrp1 leads to disruption of ARF-like1 (ARL1) targeting to the trans-Golgi in mouse embryos and HeLa cells. *Molecular membrane biology.* 23:475-485.
- Zahn, C., A. Jaschke, J. Weiske, A. Hommel, D. Hesse, R. Augustin, L. Lu, W. Hong, S. Florian, and A. Scheepers. 2008. ADP-ribosylation factor-like GTPase ARFRP1 is required for trans-Golgi to plasma membrane trafficking of E-cadherin. *Journal of Biological Chemistry.* 283:27179-27188.
- Zhao, J., M. Wang, W. Deng, D. Zhong, Y. Jiang, Y. Liao, B. Chen, and X. Zhang. 2017. ADP-ribosylation factor-like GTPase 15 enhances insulin-induced AKT phosphorylation in the IR/IRS1/AKT pathway by interacting with ASAP2 and regulating PDPK1 activity. *Biochemical and Biophysical Research Communications.* 486:865-871.
- Zhou, C., L. Cunningham, A.I. Marcus, Y. Li, and R.A. Kahn. 2006. Arl2 and Arl3 regulate different microtubule-dependent processes. *Molecular biology of the cell.* 17:2476-2487.
- Zoncu, R., L. Bar-Peled, A. Efeyan, S. Wang, Y. Sancak, and D.M. Sabatini. 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H⁺-ATPase. *Science.* 334:678-683.
- Zoncu, R., A. Efeyan, and D.M. Sabatini. 2010. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews Molecular cell biology.* 12:21-35.