**Analyzing UNC-50/GMH1 dependent membrane
trafficking in yeast and *C. elegans***

PhD Thesis

Dissertation for the award of the degree
"Doctor of Philosophy naturalium" (Ph.D.)
(Division of Mathematics and Natural Sciences)
of the Georg-August-Universität Göttingen

within the doctoral program **Grundprogramm Biologie**
of the Georg-August University School of Science (**GAUSS**)

Submitted by

Suekyoung Jeon

Born in

Seoul, South Korea

Göttingen, 2014

Thesis Committee Members:

Prof. Dr. Ralf Heinrich

Cellular Neurobiology, Schwann-Schleiden Research Centre, Uni Göttingen

Prof. Dr. Andreas Wodarz

Anatomy and Cell Biology, Center of Molecular Biosciences, Uni Göttingen

Prof. Dr. Stefan Eimer

Molecular Neurogenetics, European Neuroscience Institute, Uni Göttingen

Zelluläre Strukturbioogie, Centre for Biological Signalling Studies, Uni Freiburg

Members of the Examination Board:

Reviewer: Prof. Dr. Andreas Wodarz

Anatomy and Cell Biology, Center of Molecular Biosciences, Uni Göttingen

Second Reviewer: Prof. Dr. Stefan Eimer

Molecular Neurogenetics, European Neuroscience Institute, Uni Göttingen

Zelluläre Strukturbioogie, Centre for Biological Signalling Studies, Uni Freiburg

Additional Reviewer (if applicable):

Further Members of the Examination Board:

Prof. Dr. Ralf Heinrich

Cellular Neurobiology, Schwann-Schleiden Research Centre, Uni Göttingen

Prof. Dr. Michael Hörner

Cellular Neurobiology, Johann-Friedrich-Blumenbach-Institute

Prof. Dr. André Fiala

Molecular Neurobiology of Behaviour, Schwann-Schleiden Research Centre

Prof. Dr. Markus Zweckstetter

Biophysikalische Chemie, Max-Planck-Institute

Date of the oral examination: 3rd of December 2014

TABLE OF CONTENTS

TABLE OF CONTENTS	i
LIST OF FIGURES	v
LIST OF TABLES	ix
ABBREVIATIONS	xi
ACKNOWLEDGEMENTS	xiv

I. ABSTRACT	1
--------------------	----------

II. INTRODUCTION	3
-------------------------	----------

2.1 Intracellular trafficking pathways in eukaryotic cells	3
2.1.1 Secretory pathway	4
2.1.2 Endocytic pathway	5
2.1.3 Retrograde pathway	7
2.2 Mechanisms of intracellular trafficking by coat protein complexes	8
2.3 Bidirectional trafficking between TGNs and endosomes	11
2.3.1 The small GTPase ARF and its regulator	12
2.3.2 Retromer, GARP and COG complex	15
2.3.3 SNARE complexes	17
2.4 A conserved transmembrane protein UNC-50/Gmh1p required for nAChR sorting	18
2.5 Neurotransmission at neuromuscular junctions in <i>C. elegans</i>	20
2.6 Regulation of nicotinic acetylcholine receptors (nAChRs) in <i>C. elegans</i>	21
2.6.1 Two types of nAChRs against levamisole at NMJs	21
2.6.2 Assembly, trafficking and degradation of nAChRs	24

CHAPTER I	25
------------------	-----------

Analyzing the evolutionarily conserved function of UNC-50/GMH1 for membrane trafficking in yeast and <i>C. elegans</i>: in regulating membrane dynamics, secretion and recycling of molecular complexes at the Golgi-endosomal compartments	25
--	-----------

III. SPECIFIC INTRODUCTION	26
-----------------------------------	-----------

3.1 Subtype-specific sorting manners of nAChRs by UNC-50	26
3.2 A transmembrane receptor GMH1 of the ARF-GEFs Gea1/2p	27

3.3 Pharmacological phenotypes of <i>unc-50</i> mutants in synaptic transmission	28
3.4 Analysis of synthetic lethal interactions of the trafficking factors in the TGN	30
3.5 Aims of this study	35
 IV. RESULTS	 37
4.1 UNC-50 colocalizes with APT-9 at the <i>trans</i>-Golgi networks	37
4.2 Phenotypes of cellular/functional expression of UNC-50/Gmh1p in eukaryotes	39
4.2.1 Yeast growth phenotypes on non-fermentable and limited conditions	39
4.2.2 Analysis of intracellular organelle-specific markers in yeast <i>S. cerevisiae</i>	43
4.2.3 Analysis of intracellular organelle-specific markers in <i>C. elegans</i>	44
4.3 Morphological analysis of coelomocytes and muscles in <i>unc-50</i> mutants	48
4.3.1 <i>unc-50</i> mutants show relatively enlarged lysosomes in coelomocytes	48
4.3.2 Morphological analysis of the body wall muscles by HPF-EM	51
4.4 Intracellular traffic-dynamics to the vacuolar/lysosomal compartments	52
4.4.1 Bulk-fluid phase endocytosis functions in <i>unc-50</i> mutants	52
4.4.2 FM4-46 dyes reach fast to the vacuolar membranes in <i>gmh1</i> mutants	53
4.5 Recycling defects of vesicle components in <i>gmh1</i> mutants	57
4.5.1 SNC2-GFP localizes to the vacuolar-membranes in <i>gmh1</i> mutants	57
4.5.2 Expression of TLG2-mCherry in yeast is not affected in <i>gmh1</i> mutants	60
4.6 Requirement of Gmh1p for the trafficking of the yeast CLC protein Gef1p	61
4.6.1 Growth phenotypes and localization of GEF1-GFP in GMH1-depleted strains	62
4.6.2 GMH1 deletion induces low intensity of GEF1-GFP	65
4.7 Secretion defects of ssGFP from muscles in <i>unc-50</i> mutants	67
4.8 Interspecies-expression of <i>gmh1</i>, a yeast and human ortholog of <i>unc-50</i>, does not rescue and localize in <i>C. elegans</i>	70
 V. DISCUSSION	 72
5.1 UNC-50/GMH1 acts as a redundant factor in retrograde transport from endosomes to the Golgi	72
5.2 UNC-50/GMH1 leads to enhanced recruitments to the endosome-to-lysosomal compartments	74
5.3 Morphological analysis of coelomocytes and muscles in <i>unc-50</i> mutants	74
5.4 GMH1 may involve indirect regulation of cargo molecules	75
5.5 The ARF-dependent cascade trafficking by UNC-50/GMH1	76
 CHAPTER II	 79
Proteomic/Metabolic analysis of the UNC-50 by stable isotope labeling of amino acids in <i>C. elegans</i>	79

VI. SPECIFIC INTRODUCTION	80
6.1 Stable isotope labeling of amino acids in living cells (SILAC)	80
6.2 BioID to identify interacting proteins by Biotin protein ligase (BirA)	83
6.3 α GFP-nanobodies for functional studies in living cells	85
6.4 Studying proteome in <i>C. elegans</i>	88
6.5 Aims of this study	90
VII. RESULTS	93
7.1 SLE1 <i>E. coli</i> strains for SILAC	93
7.2 Metabolic incorporation rates of the heavy labeled-amino acids in <i>C. elegans</i>	94
7.3 Analysis of relative quantitative proteomes of <i>unc-50</i> mutants by SILAC	98
7.3.1 Quantification of protein changes in <i>unc-50</i> mutants by SILAC ratios (H/L)	99
7.3.2 SILAC proteome profiling shows trafficking factors are specifically deregulated in <i>unc-50</i> mutants	101
7.4 Metabolic analysis of the mutants of <i>unc-50</i> in <i>C. elegans</i>	105
7.5 Workflow strategy of SILAC-BioID in <i>C. elegans</i>	109
7.6 Functional specificity of the GFP nanobodies in the intracellular compartment via spatially labeling of the lumen and cytosol in <i>C. elegans</i> muscles	113
VIII. DISCUSSION	119
8.1 Metabolic SILAC labeling of proteins relies on feeding periods and concentration of the bacteria SLE1 in <i>C. elegans</i>	119
8.2 <i>unc-50</i> mutants lead to protein abundance involved in the intracellular transport	121
8.3 Specificity of the anti-GFP nanobodies based on the protein structure	123
IX. MATERIALS AND METHODS	126
9.1 Materials and Reagents	126
9.2 Methods in Molecular Biology	126
9.2.1 Maintenance of bacterial strains	126
9.2.2 Molecular biology: PCR, Cloning and Transformation	126
9.2.3 Agarose gel electrophoresis	127
9.2.4 Sequencing	128
9.3 Methods used in the nematode <i>Caenorhabditis elegans</i>	128
9.3.1 Maintenance of strains	128
9.3.2 Crossing worms	128
9.3.3 Worm PCR templates	129
9.3.4 Molecular Cloning Strategy	129
9.3.5 Transgenic lines and chromosomal integrated strains	129

9.3.6 Movement assays	130
9.3.7 Pharmacological assays	130
9.3.8 TR-BSA endocytosis assays	131
9.3.9 Confocal microscopy and imaging	131
9.3.10 Western blots	132
9.3.11 High-pressure freeze electron microscopy (HPF-EM)	133
9.3.12 Stable isotope labeling by amino acids in <i>C. elegans</i> (SILAC)	133
9.3.13 Worm stocks	134
9.3.14 Integration lines by gamma irradiation of <i>C. elegans</i>	134
9.4. Methods used in the yeast <i>Saccharomyces cerevisiae</i>	135
9.4.1 Molecular biology and strains used in yeast	135
9.4.2 Yeast growth phenotypes	135
9.4.3 Transformation of yeast cells	135
9.4.4 Creating of knockout- <i>gmh1</i> strains by PCR-based deletion cassettes	136
9.4.5 Purification of genomic DNA from yeast cells	137
9.4.6 Western blotting of yeast cellular lysates	137
9.4.7 Live cell Imaging	138
9.4.8 Glycerol Stocks	139
 X. REFERENCES	 140
 XI. APPENDIX	 158
11.1 Strains of bacteria/yeast and <i>C. elegans</i>	158
11.2 Oligonucleotides and deletion alleles	160
11.3 Plasmids and injection constructs	164
11.4 Media	168
11.5 Buffers and solutions	172
11.6 Materials and reagents	176
11.6.1 Research equipment used in this study	176
11.6.2 Chemicals	178
11.6.3 Enzymes and kits	180
11.6.4 Antibodies	181
 CURRICULUM VITAE	 182
 LIST OF PUBLICATIONS	 183

LIST OF FIGURES

Figure II.1 Overview of intracellular trafficking pathway in eukaryotic cells: Secretory pathway, endocytic pathway and retrograde pathway	3
Figure II.2 Three pathways of endocytosis in eukaryotic cells	6
Figure II.3 Transport mechanisms of coated vesicles	11
Figure II.4 The domain architecture of the large ARF-GEFs GBF/BIG subfamily and the consecutive action of ARF-GEFs for distinct ARFs activation in the entire sorting network	14
Figure II.5 Schematic representation of tethering events cooperated by GARP complex, SNAREs and Small GTPases between TGNs and carrier vesicles	16
Figure II.6 Schematic representation of cycling of SNARE complex in transport vesicles	18
Figure II.7 The evolutionarily conserved protein UNC-50 in eukaryotic systems	20
Figure II.8 Schematic cell organization and neuromuscular junctions (NMJs) in <i>C. elegans</i>	22
Figure II.9 Muscle arms at NMJs in <i>C. elegans</i>	23
Figure III.1 Intracellular trafficking routes of Lev-nAChRs by UNC-50 in the secretory pathway	26
Figure III.2 The similarity of HDS domains of the large ARF-GEFs in yeast, plant, nematode and human	28
Figure III.3 Schematic representation of cholinergic neurotransmission and pharmacological phenotypes of wild type animals on 1 mM levamisole	29
Figure III.4 Pharmacological phenotypes of <i>unc-50</i> mutants	30
Figure III.5 Schematic diagrams of research aims for understanding the intracellular trafficking of UNC-50/GMH1-dependent	36

Figure IV.1 UNC-50 localizes to the <i>trans</i> -Golgi network and endosomal interface	38
Figure IV.2 Yeast growth phenotypes on synthetic complete, non-fermentable and starvation conditions	42
Figure IV.3 <i>gmh1</i> mutants lead to enhanced trafficking to the vacuole in yeast <i>Saccharomyces cerevisiae</i>	44
Figure IV.4 <i>unc-50</i> mutants show enhanced intensity of the fluorescent-tagged organelle specific markers in the Golgi-endolysosomal compartments in <i>C. elegans</i>	47
Figure IV.5 A coat protein subunit of COPI, mCherry-εCOP, show enhanced intensity in <i>unc-50</i> mutants in the body wall muscles, but not in recycling endosomes, tagRFpt-RAB-11	48
Figure IV.6 <i>unc-50</i> mutants show relatively enlarged lysosomes in coelomocytes	50
Figure IV.7 HPF-EM analysis of the organelle morphology in <i>C. elegans</i> muscles	51
Figure IV.8 Coelomocytes show normal dynamics of endocytosis by bulk-fluid phase in <i>unc-50 (x47)</i> mutants	53
Figure IV.9 Dye structure of FM 4-64 and its schematic representation of the staining pattern in yeast cells	55
Figure IV.10 FM 4-64 dyes are rapidly reached to the vacuolar membranes at the early time points in <i>gmh1</i> depletion	56
Figure IV.11 GFP-SNC2 appears to be staining-like on the vacuolar membrane in <i>gmh1</i> mutants	59
Figure IV.12 mCherry-TLG2 in yeast cells does not affect in the <i>gmh1</i> mutants	61
Figure IV.13 Cellular-overexpression phenotypes of GEF1 tagged-GFP, GFP-GEF1 and GEF1-GFP, in wild type are slightly rescued to <i>gmh1</i> mutant strains	64
Figure IV.14 GMH1 deletion induces low intensity of GEF1-GFP, yeast CLC protein	66

Figure IV.15 Schematic representation of endocytosis by coelomocytes of adult hermaphroditocytes in <i>C. elegans</i>	68
Figure IV.16 The signal sequence GFP fusion protein (ssGFP) from the body wall muscles was not properly secreted due to be trapped in the muscles in <i>unc-50</i> mutants	69
Figure IV.17 <i>Gmh1</i> , the yeast and human ortholog of <i>unc-50</i> is not able to be functional in the body wall muscles in <i>C. elegans</i>	71
Figure V.1 Schematic representation of the synthetic lethality between the mutants of the <i>unc-50/gmh1</i> and the retrograde trafficking machineries in both yeast <i>S. cerevisiae</i> and nematode <i>C. elegans</i>	73
Figure V.2 UNC-50/GMH1 is a novel recycling factor in the retrograde pathway, which regulates in a redundant manner of the parallel pathway with the complex of GARP/COG/Retromer and the small GTPase RAB-6	75
Figure V.3 The ARF-dependent cascade transport manners by UNC-50/GMH1	78
Figure VI.1 Schematic workflow of stable isotope labeling by amino acids in living cells (SILAC)	82
Figure VI.2 Schematic representation of the enzymatic protein <i>in vivo</i> biotinylation mechanisms of the biotin ligase reaction	84
Figure VI.3 The complex structure of the antigen-GFP with the anti-GFP-nanobody fragments	87
Figure VI.4 Schematic diagrams of research aims for identifying and quantifying protein dynamics on UNC-50/GMH1-dependent in <i>C. elegans</i>	91
Figure VII.1 Schematic diagram of <i>E. coli</i> SLE1 in SILAC media for <i>C. elegans</i>	93
Figure VII.2 Stable isotope labeling by amino acids in feeding periods for <i>C. elegans</i>	97
Figure VII.3 Labeling by SILAC in wild type and <i>unc-50</i> mutants	99
Figure VII.4 Quantitative distribution of the normalized ratios of the SILAC labeling	99

Figure VII.5 Accurate quantification of practical changes of protein identified in <i>unc-50</i> mutants	100
Figure VII.6 Functional classification of the proteins identified in <i>unc-50</i> mutants	103
Figure VII.7 Schematic representation of BirA*-fusion proteins to be integrated into <i>unc-50</i> mutants	108
Figure VII.8 Functional expression of BirA*-fusion proteins in <i>C. elegans</i> muscles	108
Figure VII.9 Schematic workflow of SILAC-BioID in <i>C. elegans</i>	111
Figure VII.10 Analysis of the endogenously biotinylated proteins in <i>C. elegans</i>	112
Figure VII.11 Schematic representation of soluble α GFPNB::tagRFPt and GFP-fusion organelle specific proteins	114
Figure VII.12 Specificity of the α GFPNB to the GFP fusion proteins in the Golgi and ER	115
Figure VII.13 Specificity of the α GFPNB to the GFP/CFP fusion proteins in the mitochondria	116
Figure VII.14 Specificity of the α GFPNB to the GFP fusion proteins in the nucleus and NMJs	117
Figure VIII.1 Schematic specificity of α GFPNB complexes in the lumen and cytosol	124

LIST OF TABLES

Table III.1 Synthetic lethal phenotypes between the trafficking factors considered functionally redundant in the Golgi-endosomes	33
Table VI.1 Highly conserved essential amino acids and neurotransmitters between <i>C. elegans</i> and humans	89
Table VII.1 Metabolic incorporation rates by SILAC labeling in generation of <i>C. elegans</i>	96
Table VII.2 Quantitative protein abundance and down-regulation of intracellular transport related factors in <i>unc-50</i> mutants	105
Table VII.3 Metabolic profiles of <i>unc-50</i> mutants	106
Table XI.1.1 Bacterial <i>E. coli</i> strains used in this study	158
Table XI.1.2 Yeast <i>Saccharomyces cerevisiae</i> strains used in this study	158
Table XI.1.3 <i>C. elegans</i> strains used in this study	159
Table XI.2.1 DNA sequences of primers used in this study	160
Table XI.2.2 List of deletion alleles used in <i>C. elegans</i>	163
Table XI.2.3 Polymerase chain reaction conditions in yeast cells	163
Table XI.3.1 List of backbone vectors used in this study	164
Table XI.3.2 List of genes of interest in entry vectors	164
Table XI.3.3 List of genes of interest in injection vectors	165
Table XI.3.4 List of transgenic lines of extrachromosomal arrays (Ex.)	166
Table XI.3.5 List of plasmids used in yeast experiments	168
Table XI.4.1 List of media used in this study	169

Table XI.4.2 Amino acids of selective media	172
Table XI.5.1 List of buffers and solutions	172
Table XI.5.2 Antibiotic concentrations used in this study	176
Table XI.6.1 Laboratory equipment (Equipment/Manufacturer)	176
Table XI.6.2 Chemicals (Chemicals/Manufacturer) used in this study	178
Table XI.6.3 Enzymes or Kits (Kits/Supplier)	180
Table XI.6.4 List of antibodies used in this study	181

ABBREVIATIONS

Δ	delta; null mutation
Δ Arg	<i>Auxotroph</i> arginine (R)
Δ Lys	<i>Auxotroph</i> lysine (K)
ACh	Acetylcholine
AChE	Acetylcholinesterase
α GFPNB	Anti-GFP Nanobodies
ARF	ADP ribosylation factor
ARF-GAPs	ARF-GTPase activating proteins
ARF-GEFs	ARF-guanine nucleotide exchange factors
ARFRP	ARF-related protein
ARL	ARF-like
BAR	Bin/Amphiphysin/Rvs domains
bioAMP	Biotinyl-5'-AMP
BioID	Proximity-dependent biotinylated protein identification method
BirA	Biotin protein ligase
BPS	Batho Phenantroline diSulfate
CBS	Cystathione β -synthetase
CCV	Clathrin-coated vesicle
CDRs	Complementarity determining regions
CFP	Cyan fluorescent protein
CGN	<i>cis</i> -Golgi network
COG	Conserved oligomeric Golgi complex
COPI	Coated-protein complex in the Golgi-to-ER and internal Golgi transport
COPII	Coated-protein complex in the ER-to-Golgi
DCB	Dimerization and cyclophilin binding domain
DCG	Dense core granule
DI	Disintegrin
DIC	Differential interference contrast
DTC	Distal tip cell
DTT	Dithiothreitol
EE	Early endosome
ER	Endoplasmic reticulum
ERDA	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ESCRT	Endosomal sorting complex required for transport

F-box	One domain of ubiquitin ligase SCF (SKP1/Cullin/F-box) complex
GABA	Gamma-amino butyric acid
GABARs	GABA _A receptors
GAP	GTPase activating proteins
GARP	Golgi-associated retrograde complex
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GGA	Golgi-associated, gamma-adaptin ear homology ARF-binding protein
H-KR	Heavy-isotope lysine and arginine
Habc	N-terminal regulatory domain of <i>t</i> -SNAREs
HDS	Homology downstream of Sec7 domain
HPF-EM	high-pressure freeze electron microscopy
HRP	Horseradish peroxidase
HUS	Homology upstream of Sec7 domain
IM	Inner membrane of mitochondrion
IMS	Intermembrane space of mitochondrion
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISG	Immature secreted granules
L-KR	Light-isotope lysine and arginine
LDLR	Low-density lipoprotein receptor
LE	Late endosome
Lev-nAChRs	Levamisole-sensitive nAChRs
LGICs	Ligand-gated ion channels
M9MM	M9 minimal medium
MP	Metalloprotease
MPRs	Mannose 6-phosphate receptors
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MUL	Light-isotope labeled in the mutant
MVB	Multivesicular bodies
N-nAChRs	Levamisole-non-sensitive nAChRs
nAChRs	Nicotinic acetylcholine receptors
NGM-N	Nitrogen-free agarose worm plates
NLS	Nuclear localization signal
NMJs	Neuromuscular junctions
NSF	N-ethylmaleimide-sensitive factor
OD	Optical density
OM	Outer membrane of mitochondrion
PCI	Proteasome, COP9 and Initiation factor-3

PGK	Phosphoglycerate Kinase
PI3P	Phosphatidylinositol 3-phosphate
PLAC	Protease and lacunin
PM	Plasma membrane
POI	Protein of interest
PPIs	Protein-protein interactions
PX	Phox, phagocytic oxidase domain
RAB	Ras genes from rat brain
RE	Recycling endosome
SC	Synthetic complete
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell lines
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNX	Sorting nexin
ssGFP	Signal sequence tagged-GFP
STB	Shiga toxin-B-fragment
<i>t</i> -SNAREs	Target-membrane SNAREs
TCEP	Tris (2-carboxyethyl) phosphine hydro-chloride solution
TfR	Transferrin receptor
TGN	<i>trans</i> -Golgi network
TIP47	Tail-interacting protein of 47 kDa
TM	Transmembrane
TPM	TLP18.3, Psb32 and MOLO-1
TPR	Tetratricopeptide repeat region
TR-BSA	Texas-red conjugated to bovine serum albumin
UPR	Unfolded protein response
<i>v</i> -SNAREs	Vesicle-membrane SNAREs
VDCs	Ventral and dorsal nerve cords
VHS	Vps27, Hrs, and STAM
VPS	Vacuolar protein sorting
VTC	Vesicular-tubular cluster
WT	Wild type
WTH	Heavy-isotope-labeled in wild type
WTL	Light-isotope-labeled in wild type
YFP	Yellow fluorescent protein
YPAD	Yeast extract peptone-dextrose

ACKNOWLEDGEMENTS

I would like to appreciate all those who have helped and supported me during the season of my PhD. First of all, I would like to thank my supervisor Prof. Dr. Stefan Eimer for giving me the opportunity, support and encouragement since I started my PhD course. For last three and a half years, I learned a lot and was able to overcome hard things without any difficulties in studying and life in Germany. For my projects, I would like to appreciate my thesis committees Prof. Dr. Ralf Heinrich and Prof. Dr. Andreas Wodarz for scientific advice and help in promoting research projects. As another my boss for my projects, I would like to express my appreciation to Prof. Dr. Blanche Schwappach for collaboration and learning opportunity in her lab with scientific advice and experience for six months. Without this help, my projects in practice would not have been well finished. In addition, I am grateful to Prof. Dr. Michael Hörner for agreeing thankfully to be an additional member of my thesis committee and Prof. Dr. Dieter Heineke for useful advice and help during the six semesters.

Through the SyMBaD international research and training PhD program funded by EU consortium (Marie Curie ITN) for three years 2011 – 2013, I had many chances to learn and travel abroad in EU countries based on the program support. I appreciate Prof. Dr. Christophe Mulle, Prof. Dr. Oliver Schlüter and Dr. Antonella Caminiti for supporting and coordinating in the program and thank you all brilliant guys SyMBaD alumnus for sharing multidisciplinary knowledge and scientific discussion in beautiful venues we had annual meetings and conferences.

I am thankful to all those who helped and supported my projects in ENI (European Neuroscience Institute) and Biochemistry I (Universitätsmedizin) in Göttingen and LIC (Life imaging center) and Core Facility of Metabolomics and Proteomics of ZBSA (Center for biological systems analysis) in Freiburg. I am especially thankful to Christian Becker, Sissi, Angelika Reichinger and Eva Helmstädter coordinating administrative supports, and grateful to Sebastian Wiese and Carsten Jäger collaborating scientific help for proteomics and metabolomics.

Above all, I want to thank you all my colleagues so far for sharing friendship, knowledge and laboratory life. I would like to express my appreciation to all former members Sabine Koenig, Mandy Hennemann, Maike Kittelmann, Nora Wender, Nikhil Sasidharan, Katrin Schwarze, David Vasmer and Uta Wolke for all their helps and good memories in both Göttingen and Freiburg. Additionally, I am thankful to all members of Prof. Dr. Blanche Schwappach: Anne Clancy, Eric Arakel, Fabio Vilardi, Lena Musiol, Olga Lytovchenko, Markus Kilisch, Kirsten Unthan-Fechner and Gabriela Brodkorb. I was lucky and happy to have had all in the lab of Humboldtallee. Thanks a lot for sharing scientific knowledge, all materials, your kindness, consideration and Friday's cakes as well.

Now I would like to thank all brilliant and nice members Nathalie Göppert, Martin Piringer, Fenja Gawlas, Maria Deinert and Severine Kayser. I will never forget all the things for your help, friendship, team spirits and consideration during the tough seasons. I am also thankful to Dr. Oliver Kretz for nice EM pictures.

And I would like to express my appreciation to my friends Szi-chieh Yu, Hyunsim Min, Jungchul Kwon and Minsun Jung for sincere friendship and always cheering behind.

Last but not least, I would like to thank my family: dear my parents, dear Johan Dr. Sangyong Jung, dear Fr. Bartolomeo uncle & Sr. Catherine aunt, Maria Shooshoo and Stefano Sheunghee for their constant love, devotion and support, and I dedicate my thesis to you dear my aunt who lives in heaven since last year.

I. ABSTRACT

Although the basic principles of the intracellular trafficking of proteins and lipids within the secretory apparatus have been established, the precise regulation of molecular membrane transport events is still not well understood. The evolutionarily conserved Golgi membrane protein UNC-50 has been shown to be essential for cell surface expression of levamisole-sensitive acetylcholine receptors (Lev-nAChRs) in *C. elegans*. The yeast UNC-50 ortholog Gmh1p was demonstrated to interact with the guanine-nucleotide exchange factors for ADP-ribosylation factor GTPases (ARF-GEFs) Gea1p and Gea2p, which are required for ARF GTPase dependent transport vesicle budding and sorting. These data suggested that UNC-50/Gmh1p might be the membrane receptor that recruits Gea1/2p ARF-GEFs to the Golgi. However, the very mild phenotypes of *unc-50/gmh1* mutants yeast and *C. elegans* argue against a general transport function for UNC-50/Gmh1p.

In Chapter I, in order to find the evolutionarily conserved function of UNC-50/Gmh1p, we systematically characterized the intracellular transport *unc-50/gmh1* mutants in the two model organisms the yeast *S. cerevisiae* and *C. elegans*. We determined that the loss of UNC-50/Gmh1p function results in an enhanced traffic-flow to lysosomes/vacuoles. Yeast *gmh1* mutants display an enhanced delivery of the membrane dye FM 4-64 to the vacuole as well as recycling defects of v-SNAREs Snc2p back to the plasma membrane. In support of these observations, we also detected an enhanced recruitment of endo-lysosomal markers as well as lysosomal enlargements in *unc-50/gmh1* mutants in yeast as well as in *C. elegans*. This is in agreement with a function of UNC-50/Gmh1p in endosome-to-Golgi retrograde trafficking, which has previously been shown in synthetic lethal screens. An electron microscopic analysis revealed the accumulation of large lipid filled vesicles and mitochondrial alterations in *C. elegans unc-50* mutants. This further suggested that UNC-50/Gmh1p has a much broader role for cellular metabolism.

In Chapter II, we analyze UNC-50/Gmh1p's general function for cellular physiology by unbiased whole proteome as well as whole metabolome quantitative analysis. By metabolic SILAC labeling and quantitative proteomics, we identified proteins that have

been up or down-regulated in *unc-50* mutants. This analysis revealed that anterograde transport promoting proteins such as VPS-32.1, UNC-18 and GPB-1 as well as actin-myosin motility components ACT-2 and NMY-2 were up-regulated, whereas retrograde transport proteins such as dynein-RAB-6-mediated BICD-1 and lipid binding precursors VIT-3 and VIT-5 were down-regulated.

In order to identify UNC-50 interacting proteins by SILAC labeling and mass spectrometry we established the BioID method in *C. elegans*. This method allows to in-vivo biotinylate proteins in close proximity of target protein tagged with a mutated form of the bacterial biotin-ligase BirA. The BioID method allows the in-vivo labeling of transient or weak interactions that occur during intracellular trafficking events, which otherwise would be difficult to isolate by classical CoIP purifications. By expressing BirA-tagged anti-GFP-nanobodies (α GFPNB) we also show that GFP-fusion protein can be tagged in-vivo, extending the usability of the BioID method. This approach using α GFPNB enabled us to determine the topology of GFP-tagged integral membrane proteins in-vivo. Thus, the proteomic tools established in this work will allow us to further define the UNC-50 dependent trafficking pathway at the molecular as well as cellular level.

II. INTRODUCTION

2.1 Intracellular trafficking pathways in eukaryotic cells

In eukaryotic cells, intracellular transport of membranes and proteins is tightly regulated and highly dynamic at the same time. Intracellular membrane trafficking is mediated in large part by vesicular membrane carriers which facilitate the transport between the different intracellular organelles as well as with the plasma membrane. Three major key pathways for vesicular trafficking have evolved in eukaryotes: 1) the forward secretory pathway that delivers newly synthesized cargo to the plasma membrane, 2) the endocytic pathway that delivers cargo from the plasma membrane to the endosomal-lysosomal system, and 3) retrograde retrieval pathways that are necessary to recycle cargo and transport factors to sustain efficient sorting and transport (Fig. II.1).

Figure II.1 Overview of intracellular trafficking pathways in eukaryotic cells. The intracellular compartments ER, ERGIC and Golgi (*cis-to-trans*) are major routes in the

intracellular trafficking through the organelles endosomes-to-plasma membrane and endosomes-to-lysosome. The vesicle-coated proteins mediate the membrane-bound transport in the intracellular compartments. ISG, immature secreted granules; DCG, dense core granules; PM, plasma membrane; CCV, clathrin-coated vesicle; CGN, *cis*-Golgi network; TGN, *trans*-Golgi network; RE, recycling endosome; EE, early endosome; LE, late endosome; L, lysosome; COPII, the coated-protein complex in the ER-to-Golgi; COPI, the coated-protein complex in the Golgi-to-ER and internal Golgi transport. Source: Picture was taken from the reference (Payne, 2009). Copyright ©2009, Landes Bioscience

2.1.1 Secretory pathway

Cargo proteins that are destined to the cell surface or plasma membrane enter the secretory pathway through the endoplasmic reticulum (ER) in eukaryotic cells. They are translated directly into the ER lumen and are allowed to fold and mature in an oxidative redox environment, in contrast to the cytoplasm. Cargo proteins that leave the ER contain di-acidic motifs (e.g., Asp-X-Glu) as an ER export signal. After extensive proofreading and assessing correct folding, these di-acidic motifs are recognized by membrane-receptors which sort and package the cargo proteins into COPII-coated vesicles (Teasdale and Jackson, 1996; Wang et al., 2004). These COPII coated transport vesicles subsequently bud off the ER membrane. Through lateral fusion these ER-derived transport vesicles form the vesicular-tubular cluster (VTC) or alternatively named ER-Golgi intermediate compartment (ERGIC) (Appenzeller-Herzog and Hauri, 2006; Hauri et al., 2000; Schweizer et al., 1988). Subsequently the ERGIC matures further to form the first cisternae at the *cis*-side of the Golgi apparatus. During ERGIC maturation the COPII coat is lost as well as ER-export cargo receptors and cargo dissociate. ER-resident proteins that accidentally have escaped the ER as well as ER-export cargo receptors are recognized and retrieved back to the ER via coatomer (COPI) coated vesicles which bud off the ERGIC a subsequently fuse back with the ER (Lee et al., 2004).

After exported from the ER, cargo that stays within the ERGIC will enter the *cis*-side of the Golgi apparatus as the ERGIC continues to mature into the *cis*-Golgi by lateral fusion. The Golgi apparatus is composed of a stack of Golgi cisternae that mature from the *cis*- to the *trans*-side of the Golgi. The Golgi resident glycosylation enzymes and sorting receptors are transported in COPI coated vesicles from one Golgi cisternae in a

retrograde manner to the following one. In contrast, the cargo always stays in the identical cisternae while the cisternae slowly matures from the *cis*- to the *trans*-Golgi (Simon, 2008). At the trans-side of the Golgi through the action of multiple small GTPases, sorting adaptors and coat proteins, the most distal Golgi cisternae is transformed into another vesicular tubular network, the *trans*-Golgi network (TGN). At the TGN cargo molecules are extensively sorted and packaged into cargo vesicles (e.g. secretory granules) which are selectively transported either to the plasma membrane or further within the endosomal lysosomal system (Fig. II.1 and Summarized below in the section of II.3) (Donaldson and Lippincott-Schwartz, 2000). Thus the TGN represents the central sorting station along the secretory route.

2.1.2 Endocytic pathway

The endocytic pathway organizes the internalization of extracellular molecules as well as lipids and membrane proteins from the plasma membrane into the cell. There are different endocytic mechanisms and several pathways to enter the endocytic pathway of a cell. First, soluble cargo proteins are recognized by specific membrane receptor (e.g. megalin) in eukaryotic cells and internalized mainly by clathrin-dependent mechanisms. Clathrin belongs to the vesicular coats that are able to be together with sorting receptors and GTPases to organize transport vesicle formation and scission (Grant and Donaldson, 2009). In contrast to clathrin-dependent endocytosis, the uptake of lipids, fluid-phase cargoes, toxins and pathogens is facilitated largely by caveolin-dependent endocytic pathways (Mayor and Pagano, 2007). Alternatively, it is the third endocytic pathway that is independent of clathrin and caveolin but requires the activation of the Arf6 GTPase. Activation of Arf6 at the plasma membrane organizes the endocytosis of glycosyl phosphatidylinositol-anchored proteins, integrins and major histocompatibility complex molecules (Gould and Lippincott-Schwartz, 2009; Grant and Donaldson, 2009; Mayor and Pagano, 2007).

The endocytosis by clathrin-coated vesicles mainly delivers cargo to the endosomal-lysosomal system where it is destined for degradation (Gould and Lippincott-Schwartz, 2009). Clathrin coated vesicles coming from the plasma membrane fuse with early endosomes. From early endosomes cargoes can have two possible itineraries: i) soluble

cargo or transmembrane proteins can either recycle back to the plasma membrane through recycling endosomes or ii) be delivered to lysosomes via sorting into multivesicular bodies (MVB), late endosomes that subsequently fuse with lysosomes.

To ensure sorting, targeting and directionality during endocytic transport distinct sets of small GTPases of Rab family are critically involved in the different intracellular compartments. Rab GTPases act as molecular switches that can exist in an active GTP-bound form and an inactive GDP bound form. In their active form, GTP-bound Rab GTPases bind specific effector proteins that are thus recruited to the membrane of the active Rab GTPase, inducing downstream trafficking events such as the formation of multivesicular bodies (MVBs) during late endosomal sorting by recruitment of the ESCRT complex (Fig. II.2) (Gould and Lippincott-Schwartz, 2009; Grant and Hirsh, 1999).

Figure II.2 Three pathways of endocytosis in eukaryotic cells. Numerous cargoes can be internalized by different mechanisms that are clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent internalization. Source: Picture was taken from the reference (Gould and Lippincott-Schwartz, 2009).

2.1.3 Retrograde pathway

Intracellular vesicular trafficking between the different compartments is highly dynamic and very complex logistic. Despite multiple proofreading steps during cargo sorting into the different vesicular carriers, the trafficking process is prone to make errors leading to incorrect delivery or accidental incorporation of compartment resident proteins into transport carriers. Given that there could be errors, specific retrieval pathways are necessary for the intracellular transport, which strongly increases the efficiency of the transport processes. In terms of economic view, the recycling of cargoes and machinery components is an efficient way preventing energy waste and inappropriate protein degradation during intracellular trafficking (Donaldson and Segev, 2010; Seaman, 2005).

There are several main transport processes where recycling is of particular importance. After endocytosis at the plasma membrane particularly neurotransmitter receptors are frequently recycled and delivered back to the plasma membrane. Since the assembly, quality control and transport of multimeric receptors are inefficient, that is to say, very energy and time consuming. Therefore it is beneficial for a cell to reuse these receptors. This also allows a cell to quickly respond to different activity states that are required for membrane remodeling processes during memory formation and learning in the brain. In addition, the “Soluble N-ethylmaleimide sensitive factor (NSF) Attachment protein Receptor” (SNARE) proteins are present on the transport vesicle from stable complexes with the corresponding SNAREs on the target membrane facilitating the fusion of the vesicle with the target membrane. After the fusion event, these *cis*-SNARE complexes have to be unwound and dissociated from each other to allow further rounds of fusion events (Figure II.6). This is achieved by endocytic removal of the *cis*-SNARE complexes from the target membrane, SNARE complexes are dissociated by the ATPase NSF and the respective SNAREs are recycled back to the donor and acceptor membranes for additional rounds of fusion. In this process, Rab GTPases organize the sorting and targeting during recycling (Li et al., 2008; Sonnichsen et al., 2000); (Chen et al., 2005; de Renzis et al., 2002).

In addition, cargo receptors, which are required for cargo sorting into transport vesicles, as well as modifying enzymes such as Furin-type endopeptidases have also be retrieved in order to allow new rounds of cargo sorting and processing. After fusion of a transport

vesicle with the target membrane cargo receptors and modifying enzymes are thus constantly recognized, packed in retrograde transport vesicles and recycled back to the donor compartment to maintain transport. A well-characterized example is the retrieval of the mannose 6-phosphate receptors (MPRs), which is required to sort lysosomal hydrolases at the TGN into transport carriers destined to lysosomes. It is bound to the tail-interacting protein TIP47 and the ubiquitous endopeptidase Furin/Kex2. They are recycled back to the Golgi from late endosomes by a retrograde pathway dependent on the Rab9 GTPase. When this retrieval pathway is blocked cargo receptors and modifying enzymes are lost to lysosomes/vacuoles (Carroll et al., 2001; Ghosh et al., 2003; Wilcox et al., 1992). Similarly, TGN38 and Shiga toxin-B-fragment (STB) follow a retrograde transport route from early endosomes to the TGN that is organized by the Rab6 GTPase (Mallard et al., 1998; Mallard et al., 2002). Several different retrograde retrieval pathways exist allowing cargo receptors and enzymes to be retrieved from various intracellular compartments in a close cooperation of the transport machinery and scaffold proteins (Reviewed by Kirchhausen, 2000).

2.2 Mechanisms of intracellular trafficking by coat protein complexes

Vesicular trafficking events in eukaryotic cells follow a stereotype chain of events from the donor membrane to the acceptor membrane: selection of cargo, coat assembly, vesicle budding and scission from the donor membrane, uncoating, and vesicle fusion to the target membrane (Kirchhausen, 2000). In these processes, the coat complexes COPII, COPI and Clathrin play a central role in the transport between distinct compartments ER-to-Golgi, Golgi-to-ER, plasma membranes, endosomes and TGN (McMahon and Mills, 2004). Although the coat components between these transport modalities are multifarious in composition and structure, the underlying mechanisms are consistently in the same line of feature.

In the early secretory ER-to-Golgi pathway, transport vesicles are generated by the COPII coat complex, composed of the heterodimer SEC13-SEC31, SEC23-SEC24 dimer and small GTPase SAR1 (Barlowe et al., 1994; Fath et al., 2007; Kirchhausen, 2000). By contrast to the COPII coat complex, the COPI complex is responsible for the retrograde pathway from the Golgi to the ER and within the Golgi apparatus. It consists of the small

GTPase ARF1 and the seven-coatomer subunits divided into two groups: COPI B-subunits (α COP, β' COP and ϵ COP) clathrin-like and COPI F-subunits (δ COP, β COP, γ COP and ζ COP) a clathrin adaptor AP2-like complex (Eugster et al., 2000; Hoffman et al., 2003; McMahon and Mills, 2004). The clathrin coat complex is connected to multiple intracellular transport processes from endosomes to Golgi, Golgi to late endosomes and various endocytic pathway from the plasma membrane. The complex for clathrin coated vesicles is composed of the clathrin assembly units of heavy chains and light chains, clathrin adaptors that bind the cargo receptors and accessory proteins (McMahon and Mills, 2004). All the three coat complexes COPI, COPII and clathrin follow similar architectural principles and rules of operation concerning their regulation and coating and uncoating cycles (Fotin et al., 2004; Lee and Goldberg, 2010; Stagg et al., 2007).

Figure II.3 shows the typical steps of vesicular transport between the donor and acceptor membrane. Transport starts with the coat complex mediated trapping of cargo through its interaction with cargo receptors and subsequent budding of the donor membrane. In a concerted action of the coat complex, small GTPases of the Arf family and lipids, a transport vesicle is formed. After membrane scission the transport vesicle is transported to the acceptor membrane while the coat complexes disassemble allowing subsequent fusion of the transport vesicle with the acceptor membrane. Through multiple recognition steps the uncoated transport vesicle is tethered to the acceptor membrane. The close opposition of vesicle and acceptor membrane allows the SNAREs present on the vesicle as well as on the target membrane to zip-up. This SNARE complex formation triggers the fusion of the vesicle with the target membrane and subsequent release of the vesicular cargo into the acceptor compartment. This order of events is stereotyped for all coat complexes.

COPII and COPI coat complexes are required to generate membrane curvature that initiates the vesicle budding process. The coat protein complexes are polymerized and dissociated from the donor membrane by the small GTPase SAR1/ARF1. Thus the coat complex when the Arf-type GTPase switches back to its GDP-bound form and the uncoated transport vesicle is now able to fuse with the target membrane (Fig. II.3).

In contrast to COPI and COPII vesicles, the vesicle formation/fission of the clathrin coated complex is more complex to be involved in clathrin adaptors to gather with

accessory proteins. In this process, the clathrin-coated vesicles (CCVs) need to be formed with the clathrin-adaptor protein complexes. To form the CCVs, the adaptor proteins such as AP-2 and alternate adaptors containing PtdIns(4,5)P₂-binding sites must exist (Huang et al., 1999; Traub, 2003; Yeung et al., 1999) and other accessory proteins such as Eps-15, endophilin and amphiphysin are necessary as vesicle regulators for membrane bending and curvature in the clathrin-mediated endocytosis (Confalonieri et al., 2000; Conner and Schmid, 2003; Zhang and Zehhof, 2002). For the final formation of CCVs, the vesicle abscission requires the large GTPase dynamin to form complete clathrin-coated vesicles by the GTP hydrolysis (Merrifield et al., 2005). The scission of the nascent vesicle in the clathrin system is achieved by the large GTPase dynamin, which polymerizes and forms spirals around the neck of the budding vesicle pinching it off through rounds of GTP hydrolysis. As the next process, the CCVs are finally uncoated to be fused into the endosomes (Bonifacino and Glick, 2004). The uncoating steps of the clathrin vesicles are essential to be fused to the acceptor membrane. The ATPase Hsc70 and its cofactor auxilin are involved in the dissociation of clathrin from the coat complex. The naked vesicle to be fused is guided by tethering and docking factors SNARE complex, long coiled-coil domain proteins and the membrane-associated RAB/ARF GTPases. The machinery components used in the vesicular transport are then recycled back to the original compartment through the retrograde pathway (Bonifacino and Glick, 2004; Kirchhausen, 2000). Many recycling receptors such as the transferrin receptor (TfR), low-density lipoprotein receptor (LDLR) and yolk receptor (RME-2), and their related ligands are mediated by coated vesicle complex (Grant and Hirsh, 1999; Tam, 2009; Traub, 2003).

Figure II.3 Transport mechanisms of coated vesicles. The vesicle transport mediated by coat complexes require the seven steps: Initiation (Cargo selection), Budding (Coat assembly), Scission (Vesicle formation), Uncoating, Tethering, Docking and Fusion in the vesicle transport pathway mediated by COPII, COPI and clathrin. Source: Picture was taken from the reference (Bonifacino and Glick, 2004).

2.3 Bidirectional trafficking between TGNs and endosomes

One of the major sorting compartments within the secretory pathway is the *trans*-Golgi network (Camus et al., 1998; Ellgaard et al., 1999). Especially in highly polarized cells such as epithelial cells and neurons, the TGN is the main sorting hub for Apical versus basolateral sorting or sorting between axons and dendrites, respectively. In order to execute these multiple distinct sorting events the TGN uses a combinatorial set of coat proteins and small GTPases with their regulators. In order to maintain its sorting capacity, the TGN needs a specific retrieval machinery to recycle cargo-sorting receptors from endosomes or to retrieve TGN resident proteins that have accidentally left the TGN. Therefore, bi-directional transport routes between endosomes and TGN have been established, relying on the coordinated action of tethering factors, membrane fusion machinery and the small GTPase of the ARF and RAB families with its regulator (Donaldson and Segev, 2010; Rohn et al., 2000).

2.3.1 The small GTPase ARF and its regulator

The ADP ribosylation factor (ARF) GTPases belong to the Ras superfamily of small GTPases that act as molecular switches cycling between a membrane-attached active, GTP-bound, form and a cytosolic-inactive, GDP-bound, form. ARF GTPases act both in anterograde as well as retrograde trafficking pathways. In contrast to Rab GTPases, the main function of ARF GTPases is to induce the budding and formation of vesicular transport carriers. To activate ARF GTPases and convert them from a cytosolic-inactive form into a membrane-bound active form the bound, GDP has to be exchanged against GTP. This ARF activation i.e. the GDP-to-GTP exchange step is triggered by membrane-associated ARF-Guanine nucleotide Exchange Factors (ARF-GEFs) containing a Sec7 domain as a signature motif. In contrast, the inactivation of ARF GTPases i.e. the hydrolysis of GTP-to-GDP is promoted by ARF-GTPase Activating Proteins (ARF-GAPs) containing arginine-finger domain (Donaldson and Segev, 2010).

ARF GTPases are myristoylated at the N-terminus to promote membrane recruitments in contrast to other small GTPases that anchor to the membrane by lipid modifications at the C-terminus (Gillingham and Munro, 2007). The key to understand ARF mediated vesicular membrane transport is to understand the specific regulation of their activation and membrane recruitment by ARF-GEFs. As a membrane trafficking regulator, the ARF-GEFs consist of a conserved Sec7-domain and several non-catalytic regulatory domains: dimerization and cyclophilin binding (DCB) domain, homology upstream of Sec7 domain (HUS), guanine exchange factor or referred to Sec7 domain (GEF), homology downstream of Sec7 domain (HDS1), HDS2, HDS3 and HDS4 that are only defined by sequence homologies (Figure II.4) (Mouratou et al., 2005; Richardson et al., 2012).

All the large (>100 kDa) Sec7-domain containing ARF-GEFs are functionally and structurally conserved in eukaryotic organisms from yeast to humans. They can be divided into two major sub-families: the GBF subfamily and BIG subfamily. The GBF subfamily includes GBF/GEA/GNOM in human, yeast and plants. The BIG subfamily consists of BIG1 and BIG2 in human and SEC7 in yeast (Donaldson and Jackson, 2000; Mouratou et al., 2005).

Recently, the yeast large ARF-GEF Sec7p has been shown to be stably recruited to the TGN via its HDS1 domain interaction with activated-ARF1 suggesting a positive feedback mechanism of ARF activation (Richardson et al., 2012). The human ARF-GEF GBF1 is known to localize mainly at early *cis*-Golgi compartments for COPI complex formation required for retrograde Golgi-to-ER trafficking. However, recent studies have demonstrated that COPI is also present on the TGN domains required for the membrane recruitment of BIG1/2 GTPases (Lowery et al., 2013). These results indicate that the membrane recruitment of ARF-GEFs at the TGN is coordinated by ARF cascades similar to the well-established Rab cascades. In these ARF cascades, the early acting ARF-GEFs like GBF1 are required to activate late acting ARF-GEFs like BIG1/2 at the TGN. In addition, ARF-GEFs also interact with membrane-associated factors involved in cargo-specific sorting mechanisms suggesting that vesicle formation and cargo sorting are coordinated with each other during vesicular transport (Lowery et al., 2013; Richardson et al., 2012).

Figure II.4 The domain architecture of the large ARF-GEFs GBF/BIG subfamily and the consecutive action of ARF-GEFs for distinct ARFs activation in the entire sorting network. Structure of the Sec7 domain of the ARF-GEF GEA2 (Hydrophobic helices in yellow and essential parts for GEF activity containing a glutamic acid residue in purple) interacting with the N-terminally trimmed ARF1 (A). Schematic homology domains of large ARF-GEFs based on the model of the ARF-GEF Sec7. DCB (dimerization and cyclophilin binding domain) and HUS (homology upstream of Sec7 domain) for enhancement of GEF activity, Sec7 (guanine exchange factor conserved domain) for catalytic function of ARF-GEFs, HDS1 (homology downstream of Sec7 domain) for recruitment of the ARF-GEF SEC7 to the TGN by direct ARF1 binding, HDS2, 3 and the rest domain parts at the C-terminus of ARF-GEFs for membrane recruitment to the TGN and regulation of GEF activity (B). The HDS1 domain interaction with the ARF1-GTP and the rest of HDS domains at the C-terminus as an unknown membrane-associated additional factor

mediates stable recruitment to the TGN (C). A model of a functional cascade action of ARF-GEFs from *cis*-Golgi to *trans*-Golgi indicates in secretory pathway that early acting GBF1 activates ARFs-GTP in the *cis*-Golgi and the activated ARF4/5 by GBF1 recruits BIG1/2 to the TGN that mediates to the clathrin-adaptor complexes by the activation of ARF1/3 recruited by BIG1/2 of the TGN. The early acting ARF-GEFs may act a main regulator of the coated vesicle transport in secretory pathway (D). Source: A was taken from the reference (Jackson and Casanova, 2000). B was taken and modified from the reference (Mouratou et al., 2005). C and D were adapted and modified from the reference (Lowery et al., 2013; Richardson et al., 2012).

2.3.2 Retromer, GARP and COG complex

The mechanisms underlying the cycling of some cargo molecules are similar in eukaryotic cells and depend on the coordination of forward and retrograde vesicular trafficking (Figure II.3) (Bonifacino and Glick, 2004; Donaldson and Segev, 2010; Seaman, 2005). The retrograde transport is organized by a concerted action of several multi-subunit tethering complexes and Rab GTPases. Particularly at the TGN as the main sorting station, the need for cargo sorting-receptor retrieval is particularly high. The main retrograde trafficking complexes required for endosome-to-Golgi retrieval are the Retromer complex, Golgi-associated retrograde complex (GARP) and the conserved oligomeric Golgi (COG) complex. These complexes are cooperating with Rab GTPases and SNAREs to ensure the correct and efficient targeting of retrograde transport vesicles to the TGN (Bonifacino and Rojas, 2006). For example, the carrier vesicles containing the transmembrane mannosidase phosphate receptors (MPRs) which at the TGN are required for lysosomal sorting of hydrolases are retrieved from endosomes back to the TGN. This process depends on the retromer complex which cooperates with the small GTPases RAB-5 and RAB-7 on early and late endosomes (Rojas et al., 2008). The retromer complex consists of the cargo selection complex VPS26/35/29 and the membrane structural sorting nexin (SNX) complex (VPS5 and VPS17 in yeast and SNX1 and SNX2 in mammalian). Sorting nexins contain BAR (Bin/Amphiphysin/Rvs) domains inducing membrane curvature and PX domains that bind to the phosphatidylinositol 3-phosphate (PI3P) for membrane association (Seaman, 2005). The retromer complex organizes the formation of tubular sorting domains on endosomal compartments, the

subsequent sorting of retrograde cargo into these domains and their scission to form retrograde transport vesicles.

Once these retrograde transport carriers are formed they need to be efficiently delivered to the right target membrane for subsequent fusion and cargo delivery. This step is executed by tethering complexes such as GARP and COG. These tethering complexes consist of long coiled-coil proteins and multisubunit tethering complexes which facilitate vesicle recycling by cooperation with SNAREs and small GTPases between the TGNs and carrier vesicles (Fig. II.5) (Bonifacino and Hierro, 2011; Jackson and Casanova, 2000; Lang and Jahn, 2008; Short et al., 2005). These tethering complexes are important to mediate long-range interactions to capture retrograde transport carriers at the TGN and to support correct and efficient SNARE assembly. Multi-subunit tethering complexes are programmed or activated by Rab GTPases and hold the different target and vesicular SNAREs in a fusion competent state ensuring the right stoichiometry and efficient SNARE complex formation (Conibear et al., 2003; Hayes et al., 2009; Siniosoglou and Pelham, 2001).

Figure II.5 Schematic representation of tethering events cooperated by GARP complex, SNAREs and Small GTPases between TGNs and carrier vesicles. A heterotetramer assembled GARP complex interacting with small GTPases RAB/ARL families binds to the Habc domain of *t*-SNAREs and is recruited to the TGN. Source: Picture was taken from the reference (Bonifacino and Hierro, 2011).

2.3.3 SNARE complexes

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are mostly transmembrane proteins which mediate the membrane fusion step during membrane trafficking. Since SNAREs are constantly needed to maintain vesicular trafficking, they have to be efficiently recycled via retrograde trafficking (Bonifacino and Glick, 2004). SNARE complexes form a structurally stable four- α helix bundle by pairing of *v*-SNARE (one α -helix) and *t*-SNARE (three α -helix) in a parallel manner called *trans*-SNARE complex. These *trans*-SNARE complexes hold the vesicular membrane in very close proximity to the target membrane allowing subsequent fusion of the two membranes (Bonifacino and Glick, 2004; Sutton et al., 1998). After membrane fusion all SNAREs, the target SNAREs as well as the vesicular SNAREs, are residing in the same membrane forming the so-called *cis*-SNARE complex. To be able to reuse the SNARE molecules for subsequent rounds of fusion, the *cis*-SNARE complex has to be disassembled. Through rounds of ATP hydrolysis, NSF/ α -SNAPs proteins dissociate the *cis*-SNARE complex regaining the monomeric SNAREs (Fig. II.6).

In yeast, it has been shown that the *v*-SNAREs SNC1/2 are endocytosed and recycled back to the TGN after fusion with the plasma membrane. This recycling relies on retrograde tethering factors such as the GARP complex, the retromer complex and the sortin nexin VPS5 as well as on the yeast Rab6 homologue YPT6 (Galan et al., 2001; Lafourcade et al., 2004; Lewis et al., 2000; Quenneville et al., 2006). In the absence of these retrograde trafficking factors, the recycling of *v*-SNAREs SNC1/2 is defective and SNC1/2 are trafficked to the vacuole for subsequent proteolysis.

Figure II.6 Schematic representation of cycling of SNARE complex in transport vesicles. The pairing of v-SNARE (one α -helix) and t-SNARE (three α -helix) facilitates the membrane fusion of vesicle which is dissociated by using ATP-driven hydrolysis via binding of NSF/ α -SNAP to the SNARE complex to be recycled in transport vesicles. Source: Picture was taken from the reference (Bonifacino and Glick, 2004).

2.4 A conserved transmembrane protein UNC-50/Gmh1p required for nAChR sorting

Assembly and transport of multimeric ion channels such as nicotinic acetylcholine receptors (nAChRs) are tightly controlled. Particularly the sorting of nAChRs is a highly dynamic process and tightly regulated by a set of regulatory proteins. Especially heteropentameric nAChRs have to be assembled from up to five different subunits. This requires a close monitoring of the assembly status, subunit composition and stoichiometry before ER exit. Similarly, at the late Golgi/TGN level, nAChRs have to be sorted and delivered to the right postsynaptic compartment. One of the factors required for nAChRs sorting at the Golgi is the integral membrane protein UNC-50 in *C. elegans*. UNC-50 localizes at the Golgi, which is ubiquitously expressed in *C. elegans*, and evolutionarily conserved from yeast to humans. UNC-50 is essential for trafficking of

levamisole-sensitive nAChRs (Lev-nAChRs) to the cellular surface at the neuromuscular junctions (NMJs) (Fig. II.9 and III.1) (Eimer et al., 2007). Levamisole is an anthelmintic drug that acts as a nicotinic agonist and open channel blocker of a specific nAChR, the Lev-nAChR, present at NMJs. UNC-50 was first isolated from the genetic screens of levamisole resistance in *C. elegans*. The mutants of *unc-50* show phenotypically uncoordinated movement due to cholinergic neurotransmission defects by mis-routed trafficking of the complete-assembled Lev-nAChRs in *unc-50* mutants (Fig. III.1) (Eimer et al., 2007; Lewis et al., 1980). In case of the mammalian ortholog of UNC-50, named as UNCL, it has been suggested to localize in the ER and the inner nuclear membrane involved in fundamental cellular functions and maintaining of periodontal ligament fibroblasts (Fitzgerald et al., 2000; Kim et al., 2007). The yeast/human orthologous protein is called as Gmh1p, likewise-UNC-50 of *C. elegans*, which interacts with the large ARF-GEFs Gea1/2p of the GBF subfamily that includes GBF/GEA/GNOM in human, yeast and plant (Chantalat et al., 2003; Donaldson and Jackson, 2000; Eimer et al., 2007; Mouratou et al., 2005). The Gmh1p was initially identified as a membrane-associated suppressor of the large ARF-GEFs *gea1-6* in yeast *Saccharomyces cerevisiae*. In the study, the thermosensitive growth defects of *gea* mutants were appeared to be rescued by overexpression of the *gmh1* (Chantalat et al., 2003). This suggests that UNC-50/Gmh1p may act as a highly conserved regulator involved in the activation of the small GTP binding proteins in the intracellular trafficking in eukaryotic organisms.

Figure II.7 The evolutionarily conserved protein UNC-50 in eukaryotic systems. From yeast to humans, UNC-50/GMH1 appears to be highly conserved with five putative transmembranes in the all species shown in the ClustalX alignment using the genomic sequence of UNC-50/GMH1. Source: Picture was provided from Stefan Eimer (Eimer et al., 2007).

2.5 Neurotransmission at neuromuscular junctions in *C. elegans*

At neuromuscular junctions (NMJs) in *C. elegans* as well as in vertebrates the neurotransmitter acetylcholine (ACh) mediates fast activatory neurotransmission (Hulme and Whitesides, 2011; Richmond and Jorgensen, 1999). Postsynaptically in bodywall muscles acetylcholine induces the opening of ligand gated ion channels of the nicotinic acetylcholine receptor (nAChR) type. At NMJs, the acetylcholine released from cholinergic motor neurons causes body wall muscle contraction. At the same time, the body wall muscles on the opposite side of the animal are simultaneously inhibited by release of the neurotransmitter gamma-amino butyric acid (GABA) from the inhibitory neurons. GABA activates GABA_A receptors of postsynaptic membranes, which leads to

chloride influx and muscle relaxation. As such, the coordinated reciprocal excitation and relaxation of the body wall muscle by cholinergic and GABAergic neurotransmission facilitates locomotion in *C. elegans* as well as other behaviors such as eating and mating (Garcia et al., 2001; Gotti and Clementi, 2004; Kim et al., 2001; McKay et al., 2004; Schuske et al., 2004; Wessler et al., 2003). In contrast to vertebrates where the motoneurons grow into the muscle fibres, *C. elegans* body wall muscles send out projections, so-called muscle arms, growing into the motoneuron bundles to form NMJ synapses (Fig. II.8 and Fig. II.9) (Pinan-Lucarre et al., 2014).

2.6 Regulation of nicotinic acetylcholine receptors (nAChRs) in *C. elegans*

2.6.1 Two types of nAChRs against levamisole at NMJs

nAChRs are highly conserved in both vertebrate and invertebrate (Touroutine et al., 2005). *C. elegans* NMJs contain two types of nAChRs at the postsynaptic membranes, levamisole-sensitive nAChRs (Lev-nAChRs) and levamisole-insensitive nAChRs (N-nAChRs) (Fig. II.8D left). The Lev-nAChRs are hetero-pentameric ligand-gated ion channels (LGICs) encoded by α -subunits UNC-38, UNC-63 and LEV-8 and non- α subunits UNC-29 and LEV-1 (Lewis et al., 1980). In contrast, N-nAChRs are homo-pentameric $\alpha 7$ -like nAChRs encoded by ACR-16 subunit (Brown et al., 2006; Touroutine et al., 2005). At NMJs, the two-type of nAChRs can also be distinguished by electrophysiological and pharmacological experiments (Eimer et al., 2007; Le Novere et al., 2002; Thany et al., 2007; Touroutine et al., 2005). Lev-nAChR mutants exhibit a slow and uncoordinated locomotion, while *acr-16* mutant animals display only a mild locomotion defect. This suggests that the Lev-nAChR is the main nAChR used for normal locomotion (Touroutine et al., 2005). However, animals lacking both types of nAChRs at NMJs exhibit a strongly increased locomotion defect as compared to Lev-nAChR single mutants showing that both nAChR are used for locomotion. Interestingly, in *unc-50* mutants only the Lev-nAChR is mistrafficked and degraded while the ACR-16 type of nAChR is properly delivered to the synapse (Eimer et al., 2007).

Figure II.8 Schematic cell organization and neuromuscular junctions (NMJs) in *C. elegans*. Adult hermaphrodites consist of 302 neurons and 95 muscles in *C. elegans*, a nice model system to study synaptic transmission, protein function and intracellular transport as a eukaryote system, in which both excitatory and inhibitory innervations exist. As a simple organism of 1 mm transparent roundworm, the body organization of *C. elegans* represents (A). It has organs,

differentiated tissues and innervation system along with the nerve cords, the dorsal and the ventral containing motor neurons. The body wall muscles are composed of quadrants along the sides of ventral and dorsal nerve cords (VDCs) (B). Red dashed boxes in order (A-C) show cross-sectional cut of the worm body indicating that the VDCs form functional synapses with the body wall muscles nearby by stretching muscle arms to the nerve cords (C). The excitatory and inhibitory innervations at the NMJs are mediated by two-type nAChRs (levamisole-sensitive and non-sensitive receptors) and GABA_A receptors, respectively, by which the behaviors such as the locomotion of the sine-wave movement forward/backward of the worm are balanced by muscle contraction and relaxation at the NMJs (D). Source: A and B were provided from Stefan Eimer, C was taken from WormAtlas (Altun, 2002-2012).

Figure II.9 Muscle arms at NMJs in *C. elegans*. In wild type, the body wall muscles (in red) along with the ventral nervous cord (VNC in green) containing the motor neurons (MN) were shown by using the strain *sjEx [InJ13;InJ64]* expressing green fluorescent-fusion protein (GFP) in neurons (*prab-3::gfp*) and red fluorescent-fusion protein in muscles (*pmyo-3::tagRFPt*) (A). A receptor subunit, UNC-29-RFP, of Lev-nAChRs is shown to cluster at the postsynaptic membrane at the NMJs. The muscle arms (MA) in green are projected out to the synaptic terminal of the motor neurons (B). Scale bars indicate 20 μ m (A) and 10 μ m (B), respectively. Source: B was taken from the reference (Pinan-Lucarre et al., 2014).

2.6.2 Assembly, trafficking and degradation of nAChRs

nAChR pentamers are synthesized and assembled in the endoplasmic reticulum (ER) and transported through the Golgi apparatus and TGN en route to the plasma membrane (Camus et al., 1998; Ellgaard et al., 1999; Green and Millar, 1995; Richard et al., 2013; Smith et al., 1987). During their biosynthesis in the ER, nAChRs are tightly regulated in their folding, assembly and maturation by multiple factors such as the chaperones BiP, calnexin, RIC-3 and ER membrane protein complex (EMC)-6 (Forsayeth et al., 1992; Gelman et al., 1995; Halevi et al., 2002; Richard et al., 2013). The nAChR folding and assembly have been shown to be very inefficient due to the degradation of a large part of nAChRs by the ER-associated degradation (ERAD) machinery (Christianson and Green, 2004; Richard et al., 2013; Wanamaker et al., 2003; Wang et al., 2002). In mouse muscle cells, the nAChR that failed to assemble properly in the ER are rapidly degraded and only about 30% of newly synthesized subunits reach the cell surface, indicating that nAChRs undergo tight regulation in the ER (Merlie and Lindstrom, 1983). For example, the unassembled subunits of nAChRs containing retention signals PL(Y/F)(F/Y)XXN of the first transmembrane (TM1) fragment are retained in the ER. In case of the exported receptors out of the ER, a conserved Arg³¹³-Lys³¹⁴ ER motif in the TM3-TM4 is present for COPI-associated transport in the unassembled α -subunits of nAChRs (Keller et al., 2001; Ren et al., 2005).

After passing the ER quality control, functionally assembled nAChRs are delivered to the Golgi apparatus where they are sorted to the synapse (Ellgaard et al., 1999; Gu et al., 2001; Rohn et al., 2000; Rouille et al., 2000; Zerangue et al., 1999). In order to maintain the efficient expression of the assembled Lev-nAChRs *in vivo*, the auxiliary proteins UNC-50, UNC-74 and RIC-3 are required for functional expression of Lev-nAChRs in the plasma membrane of *Xenopus laevis* oocytes (Boulin et al., 2008).

These studies indicate that multimeric protein complexes in eukaryotic cells, even though they are similar, have subtype-specific sorting and trafficking pathways (Bruneau et al., 2005; Mallet and Maxfield, 1999; Rohn et al., 2000).

CHAPTER I

Analyzing the evolutionarily conserved function of UNC-50/GMH1 for membrane trafficking in yeast and *C. elegans*: in regulating membrane dynamics, secretion and recycling of molecular complexes at the Golgi-endosomal compartments

III. SPECIFIC INTRODUCTION

3.1 Subtype-specific sorting manners of nAChRs by UNC-50

UNC-50 deficiency causes transport of Lev-nAChRs to the lysosome (Eimer et al., 2007). Consequently, the complex of Lev-nAChRs then does not arrive at the postsynaptic membrane (Fig. III.1). The mutants of *unc-50* in *C. elegans* show strong levamisole resistance and locomotion defects, but are mostly fine for normal growth. These phenotypes support that the second type of nAChRs covers alternatively functional needs of the regular synaptic transmission to compensate for the levamisole receptor loss (Eimer et al., 2007). In contrast to the heteromeric Lev-nAChRs, trafficking of N-nAChRs and GABA_A receptors (GABARs) for inhibitory innervations are not affected in *unc-50* mutants. The misfolded nAChRs and GABARs are treated by regulation of rapid ERAD through the ubiquitin-proteasome system (Christianson and Green, 2004; Eimer et al., 2007; Gallagher et al., 2007).

Figure III.1 Intracellular trafficking routes of Lev-nAChRs by UNC-50 in the secretory pathway. Intracellular transport of assembled Lev-nAChRs from the Golgi to the cell surface are regulated by the conserved Golgi membrane protein UNC-50 which acts as a sorting factor for subtype-specific Lev-nAChRs. In *unc-50* mutants, unassembled receptors of Lev-nAChRs are retained in the ER whereas the assembled complex is delivered to the lysosome to be degraded through the UNC-50-dependent secretory pathway. Source: Picture was represented by the author based on the reference (Eimer et al., 2007).

3.2 A transmembrane receptor GMH1 of the ARF-GEFs Gea1/2p

Membrane-mediated cargo targeting of ARF activation is controlled by ARF-GEFs that consist of multi-domain proteins to coordinate tight regulation of membrane traffic (Chardin et al., 1996; Goldberg, 1998; Mouratou et al., 2005). As regulation mechanisms of ARF-GEFs through the multi-domains containing the catalytic Sec7-domain, it has been recently discovered that the large ARF-GEF Sec7 domain in yeast is recruited to the TGN via the HDS1-domain direct interaction with ARF1-GTP (Richardson et al., 2012). In addition to the Sec7 ARF-GEFs, the human large ARF-GEF GBF1, known as localized in the early- and internal-Golgi, has also been present in the TGN for the membrane recruitment of BIG1/2 GTPases through the distinct small GTPase-cascade activation from early-Golgi to the TGN (Lowery et al., 2013; Richardson et al., 2012). The membrane recruitment of ARF-GEFs at the TGN is dependent on the non-catalytic HDS domains by its consecutive acting from the early-to-final sorting venue. However, the ARF-GEFs-related membrane-associated factors of the membrane are little unknown that may act as critical regulators of the cargo-specific sorting routes (Lowery et al., 2013; Richardson et al., 2012). To date, UNC-50/Gmh1p has only been proposed as a transmembrane receptor to recruit ARF-GEFs Gea1/2p to the Golgi membrane since the Gmh1p was identified by Chantalat et al. in 2003. From the study, UNC-50/Gmh1p is known as a physical interaction partner of the ARF-GEFs Gea1/2p via the HDS domains at the C-terminus of Gea1/2p (Fig. III.2), and the interaction between Gmh1p and Gea1/2p has been observed not only in yeast, but also in nematode and human (Chantalat et al., 2003; Eimer et al., 2007). These data indicate that it is likely to be a conserved mechanism involved in the small GTPase ARF, its regulator ARF-GEF and a transmembrane receptor in the intracellular transport of eukaryotic organisms.

Figure III.2 The similarity of HDS domains of the large ARF-GEFs in yeast, plant, nematode and human. Schematic domains of GBF subfamily (GBF/GEA/GNOM) of the large ARF-GEFs represent (Refer to the Fig. II.4) The HDS domains of GEA1 (749–1408) are GMH1-binding sites (Dashed box in red) by which the SEC7 is stably recruited to the TGN and positively regulated via the HDS domains (Richardson et al., 2012). Source: Picture was taken from the reference (Chantalat et al., 2003).

3.3 Pharmacological phenotypes of *unc-50* mutants in synaptic transmission

The synaptic transmission in *C. elegans* can be phenotypically visualized by pharmacological assays by using levamisole, a cholinergic agonist of nAChRs, and aldicarb, an inhibitor of acetylcholinesterase (AChE) which is a hydrolytic enzyme of ACh in synaptic cleft (Fig. III.3A) (Mahoney et al., 2006). The mutants of *unc-50* were initially identified on movement defects (Brenner, 1974) and shown to be extremely resistant on 1 mM levamisole that, in wild type, causes muscle hyper-contraction, paralysis of worm body and death (Fig. III.3B). In order to clarify pharmacological phenotypes about the synaptic transmission of the *unc-50* mutants (*x47* and *ok1847*), *unc-29* (*e1072*) as a subunit of Lev-nAChRs (Fleming et al., 1997) and *ric-3* (*hm9*) as a chaperone required for maturation of multiple nAChRs (Halevi et al., 2003) were used as positive-control mutant strains to compare resistant phenotypes. The *unc-50* mutants were shown to be strong resistant on levamisole and aldicarb plates in both 100 ± 0.00 % at the time-points (Fig. III.4A and B), supporting that if a mutant is resistant to in both

chemicals the gene mainly operates in the postsynaptic area, whereas if a mutant is resistant to aldicarb and sensitive to levamisole the gene is likely to operate in the presynaptic area (Mahoney et al., 2006). After four hours on aldicarb plates, 50% of *unc-50* mutants appeared to be kinker phenotypes (Fig. III.4C), indicating that the impaired neurotransmission in the synaptic cleft causes the mutant *unc-50* to be severe kink and dead on the aldicarb plates (observed by the author). These results support that the UNC-50 is mainly required for expression of certain molecules in postsynaptic membranes such as Lev-nAChRs, not in presynaptic areas (Eimer et al., 2007).

Figure III.3 Schematic representation of cholinergic neurotransmission and pharmacological phenotypes of wild type animals on 1 mM levamisole. Synaptic transmission results in the activation of postsynaptic receptor-expressed membranes by neurotransmitters, which are released from presynaptic nerve terminal. *C. elegans* like mammals uses acetylcholine (ACh) which is released from cholinergic motor neurons to the postsynaptic membranes that allows the worm to make a sinusoidal movement by muscle contraction (Von Stetina et al., 2006). Levamisole, a cholinergic agonist of nAChRs, binds to one subtype of nAChRs (A), which leads to muscular hyper-contracted paralysis and death of wild type nematodes (B). Aldicarb is an inhibitor of acetylcholinesterase (AChE) that blocks the enzymatic hydrolysis of AChE to recycle choline back into presynaptic neurons in synaptic cleft (A). Therefore, impaired pre- and post-synaptic transmission in *C. elegans* can be easily distinguished by two chemical compounds, levamisole and aldicarb, that shown to be resistant and/or sensitive phenotypes. Source: Worm pictures of B were provided from Stefan Eimer.

Figure III.4 Pharmacological phenotypes of *unc-50* mutants. UNC-50 is necessary for trafficking of levamisole-sensitive nAChRs (Lev-nAChRs) in the postsynaptic membranes. Pharmacological phenotypes of the *unc-50* mutants were shown to be strong resistance, $100 \pm 0.00\%$, in both 1 mM levamisole and 2 mM aldicarb plates (A and B). However, in the aldicarb assays, the approximately 50% worms of *unc-50* mutants appeared to be kinker phenotypes after four hours and, in the following day (24 hours), the rest of worms (100%) were shown to be severe uncoordinated phenotypes (Kink) with treatment on the 2 mM aldicarb. In contrast, *unc-50* mutants were not affected in the levamisole plates (C). Three independent experiments were performed on each strain. The number of worms was thirty per strain for analyzing.

3.4 Analysis of synthetic lethal interactions of the trafficking factors in the TGN

In the secretory pathway, dynamic sorting and trafficking of proteins take place at the *trans*-Golgi network (TGN) in which many critical factors related to the regulation of membrane trafficking are redundant in cellular processes (Altschuler et al., 1998; Baugh and Hunter, 2006; Ishizaki et al., 2008; Lode et al., 2002; Naor et al., 2005). In the case that redundant pathways exist in parallel at the TGN, functionally redundant genes in the pathway are able to compensate for the loss of gene. On the basis of the idea, *unc-50* mutants have been examined by synthetic lethality analysis on the intracellular trafficking

factors involved in the bidirectional anterograde and retrograde pathways in the interface of the TGN-endosomes (Table III.1A and 1B) where UNC-50 seems to mainly localize and function. Synthetic lethal phenotypes of the genetic interactions with *unc-50* mutants obviously appeared in the mutant combinations with the retrograde trafficking factors (Table III.1B). This indicates that UNC-50 is likely to be functionally redundant with the factors Golgi associated retrograde protein complex (GARP), conserved oligomeric Golgi complex (COG), retromer complex and a small GTPase RAB-6, in the retrograde pathway. Synthetic interaction needs to be clarified precisely between the retrograde factors tested in the study.

To verify the synthetic lethality of the retrograde trafficking factors that function in parallel pathway, we examined the genetic interactions by synthetic lethal combinations between the retrograde factors: *cogc-3* (*k181*) as a subunit of COG complex, *vps-35* (*hu68*) as a subunit of retromer complex, *vps-53* (*ok2864*) as a subunit of GARP complex. They are required for sorting and recycling cargoes and tethering vesicles at the Golgi-endosomal interface (Bonifacino and Rojas, 2006; Conibear and Stevens, 2000; Seaman, 2005). As an upstream regulator for ARF-like 1 (ARL1), ADP-ribosylation factor (ARF)-related protein 1 (ARFRP1, *arfrp1* (*tm2779*)) is needed for GRIP-domain recruitment to the TGN in both anterograde and retrograde pathway (Nishimoto-Morita et al., 2009; Panic et al., 2003; Setty et al., 2003; Shin et al., 2005; Zahn et al., 2006). Therefore, we tested these mutants to be lethal by crossing. Being seen in Table III.1C, the genetic interactions of ARFRP1 with VPS-53 (a subunit of GARP complex (*arfrp1*; *vps-53*)) and VPS-35 (a subunit of retromer complex (*arfrp1*; *vps-35*)) were shown to be severe defects in growth and egg-laying defects as well. However both are not embryonic lethality in the double mutant. On the other hand, the double mutant *cogc-3* (*k181*); *vps-35* (*hu68*) was genetically lethal (Table III.1C). As tested initially, *cogc-3* (*k181*); *unc-50* (*x47*) and *vps-35* (*hu68*); *unc-50* (*x47*) resulted in embryonic lethal phenotypes, respectively (Table III.1B). This is consistent with the results that the double mutant in the GARP-COG complex (*vps-52*; *cogc-1* and *vps-52*; *cogc-3*) and GARP-retromer complex (*vps-54*; *vps-35*) were synthetic lethal phenotypes conducted by Ling Luo (Luo et al., 2011). In both organisms yeast and *C. elegans*, the genetic interactions were consistently shown to be lethal in combination with *unc-50* mutants, but not in the

anterograde trafficking factors, even the triple mutant *syn-13 (tm2037); syn16 (tm1506); unc-50 (x47)* was no defects (Table III.1B). Taken together, these results from the genetic interactions between the TGN genes, such as GARP, COG and Retromer complexes that are functionally redundant, support that UNC-50/GMH1 plays a functionally redundant role in the retrograde trafficking pathway in the TGN-endosomes.

Mutation	Gene identity	Synthetic phenotype
<u>Anterograde factors with unc-50 mutants</u>		
A	Adaptor proteins	
<i>unc-101 (sy108)</i>	μ1 subunit of adaptor protein complex 1 (AP-1)	Normal growth
<i>dpy-23 (e840)</i>	μ2 subunit of adaptor protein complex 2 (AP-2)	Normal growth
<i>apb-3 (ok429)</i>	β3 subunit of adaptor protein complex 3 (AP-3)	Normal growth
<i>apt-9 (tm3776)</i>	GGA, γ-ear-containing ARF-binding proteins	Normal growth
<i>dab-1 (gk291)</i>	DISABLED, monomeric adaptor proteins in endocytosis of lipoprotein receptors	Normal growth
	SNARE protein	
<i>syn-13 (tm2037)</i>	Syntaxin-13	Normal growth
	Small GTPases	
<i>arl-3 (tm1703)</i>	ADP-ribosylation factor (ARF)-like protein	Normal growth
	Novel domain containing trafficking factors	
<i>rme-1 (b1045)</i>	EHD protein required for endocytosis	Normal growth
<i>rme-8 (b1023)</i>	J-domain protein required for endocytosis	Normal growth
	Motor protein	
<i>klp-4 (tm2114)</i>	kinesin-like motor protein	Normal growth
<u>Retrograde factors with unc-50 mutants</u>		
B	Tethering complex	
<i>vps-52 (ok853)</i>	Subunit of GARP complex	Lethal
<i>vps-54 (ok1463)</i>	Subunit of GARP complex	Lethal
<i>vps-51 (tm4275)</i>	Subunit of GARP complex	Normal growth
<i>cogc-1 (k179)</i>	Subunit of COG complex	Lethal
<i>cogc-3 (k181)</i>	Subunit of COG complex	Lethal
	Vesicle coat protein	
<i>vps-35 (hu68)</i>	Subunit of retromer complex	Lethal
<i>snx-1 (tm847)</i>	Subunit of retromer complex	Slow growth
	Small GTPase	
<i>rab-6.2 (ok2254)</i>	RAB GTPase	Lethal
	SNARE protein	
<i>syn-16 (tm1560)</i>	Syntaxin-16	Normal growth
	Adaptor proteins	
<i>rsd-3 (pk2010)</i>	EpsinR	Normal growth
<i>rsd-3 (pk2013)</i>	EpsinR	Normal growth
<u>Triple mutants with unc-50 mutants</u>		
<i>syn- 13 (tm2037) ;syn-16 (tm1506)</i>	Syntaxin-13 and Syntaxin-16	Normal growth

C		
<u>Synthetic lethal interactions in TGNs</u>		
<i>arfrp1 (tm2779)</i> ; <i>vps-53 (ok2864)</i>	Arf-related protein, Arfrp1 and a subunit of GARP complex	Growth defect, unhealthy and dead
<i>cocg-3 (k181);</i> <i>vps-35 (hu68)</i>	Subunits of COG complex and retromer complex	Lethal
<i>vps-35 (hu68)</i> ; <i>arfrp1 (tm2779)</i>	Subunits of retromer complex and Arf-related protein, Arfrp1	Egg-laying defect, paralyzed and dead
D		
<u>Tests for synthetic lethality with <i>gmh1</i> in yeast</u>		
GMH1	ARF1, GGA1, GGA2, GEA1, VMA1, VMA3, VMA6, ARL1, ARL3, SYS1, IMH1, TLG1, VPS35, VPS5, VPS17, VPS41, APM1, SAC1, INP51, INP52, INP53, PEP3, VPS33	Normal growth
	VPS53, RIC1	Growth defect
	COG5, COG6, COG7, COG8, YPT6, SGM1, VPS51, VPS52, VPS54	Lethal

Abbreviations used in this table: Arf, ADP-ribosylation factor; GGA, Golgi localized, gamma-ear-containing Arf-binding protein; SNAREs, Soluble N-ethylmaleimide-sensitive factor attachment protein receptors; TGN, *trans*-Golgi network; EHD, Eps15 homology domain as epidermal growth factor receptor substrates.

Table III.1 Synthetic lethal phenotypes between the trafficking factors considered functionally redundant in the Golgi-endosomes. In the parallel pathway, synthetic lethality by double-mutants represents genetic interactions between two genes in the Golgi-endosomes. If there are only two pathways in function and both pathways are inhibited, it would show lethal phenotypes because there is no way to compensate the function by the other player. In the TGN, as a positive genetic interaction in parallel pathway, the genetic interactions of two genes *cocg-3*; *vps-35*, that act as a retrograde trafficking factor in COG and retromer complex, respectively, were shown to be lethal (C). This supports that genes in parallel pathway which are functionally redundant appear synthetic lethal phenotypes in the genetic combination. The tested genes shown to be lethal with *unc-50/gmh1* mutants are all involved in the retrograde trafficking pathway in the endosomes-to-Golgi retrieval in two model organisms the yeast and *C. elegans*. Source: The

experiments of synthetic lethal analysis were conducted by and Ling Luo (PhD dissertation, 2010) (A and B), the author (C) and Katrin Hartwich (D).

3.5 Aims of this study

Highly conserved proteins required for intracellular trafficking play fundamentally important roles in eukaryotic cells. One of the conserved Golgi membrane proteins, UNC-50 is required for intracellular trafficking of Lev-nAChRs to the postsynaptic membrane at NMJs in *C. elegans*. The protein UNC-50 is highly conserved from yeast to humans, but the yeast *S. cerevisiae* and plant *Arabidopsis* do not have nAChRs. The yeast orthologous protein Gmh1p of the UNC-50 was not yet revealed about functional defects even it seems to be a critical transmembrane receptor as a binding partner of the ARF-GEFs that regulates vesicle trafficking by activating ARF GTPases. To date, little is known about its conserved protein function of UNC-50/GMH1 as shown to be evolutionarily conserved in eukaryotic cells (Fig. II.7).

In case of the transport of Lev-nAChRs, due to the complexity of the multiple cargoes trafficking within intracellular interface, the selectivity of Lev-nAChRs by UNC-50 contributes to further insight into the regulation of the subtype-specific receptor trafficking since how UNC-50/GMH1 functions and its regulation mechanism remain unclear. To gain the insight of the specific-cargo trafficking by the ARF-GEFs membrane-mediated interaction partner UNC-50/GMH1, we focused on its function and intracellular phenotypes between the intracellular compartments where it works.

In Chapter I (Fig. III.5: STEP1-6), we show that UNC-50 localizes to the Golgi-endosomal interface and functions as a recycling factor. *unc-50/gmh1* mutants appear to be lethal or growth defects in combination with retrograde trafficking factors, GARP, COG, retromer complex and RAB6, which can be considered as a redundant function of the protein required for retrieving cargoes back to the TGN. As a visual phenotype of *unc-50* mutants shown in previous studies that the migration of posterior gonads was defected by regulating the secretion of MIG-17 through UNC-50-dependent pathway, the secretion defects of the signal sequence GFP fusion protein (ssGFP) from muscles were evident in *C. elegans*. Furthermore, inquiring aspects of intracellular organelles lacking UNC-50/GMH1 result in the targeting to be the lysosomal/vacuolar-bound phenotypes and enhanced recruitments as shown by using subcellular-organelle markers labeled with

fluorescence from endosomes to lysosomes. In conclusion, we propose that UNC-50/GMH1 as a membrane-mediated retrograde trafficking factor is required for recycling, secretion and trafficking of cargo proteins in the endosomal-lysosomal/vacuolar compartments.

Figure III.5 Schematic diagrams of research aims for understanding the intracellular trafficking of UNC-50/GMH1-dependent. The project that has been done and doing by the author consists of eight parts divided into individual and collaborated works in two model organisms the nematode *C. elegans* and yeast *S. cerevisiae*. The STEP1-STEP6 belongs to the Chapter I: *Analyzing the evolutionarily conserved function of UNC-50/GMH1 for membrane trafficking in yeast and C. elegans*. The STEP1-STEP2 includes the data conducted by the author and Ling Luo. The STEP3, STEP4 and STEP6 were conducted by the author. The STEP5 was done by the author and Oliver Kretz. The STEP7-STEP8 belongs to the Chapter II: *Proteomic/Metabolic analysis of the UNC-50 by stable-isotope labeling of amino acids in C. elegans* which is ongoing experiments by the author and the collaborators in proteomics/metabolomics Core Facility of ZBSA, Uni-Freiburg (Carsten Jäger in metabolomics, Sebastian Wiese in proteomics and the group of Jörn Dengjel in proteomics).

IV. RESULTS

The evolutionarily conserved Golgi protein UNC-50/Gmh1p has been reported to be required for specific intracellular membrane trafficking events in yeast and *C. elegans*. However, the precise function of UNC-50/Gmh1p for intracellular membrane transport has yet to be determined (Chantalat et al., 2003; Eimer et al., 2007). In Chapter I, we focused on two model organisms yeast *Saccharomyces cerevisiae* and nematode *Caenorhabditis elegans* in order to identify the fundamentally conserved role of UNC-50/Gmh1p by analyzing cellular phenotypes.

4.1 UNC-50 colocalizes with APT-9 at the *trans*-Golgi networks

In *C. elegans*, UNC-50 has been localized at the Golgi apparatus as reported previously by using a medial-Golgi marker alpha-Mannosidase II-GFP fusion protein (Eimer et al., 2007). In yeast, Gmh1p has been functionally linked to the early Golgi through its interaction with the ARF-GEF Gea1p which is required for COPI dependent vesicular trafficking (Chantalat et al., 2003). However, UNC-50 does not colocalize with COPI coat components such as ϵ -COP in *C. elegans* as shown (Ling Luo). This suggests that UNC-50 might rather localize to late Golgi compartments or the TGN. To test this, we analyzed the localization of UNC-50 relative to APT-9 at the TGN.

APT-9 is the single *C. elegans* ortholog of GGA (Golgi-associated, gamma-adaptin ear homology and ARF-binding proteins) clathrin adaptor protein that is localized at the TGN. GGA adaptors facilitate ARF (ADP-ribosylation factor)-dependent clathrin recruitments (Boehm and Bonifacino, 2001; Boman et al., 2000; Dell'Angelica et al., 2000; Puertollano et al., 2001b; Robinson, 2004) and trafficking of sortilin and mannose 6-phosphate receptors (MPRs) from the TGN to the endosomal or lysosomal compartments (Ni and Morales, 2006; Nielsen et al., 2001; Puertollano et al., 2001a).

The N-terminus of APT-9 was fused to tagRFpT, a photostable red-fluorescent protein, and co-expressed with UNC-50::mYFP under the muscle specific promoter *myo-3* (Table XII.3.3) in *C. elegans*. As shown in Figure IV.1, UNC-50::mYFP and tagRFpT::APT-9

appeared to be highly colocalized at the TGN. This is consistent with the colocalization of GFP::GRIP domain at the TGN as previous experiments (Ling Luo) in which mRFP::UNC-50 was shown to be largely colocalized with an endosomal marker GFP::2xFYVE.

Taken together with our previous experiments, UNC-50 is likely to be localized at the TGN and/or Golgi-endosomal interface. At the TGN, UNC-50 guides the transport of the Lev-nAChRs to the plasma membrane and prevents their delivery to the lysosomes.

Figure IV.1 UNC-50 localizes to the *trans*-Golgi network and endosomal interface. Confocal images expressing the fluorescent-labeled organelle markers at the *trans*-Golgi network, tagRFPt::APT-9, and an early endosome, GFP::2xFYVE domain, are shown in the body wall muscles. UNC-50::YFP appears to be colocalized in both tagRFPt::APT-9 (A-C) and GFP::2xFYVE domain (D-F), respectively. The insets in the bottom of the figure were magnified from white box containing an arrow of each picture. Scale bars, 5 μ m. Source: Above experiments were conducted by the author (A, B and C) and Ling Luo (D, E and F).

4.2 Phenotypes of cellular/functional expression of UNC-50/Gmh1p in eukaryotes

The cellular phenotypes of UNC-50 reflect colocalization to the Golgi-endosomal interface. To find out the evolutionarily conserved function of UNC-50/Gmh1p, we further analyzed cellular phenotypes in yeast and *C. elegans*.

4.2.1 Yeast growth phenotypes on non-fermentable and limited conditions

The yeast ortholog of UNC-50, Gmh1p, has been shown to interact with Sec7-domain containing ARF-GEFs Gea1p and Gea2p (Gea1/2p) that are required for COPI-coated vesicular trafficking. This indicates that Gmh1p recruits Gea1/2p to the Golgi to locally activate Arf1 GTPases required for the generation and sorting of COPI-coated transport vesicles that mediate transport between the ER and Golgi compartment (Chantalat et al., 2003; Eimer et al., 2007). However, a more precise phenotypic analysis has not yet been done with *gmh1* mutants. In order to access the fundamental function of Gmh1p for *S. cerevisiae* phenotypes, we analyzed the cellular growth phenotypes at different growth conditions. We found that yeast growth in the integrated *gmh1*-deleted strains ($\Delta gmh1$) grew better than the wild type strain (BY4741) under iron-limited and starvation conditions (Data not shown).

In order to make sure the phenotype of Gmh1p, the screening for yeast growth phenotypes were designed with the iron chelators, Batho Phenantroline diSulfate (BPS) and Ferrozine (Coward et al., 1993), non-fermentable condition plates and nutrient starved condition plates using Rapamycin, which is a component of mammalian target of rapamycin (mTOR) pathway inhibitor that involves in mitochondrial functions in energy and nutrient homeostasis (Cunningham et al., 2007) (All yeast media used in this study are listed in the Appendix). According to the growth phenotypes on iron-limited and starvation conditions, there was a strong possibility that Gmh1p would be involved in iron metabolisms and affected in nutrient regulations in yeast. To address these questions, the iron metabolism-related genes were assessed with *gmh1*. GEF1 known as yeast CLC proton-chloride exchangers in *Saccharomyces cerevisiae* is an evolutionarily conserved integral membrane protein. In the steady state it localizes in the late Golgi and prevacuolar compartments that depends on a soluble multicopper oxidase required for iron uptake, FET3, which accumulates the apo-FET3 in *gef1* deletion (Davis-Kaplan et

al., 1998; Gaxiola et al., 1998; Greene et al., 1993; Schwappach et al., 1998; Yuan et al., 1997; Yuan et al., 1995). In particular, the GEF1 is known to be involved in cellular homeostasis for balancing the proton-chloride exchange in endosomal/lysosomal pathway for which it functions in the late Golgi and prevacuolar compartments (Gaxiola et al., 1999; Gaxiola et al., 1998; Scheel et al., 2005; Schwappach et al., 1998; Wachter and Schwappach, 2005). A vacuolar protease essential protein, PEP4, for protein degradation in the vacuole of yeast, was used to show the consistent hypothesis about the endo-lysosomal pathway. In the double-deletion of *unc-50* with *cup-5* (a lysosomal formation protein induced from endo-lysosomal organelles in *C. elegans*), the subunits of Lev-nAChRs were rescued as detectable levels by Western blots (Eimer et al., 2007). This means Lev-nAChRs target to the mis-routed way into the lysosome in the absence of UNC-50 in *C. elegans*.

In this study, the yeast strains used for growth phenotypes, wild type (BY4741), $\Delta gmh1$, $\Delta gef1$, $\Delta gmh1;\Delta gef1$, $\Delta pep4$, $\Delta fet3$ and $\Delta gmh1;\Delta pep4$ (Appendix), were inoculated in yeast extract peptone-dextrose (YPAD) and/or synthetic complete (SC) liquid media (Appendix). For the assessment by spot dilutions on the solid media, yeast agar plates were prepared as follows: Synthetic Complete (SC) plates (D-glucose added), SC with BPS (40 μ M) plates, SC medium with Ferrozine (3 mM) plates, yeast extract with 2% glycerol-lactate (YP.Gly/Lac) plates and YP.Gly/Lac with Ferrozine (3, 1, 0.5 to 0.25 mM) plates. The yeast strains were grown in YPAD or SC media for overnight at 30°C and normalized to an optical density (OD) 600 nm (OD₆₀₀) of 0.2 for six serial dilutions (until 2×10^{-6} , OD₆₀₀) on each testing plate. Yeast growing phenotypes were no difference in either YPAD or SC. Instead, the growth phenotypes appeared to be depending on the fermentable (Fig. IV.2A-C) or non-fermentable carbon source plates (Fig. IV.2D-G). In *gmh1* deletion strains, the growth phenotypes were consistently shown to be better than wild type on the non-fermentable conditions (Fig. IV.2A-C vs D). Thus, these results led us to thought that GMH1 might have effects on respiration and iron-limited growth involved in mitochondria functions.

As the results, the yeast growth phenotypes on the YP.Gly/Lac plates containing Ferrozine (3, 1, 0.5 and 0.25 mM shown in Figure IV.2E) were shown to be similar in comparison with the YP.Gly/Lac plates without iron chelators (Fig. IV.2D-E). With this

point in view, the growth phenotype of the double deletion strain $\Delta gmh1;\Delta pep4$ was also similar phenotypes to a single mutant ($\Delta pep4$), indicating that cellular/functional expression of proteins in yeast might be more tightly regulated in phenotype assays as shown in higher biological network systems (Eddy et al., 2010). In order to address the growth phenotypes of non-fermentable conditions (Fig. IV.2F), it was compared with the YP. Gly/Lac plates containing Rapamycin (4, 2, 1 and 0.5 nM shown in Fig. IV.2G), considering that Gmh1p would be involved in nutrient metabolisms as shown to be resistant in starved conditions on YP.Gly/Lac plates. As shown in Figure IV.2G, the growth phenotypes of Gmh1p-depleted strains were not different from the phenotype of YP.Gly/Lac as a control (Fig. IV.2F). However, it has been shown to be much decreased in growth due to the non-fermentable-starved complex conditions. These results suggest that Gmh1p may not be closely or directly linked to involve iron-dependent pathways due to the indistinguishable phenotypes between non-fermentable and/or iron limited conditions by using YP. Gly/Lac plates.

Figure IV.2 Yeast growth phenotypes on synthetic complete, non-fermentable and starvation conditions. The yeast cells used were grown in synthetic complete medium on fermentable carbon source plates (A, B and C) and non-fermentable carbon source plates (2% Glycerol-Lactate yeast extract, YP.Gly/Lac) (D, E, F and G) for overnight at 30°C and normalized to an OD₆₀₀ of 0.2 for six serial dilutions. Yeast growth phenotypes were shown to be better in $\Delta gmh1$ strains on YP.Gly/Lac plates (D, E, F and G). Ferrozine (3, 1, 0.5 to 0.25 mM), Batho Phenantroline diSulfate (BPS) (40 μ M) and rapamycin (4 to 0.5 nM) were used to be iron-limited and starvation conditions. The serial diluted cells (0.2 to 2×10^{-6} , OD₆₀₀) were spotted and grown on the testing plates and incubated at 30°C for three days. Asterisks represent similar growth phenotypes in both ‘with or without Ferrozine’ (D and E) and the growth phenotypes of the red box (F) are comparable to the rapamycin-containing plate (G).

4.2.2 Analysis of intracellular organelle-specific markers in yeast *S. cerevisiae*

The improved growth rates on a non-fermentable carbon source would give the possibility that *gmh1* mutants contain a more active metabolism relying on mitochondrial respiration. Therefore, we analyzed the morphology and distribution of cellular compartments in *gmh1* mutants of yeast cells.

In order to assess the effect of loss of Gmh1p function causes on intracellular trafficking between organelles, we used compartment specific markers such as ANP1-RFP (Golgi marker), CHC1-RFP (endosomal marker), SNF7-RFP (prevacuolar marker) (Huh et al., 2003) and the dye FM 4-64 as a vacuolar marker in the *gmh1*-knockout background. *gmh1* mutants and wild type yeast strains containing the plasmid pYESmt-GFP by transformation as a mitochondria marker (Westermann and Neupert, 2000) were grown in 2% galactose to induce fluorescent protein expression.

To confirm the difference from the observation, three independent experiments at least were repeated by microscopy and Western blots in the same batch. As shown in Figure IV.3, CHC1-RFP (endosomes) and SNF7-RFP (prevacuoles) were shown to be strongly vacuolar-bound forms in *gmh1* mutants (White arrows, Fig. IV.3). In contrast, ANP1-RFP and pYESmt-GFP were not different when compared to the wild type (Fig. IV.3A). The vacuolar staining marker FM 4-64 showed that the vacuolar morphology in size as well as position were not affected in *gmh1* mutant cells. Expression levels of the markers as relative values were similar in amount and shown as non-significant, even though CHC1-RFP was destabilized in *gmh1* deletion by Western blots (Fig. IV.3B). Interestingly, these results are consistent with the observation of intracellular markers by fluorescence in the Golgi-endolysosomal compartments of *C. elegans* (Fig. IV.4). These results indicate that UNC-50/Gmh1p deletion induces enhanced recruitments of intracellular organelle markers related in trafficking to the endo-lysosomal pathway and UNC-50/Gmh1p is required for trafficking regulation in the intracellular compartments.

Figure IV.3 *gmh1* mutants lead to enhanced trafficking to the vacuole in yeast *Saccharomyces cerevisiae*. Intracellular markers tagged by fluorescence were observed in different organelles as follows: pYESmt-GFP (mitochondria), Golgi (ANP1-RFP), endosomes (CHC1-RFP), prevacuoles (SNF7-RFP) and vacuoles (FM4-64) in wild type and *gmh1* mutants ($\Delta gmh1$) (A). It has been shown that CHC1-RFP and SNF7-RFP in which the RFP was endogenously expressed in each strain and clearly showed strong intensity to vacuolar membrane-bound forms in *gmh1* mutants. The total amount of the protein labeled by fluorescence was similar in Western blots (B). Three independent experiments were performed and error bars represent $\pm$ s.e.m. ns, non-significant by Student's *t* test. Scale bars, 5 μ m.

4.2.3 Analysis of intracellular organelle-specific markers in *C. elegans*

To assess the trafficking interference by lacking UNC-50 in the intercellular compartments, the fluorescent-tagged markers characterized as the representative of each subcellular organelle in the Golgi-endolysosomal interface were analyzed in body wall muscles in *C. elegans*. The transgenic worms expressing each fluorescent-tagged gene

under the control of the muscle specific *myo-3* promoter in pPD115.62 were generated by microinjection of DNA constructs into the gonads (Mello et al., 1991) in wild type and *unc-50* mutants (*x47* and *ok1847*). As shown in Figure IV.4, alpha-mannosidase II as a medial Golgi marker (Mans::tagRFP) (van den Elsen et al., 2001), 2xFYVE domains binding PI3P (GFP::2xFYVE) (Gillooly et al., 2000) and a small GTPase RAB-5 (RAB-5::YFP) as early endosomal markers (Rink et al., 2005), a small GTPase RAB-7 as a late endosomal marker (mCherry::RAB-7) (Chavrier et al., 1990; Rink et al., 2005), APT-9 known as an ortholog of GGA (Golgi-localized, gamma-adaptin ear homology and ARF-binding protein) adaptor localized at TGN (tagRFPT::APT-9) (Robinson, 2004) and CTNS-1 as a lysosomal marker that encodes a homolog of human cystinosis as a cysteine transporter in *C. elegans* (Kalatzis et al., 2001; Town et al., 1998) were used in this study. It has been shown that enhanced intensity of the fluorescent-labeled markers in *unc-50* mutants was observed and especially the small GTPase RAB-5::YFP and CTNS-1 appeared to be increased intensities and much clustered punctate structures compared to the wild type by confocal microscopy (Fig. IV.4A). Quantitative analysis of punctate size and the intensity was measured in the body wall muscles. Except for the markers of the Golgi and FYVE domain, the fluorescent-tagged markers RAB-5, RAB-7, APT-9 and CTNS-1 in TGN to endolysosomal compartments were shown to be significantly enhanced intensities in the *unc-50* depletion strains (Fig. IV.4B and C).

A cytosolic coatamer, COPI, highly conserved from yeast to mammals is an ARF-dependent adaptor protein complex for coating vesicles involved in the retrograde trafficking in the Golgi-ER intermediate compartments and vesicle sorting from the late-endosomes to the lysosome (Cosson and Letourneur, 1997; Gabriely et al., 2007; Letourneur et al., 1994; Schekman and Orci, 1996; Serafini et al., 1991; Sohn et al., 1996). RAB-11, a small GTPase, mediates mainly recycling endosomes in endocytosis and exocytosis (Grant and Donaldson, 2009; Stenmark, 2009; Takahashi et al., 2012; van Ijzendoorn, 2006). One subunit of COPI consisting of seven subunits (α , β , β' , γ , δ , ϵ and ξ) mCherry- ϵ COP and tagRFPT-RAB-11 were monitored in the body wall muscles of young adults in wild type and *unc-50* mutants (*x47* and *ok1847*). As a coated protein organelle marker, mCherry- ϵ COP was shown to be strong intensity of the fluorescence in

unc-50 mutants (Fig. IV.5A), whereas the recycling endosomal marker, tagRFpt-RAB-11, was not significantly different from the wild type (Fig. IV.5B).

These results suggest that UNC-50 is likely to be involved in COPI mediated-ARF-dependent and RAB small GTPase-dependent vesicle trafficking in the Golgi-endolysosomal interface. Therefore, in the absence of UNC-50, cargoes such as the receptor subunits of Lev-nAChRs seem to be re-routed or mis-localized to the lysosome under the coated vesicles with the consecutive ARF/RAB small-GTPases membrane-mediated trafficking by UNC-50-dependent-pathway.

Figure IV.4 *unc-50* mutants show enhanced intensity of the fluorescent-tagged organelle specific markers in the Golgi-endolysosomal compartments in *C. elegans*. Confocal images expressing the fluorescent-labeled markers in organelles are shown in wild type and *unc-50* mutants (*x47* and *ok1847*). It has been observed that the Golgi-endolysosomal interface in which Golgi (α -mannosidase II labeled-tagRFP), early endosomes (GFP-tagged 2xFYVE and RAB-5 tagged-YFP), late endosomes (mCherry-tagged RAB-7), a *trans*-Golgi network (tagRFpt-tagged APT-9) and lysosomes (CTNS-1 tagged by YFP) were monitored in the body wall muscle cells of young adults. Scale bars, 5 μ m (A). Analysis of the punctate size of the corresponding fluorescent-labeled organelle markers (B) and the relative intensity were assessed in the endolysosomal pathway (C). At least three independent experiments were performed on each

strain. Total number of worms analyzed is presented in the graph bars. Error bars represent the standard error of the mean ($\pm$ s.e.m). Asterisks indicate significant between wild type and *unc-50* mutants as follows: respective *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, non-significant by Student's *t* test.

Figure IV.5 A coat protein subunit of COPI, mCherry-εCOP, show enhanced intensity in *unc-50* mutants in the body wall muscles, but not in recycling endosomes, tagRFPT-RAB-11. Confocal images expressing the fluorescent-labeled markers in a coated organelle marker, mCherry-εCOP (A) and a recycling endosomal marker, tagRFPT-RAB-11 (B), in the body wall muscles of young adults in wild type and *unc-50* mutants (*x47* and *ok1847*). Three independent experiments were performed on each strain. Total number of worms analyzed is presented in the graph bars. Error bars represent the standard error of the mean ($\pm$ s.e.m). Asterisks indicate significant between wild type and *unc-50* mutants as follows: respective *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, non-significant by Student's *t* test. Scale bars, 5 μ m

4.3 Morphological analysis of coelomocytes and muscles in *unc-50* mutants

4.3.1 *unc-50* mutants show relatively enlarged lysosomes in coelomocytes

In *unc-50* mutants, the positive vesicles of the lysosome were observed and compared in size between wild type and *unc-50 (x47)* by using an integrated transgenic worm strain [*pwIs50*] expressing a lysosomal marker tagged by GFP (LMP-1::GFP) in coelomocytes. Given that the trafficking aspects of the enhanced recruitments were observed by fluorescent-tagged subcellular markers in the Golgi-endolysosomal interface (Fig.

IV.4A), we compared the lysosomal size in wild type with *unc-50* mutants. As can be seen in Figure IV.6A, LMP-1::GFP was shown to be significantly enlarged GFP-positive vesicles in size in *unc-50 (x47)* mutants when compared to wild type. The ratio of the vesicles expressing GFP was either analyzed in size, in which they were divided into two groups: less than 1 μm and more than 1 to 3 μm (Fig. IV.6B). Most of the GFP-positive vesicles were shown to be less than 1 μm in $70\% \pm 0.16$ of the wild type and no GFP-positive vesicles as more than 3 μm were found in the wild type unlike *unc-50* mutants. According to recent studies related to the lysosomal morphology in size, the absence of GARP complex subunits *vps-52*, *vps-53*, *vps-52* and *vps-51*, which are known to be required in recycling and tethering vesicles with SNARE complexes through a retrograde trafficking manner in the Golgi-endosomal interface, causes enlarged lysosomes (Luo et al., 2011). The LMP-1::GFP in *unc-50 (x47)* was much similar to the *vps-51 (tm4275)* rather than other subunits in the size of the positive vesicles. This suggests that, with the genetic interaction experiments shown as lethal phenotypes between *unc-50/gmh1* and retrograde trafficking factors such as COG, GARP, retromer and RAB-6 in the two model systems (Table III.1), this finding is consistent with the expectation that UNC-50 and GARP complex have redundant functions in the Golgi-endosomal interface.

A**Lysosomes in coelomocytes** **B****Ratios of GFP vesicles by size**

Figure IV.6 *unc-50* mutants show relatively enlarged lysosomes in coelomocytes. The lysosomal marker LMP-1 expressing GFP (LMP-1::GFP) appears to be relatively enlarged positive vesicles in *unc-50 (x47)* mutants by phase contrast and confocal microscopy. Scale bars, 5 μm (A). The positive vesicles of LMP-1::GFP were divided into two groups by size as follows: the positive vesicle < 1 μm and 1 – 3 μm or more (B). Total number of analyzed animals is 23 of wild type (the average of positive vesicles: 10.3 ± 2.1) and 21 of *unc-50* mutants (the average of positive vesicles: 10.5 ± 2.6), respectively. Each group was shown by percentages of the GFP

positive vesicles. Error bars represent $\pm$ s.e.m and the significant **, $P \leq 0.01$ values are analyzed by Student *t*-test.

4.3.2 Morphological analysis of the body wall muscles by HPF-EM

Typical somatic muscle cells in *C. elegans* can be divided into three parts: contractile, non-contractile and muscle arms. The contractile filament lattice contains sarcomeres, a non-contractile body is called as a muscle belly including mitochondria and organelles in the cytoplasm (Bird and Bird, 1991) (www.wormatlas.org). For the morphological analysis of the body wall muscles, young adults of *unc-50 (x47)* mutants were compared to wild type animals, which were analyzed by using high-pressure freeze electron microscopy (HPF-EM). In the longitudinal sections, the unusual vesicles (lipid-like) in various sizes near mitochondria in the muscle belly were largely observed in *unc-50* mutants unlike wild type (Fig. VI.7), suggesting UNC-50 may affect in lipid metabolisms of the worm.

Figure IV.7 HPF-EM analysis of the organelle morphology in *C. elegans* muscles. The longitudinal section views are shown in wild type and *unc-50* mutants. In the muscle belly as a non-contratile body, mitochondria and organelles are commonly observed in both strains by HPF-EM. Scale bars, 1 μ m. Source: The HPF-EM was conducted by Oliver Kretz and the pictures were kindly provided from him.

4.4 Intracellular traffic-dynamics to the vacuolar/lysosomal compartments

Through the synthetic lethal assays, it has been revealed that UNC-50 was shown to be involved in the retrograde transport such as GARP, COG, retromer complexes and RAB-6 at the Golgi-endosomal interface. Interestingly, *unc-50/gmh1* mutants had no defects and shown to be normal growth with the absence of the factors involved in the anterograde transport factors or endocytosis by the synthetic lethal analysis (Table III. 1A). These results indicate that UNC-50/GMH1 may be not required for the cellular processes in the anterograde transport as well as endocytosis, but that needs to be verified, in the intracellular compartment.

4.4.1 Bulk-fluid phase endocytosis functions in *unc-50* mutants

In order to know the capability of endocytosis in the depleted status of UNC-50, the coelomocyte that is a macrophage-like scavenger cell in *C. elegans* were observed for checking endocytosis in the bulk-fluid phase with Texas Red labeled BSA (TR-BSA, 1mg/ml). The coelomocyte is known as highly active in membrane transport by bulk endocytosis from pseudocoelomic body fluids into the cell in *C. elegans* (Fares and Greenwald, 2001). For this test, the TR-BSA was injected into the body cavity of the pharyngeal region of the transgenic worm strains, *bIs34 [prme-8::rme-8::GFP]* expressing GFP as endocytic markers, and observed uptake in coelomocytes as time series (10, 30 and 50 minutes). After injection of TR-BSA, worms were mounted on ice-chilled 2 % agarose pads to observe the endocytic dynamics at the indicated time points in the same conditions by confocal microscopy (Figure IV.8). The endocytic aspects were shown to be similar in both wild type and *unc-50 (x47)* mutants. After 10 minutes of the injection, the positive structures were already shown in late endosomes by endocytosis. After 30 minutes, TR-BSA was shown in the inside and the outside of RME-8::GFP vesicles in coelomocytes. At 50 minutes, most of TR-BSA appeared in later structures. These results suggest that endocytosis seems not to be affected by *unc-50* mutants, in particular, in the bulk-fluid phase and these results are consistent with the fact that UNC-50 is not required for the trafficking pathway involved in the general endocytosis.

Figure IV.8 Coelomocytes show normal dynamics of endocytosis by bulk-fluid phase in *unc-50 (x47)* mutants. Confocal images of coelomocytes expressing late endosomal marker RME-8::GFP after injection of TR-BSA (1 mg/ml) at the indicated time points (10, 30 and 50 minutes). Scale bars, 5 μ m.

4.4.2 FM4-46 dyes reach fast to the vacuolar membranes in *gmh1* mutants

To characterize the endocytic aspects of the intracellular trafficking in *gmh1* mutants, we examined membrane dynamics of endocytosis by tracking FM 4-64, a lipophilic styryl dye as a vital stain of yeast cells (Fig. IV.9). The internalized trafficking way of FM 4-64 from plasma membranes to endosomes and finally vacuolar membranes in the cytoplasm is likely to be affected by temperature, time, energy dependent and transport factors while they are in the process of being internalized by endocytosis (Vida and Emr, 1995).

UNC-50 is known as involved in sorting and trafficking of Lev-nAChRs and its depletion was shown that the receptor's subunits were degraded in lysosome in *C. elegans* (Eimer et al., 2007). We found in this study that *gmhl/unc-50* mutants lead to the enhanced trafficking to the vacuole/lysosome through the recruitments of interacellular organelle markers of the Golgi-endolysosomal compartments in both yeast *S. cerevisiae* and *C. elegans* (Fig. IV.3 and Fig. IV.4). This indicates that *gmhl* depletion would evoke transportation mobility of certain molecules to the vacuolar membranes by its endocytosis of FM 4-64. In order to determine that how *gmhl* deletion affects in endocytosis of yeast cells under steady state conditions, FM 4-64 was used for visualizing membrane dynamics and observed by chasing the red-fluorescent labeling dyes of the membrane as the indicated time points (10, 20, 30 and 50 minutes) (Fig. IV.9C and 10A). Before checking the mobility of the dye, yeast cells were treated for 30 minutes at 0°C in FM 4-64 as a final conc. 40 μ M, and then the cells with FM 4-64 were analyzed by microscopy at each time point (Fig. IV.10). In *gmhl* mutants, dynamics of the endocytosis was observed to be rapidly reached to the vacuolar membrane compared to the wild type at early time point within 20 minutes. To analyze the difference from the control, the images were divided into three patterns (Fig. IV.10B): Ring (plasma membranes), Dots (small punctate structures in endosome-like membranes) and Vacuoles (vacuolar membranes) at the indicated time points, and by which the staining cells were measured as four time-intervals at 10 (1 to 15 minutes), 20 (15 to 25 minutes), 30 (30 to 40 minutes) and 50 (50 to 60 minutes) that were repeated by four independent experiments (Fig. IV.10B). After treatment of the dye, the ring-staining pattern of the styryl dye was shown to appear at the outer membrane and taken up to rapidly endosome-like membranes shown as small punctate structures in the cytoplasm. It was then eventually attached to the vacuolar membrane in both strains. Interestingly, at early time-points (10 and 20), vacuolar-staining patterns came out in *gmhl* mutants first and its significance was shown to appear continuously in the time point at 20 (15 to 25 min) (Fig. IV.10B). After 30 minutes, they were shown to be not significant difference anymore and most of them were observed on the vacuolar membranes in both after 50 minutes. Thus, these data suggest that the endocytosis not only functions normal in *gmhl*

mutants but also its dynamics is affected and rather faster in the trafficking to the vacuolar membrane in the absence of GMH1.

Figure IV.9 Dye structure of FM 4-64 and its schematic representation of the staining pattern in yeast cells. The Styryl dye, FM 4-64, has two lipophilic carbon tails and charged head regions. Its fluorescent property depends on the insertion of the hydrocarbon tail into the cell membrane and two aromatic rings by which the spectral properties are determined by the double bond in the center region (A). Increasing dye concentration (0 – 20 μM) does affect the fluorescent intensities in the dye/liposome* complexes indicated as the solid squares. The open squares represent lacked liposomes with the dye (B). The emission spectra of FM 4-64 when bound to the surface of membrane (i.e. the dye-membrane complex means the status of the insertion of the tails into lipid bilayers) exhibits in 550 – 750 nm red-shifted by microscopy. The staining pattern of FM 4-64 can be divided into three patterns: Ring (plasma membranes), Dots (small punctate structures in endosome-like membranes) and Vacuoles (vacuolar membranes) at the represent time points (C). Therefore, the dynamic fluids from the outer membrane into the

vacuole can be pulse-chased with the FM 4-64 by endocytosis in yeast cells. Source: Dye structure of FM 4-64 and fluorescence intensities of dye-membrane complexes related to the dye concentration (A and B) were taken and adapted from the reference (Wu et al., 2009). The asterisk represents that the liposome consists of 30% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE) / 70% 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC).

Figure IV.10 FM 4-64 dyes are rapidly reached to the vacuolar membranes at the early time points in *gmh1* depletion. Live cell images indicating membrane dynamics were displayed at the indicated minutes (m) by FM 4-64. In *gmh1* mutants, the membrane dynamics was rapidly reached to the vacuolar membrane compared to the wild type at early time points (A). To obtain the numeric information, the images were divided into three patterns: Ring (plasma-outer membranes), Dots (small punctate structures in endosome-like membranes) and Vacuoles (vacuolar membranes), and by which the staining cells were measured as four time-intervals at 10 (1 to 15 min), 20 (15 to 25 min), 30 (30 to 40 min) and 50 (50 to 60 min) (B). Quantification of the staining types from four independent experiments was shown at each time point by measuring the membrane-associated staining shapes of the cells stained by FM 4-64 (B). Total number of cells divided by four groups as represent time-points (10, 20, 30 and 50) is shown as follows: wild type (943 cells at 10, 491 cells at 20, 461 cells at 30 and 555 cells at 50) and *gmh1* mutants

(791 cells at 10, 937 cells at 20, 617 cells at 30 and 705 cells at 50). Significant differences were shown in the represent time-points at 10 (***, $P \leq 0.001$) and 20 (** $P \leq 0.01$), respectively. Error bars represent $\pm$ s.e.m and data were analyzed by Student *t*-test. Scale bars, 5 μ m.

4.5 Recycling defects of vesicle components in *gmh1* mutants

In the secretory pathway, carrier vesicles known as synaptobrevins or *v*-SNAREs are thought to facilitate its membrane recognition and vesicle fusion (Couve et al., 1995; Rothman and Warren, 1994; Takizawa and Malhotra, 1993). The *v*-SNAREs localized in the secretory vesicles play a role in vesicle docking, fusion and endo/exocytosis as vesicle-associated membrane proteins that interact physically with the targeting-membrane SNAREs (*t*-SNAREs) from the plasma membrane (Baumert et al., 1989; Gerst et al., 1992; Gurunathan et al., 2000; Protopopov et al., 1993; Trimble et al., 1988). In addition, SNC1/2 *v*-SNAREs are involved in multiple trafficking steps and recycled to the *trans*-Golgi network via early endosomes in which SNC1/2 and GCS1 (ARF GTPase-activating protein) interact in sorting cargo molecules back to the TGN through collaboration with coatomers in the Golgi-endosomal sorting activity (Hettema et al., 2003; Lewis et al., 2000; Robinson et al., 2006).

4.5.1 SNC2-GFP localizes to the vacuolar-membranes in *gmh1* mutants

GMH1 was first identified as a *geal-6* (ARF-GEFs) membrane-associated suppressor in yeast (Chantalat et al., 2003). Thus, if GMH1/UNC-50 functions as a retrograde trafficking factor shown in the synthetic lethality experiments (Table III.1B) in the Golgi-endosomal interface (Fig. IV.1), the transport factors in the multiple trafficking steps are enhanced recruitments to the Golgi-endosomal interface on behalf of the GMH1/UNC-50 in its depleted conditions. As shown in the intracellular organelle markers in *C. elegans* and yeast (Fig. IV.3 and Fig. IV.4), *gmh1/unc-50* mutants lead to an enhanced delivery to the vacuole/lysosome by the intracellular traffic-flow. In order to address close to the intracellular trafficking between the Golgi-endolysosomal interface on GMH1/UNC-50-dependent pathway, we examined SNC2 labeled-GFP and overexpressed in yeast cells. The intracellular phenotypes of GFP-SNC2 observed was divided into three groups as

follows: Vacuolar-membrane (VM), Punctates on VM (P.VM) and Spot-like in cells (Puncta). GFP-SNC2 was observed to be the vacuolar-associated membrane forms ($82 \pm 11\%$) as the form of VM and P.VM in *gmh1* mutants (Fig. IV.11A). Interestingly, GFP-SNC2 in the VM seems as if a *sausage chain* and its punctate structures on vacuolar membrane (P.VM) were clustered to the vacuole in *gmh1* mutants. In contrast, the wild type in which most of cell types ($77 \pm 17\%$) was shown in punctate structures (Fig. IV.11A). Quantification analysis of the GFP-SNC2 was similar amount between wild type and *gmh1* mutants by Western blots (Fig. IV.11B). As shown in VM and P.VM of the GFP-SNC2 in *gmh1* mutants, we examined whether GFP-SNC2 colocalizes with FM 4-64 in the vacuolar membranes (Fig. IV.11C). It has been shown that the GFP-SNC2 was shown to be largely colocalized with FM 4-64 in the vacuolar membranes and obviously shown to be vacuolar-bound and membrane-attached types in *gmh1* mutants but not in the wild type (Fig. IV.11C). Together, these results suggest that GFP-SNC2 is recruited by GMH1-dependent pathway that would be considered as a cargo molecule and/or a fundamental factor involved in a manner dependent on GMH1-sorting/trafficking pathway, supporting that a role of GMH1 is likely to be a regulator in recycling machinery components in the intracellular trafficking.

A

Vacuolar-associated forms of GFP-SNC2 in yeast cells

Figure IV.11 GFP-SNC2 appears to be staining-like on the vacuolar membrane in *gmh1* mutants. GFP-SNC2 were overexpressed and the results were divided into three groups as follows: Vacuolar-membrane (VM), Punctates on VM (P.VM) and Spot-like in cell (Puncta) in wild type and *gmh1* mutants (A). Most of GFP-SNC2 in the VM seem as if a sausage chain and punctate structures clustered to the vacuolar membrane in *gmh1* mutant. Total number of cells used in this study was wild type (523) and *gmh1* mutant (466) by three colonies as independent experiments (A). Quantification analysis was shown to be non-significant difference between wild type and *gmh1* mutant in amount and the primary antibody used was anti-GFP and then normalized as a loading control, anti-BMH1p by Western blots. The ns represents non-significant by Student's *t* test (B). Showing images appear to be colocalized to the vacuolar membrane in the *gmh1* mutant between GFP-SNC2 and FM 4-64. Images taken after FM 4-64 staining for one hour were analyzed by microscopy (C). Scale bars represent 5 μ m.

4.5.2 Expression of TLG2-mCherry in yeast is not affected in *gmh1* mutants

The SNARE complexes are assembled to the target membrane-mediated vesicle recognition that drives vesicle fusion in eukaryotic cells and involved in many cellular processes as essential trafficking regulators (Jahn and Scheller, 2006). TLG2, ν -SNARE, of yeast *S. cerevisiae* belongs to the syntaxin protein family and localizes to the TGN and endosomes (Abeliovich et al., 1998), which directly binds to vacuolar protein sorting (vps) VPS45, a yeast ortholog of Sec1/Munc18 (SM) protein, and is regulated with ν -SNAREs SNC2 in the cellular expression levels (Nichols et al., 1998; Shanks et al., 2012). Due to the vacuolar membrane-targeting phenotypes of GFP-SNC2, the intracellular phenotypes of TLG2-mCherry were examined whether there are any defects of the intracellular phenotype in the absence of GMH1. As shown in Figure IV.12, TLG2-mCherry was overexpressed into the strains wild type and *gmh1* mutants. Both were shown to be similar aspects unlike the intracellular phenotypes of GFP-SNC2 which had appeared to be vacuolar membrane-targeting in the GMH1-deletion (Fig. IV.11). In the synthetic lethal assays, t -SNAREs *tlg1* and *syn-16 (tm1506)* have been shown to be viable with *unc-50 (x47)* mutants in yeast and *C. elegans*, respectively, even the triple combination mutants of *unc-50 (x47); syn-13 (tm2037); syn-16 (tm1506)* appeared to be normal growth phenotypes (Table III.1). This indicates that UNC-50/GMH1 seems not to be redundant in functioning SNAREs complex either in the anterograde or retrograde transport. This means that it may exist largely redundant in both pathways with distinguished mechanisms because *gmh1* mutants do not cause phenotypic defects of TLG2-mCherry by microscopy.

Figure IV.12 mCherry-TLG2 in yeast cells does not affect in the *gmh1* mutants. The *t*-SNARE TLG2 tagged-mCherry was transformed and overexpressed in both wild type and *gmh1* mutants ($\Delta gmh1$). They are shown to express similar and no significant difference between two strains wild type (BY4741) and *gmh1* mutants. The images were taken by a Coolsnap HQ camera of a Delta Vision RT (Applied Precision) microscopy by using 100x/0.35-1.5 Uplan Apo objective with filter sets of mCherry (Ex: 575/25, Em: 632/60) and differential interference contrast (DIC) fields were shown in wild type and $\Delta gmh1$, respectively. Scale bars represent 5 μ m.

4.6 Requirement of Gmh1p for the trafficking of the yeast CLC protein Gef1p

Growth phenotypes of an organism depend on expressed genomes and growth conditions. In yeast, the relevance of genes in the intracellular trafficking can also be determined by yeast growth phenotypes. GMH1 is known as a membrane-mediated ARF-GEFs GEA1/2 interaction partner and its overexpression can rescue the thermosensitive growth defect of *gea 1-6* in yeast growth experiments and it would be conceivable as a transmembrane receptor for recruitment of ARF-GEFs GEA1/2 to the membrane of Golgi (Chantalat et al., 2003).

4.6.1 Growth phenotypes and localization of GEF1-GFP in GMH1-depleted strains

To find out the relative molecules on the *gmh1*-dependent manner, yeast growth phenotypes were tested as shown in Figure IV.2. We found that the definitive growth phenotypes of *gmh1*-depleted strains appeared to grow better than wild type on the YP.Gly/Lac plates regardless of iron-limited and/or starvation conditions (Fig. IV.2D-G). In order to see the growth interactions between GMH1 and GEF1 as a yeast ortholog of the CLC proton-chloride transporter related to the iron metabolism (Braun et al., 2010; Greene et al., 1993), we investigated GEF1 in the growth phenotypes, expression levels and localization in the intracellular compartments. The conserved integral Golgi membrane protein, GEF1, is likely to be in the endo-lysosomal pathway for acidification of vesicle and balancing the proton-chloride exchange in the intracellular compartments (Gaxiola et al., 1999; Gaxiola et al., 1998; Scheel et al., 2005; Schwappach et al., 1998; Wachter and Schwappach, 2005). Even though GMH1 does not seem to be directly involved in iron metabolisms through the growth phenotypes of cellular expressions (Fig. IV.2), GFE1 might be regulated by GMH1 as a minor factor in cellular metabolic conditions.

In order to test whether GEF1 is affected in *gmh1* mutants, N- and C-terminally tagged GFP (GFP-GEF1 and GEF1-GFP in P416MET25) (Braun et al., 2010; Schwappach et al., 1998) were overexpressed and observed in wild type (BY4741) and *gmh1* mutant strains by microscopy (Fig. IV.13). Cellular expression intensity of the GFP-GEF1 and GEF1-GFP were relatively dimmed in *gmh1* mutants (Fig. IV.13C). To access certain genetic interaction in regulating GEF1 on GMH1-dependent pathway, yeast growth phenotypes were assessed by spot serial dilutions on YP.Gly/Lac plates. The idea was that GMH1 would regulate GEF1 in the intracellular compartments. In the yeast phenotypes, GMH1-deletion strains appeared growth better than wild type on non-fermentable and iron-limited conditions, even though the growth phenotypes of *gmh1* mutants were shown to grow better in both cases with/without iron chrators on YP.Gly/Lac (Fig. IV.2D-E). When overexpressed-GEF1 (GFP-GEF1 and GEF1-GFP), the growth phenotypes of the spot dilutions were observed in the wild type (WT* means that GMH1 exists in the cells). The growth phenotypes of WT* were shown to grow better than the wild type where the empty vector (P416MET25) was overexpressed. As shown in Figure IV.13D, the spot

dilution lines of red-arrows marked were shown to be growth better which appeared to 1.7-fold in GFP-GEF1 and 1.3-fold in GEF1-GFP, respectively, remarked by surface plots and relative intensities dashed-red lines. The difference of growth phenotype was in subtle than expected, indicating that cellular growth phenotypes by balancing metal-ions homeostasis may be affected by tight regulation in the intracellular organisms. These results suggest that cellular expression of GEF1, GFP-GEF1 and GEF1-GFP, is affected by GMH1 in yeast cells, even though in subtle.

Figure IV.13 Cellular-overexpression phenotypes of GEF1 tagged-GFP, GFP-GEF1 and GEF1-GFP, in wild type are slightly rescued to *gmh1* mutant strains. Structure of GEF1, based on the crystal structure of *E. coli*, in which a smaller N-terminal and large C-terminal fragment faces in cytosol and the cleavage site by Kex2p, that processes in the late-Golgi, is indicated in the lumen (A). Schematic diagrams represent the N-terminal tagged-GFP of GEF1, GFP-GEF1, and C-terminal tagged-GFP of GEF1, GEF1-GFP (B). Cellular expression phenotypes of GFP-GEF1 and GEF1-GFP show less intensity of the fluorescence in *gmh1*

deletion strains by microscopy (C). The images were taken by a Coolsnap HQ camera of a Delta Vision RT (Applied Precision) microscopy by using 100x/0.35-1.5 Uplan Apo objective with filter sets of GFP (Ex: 470/40, Em: 525/50) and differential interference contrast (DIC) fields were shown in wild type and $\Delta gmh1$, respectively. Scale bars represent 5 μm (C). Yeast growth phenotypes by spot serial dilutions from 0.4 OD (optical density, OD_{600 nm}) to 4×10^{-5} OD (D). WT* represents the overexpression of GFP-GEF1 and GEF1-GFP in wild type and EV indicates the overexpression of the empty vector, P416MET25, as a control in wild type and *gmh1* mutants ($\Delta gmh1$). The arrows and dashed lines, red color, indicates the difference of growth phenotypes (D). Source: The pictures (A and B) were taken and modified from the reference (Braun et al., 2010; Schwappach et al., 1998).

4.6.2 GMH1 deletion induces low intensity of GEF1-GFP

In addition to the cellular expression phenotypes of GFP-GEF1 and GEF1-GFP shown to express less intensities of the fluorescence in *gmh1* deletion strains by microscopy (Fig. IV.13C), the six strains used for growth phenotypes (Fig. IV.2), wild type (BY4741), $\Delta gmh1$, $\Delta gef1$, $\Delta gmh1;\Delta gef1$, $\Delta pep4$, $\Delta fet3$ and $\Delta gmh1;\Delta pep4$ were transformed with GFP-GEF1 and GEF1-GFP, respectively, and Western blots were performed to reveal biochemical protein levels in each strain. It has been interestingly shown that the overexpression of GFP-GEF1 and GEF1-GFP representing the full-length at around 100 kDa (Wachter and Schwappach, 2005) was rarely detected in $\Delta gmh1$ and $\Delta fet3$ strains (Fig. IV.14A). The $\Delta gef1$ and $\Delta gmh1;\Delta gef1$ strains appeared to be rescued in the blots, in particular, when overexpressed GEF1-GFP, the C-terminally tagged GFP of GEF1. As you can see the structure of GEF1 (Fig. IV.13A), the C-terminal end of GEF1 containing two copies of cystathione β -synthetase (CBS) domains is largely indicated in cytosol.. It has been known that the CBS domains bind to ATP as an energy sensor of adenosine-ligands in the C-terminus of CLC-5 in mammalian (Meyer et al., 2007; Scott et al., 2004). This suggests that the GEF1-GFP is likely to be recruited by the cytosolic effectors in the intracellular compartments and stably kept in the biochemical approach detecting protein levels. In contrast, the N-terminal end of GEF1 has the proteolytic cleavage sites by Kex2, which processes in the post-Golgi, at the first luminal loop (Fig. IV.13A). Based on the structure of GEF1, we observed the C-terminal tagged GFP of GEF1 (GEF1-GFP) with the following experiments Western blots.

In the previous study, *unc-50* mutants in *C. elegans* lead to mis-sorting of Lev-nAChRs into the lysosome, by which the assembled receptors of the Lev-nAChRs were back to detectable intensities by Western blots in the double mutants *unc-50* and *cup-5* essential for lysosomal formation (Eimer et al., 2007). These results led us to test double-deletion mutant *pep4;gmh1*. PEP4 is known as a vacuolar protease essential for protein degradation in yeast cells (Teichert et al., 1989). As shown in Figure IV.14B, the GEF1-GFP in the *pep4* single mutant was shown to be similar expression levels to the wild type. In the result, the relative concentration of the GEF1-GFP in *pep4;gmh1* mutants was much higher which means GEF1-GFP would be mis-routed to the vacuole/lysosome pathway in the absence of GMH1 (Fig. IV.14B). This suggests that GEF1-GFP may be regulated through a GMH1-dependent manner so that in the same way certain molecules would not be recycling in the intracellular organelles and induced to the vacuolar/lysosomal targeting as shown in both experiments (Fig. IV.3 and Fig. IV.4).

Figure IV.14 GMH1 deletion induces low intensity of GEF1-GFP, yeast CLC protein. The six strains, wild type (BY4741), $\Delta gmh1$, $\Delta gef1$, $\Delta gmh1;\Delta gef1$, $\Delta pep4$, $\Delta fet3$ and $\Delta gmh1;\Delta pep4$, which were assessed by yeast growth phenotypes (Fig. IV.2), were transformed with GFP-GEF1, the N-terminal tagged GFP of GEF1, and GEF1-GFP, the C-terminal tagged GFP of GEF1, respectively, in the plasmid P416MET25 (Braun et al., 2010; Schwappach et al., 1998). The GFP-GEF1 and GEF1-GFP of the full length were much less detected in the GMH1-depleted strains (A). The intensity of GEF1-GFP was confirmed by Western blots. Three independent experiments were performed in wild type (BY4741), $\Delta gmh1$, $\Delta pep4$ and $\Delta pep4;\Delta gmh1$. GMH1

deletion strains were much destabilized-intensity of GEF1-GFP, but the intensity of GEF1-GFP was shown to back higher intensity than wild type and $\Delta pep4$ in a double mutant, $\Delta pep4;\Delta gmhl$ (B). The full length of GFP-GEF1 and GEF1-GFP is indicated at around 100 kDa by using anti-GFP mouse monoclonal B-2 (0.2 mg/ml, Santa Cruz), 1:1000 dilution and anti-PGK1 (Phosphoglycerate Kinase 1) mouse monoclonal 22C5 (1 mg/ml, Molecular Probes), 1:1000 dilution, was used as a cytosolic control to be normalized. Error bars represent the standard error of the mean ($\pm$ s.e.m). Asterisks indicate significant between the wild type and *gmhl* mutants as follows: ****, $P \leq 0.0001$; ns, non-significant by Student's *t* test.

4.7 Secretion defects of ssGFP from muscles in *unc-50* mutants

Apart from the levamisole-resistant phenotype of *unc-50* mutants in *C. elegans*, the distal tip cell (DTC) migration defects of the posterior gonads were observed in *unc-50* mutants that cause gonad migration defects similar to the defective phenotype of *mig-17* mutants (Ling Luo, PhD dissertation, 2010). MIG-17, a soluble metalloprotease, is secreted from the body wall muscle cells as a proform with a glycosylated N-terminal prodomain and localizes to the gonadal basement membranes as a metalloprotease (MP) domain, a disintegrin (DI) domain and a protease and lacunin (PLAC) domain at the C-terminal (Ihara and Nishiwaki, 2007). We found in the previous study that MIG-17 and UNC-50 in the same genetic pathway play a role to regulate DTC migration and, in *unc-50* mutants, MIG-17 can be expressed and normally localized at the gonadal basement membrane as revealed by immune-staining and Western blots, respectively. However, when overexpressed MIG-17::VENUS in *unc-50* mutants, the ratio (3:1) analyzed between the proform and the mature form of MIG-17 was different from the ratio (1:1) in wild type, indicating that MIG-17 might be trapped in the body wall muscles in the absence of UNC-50 in muscles. Thus, we tested the secretion from the body wall muscles, in order to verify the secretion pathway on UNC-50-dependent, by using the transgenic worm line, *arl37 [pmyo-3::ssGFP]*, expressing signal sequence-GFP fusion protein (ssGFP) from muscle cells to the body cavity in which the ssGFP is taken up by coelomocytes, six macrophage-like scavenger cells in *C. elegans*, in the pseudocoelom (Fig. IV.15) (Fares and Greenwald, 2001; Girard et al., 2007; Grant and Sato, 2006). Before testing the secretion of ssGFP in *unc-50* mutants, we confirmed that secretion of

MIG-17 from muscles was not affected in secretion of ssGFP from muscles as well as secretion of ssGFP from the body wall muscles was also not related to the gonad morphological defects by mis-migration of DTCs.

In the secretion tests of ssGFP, the wild type in coelomocytes was fulfilled inside as green signals, ssGFP, whereas in *unc-50* mutants the ssGFP was much less in coelomocytes. Instead, it was shown to be trapped in the body wall muscles (Fig. IV.16A). The relative signal intensity of ssGFP between coelomocytes and body wall muscles was quantitatively analyzed and shown to be significantly different in coelomocytes and nearby muscles between the wild type and *unc-50* mutants (Fig. IV.16B). This supports that UNC-50 may be required for the proper secretion of MIG-17 from the body wall muscles. It is a rising possibility that, in *unc-50* mutants, MIG-17 can be mis-sorted to the lysosomal pathway. These results suggest that UNC-50 is involved in trafficking pathway as a secretary or general factor in the Golgi-endolysosomal compartments.

Figure IV.15 Schematic representation of endocytosis by coelomocytes of adult hermaphrodites in *C. elegans*. The signal sequence-tagged GFP, ssGFP, is produced in muscles and internalized into six coelomocytes in *C. elegans*. The transgenic worm strain *arIS37 [pmyo-3::ssGFP]* shows ssGFP in coelomocytes (Fares and Greenwald, 2001; Grant and Sato, 2006). Source: Pictures used were taken and modified from the references (Fares and Greenwald, 2001;

Grant and Sato, 2006) and the image of *C. elegans*, coelomocytes shown in adult hermaphrodites, was taken from WormAtlas.org.

Figure IV.16 The signal sequence GFP fusion protein (ssGFP) from the body wall muscles was not properly secreted due to be trapped in the muscles in *unc-50* mutants. Confocal images of the ssGFP in both wild type and *unc-50 (x47)*, in which the strain *arIS37 [pmyo-3::ssGFP]* secretes ssGFP from the muscle cells to the body cavity, are shown to be taken up by the immune system cell coelomocytes of *C. elegans*. White arrow shows ssGFP in the muscles in *unc-50 (x47)* and scale bars represent 5 μ m (A). Comparison of the relative intensities of the ssGFP in wild type and *unc-50 (x47)* was shown in both coelomocytes and muscles nearby posterior side of the gonad. Error bars mean the standard error of the mean ($\pm$ s.e.m) by three independent experiments. The significance of the different values between wild type and *unc-50* was shown as asterisks (**, $P \leq 0.01$; ***, $P \leq 0.001$) by Student's *t* test (B).

4.8 Interspecies-expression of *gmh1*, a yeast and human ortholog of *unc-50*, does not rescue and localize in *C. elegans*

Since UNC-50 has been identified in various species, we tried to confirm whether a yeast and human ortholog of UNC-50, GMH1 and hGMH1, are able to be functional in the body wall muscles of *C. elegans*. This idea was that it might be able to interact or be functional with the yeast/human orthologs of UNC-50 as a traffic-machinery to be alternately expressed in interspecies. It has also been previously reported that in the interspecies *Drosophila* subunits of nAChRs can be reconstituted and expressed in *Xenopus oocytes* with the subunits of vertebrate (Bertrand et al., 1994; Ihara et al., 2003). In this study, as a trafficking machinery, we tested UNC-50/hGMH1/GMH1 to be expressed in the interspecies.

In order to test whether the hGMH1 and GMH1 can be functional in *C. elegans*, we overexpressed the hGMH1::mYFP and GMH1::mYFP under the muscle specific promoter *myo-3* into *C. elegans*, respectively. Unlike the previous studies about the expression of the hybrid-nAChRs in *Xenopus oocytes*, they did not restore the phenotype of levamisole-sensitivity in both hGMH1::mYFP and GMH1::mYFP in *C. elegans*. The overexpression of *C. elegans* UNC-50::mYFP in the body wall muscles did normally rescue the phenotype levamisole-sensitive and show much improved movement of the body bends like the wild type (Fig. IV.17A and B). Moreover, the body bends per minute and the localization in the intracellular compartments of hGMH1::mYFP and GMH1::mYFP were not shown to be restored at all in *C. elegans* (Figure IV.17A and B). In contrast, UNC-50::mYFP of *C. elegans* was explicitly rescued in the body bends, the phenotype of levamisole-sensitivity and the localization as shown to be colocalized with alpha-mannosidase II as a medial Golgi marker (Mans::tagRFP) (Fig. IV.17C). The localization of hGMH1::mYFP and GMH1::mYFP was not indicated in the body wall muscles of *C. elegans* by using confocal microscopy. These data indicate that hGMH1::mYFP and GMH1::mYFP may not be functionally expressed and properly localized in *C. elegans*. The foreign proteins and machineries may be subsequently degraded in *C. elegans*.

Figure IV.17 *Gmh1*, the yeast and human ortholog of *unc-50* is not able to be functional in the body wall muscles in *C. elegans*. Interspecies-expression of *unc-50* and *gmh1* in the body wall muscle of *C. elegans* was tested by extrachromosomal arrays with three constructs as follows: 1. *Unc-50* tagged-mYFP (UNC-50::mYFP) of *C. elegans* *Ex [pmyo3::unc-50::mYFP]* 2. The human ortholog of *unc-50*, *hGmh1* *Ex [pmyo3::hGmh1::mYFP]* 3. The yeast ortholog of *unc-50*, *Gmh1* *Ex [pmyo3::Gmh1::mYFP]*. Each construct was expressed in the body wall muscles and functionally evaluated in *C. elegans*. Body bends per minute and phenotype tests on 1 mM levamisole were shown and the UNC-50::mYFP of *C. elegans* was rescue, but not with one of the human (*hGmh1*) and yeast (*Gmh1*) (A and B). Confocal pictures showing the normal localization of UNC-50::mYFP as rescued in *C. elegans* and colocalization of UNC-50::mYFP with the medial Golgi marker, α -mannosidase II labeled with tagRFP, in young adult body wall muscles in *C. elegans* (white arrowheads) (C). The yeast and human ortholog of *unc-50*, *Gmh1* and *hGmh1*, were not able to be functional and localized in the body wall muscles in *C. elegans*. Scale bar, 5 μ m.

V. DISCUSSION

5.1 UNC-50/GMH1 acts as a redundant factor in retrograde transport from endosomes to the Golgi

We have studied that on the subtype-specific trafficking manner how UNC-50 is required for intracellular trafficking in two organisms, *C. elegans* and *S. cerevisiae*. Although previous studies have placed UNC-50 and Lev-nAChRs as a specific pair for the receptor trafficking to the cellular surface (Eimer et al., 2007), we propose that the UNC-50 is not only for Lev-nAChRs trafficking but also plays a role in an unspecific cargo transport as a retrograde trafficking factor from endosomes to the TGN as shown in Table IV.1. In addition, UNC-50 has a function in controlling traffic-flow of machinery components mediated by RAB and ARF GTPases as intracellular organelle markers in the endo-lysosomal pathway (Fig. IV.3 and IV.4).

An evolutionarily conserved protein UNC-50 was initially observed in an inner nuclear membrane, ER, and Golgi apparatus in previous studies (Chantalat et al., 2003; Eimer et al., 2007; Fitzgerald et al., 2000). In order to find out the reason for the existence of UNC-50 in all eukaryotic cells, we sought to determine its role in sub-cellular interfaces and became aware of intracellular features which are likely to be a cargo specific or unspecific pathway on UNC-50-dependent. Among the eukaryotic cells, plant *Arabidopsis thaliana* and yeast *S. cerevisiae* do not have nAChRs, but they are likewise highly conserved the protein UNC-50 in species. Therefore, UNC-50 may play a fundamental role in the intracellular compartments in where UNC-50 localizes.

Our synthetic lethal experiments by the mutant combination between the trafficking factors of the Golgi-endosomal interface and *unc-50/gmh1* showed that the retrograde trafficking factors GARP, COG, retromer complex and RAB-6 appeared to be lethal with *unc-50/gmh1* mutants whereas the anterograde trafficking factors were viable and normal growth (Fig. V.1). It has been known that the retrograde trafficking factors disrupt protein retrieval to the TGN from endosomes and disturb the recycling of SNARE complexes in the TGN-endosomes (Conibear et al., 2003; Hayes et al., 2009; Quenneville et al., 2006; Rojas et al., 2008; Siniosoglou and Pelham, 2001). These reports support that UNC-50

may act as an unspecific/fundamental factor to be recycling protein components in the retrograde trafficking. In addition, as a membrane-mediated ARF-GEFs GEA1/2 interaction partner, GMH1 has only been proposed as a transmembrane receptor for recruiting the ARF-GEFs GEA1/2 at the early-Golgi complex (Chantalat et al., 2003). However, GMH1 does not seem to be functionally essential in cellular processes due to alternative factors to regulate membrane trafficking in the TGN. The retrograde factors would provide redundant roles to compensate each other when individual sorting factors are lost in close proximity. In this respect, recycling mechanisms and certain factors would also be conserved in similar aspects in eukaryotic cells (Donaldson and Segev, 2010; Seaman, 2005).

Figure V.1 Schematic representation of the synthetic lethality between the mutants of the *unc-50/gmh1* and the retrograde trafficking machineries in both yeast *S. cerevisiae* and nematode *C. elegans*. *unc-50/gmh1* mutants cause synthetic lethal in combination with the retrograde trafficking factor mutants GARP, COG, Retromer complex and RAB-6 involved in the cargo retrieval of the endosome-to-Golgi. The synthetic lethal phenotypes either appeared in the genetic combinations between the retrograde trafficking factors as well as a physical interaction.

5.2 UNC-50/GMH1 leads to enhanced recruitments to the endosome-to-lysosomal compartments

The intracellular phenotypes of the *unc-50/gmh1* mutants are sufficient to explain that intracellular organelle markers fluorescent-labeled APT-9, RAB-5, RAB-7 and a lysosomal protein CTNS-1 were probably recruited by UNC-50-dependent because they were shown to be increased punctate structures and strong intensity in *unc-50* mutants (Fig. IV.4). It is likely to fail to control the intracellular traffic-regulation due to the absence of UNC-50. This suggests that UNC-50 might be necessary to get ready for tight traffic-regulation in the endo-lysosomal pathway. It is consistent that the yeast strains lacking GMH1 were shown to be enhanced recruitments to the vacuolar membrane targeting shown in CHC1-RFP, SNF7-RFP and GFP-SNC2 (Fig. IV.3). Moreover, we found that UNC-50 functions in secretion as a regulator of ssGFP in muscles (Fig. IV.16), supporting that UNC-50 may operate an efficient secretion of MIG-17 that causes gonadal migration defects as a visual phenotype. As demonstrated by membrane-dynamics, the endocytic-flow aspects to the vacuolar membrane appeared to be rapidly reached in GMH1 deletion strains at the early time stages (Fig. IV.10), which indicates that UNC-50/GMH1 does affect in general transport pathway as an unspecific/regulating manner.

5.3 GMH1 is a novel recycling factor of the vesicle fusion machinery SNC ν -SNAREs

To make an efficient traffic-flow in intracellular organelles, cargoes and machinery components are needed to be matched each other in sorting and trafficking. The machinery components SNC1/2 ν -SNAREs are involved in multiple trafficking steps and recycled between the TGN and endosomes (Hettema et al., 2003; Lewis et al., 2000; Robinson et al., 2006). We found that the SNC2-GFP was shown to the vacuolar membrane-bound forms in *gmh1* mutants instead of recycling in the intracellular compartments. As the consistent results with the retrograde trafficking factors, it has been known that the *vps54* mutants of GARP complex lead to recycling defects of the SNC1 ν -SNARE from endosomes back to the TGN retrieval (Quenneville et al., 2006), suggesting

that UNC-50/GMH1 as a membrane-mediated trafficking factor might be required for recycling proteins as one of machinery components in the endosomal-lysosomal/vacuolar compartments (Fig. V.2) (Donaldson and Segev, 2010).

Figure V.2 UNC-50/GMH1 is a novel recycling factor in the retrograde pathway, which regulates in a redundant manner of the parallel pathway with the complex of GARP/COG/Retromer and the small GTPase RAB-6. In the absence of UNC-50/GMH1, the multimeric proteins such as Lev-nAChRs follow the endolysosomal pathway that leads to enhanced recruitments of the membrane-mediated machinery components SNC2, RAB5/7, APT-9 and coated proteins COPI/Clathrin on the UNC-50/GMH1-dependent manners.

5.4 GMH1 may involve indirect regulation of cargo molecules

Based on the depletion phenotype of UNC-50/GMH1, *gmh1* mutant strains have a resistance on non-fermentable conditions in yeast growth (Fig. IV.2). GMH1 would be involved in cellular homeostasis that has to be tightly regulated in intracellular organisms. Our finding showed that GEF1-GFP, required for iron homeostasis in the endo-lysosomal pathway, is dramatically reduced in GMH1 depletion (Fig. IV.14). Thus, GEF1 may recruit GMH1 to the endo-lysosomal pathway because the deletion of an

essential protease PEP4 into *gmh1* mutants exhibits strong intensity of GEF1-GFP compared to the single *gmh1* mutant such as Lev-nAChR subunit UNC-29 that came back in a lysosomal formation protein *cup-5* deletion in a similar manner (Eimer et al., 2007). How would GMH1 be connected to GEF1? They localize at the Golgi-endosomal interface, however, the localization of GEF1-GFP seems not to be closely affected in GMH1 deletion, although it appears to be unstable positioned as culture conditions in yeast (controversial to be determined by the colocalization microscopy). Thus, GEF1 may be recruited indirectly by GMH1 and still remains ambiguous relevance in the trafficking route that has to be identified with further studies.

5.5 The ARF-dependent cascade trafficking by UNC-50/GMH1

Why does UNC-50 exist in eukaryotic cells? Previous studies showed that UNCL, the mammalian orthologous of UNC-50, is required in development, differentiation and maintaining of periodontal ligament fibroblasts and, found as an inner nuclear membrane protein (Fitzgerald et al., 2000; Kim et al., 2007). Alternatively, on the basis of the trafficking of Lev-nAChRs in *C. elegans* and the ARF-GEFs binding partner as a transmembrane receptor in yeast (Chantalat et al., 2003; Eimer et al., 2007), UNC-50 may be routinely recruited in the cascade trafficking network to sort cargoes. Also it regulates membrane protein secretion and cycles machinery components by sorting away from TGN into the ARF-GEFs-dependent retrograde route to be discarded or not into lysosomes (Fig. V.2).

As a cascade trafficking model, the ARF-GEFs for the activation of ARF GTPases would occur functionally consecutive events from early-Golgi (GBF/GEA/GNOM subfamily) to the post-Golgi (BIG/SEC7 subfamily) with the activation of the distinct ARF GTPases, respectively. The ARF GTPases can be stably recruited by the consecutive-ARF-GEFs and its HDS C-terminal domains for membrane recruitments to the TGN (Lowery et al., 2013; Richardson et al., 2012). The recent two publications (Lowery et al., 2013; Richardson et al., 2012) support the cascade trafficking model (Fig. V.3). In their results, the large ARF-GEFs GBF1, the mammalian ortholog of GEA1/2, is present in the entire Golgi (early-medial-TGN) and required for the activation of the

distinct ARF GTPases. They also showed that the ARF-GTP form was recruited at the membrane through the binding of HDS domains of the SEC7 ARF-GEF and its membrane regulator. This indicates that it is likely to be a physical binding transmembrane receptor such as UNC-50/GMH1.

As such, studying UNC-50/GMH1 involved in ARF-dependent vesicle trafficking could contribute to better understanding how cargoes sort and transport to the destinations via the Golgi-endosomal interface in the secretory pathway. Despite the complexity of multimeric-protein trafficking pathway, the selectivity of Lev-nAChRs has contributed to reveal not only the functional roles of ligand-gated ion channels but also how its intracellular trafficking is regulated by UNC-50-dependent. Our analysis in two organisms establishes a role as a trafficking regulator as important features of UNC-50 in eukaryotic cells. Therefore this study will enable a means to further decipher the mechanism of intracellular trafficking and provide clues to develop therapeutic strategies in neuro-pathological disorders.

Figure V.3 The ARF-dependent cascade transport manners by UNC-50/GMH1. The functional cascade ARF-GEFs from early-Golgi to TGN represent in secretory pathway that GBF1 recruits ARFs-GTP in the early-Golgi and the ARF4/5 activated by GBF1 recruits again the distinct ARF-GEFs BIG1/2 to the TGN that mediates to the clathrin-adaptor complexes by the activation of ARF1/3 recruited by BIG1/2 of the TGN. The early acting ARF-GEFs may play a key regulator of the coated vesicle transport in secretory pathway (A). The early acting ARF-GEFs affect in the TGN with the distinct ARFs but in the same manner (B). The membrane recruitments of the ARFs-GTP are mediated by the GBF subfamily ARF-GEFs (GBF/GEA/GNOM) through the binding of the HDS domains and the transmembrane receptor UNC-50/GMH1 may act as a membrane regulator (C). Source: A was taken from the reference (Lowery et al., 2013) and B and C were modified and depicted from the reference (Richardson et al., 2012).

CHAPTER II

**Proteomic/Metabolic analysis of the UNC-50 by stable isotope labeling
of amino acids in *C. elegans***

VI. SPECIFIC INTRODUCTION

To compare changes in protein levels or modifications between mutants or different environmental conditions, quantitative proteomics is used in combination with mass spectrometry (MS) (Schmollinger et al., 2012). In order to compare quantitative proteomes between two samples, one of them needs to be labeled. Metabolic labeling *in vivo* brings minimized variations, clear incorporation and no side reactions between two cell populations that are mixed equally during sample preparations. In contrast to the labeling in the metabolic process, samples of chemical labeling are isolated after the metabolic process that remains incomplete labeling of the sample and potential quantitation errors of the separate sample treatments in two different or multiplex samples (Craft et al., 2013; Mann, 2006).

6.1 Stable isotope labeling of amino acids in living cells (SILAC)

Metabolic stable-isotope labeling is limited in living cells. Labeling of heavy stable isotope in carbon (^{13}C) and/or nitrogen (^{15}N) and stable isotope labeling by amino acids in cell lines, termed as SILAC, have been widely applied for isotopic labeling to incorporate into biological samples comprising microorganisms (e.g., bacteria and yeast), plants, mammalian cells, mice, fruit flies and nematode *C. elegans* (Findlay et al., 2008; Fredens et al., 2011; Gruhler et al., 2005; Krijgsveld et al., 2003; Kruger et al., 2008; Lahm and Langen, 2000; Larance et al., 2011; Oda et al., 1999; Ong et al., 2002).

In order to incorporate heavy isotope-labeled atoms (e.g., ^2H , ^{13}C , ^{15}N or ^{18}O) of amino acids into proteomes by SILAC, the minimal media including heavy-labeled essential amino acids (e.g., arginine and/or lysine) is supplied to the cell and organisms in which the light/natural-isotopes can be substituted by labeling heavy-nitrogen and/or heavy-carbon in essential amino acids. Due to the incorporation of ‘heavy’ amino acids, the proteins digested into the peptides after trypsin digestion are shifted to higher masses in the mass spectrometry. If the ‘heavy’ protein extract is mixed with a metabolically unlabeled control extract at equal amounts, the peptides can be unambiguously identified and quantitatively compared. (Ong and Mann, 2006). For example, labeling the amino

acid lysine- $^{13}\text{C}_6$ as a form of heavy-isotope can be metabolic incorporated into proteomes *in vivo* through the intrinsic cycling of protein synthesis and turnover in cellular growth during culture in the treated/feeding media. In the results, it appears a 6-Da mass shift of the lysine- $^{13}\text{C}_6$ from the light/natural-isotope (lysine- $^{12}\text{C}_6$) labeled samples and differentiates relative protein quantification by measuring peak ratios through MS analysis (Fig. VI.1) (Ong and Mann, 2006).

In addition to the typical SILAC whole proteome analysis, it can be used as internal standards in both relative and absolute quantification. For using a standard, non-labeled or soluble-tagged cultured cells to be purified in multiple conditions are separately combined with the SILAC standard. In the process, SILAC standards can be stable isotope-labeled as well as measurement of absolute amount to be divided by the relative ratio of the other sample to the standard (Deeb et al., 2012; Geiger et al., 2010; Geiger et al., 2011; Zeiler et al., 2012).

With the accurate quantification by SILAC labeling, the affinity purifications of protein complexes using the endogenous/exogenous tags of a specific protein of interest (POI) allow a powerful platform combined with MS. The tagging of POI is to identify relative-interacting proteins and protein-protein interactions including direct, indirect and low-affinity interactions in which non-specific contaminants can also be distinguished in representing equal abundances in both control and experiment samples (Emmott and Goodfellow, 2014; Emmott et al., 2013; Mittler et al., 2009; Rhee et al., 2013).

In order to identify specific protein interaction partners with quantitative approaches, the combination of SILAC labeling with affinity pull-downs of a protein of interest follows principally experimental procedures in order: i) isotope-labeling in metabolic conditions and tagging of an interesting-protein by using GFP, FLAG, biotin and primary antibodies (Dobrev et al., 2008; Mittler et al., 2009; Rothbauer et al., 2008; Selbach and Mann, 2006; Trinkle-Mulcahy et al., 2008), ii) co-immunoprecipitations with affinity bead-matrices against tags of proteins, iii) purifying protein complexes of either endogenous or tagged fusion proteins, iv) equally mixes of eluted protein complexes, v) digesting peptides with trypsin and vi) identifying interaction proteins with quantitative ratios of isotopic heavy- and light-labeled peptides (Dobrev et al., 2008; Emmott and Goodfellow, 2014; Mittler et al., 2009; Rothbauer et al., 2008; Schmollinger et al., 2012;

Selbach and Mann, 2006; Trinkle-Mulcahy et al., 2008). In the process, stable isotope labeling strategies and particular protein-tags can be modified in accordance with various cellular/biological processes and validated in reverse isotope labeling in replicated experiments about targeted molecules, sample treatments and genetic conditions, which provides accurate identification and quantification of proteomes in living cells and organisms (Gouw et al., 2010; Gouw et al., 2011; Krijgsveld et al., 2003; Mann, 2006; Ong et al., 2002).

Figure VI.1 Schematic workflow of stable isotope labeling by amino acids in living cells (SILAC). Stable isotope labeling in living cells is applied for accurate quantification of proteins in two or multiple populations eluted in one-mixed samples prior to the MS analysis. For the SILAC labeling, the isotopes heavy and light forms of amino acids are supplied in SILAC media which contain essential amino acids to be incorporated into proteomes through *in vivo* occurring turnover of proteins. Equal amounts of protein extracts are mixed and, if applicable, immunoaffinity purification of tagged fusion proteins of interest using proper binders covalently coupled to beads at an equal concentration are subjected to eliminate non-specific contaminants

before separation steps on SDS-PAGE and the proteins digested with trypsin are fragmented to isotopic peptides. Thus, differentially labeled peptides are quantified in SILAC ratios and specific proteins can be represented by fold changes of ratios in certain conditions in which non-specific or environmental contaminants (Protein A and Protein B) in quantitative abundances are close to 1:1 ratios. The peptides of Protein C can be considered as a specific interaction partner in abundance of relative levels by SILAC ratios. Source: Picture was modified and taken from the references (Geiger et al., 2011; Oda et al., 1999; Ong and Mann, 2006).

6.2 BioID to identify interacting proteins by Biotin protein ligase (BirA)

Physical binding proteins *in vivo* of a protein of interest (POI) are generally identified by high-affinity protein capture techniques to be applied for mass spectrometry (MS)-based proteomics. In order to detect low affinity or very dynamic interaction that can not be stable purified by co-immunopurification experiments such as protein interactions of membrane trafficking events, a high-affinity method is necessary for identification of proteins. As a unique and stable labeling used in living cells, BioID is based on a proximity-dependent protein identification method by using biotin protein ligase (BirA), by which interaction proteins of POI can be targeted as proximal biotinylated proteins in living cells (Choi-Rhee et al., 2004; Roux et al., 2013).

BirA (35.5 kDa) is the *Escherichia coli* biotin ligase that regulates biosynthetic biotin transcription as an inducible repressor and catalyzes the biotinylation of POI by covalently attaching biotin to a specific target lysine residue of carboxylase substrates (Chapman-Smith and Cronan, 1999). In the process, biotin molecules are activated by ATP-dependent manners to form a biotinyl-5'-AMP (bioAMP) as a reactive intermediate which is tightly bound to the BirA active sites and directly transferred to the ϵ -amino group of the specific lysine of the protein (Fig. VI.2A) (Chapman-Smith and Cronan, 1999; Roux et al., 2012).

To label binding partners of a given POI, the protein is expressed as a fusion protein with BirA. Since BirA only transfer biotin to specific target protein, the BirA-tag has to be modified for efficient proximity *in vivo*. A mutated residue of BirA (called BirA*_R118G, highly promiscuous form) is used instead that has a reduced capacity to hold on the activated AMP-biotin intermediate which is able to diffuse out of the BirA*

enzyme and react with proteins in close proximity. Based on the promiscuous reaction, the BirA* is usefully applied in various cell types for identification of promiscuously neighboring protein interactions to avoid in both unspecific DNA binding and self-association as well (Kwon and Beckett, 2000; Roux et al., 2013; Roux et al., 2012). Thus, BirA*-fusion protein catalyzing the reactive bioAMP between the carboxyl group of biotin and ATP leads to promiscuously be biotinylated in interacting proteins of POI that can be eluted by high-affinity biotin-streptavidin purification methods prior to MS analysis (Fig. VI.2B). The fusion protein of BirA* can also be endogenously expressed in living cells containing the supplement biotin.

Figure VI.2 Schematic representation of the enzymatic protein *in vivo* biotinylation mechanisms of the biotin ligase reaction. Biotin (vitamin H) is an essential organic cofactor known as covalently attached to several metabolic enzymes carboxylases, decarboxylases and transcarboxylases and active when the protein is coupled in biological processes involved in carbohydrate metabolisms found in plants, fungi and bacteria. Biotinylation of proteins, a rare

modification in nature, is catalyzed by the enzyme substrate BirA in which an oxygen atom of carboxyl group of biotin and the alpha phosphate ($P\alpha$) of ATP are formed to the Biotinyl-5'-AMP (bioAMP) plus pyrophosphate (PPi). The reactive bioAMP is then attached to a primary amine (NH_3^+) of lysine residues of the biotin acceptor domain of protein substrates. Once the linkage between the amide group and the bioAMP is formed it sustainably remains the attached state in life of the proteins (A). As a highly promiscuous form, BirA* (R118G) is used to identify proximal/interacting proteins of POI. The BirA*-fusion protein of POI is formed to the reactive bioAMP as promiscuously biotinylation proteins with interacting proteins *in vivo* reaction and the putative binding partners of POI can be purified by biotin affinity captures of streptavidin coated beads ($\sim 10^{-14}$ M of K_d , dissociation constant) and identified by mass spectrometry (B). Source: A and B were taken and modified from the references in order (Chapman-Smith and Cronan, 1999; Roux et al., 2012).

6.3 α GFP-nanobodies for functional studies in living cells

Direct protein tagging and modifications *in vivo* are difficult to execute since proteins, especially large complexes or attached/embedded in membranes, might not accept every fusion tag. GFP has a tractable probe as a good fusion tag allowing the tracking and localization of proteins by fluorescence microscopy. Thus GFP fusion proteins have been widely used for protein labeling and large numbers of GFP-fusion constructs are directly available. In addition, by using anti-GFP nanobodies-GFP fusion the complexe can be tagged further studies *in vivo* (Chakravarty et al., 2014; De Meyer et al., 2014; Kubala et al., 2010). As functional approaches to manipulate and verify proteins in cellular processes, biological nano-complexes of the GFP-nanobody in studying the intracellular localization, binding dynamics and affinity isolation of proteins have been developed to be applied in living cells (Kirchhofer et al., 2010; Rothbauer et al., 2008; Rothbauer et al., 2006; Schornack et al., 2009) and also efficiently used in immunological applications (Groot et al., 2006; Jobling et al., 2003; van Koningsbruggen et al., 2003). In preclinical purposes, the molecular imaging studies in human cancers using various imaging techniques requiring radiolabeled nanobodies as proves have also been extensively investigated (Chakravarty et al., 2014).

Origin of the nanobodies came from the Camelidae family that possesses unique heavy chain-only antibodies containing a single variable domain antibody fragment (V_{HH} , variable domain of heavy chain antibody) unlike typical antibodies that consist of heavy and light chains (Fig. VI.3) (Hamers-Casterman et al., 1993; Muyldermans et al., 2009).

The GFP-binding antibody or GFP-nanobody (α GFPNB V_{HH} , 12 – 15 kDa) indicating high affinity and specificity binding of the target fluorescent proteins (GFP and some variants) is a highly soluble, smallest size (2.5 nm in diameter and 4.2 nm in length) naturally derived and stable protein fragment that contains three hypervariable loops called as complementarity determining regions (CDRs) to be genetically altered as a primary antigen-specific interacting region for the fluorescent proteins (Fig. VI.3) (Chakravarty et al., 2014; Kubala et al., 2010; Rothbauer et al., 2008; Rothbauer et al., 2006).

As crucial advantages in living cells, the GFP-nanobodies can be fused with cellular proteins of interest to trace GFP fusion proteins with their interacting proteins to be detected in biochemical and proteomic analyses as endogenous antigenic epitopes *in vivo* that provide multifaceted information based on microscopic, biochemical and functional aspects about cellular functions, structures and processes (Rothbauer et al., 2008; Rothbauer et al., 2006). The α GFP nanobody (α GFPNB) may also be combined with the BioID technology by using a α GFPNB-BirA* fusion to attach the BirA* tag to GFP-fusion protein to label their binding partners *in vivo*.

Figure VI.3 The complex structure of the antigen-GFP with the anti-GFP-nanobody fragments. The heterodimeric complex of GFP:GFP-nanobody showing in the asymmetric unit by X-ray crystallography (GFP in green-gold and GFP-nanobody in pink-red) (A). Alignment of the GFP-nanobody sequences with the known camelid-antibody fragments. Three CDR regions are indicated as boxed and a disulfide bridge is marked as a dashed line. Physical GFP-binding sites of the nanobody are shown as green arrowheads in which CDR3 is indicated as major contact points for GFPs. The fragments of the GFP-nanobodies are composed of nine antiparallel β -strands configuring two sandwiching β -sheets (B). Schematic representations of conventional structures of typical antibodies and camelid nanobodies with the binding surface shown by cutting-away approach between GFP and GFP-nanobody (C). Source: A and B were taken the reference (Kubala et al., 2010) and C was taken and modified from the references (Kubala et al., 2010; Rothbauer et al., 2006).

6.4 Studying proteome in *C. elegans*

The nematode *C. elegans* as a simple model organism to understand higher eukaryotic systems is about 60-80% homologous to humans in genomes and highly classical aspects like mammals in organization and function of intracellular organelles, biosynthetic pathways and molecular mechanisms (Hulme and Whitesides, 2011; Kaletta and Hengartner, 2006).

In the metabolic processes, biosynthesis and breakdown of dietary sources, needs of essential/non-essential amino acids and energy storage as lipid-formed, are substantially similar with humans (Table VI.1) (Braeckman et al., 2009; Szewczyk et al., 2003). Its dietary source contains proteins and cofactor molecules required for fundamental pathways in cellular respirations such as β -oxidation of fatty acids to generate acetyl-coA that goes to the electron transport chain and ATP synthesis (Hulme and Whitesides, 2011; Szewczyk et al., 2003). Moreover, the proteomes related to the neurobiological mechanisms for synaptic transmission in biosynthesis, secretion and elimination of neurotransmitters are highly conserved between humans and *C. elegans* (Table VI.1) (de Bono and Maricq, 2005; Husson et al., 2007). As such, studying proteomes of *C. elegans* based on the metabolic processes can be powerful tools to understand function and mechanisms of cellular processes in eukaryotic systems.

In terms of studying proteome in *C. elegans*, MS-based quantification methods with isotope (^{15}N)-labeled and/or SILAC labeling into proteomes in the metabolic stages via feeding bacteria have allowed accurate quantitative identification of relative proteomes in *C. elegans* (Fredens et al., 2011; Krijgsveld et al., 2003; Larance et al., 2011). To complete metabolic labeling into proteomes by SILAC, *C. elegans* is a suitable model organism that requires essential amino acids like humans, in which protein complexes synthesized by metabolic processes are labeled by feeding nutrients containing the essential amino acids such as lysine and arginine during the growth in the SILAC plates (Fredens et al., 2011; Larance et al., 2011). Furthermore, isolation techniques of a protein of interest using high-affinity purifications combined with SILAC labeling in *C. elegans* can be spotlighted in versatile pathways for studying proteomes based on functional model organisms.

Amino acids	<i>C. elegans</i>	Humans
arginine	essential	essential in development
histidine	essential	essential
lysine	essential	essential
aspartic acid	non-essential	non-essential
glutamic acid	non-essential	non-essential
serine	non-essential	non-essential
threonine	essential	essential
asparagine	non-essential	non-essential
glutamine	non-essential	non-essential
alanine	non-essential	non-essential
isoleucine	essential	essential
leucine	essential	essential
methionine	essential	essential
phenylalanine	essential	essential
tryptophan	essential	essential
tyrosine	non-essential	essential in development
valine	essential	essential
cysteine	non-essential	E in development
glycine	non-essential	non-essential
proline	non-essential	non-essential
Neurotransmitters	Used in <i>C. elegans</i> ?	Used in Humans?
acetylcholine	Yes	Yes
GABA	Yes	Yes
nitric oxide	Yes	Yes
serotonin	Yes	Yes
dopamine	Yes	Yes
glutamic acid	Yes	Yes
octapamine	Yes	No
tyramine	Yes	No
epinephrine	No	Yes
norepinephrine	No	Yes
histamine	No	Yes
neuropeptides (different types)	Yes	Yes

Table VI.1 Highly conserved essential amino acids and neurotransmitters between *C. elegans* and humans. Source: It was taken from the reference (Hulme and Whitesides, 2011).

6.5 Aims of this study

Intracellular trafficking in eukaryotic systems is orchestrated by protein dynamics that occurs in protein-protein interactions (PPIs), in which the protein assembled trafficking factors such as cargo-machinery complexes are fundamentally regulated by PPIs on the particular transport pathways that can be identified in quantitative protein levels by mass spectrometry (MS)-based proteomics (Helsens et al., 2011). The conserved transmembrane protein UNC-50/GMH1, a kind of ARF-GEFs binding proteins, undergoes highly dynamic and very transient things in subcellular compartments. As shown in Chapter I, UNC-50 acts as a recycling regulator that is required for endo-lysosomal traffic flows and function in retrograde pathways of the endosome-to-TGNs.

To identify the related molecules and protein-binding partners on UNC-50-dependent pathways, powerful proteomic technologies were adopted and optimized for accurate quantification and identification of proteomes. In this study, several methods have been accomplished in *C. elegans* as follows: stable isotope labeling by amino acids (SILAC), proximity-dependent enzymatic fusion protein tags (BioID), tracing anti-GFP nanobodies conjugated with soluble fluorescent proteins in the spatially restricted protein structures and SILAC-combined immuno-affinity purifications (SILAC-BioID).

In Chapter II (Fig. VI.4: STEP7-8), we analyze UNC-50/GMH1 in metabolic stages with minimally disturbed conditions. In order to identify the membrane protein-binding partners that undergo dynamic and transient interacting processes, capture methods of high-affinity protein interactions were used to validate relative quantification and protein identification based on the metabolic labeling by SILAC. From this study, proteomic experiments in *C. elegans* were optimized to identify quantitative proteins. We found that the anterograde transport promoting proteins ACT-2, NMY-2, GPB-1, ERFA-3, UNC-18 and VPS-32.1 were shown to be quantitative protein abundance. Whereas retrograde transport Rab6-dynein mediated BICD-1 and secretory yolk genes VIT-3 and VIT-5 taken by gonad in the body cavity of *C. elegans* were shown to be down-regulated in *unc-50* mutants. This is consistent with the results of Chapter I that UNC-50 acts as a trafficking regulator in eukaryotes yeast and *C. elegans* in which the absence of UNC-50

leads to enhanced membrane-dynamic flow to the endo-lysosomal/vacuolar compartments that disturbs the recycling of vesicle trafficking machineries in the endosomes-to-TGN.

Additionally the BioID using biotinylated-dependent enzymatic protein conjugation methods was endogenously applied and functionally expressed *in vivo* with UNC-50 and other soluble proteins GFP and anti-GFP nanobodies in *C. elegans*. In this study, the advanced proteomic methods were adopted and being established in MS-combined neurogenetic projects that have been done and ongoing experiments in collaboration with the proteomics/metabolomics laboratories. Thus this study will guide as an accurate quantitative and intuitive protein identification approach to be driven in the filed of versatile protein dynamics in order to understand intracellular trafficking mechanisms.

Figure VI.4 Schematic diagrams of research aims for identifying and quantifying protein dynamics on UNC-50/GMH1-dependent in *C. elegans*. As on-going projects, it consists of parts in proteomics and metabolomics in two model organisms the nematode *C. elegans* and yeast *S. cerevisiae*. Major aims of Chapter II are to identify interaction proteins and accurate quantitative protein profiles of the UNC-50-dependent trafficking pathway for understanding the intracellular transport in proteomes. The STEP7 and STEP8 belong to the Chapter II:

Proteomic/Metabolic analysis of the UNC-50 by stable isotope labeling of amino acids in C. elegans. In this study, rising experimental technologies in proteomics were adapted and carried out by the author. After samples optimized in accordance with the research purpose in the model organisms, proteome mass spectrometric data mining were conducted by our collaborators Sebastian Wiese and Carsen Jäger in Core Facility of Proteomics and Metabolomics of ZBSA, Uni-Freiburg and Uni-Ulm.

VII. RESULTS

7.1 SLE1 *E. coli* strains for SILAC

Quantitative proteomics in living organisms is gradually applied for which strategic labeling procedures of heavy and/or light amino acids into newly synthesized proteins are facilitated by using the SILAC method that is stable isotope labeling of amino acids in living cells (Gouw et al., 2010; Krijgsveld et al., 2003; Larance et al., 2011; Ong et al., 2002).

In *C. elegans*, the SILAC can be done by metabolic labeling through feeding worms the *E. coli* SLE1 strain that is auxotroph for lysine (Lys or K) and arginine (Arg or R). The growth medium then has to be supplied with either light or heavy amino acids. In addition, SLE1 carries the plasmid pAG608 containing the *orn-1* targeted RNAi feeding vector to prevent arginine-to-proline (Arg-to-Pro) conversion through the biosynthetic pathway (Fig. VII.1) (Larance et al., 2011).

Figure VII.1 Schematic diagram of *E. coli* SLE1 in SILAC media for *C. elegans* feeding. *E. coli* SLE1 driven from the HT115 (lambda DE3) RNase-deficient *E. coli* strain which is generally used for RNAi via feeding in *C. elegans* contains the *orn-1* targeted RNAi feeding vector and the amino acids arginine and lysine auxotrophy for metabolic labeling by using a given SILAC media. During the growth in SILAC media, 1 mM IPTG is added to induce dsRNA transcription for *orn-1* knockdown targeted by RNAi and the SILAC media are concentrated to be seeded on the nitrogen-free agarose pads. Source: Picture was taken and modified from the pictures of the

references (Kamath et al., 2001; Larance et al., 2011). SLE1 was made in the group of Lamond and applied in this study as described in the reference (Larance et al., 2011).

To label the amino acids (K and R) metabolically into the *E. coli* for *C. elegans*, the SLE1 *E. coli* strains were prepared to confirm rates of incorporation of heavy-isotope amino acids into peptides and Arg-to-Pro conversion before feeding worms as described previously (Larance et al., 2011). The SLE1 was streaked out on LB plate from a frozen stock and one colony was grown on the LB containing Amp^r (100 µg/ml) and Tet^r (12.5 µg/ml) plates at 37 °C for overnight. A single colony was inoculated into M9 minimal medium (M9MM, See the Appendix) containing each 40 µg/ml (220 nM) of light (L-KR; ¹⁴N₂-¹²C₆-lysine and ¹⁴N₄-¹²C₆-arginine) and heavy (H-KR; ¹⁵N₂-¹³C₆-lysine and ¹⁵N₄-¹³C₆-arginine) amino acids, respectively (Larance et al., 2011).

In order to eliminate the amino acid conversion Arg-to-Pro, the *orn-1* targeted RNAi-feeding vectors of the SLE1 strains were expressed in the medium M9MM (L-KR and/or H-KR) containing 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and carbenicillin (1 µg/ml) as a selective marker during the growth until OD_{600nm} of 1 at 37°C. Before feeding *C. elegans* using the *E. coli* SLE1 for metabolic labeling, the incorporation of the heavy-arginine and lysine into the SLE1 *E. coli* grown in the media was confirmed to be fully labeled as heavy-isotopic forms and no conversion of the Arg-to-Pro as shown in the previous study that a heavy proline containing peptides does not appear in the bacteria (Larance et al., 2011).

7.2 Metabolic incorporation rates of the heavy labeled-amino acids in *C. elegans*

In order to apply SILAC labeling in *C. elegans*, nitrogen-free conditions except for isotope-labeling supplements in the media and growing plates are necessary for efficient metabolic labeling without surplus nitrogen sources. For this reason, nitrogen-free agarose worm plates (NGM-N) were prepared (Appendix) and the *E. coli* SLE1 grown in the M9MM L-KR and/or H-KR were concentrated as a 10-fold in OD_{600nm} of 1 after overnight culture (Gouw et al., 2011; Krijgsveld et al., 2003; Larance et al., 2011).

To test whether feeding periods with SLE1 bacteria are affected in rates of incorporation of heavy isotopes into *C. elegans*, different worm generations were used to find proper generations in feeding periods to be incorporated efficiently into the proteomes. For feeding worms on the SLE1 seeded plates which were concentrated as a 10-fold in OD_{600nm} of 1, worms were transferred onto non-seeded NGM-N plates for metabolic labeling with the given amino acids and allowed to lay eggs at 20°C. All the synchronized worm generations were transferred onto the SLE1-seeded plates (L-KR and/or H-KR) and maintained at 20°C on the heavy-amino acids lawn pads (Fig. VII.2A). The worms of F2, F5 and F8 generations in order to confirm metabolic incorporation rates after feeding heavy-isotope amino acids (H-KR) were collected as described previously by flushing the plates in PBS and immediately frozen at liquid nitrogen in cold-PBS solution containing protease inhibitors and stored at -80°C (Larance et al., 2011). The frozen sample was thawed on ice and powdered guanidine-HCl (76 mg, bond breakers) and 25 mM Tris (2-carboxyethyl) phosphine hydro-chloride solution (TCEP, reducing agents) were added, vortexed vigorously and heated at 65°C for 10 minutes. Before the centrifugation for 10 minutes at 17,000 g at room temperature, the worm lysates were bead-beated with glass-beads (0.7 mm, 50% vol. slurry). As an alternative protocol, whole worm lysates frozen with protease inhibitors were boiled at 95°C for 10 minutes in SDS loading buffer (Appendix) containing reducing agents such as dithiothreitol (DTT) or TCEP and resolved on SDS-PAGE gels (Appendix, 10% lab-made gels or 4 – 15% Mini-PROTEAN® TGX™ Gel, Bio-rad). The staining protein bands with Coomassie solution (Appendix) after destaining were tryptic digested and analyzed by MS.

As the results, the incorporation rates of F2 have been shown to be 50% in light (Lys0, Arg0) and heavy (Lys8, Arg10) labeling in peptides and either Arg-to-Pro conversions as red peaks (Fig. VII.2B). The F5 generations as longer-period fed worms on the given heavy-isotope plates were shown to be much higher incorporation of 90% and less proline conversions shown in black peaks (Fig. VII.2B). Therefore, the feeding periods can not be negligible to be incorporated amino acids into proteins for metabolic incorporations through biosynthetic pathway, even if there are contaminants of some

natural amino acids from their parents or others that actually can not be possible in the medium nitrogen-free used in this study.

To get complete or higher incorporation of the given amino acids, we found that the feeding periods were not only considered in labeling rates because there was no significant difference between F5 and F8. From these results, the bacteria SLE1 lawn pads were tested whether the concentrations do affect in metabolic incorporation rates via feeding for *C. elegans*. The concentration of the bacteria can be used a 10-to-50-fold in OD_{600nm} of 1 on the seeding plates (Gouw et al., 2011; Krijgsveld et al., 2003; Larance et al., 2011). After feeding worms in the 50-fold (OD_{600nm} of 1) seeded plates, the incorporation rates were shown to be improved (Table VII.1) and minimal proline conversions (Sebastian Wiese). These results suggest that the metabolic incorporation rates of the given light or heavy amino acids in the nematode were affected in the treated periods and the concentration of feeding SLE1 bacteria to be incorporated into proteins *in vivo*. In order to do accurate quantitative studies prior to the protein comparison in certain conditions, the incorporation values of SILAC labeling should be fundamentally concerned in metabolic circumstances of living organisms.

Generation of <i>C. elegans</i> wild type	F2	F5	F8	F8
<i>E. coli</i> SLE1 concentration (OD ₆₀₀ of 1)	10-fold	10-fold	10-fold	50-fold
Incorporation (%) of heavy-isotope (Lys8, Arg10)	50	90	90	93

Table VII.1 Metabolic incorporation rates by SILAC labeling in generation of *C. elegans*.

SLE1 strains are inoculated in 100 – 500 ml SILAC heavy medium (Lys8, Arg10) and concentrated into 10 – 50 ml (OD_{600 nm} = 1, 10-to-50-fold) as a final volume after centrifugation for seeding SLE1 on nitrogen free plates to feed *C. elegans*.

Figure VII.2 Stable isotope labeling by amino acids in feeding periods for *C. elegans*. Stable isotope labeling by amino acids in *C. elegans* are shown as workflows: *E. coli* SLE1 strains containing the *orn-1* targeted RNAi feeding vectors are grown in the minimal media with 1 mM IPTG to prevent Arg-to-Pro conversions and seeded on NGM-N plate for feeding worms to be metabolically labeling of amino acids into proteins newly synthesized. Worm lysates treated with protease inhibitors are subjected to resolve on SDS-PAGE, digest proteins with trypsin and detect in mass spectrometry (A). Metabolic incorporation rates of SILAC labeling in *C. elegans* are affected in prolonged-fed periods and seeded concentrations of the bacteria grown in the given SILAC media. The incorporation rates of F2 (red peaks, 50:50 ratios in the heavy labeling) were much improved to ~ 90% and less proline conversions in F5 (black peaks) which was fed in the longer periods on the given heavy-source plates (B). Source: All experiments in SILAC labeling

in *C. elegans* were conducted by the author and raw data of peptide abundances by MS shown in B were performed in collaboration with Sebastian Wiese.

7.3 Analysis of relative quantitative proteomes of *unc-50* mutants by SILAC

Since the SILAC labeling was proven in rates of incorporation in this study, *unc-50* mutants were investigated in quantitative proteins compared with the relative abundance profiles of wild type controls. For this purpose, we have referenced two experimental protocols as performed in *C. elegans* (Fredens et al., 2011; Larance et al., 2011). The light-(Lys0, Arg0) and heavy-(Lys8, Arg10) by SILAC were labeled in wild type controls (WTH, heavy-labeled in wild type and WTL, light-labeled in wild type) and *unc-50* (*x47*) mutant strains (MUL, light-labeled in the mutant) via feeding 50-fold SLE1 bacteria (OD_{600nm} of 1), in which the labeling procedures that have been done previously in metabolic incorporations were used in this study (Fig. VII.3).

Figure VII.3 Labeling by SILAC in wild type and *unc-50* mutants. To comparison quantitative protein abundances by metabolic labeling, wild types (WT) and mutants (MU, *unc-50*) were labeled as follows: heavy-WT (WTH) and light-WT (WTL) as controls and light-MU (MUL) as samples. The labeling worms in SILAC bacteria plates were mixed equally (1:1) as the control (WTH:WTL) *versus* sample (WTH:MUL). In order to equal proportions of proteins after the extraction as described previously, a Bradford assay was performed in the supernatant of whole lysates prior to be resolved on 10% SDS-PAGE gels (Larance et al., 2011).

7.3.1 Quantification of protein changes in *unc-50* mutants by SILAC ratios (H/L)

As the labeling workflow of the SILAC summarized in Figure VII.3, we assayed quantitative protein abundances in samples driven from whole worm lysates as equal amounts mixed. In this study, the proteins identified were a total of 2307 which was normalized in mean ratios close to one because of, in practice, the experimental values in SILAC ratios heavy:light vary in either WT/WT and/or WT/MU that theoretically should closely be ‘0’ at the center in the normal distribution. Thus the intensity plots of replicates were normalized shown in both controls and test samples (Fig. VII.4), and then quantification of protein changes was represented on the 2-dimensional scatter-plots of the control replicates (C1 and C2, WT/WT) and test replicates (T1 and T2, WT/MU) (Fig. VII.5).

Figure VII.4 Quantitative distribution of the normalized ratios of the SILAC labeling. Experimental replicates in a control (WTH/WTL) and query (WTH/MUL) are shown as intensity

plots (A) and distribution of histogram data (B) normalized to be centered (0) by SILAC ratios. The control replicates (C1 and C2) and sample replicates (T1 and T2) were used in this study. Source: Data were provided from a collaborator, Sebastian Wiese, Laboratory of Proteomics, Uni-Ulm).

Figure VII.5 Accurate quantification of practical changes of protein identified in *unc-50* mutants. Quantitative proteins identified by SILAC labeling H/L are shown in 2D-scatter plots of normalized ratios of control-SILAC (C1 and C2) (A) and test-SILAC (T1 and T2) (B). Data visualized in the plots, clustered at the center that is close to zero marked as an asterisk (*), can be considered as putative experimental contaminants and either external/internal environmental contaminants can be distinguished in SILAC ratios (close to ~ zero) (Boulon et al., 2010). In

addition to the non-specific contaminants, a few particular proteins based on the experimental conditions such as a targeted-RNAi and its related abnormal expressions should be confirmed in ratios due to probable experimental errors and/or incomplete incorporates. In order to choose a ratio as an arbitrary threshold for the test-SILAC (WTH/MUT), a fold change in SILAC ratios as being significantly regulated were applied to identify specific proteins in *unc-50* mutants. The SILAC ratios of less than 0.5 (i.e., a value $< 0.5 - 1$) indicate up-regulated proteins whereas a value of ratio more than 2 (i.e., $1 - 2 < \text{a ratio}$) means relatively down-regulated proteins against the control-SILAC (C). The 2D-scatter plots merged as all-in-one-SILAC replicates (Control:Test) are visualized in which non-specific and environmental contaminants are distinctly recognized in color plotted (C). From this study, we identified 24 proteins to be changed as a fold ratio except for non-specific and environmental contaminants that include the ornithine aminotransferase of the *orn-1* targeted-RNAi feeding vectors used in both SILAC labeling. Up-regulated proteins of 14 as a ratio < 0.5 and down-regulated proteins of 10 as a ratio > 2 were identified at least in one replicate in the test-SILAC of *unc-50* mutants. Source: A and B were provided from the collaborator Sebastian Wiese (Laboratory of Proteomics, Uni-Ulm) and C was annotated by the author in merged scatter-plots of normalized controls and test replicates that all are based on the proteomic results of SILAC labeling conducted by the author.

7.3.2 SILAC proteome profiling shows trafficking factors are specifically deregulated in *unc-50* mutants

As the results of quantitative protein abundance in *unc-50* mutants, we found that protein changes (0.5 % of total proteins identified) were largely involved in the function of the intracellular transport related and regulatory categories (Figure VII.6).

The proteins shown to be up-regulated as a fold change of SILAC ratio at least in one replicate include NASP-2 (transport of histones) (Nabeel-Shah et al., 2014), ACT-2 (contractile actin filament components), NMY-2 (non-muscle myosin motor proteins), GPB-1 (guanine nucleotide-binding protein subunit beta-1 and anterograde transport promoting factors in early cell divisions) (Thyagarajan et al., 2011), ERFA-3 (Eukaryotic release factor homolog, putative GTP binding and GTPase activity in Gene Ontology), UNC-18 (anterograde promoting transport as a syntaxin-binding protein 1) (McEwen and Kaplan, 2008) and VPS32.1 (vacuolar protein sorting related factors) (Table VII.2A and B). In addition to the protein abundance of the anterograde promoting transport, unknown and/or poorly characterized proteins identified (Protein ID: G5EBP5, Q9N4I3 and

Q8MXD9) were found to be up-regulated, in which Q8MXD9 (E02D9.1, isoform b) uncharacterized was represented as a physical interaction partner of GPB-1, shown to be up-regulated in this study, in humans and yeast by the program of functional protein association networks (STRING 9.1). The other Q9N4I3 has been predicted to regulatory function ubiquitin-associated domains (UBA)-like in Gene Ontology. With this, RAD-23 known as a protein of Radiation Sensitivity 23 family of proteasomal ubiquitin receptors was quantitatively up-regulated in *unc-50* mutants. It also interacts physically with PNGases (Peptide:N-glycanases, cytoplasmic de-N-glycosylation enzymes) facilitating the degradation of misfolded protein substrates during endoplasmic reticulum-associated degradation (ERAD) in yeast and mammals and regulates neuronal axon branching in the vulva of *C. elegans* (Habibi-Babadi et al., 2010). Besides, UCR-2.3 (Ubiquinol-Cytochrome c oxidoreductase known as mitochondrial electron transport complex III), PBRM-1 (Polybromo 1 involved in transcriptional regulation of select genes by chromatin remodeling and metal ion binding in Gene Ontology) and H2B1 (Histone H2B1 as chromatin components) were appeared to be up-regulated in the SILAC ratios of *unc-50* mutants (Table VII.2A and B).

In contrast, the proteins shown to be down-regulated in the SILAC ratios were represented in Table VII.2C and D. VIT-3 (Vitellogenin-3, yolk and lipid binding proteins), C27F2.10 (Uncharacterized, proteasome component (PCI) domain-containing protein 2) (Wei et al., 2008) and BICD-1 (Bicaudal D homolog dynein binding proteins) required for the retrograde migration of Rab6-positive vesicles to the perinuclear region and the Golgi-to-ER as a dynein-mediated transport along microtubules were down regulated in both replicates (T1 and T2). As a fold change of SILAC ratio at least in one replicate shown to be down-regulated, VIT-5 (Vitellogenin-5, yolk and lipid transport proteins), Q22531 (Unknown, putative metallopeptidase activity and zinc ion binding in Gene Ontology), CTH2 (Putative cystathionine gamma-lyase 2, function of iron savings and adaptive advantages to iron deficiency in loss of function) (Cyert and Philpott, 2013), P34385 (Uncharacterized, probable methylcrotonoyl-CoA carboxylase beta chain), Q6BEV6 (Unknown, F49E2.5), DOD-19 (Lifespan extension related proteins) (Murphy et al., 2003) and O61880 (Uncharacterized, lifespan extension related proteins) (Li et al., 2004) were included (Table VII.2C).

These results indicate that the lack of UNC-50 in *C. elegans* causes quantitative protein changes of transport-related factors, in which the active forward movement of vesicles and/or organelles seems to be dependent on the motor-driven actin-myosin pathways. Whereas the dynein-mediated retrograde-oriented movement factors are suppressed in the absence of UNC-50, supporting that UNC-50 does act as a trafficking regulator between intracellular compartments.

Moreover, quantitative protein abundance of GPB-1, UNC-18 and VPS-32.1, which is involved in the anterograde promoting transport as endosome-mediated trafficking manners in *C. elegans* and yeast (McEwen and Kaplan, 2008; Thyagarajan et al., 2011), is likely to disturb the balance of traffic-dynamic flow in the subcellular compartments due to the lack of the retrograde regulators.

Taken together, the absence of UNC-50 leads to quantitative protein abundance of anterograde pronounced transport and, at the same time, protein poverty of retrograde transport regulators, occurring that protein recycling, secretion and membrane-mediated trafficking are affected in *unc-50* mutants.

Figure VII.6 Functional classification of the proteins identified in *unc-50* mutants. Pie charts showing the groups of proteins un-regulated (A) and down-regulated (B) in *unc-50* mutants. Based on the experimental evidence and functional protein information of UniProt (the Universal Protein Resource, www.uniprot.org), WormBase, yeast genome database (SGD) and/or Ensemble, functional categories of proteins were classified into seven groups: Transport, Regulatory, Structural, Contractile, Catalytic, Immunological and Secretory.

	Protein ID	Name	Description	Functional Categories	Source	Peptides	Norm. Ratio WTH/MUL	Norm. Ratio WTH/MUL
A	Proteins with a fold change of SILAC ratios < 0.5 in both replicates T1 and T2 (up-regulated)						replicate 1	replicate 2
	O17687	NASP-2	Nuclear autoantigenic sperm protein (histone-binding protein); highly conserved proteins in eukaryotes; transport of histons ; SHNi-TPR (Sim3-HiFl-NASP interrupted TPR) protein family (Ref. 1)	Transport	Ensembl	14	0,39	0,47
	G5EBP5	ZC247.1	Unknown	–	–	11	0,39	0,32
B	Proteins with a fold change of SILAC ratios < 0.5 in one replicate and a fold change of SILAC ratios < 1 in the other (up-regulated)							
	P10984	ACT-2	Actin-2; cell motility; ubiquitously expressed in all eukaryotic cells; contractile filaments in muscles	Contractile / Transport	UniProt, WormBase	24	0,85	0,49
	G5EBY3	NMY-2	Non-muscle myosin; motor proteins; coiled-coil and myosin-like IQ motif-containing domains	Contractile / Transport	UniProt, WormBase	19	0,49	0,79
	Q9N4I3	Y71F9AL.9	Uncharacterized; UBA (ubiquitin-associated domains)-like (Molecular function, IEA*)	Regulatory	Ensembl	8	0,44	0,77
	P17343	GPB-1	Guanine nucleotide-binding protein subunit beta-1; endosomal membranes; trafficking to the anterior-side in early cell division axes (Ref. 2)	Regulator / Transport	UniProt, WormBase	4	0,77	0,42
	O45622	ERFA-3	Eukaryotic Release FActor homolog; phi-19; GO_ (Molecular function, IEA*), GTPase activity, GTP binding	Regulator / Transport	UniProt, WormBase	7	0,88	0,49
	Q9TZ33	UCR-2.3	Ubiquinol-Cytochrome c oxidoreductase complex; bc1 complex; mitochondrial electron transport complex III	Regulatory	WormBase	10	0,49	0,58
	Q8MXD9	E02D9.1	Unknown; E02D9.1, isoform b	–	–	8	0,47	0,69
	Q23451	RAD-23	DNA repair; ubiquitin-like N terminus; Peptide:N-glycanases (PNGases) binding proteins	Regulatory	SGD, WormBase	5	0,28	0,61
	P34815	UNC-18	Putative acetylcholine regulator; Syntaxin-binding protein 1; Promoting anterograde trafficking of syntaxin (Ref. 3)	Transport	UniProt	6	0,53	0,44
	G5EEY5	PBRM-1	Polybromo 1; transcriptional regulation of select genes by chromatin remodeling	Regulatory	UniProt, WormBase	2	0,38	0,67
	Q18886	VPS-32.1	Related to yeast vacuolar protein sorting factor	Transport	WormBase	3	0,67	0,36
	P04255	H2B1	Histone H2B family; Core histones; his-11; his-4; his-48; his-41; his-39; components of chromatin	Structural	UniProt	2	0,60	0,43
C	Proteins with a fold change of SILAC ratios > 2 in one replicate and a fold change of SILAC ratios > 1 in the other (down-regulated)							
	P06125	VIT-5	VITellogenin-5; VITellogenin structural genes (yolk protein genes); lipid transport proteins; Secreted into the body cavity from intestine hermaphrodite and subsequently taken up by the gonad (oocytes) (Ref. 4 and 5)	Structural / Secretory	UniProt, WormBase	94	1,90	2,06
	Q22531	T16G12.1	Unknown; GO_ (Molecular function, IEA*), metalloproteinase activity, zinc ion binding	Catalytic / Secretory?	UniProt	14	2,07	1,77
	P55216	CTH-2	Putative cystathionine gamma-lyase 2; mRNA-binding protein expressed during iron starvation	Regulatory	UniProt	6	2,86	1,69
	P34385	F02A9.4	Uncharacterized; Probable methylcrotonoyl-CoA carboxylase beta chain; mitochondrial	Regulatory	UniProt	8	4,41	1,08
	Q6BEV6	F49E2.5	Unknown; F49E2.5, isoform h	–	–	7	2,49	1,95
	O61874	DOD-19	Membrane raft; related to lifespan extension (Ref. 6)	Regulatory	UniProt	4	4,83	1,00
	O61880	F59B1.2	uncharacterized; dll-1 binding proteins related to lifespan (Ref. 7)	Regulatory	WormBase	1	2,09	1,96
D	Proteins with a fold changes of SILAC ratios > 2 in both replicates T1 and T2 (down-regulated)							
	Q9N4J2	VIT-3	VITellogenin-3; VITellogenin structural genes (yolk protein genes); a precursor of the lipid-binding protein; Secreted into the body cavity from intestine hermaphrodite and subsequently taken up by the gonad (oocytes) (Ref. 4)	Structural / Secretory	UniProt, WormBase	89	3,06	3,50
	Q95QU0	C27F2.10	Proteasome component (PCI) domain-containing protein 2 homolog; COP9 signalosome (CSN) 12-like protein; ubiquitin-proteasome pathway (Ref. 8)	Regulatory	UniProt	3	2,66	4,11
	V6CK55	BICD-1	Bicaudal D (Drosophila) homolog; dynein-mediated transport; dynein regulators; nuclear migrations; microtubule anchorage at the centrosome; Rab6-secretory vesicles to the pericentrosome and Golgi-ER retrograde transport (Ref. 9, 10 and 11)	Transport / Secretory	WormBase	3	2,79	2,88

Table VII.2 Quantitative protein abundance and down-regulation of intracellular transport related factors in *unc-50* mutants. Twenty-four proteins were identified with a fold change in SILAC ratios in the absence of UNC-50. The quantitative protein abundances normalized in the control are shown in replicator 1 and replicator 2. Proteins identified were sorted in order (A – D) as up-regulated proteins (total 14) of a fold change (SILAC ratios < 0.5) and down-regulated proteins (total 10) of a fold change (SILAC ratio > 2) from at least one experimental replicator. IEA* stands for Inferred from electronic annotation in Gene Ontology (GO). Ref. 1: (Nabeel-Shah et al., 2014), Ref. 2: (Thyagarajan et al., 2011), Ref. 3: (McEwen and Kaplan, 2008), Ref. 4: (Spieth and Blumenthal, 1985), Ref. 5: (Kamath et al., 2003), Ref. 6: (Murphy et al., 2003), Ref. 7: (Li et al., 2004), Ref. 8: (Wei et al., 2008). Ref. 9, 10 and 11: (Matanis et al., 2002; Schlager et al., 2010; Terenzio and Schiavo, 2010). Source: The SILAC ratios normalized were provided from a collaborator, Sebastian Wiese, Laboratory of Proteomics, Uni-Ulm.

7.4 Metabolic analysis of the mutants of *unc-50* in *C. elegans*

C. elegans metabolic processes are highly similar to humans in the process of biosynthesis and breakdown of dietary sources and energy storage as lipids (Table VI.1) (Braeckman et al., 2009; Szewczyk et al., 2003).

Based on the phenotypes by HPF-EM, unusual large lipid-droplets like vesicular structures are observed in the muscle cytoplasm in *unc-50* mutants that may indicate defects of metabolic processes. In order to see differential metabolic intermediates between wild type and *unc-50* mutants, the unbiased whole metabolome profiling was initiated in collaboration with the ZBSA Core Facility Metabolomics, Uni-Freiburg. As shown in Table VII.3, an initial metabolic profiling of *unc-50* mutants revealed that the levels of a total of 47 metabolites were significantly altered compared to wild type, in which the ratios of fold-change in order were present from large to small values. In the results, major metabolic changes in KEGG pathways indicated in carbohydrate/nucleotide/amino acid metabolisms that may be affected in *unc-50* mutants in *C. elegans*. In particular, we detected strong increase in the levels of phosphoenolpyruvate (PEP), urate, L-asparagine and alpha-trehalose whereas (S)-allantoin, sucrose, GMP, fumarate, anthranilate and xanthine were found to be decreased. This suggests that the catabolic pathway from urate to allantoin through urate oxidase

reaction may be blocked so that it causes increased urate and decreased allantoin in *unc-50* mutants. Therefore UNC-50 may affect in the urate metabolism.

KEGG_NAME	KEGG_ENTRY	Fold-change	SD	p value (t-test)
Phosphoenolpyruvate	C00074	2,94	0,19	0,03
Urate	C00366	2,47	0,10	0,00
L-Asparagine	C00152	1,84	0,11	0,01
alpha,alpha-Trehalose	C01083	1,74	0,06	0,00
L-Glutamine	C00064	1,45	0,15	0,05
L-Serine	C00065	1,28	0,06	0,01
Ethanolamine phosphate	C00346	1,21	0,10	0,04
sn-Glycerol 3-phosphate	C00093	0,92	0,05	0,03
Pantothenate	C00864	0,91	0,04	0,01
Guanosine	C00387	0,90	0,03	0,01
Citrate	C00158	0,90	0,05	0,04
Sedoheptulose 7-phosphate	C05382	0,88	0,05	0,00
Benzoate	C00180	0,87	0,06	0,05
D-Mannose 6-phosphate	C00275	0,86	0,03	0,00
beta-D-Glucose 6-phosphate	C01172	0,84	0,05	0,00
L-Isoleucine	C00407	0,84	0,03	0,05
(9Z)-Octadecenoic acid	C00712	0,83	0,04	0,00
L-Ornithine	C00077	0,81	0,05	0,01
L-Aspartate	C00049	0,80	0,03	0,00
Icosanoic acid	C06425	0,79	0,10	0,02
Octadecanoic acid	C01530	0,78	0,07	0,02
Nicotinate	C00253	0,78	0,10	0,04
Cadaverine	C01672	0,76	0,06	0,00
L-Methionine	C00073	0,75	0,11	0,02
(S)-Lactate	C00186	0,74	0,07	0,00
D-Ribose 5-phosphate	C00117	0,74	0,06	0,00
Diphosphate	C00013	0,74	0,04	0,01
beta-D-Fructose	C02336	0,74	0,03	0,00
AMP	C00020	0,74	0,03	0,00
L-Tyrosine	C00082	0,73	0,06	0,03
Hexadecanoic acid	C00249	0,72	0,06	0,00
6-Phospho-D-gluconate	C00345	0,71	0,05	0,00
Putrescine	C00134	0,70	0,06	0,00
Spermidine	C00315	0,70	0,14	0,02
Linoleate	C01595	0,70	0,09	0,00
(9Z,12Z,15Z)-Octadecatrienoic acid	C06427	0,69	0,07	0,00
D-Ribose	C00121	0,68	0,06	0,00
Pyruvate	C00022	0,64	0,07	0,00
Adenine	C00147	0,62	0,04	0,00
Hydroxylamine	C00192	0,62	0,12	0,03
Oxalate	C00209	0,62	0,10	0,00
Xanthine	C00385	0,60	0,05	0,00
Anthranilate	C00108	0,53	0,08	0,00
Fumarate	C00122	0,47	0,02	0,00
GMP	C00144	0,43	0,18	0,00
Sucrose	C00089	0,28	0,23	0,04
(S)(+)-Allantoin	C02350	0,02	0,00	0,00

Table VII.3 Metabolic profiles of *unc-50* mutants. *C. elegans unc-50* mutants were compared to wild type in a fold change, in which forty-seven compounds were observed to be significantly

changed. Source: Results sorted by the author were provided from Carsen Jäger in ZBSA, Core Facility of Metabolomics, Uni-Freiburg.

7.5 Functional expression of BirA* fusion proteins in *C. elegans* muscles

Protein-protein interactions in intracellular trafficking pathways are very dynamic as both core machinery components and cargo proteins interact only transiently during their transport. As described in Chapter I, UNC-50 may act as a trafficking regulator involved in the ARF-dependent retrograde membrane trafficking at the Golgi-endosomal interface. To identify UNC-50 interacting proteins in *C. elegans*, *in vivo* UNC-50 has to be tagged and purified with the interacting proteins. To reduce the number of non-specific contaminants, a high-affinity purification system has to be applied that is compatible with SILAC labeling for MS analysis.

For this purpose, we chose to use BioID, a proximity-dependent biotinylation method to identify interacting protein. We adopted this technique to isolate UNC-50 interaction partner and expressed the UNC-50::BirA* fusion protein specifically in the muscle cell of *unc-50 (x47)* mutant animals (Fig. VII.7).

To evaluate the functional expression of BirA* fusion proteins that were integrated into *unc-50 (x47) III* mutants, we investigated the worms by 1 mM levamisole plates, microscopy and biochemical assays to confirm whether they are functional endogenously in *C. elegans*. The strains of UNC-50::BirA* were shown to be paralyzed like wild type after two-hour on 1 mM levamisole plates, meaning that the *unc-50* mutants were rescued by the UNC-50::BirA* to be hyper-contracted on 1 mM levamisole plates (Fig. VII.8A). This suggests that the UNC-50::BirA* fusion construct is functional in *C. elegans*.

As control constructs, we tested BirA* to be soluble when fused to GFP and anti-GFP nanobody (α GFPNB), respectively, whether they are functionally expressed without any proteolytic processing. As shown in Figure VII.8B and B', GFP::BirA* were apparently expressed in the body wall muscles as shown by microscopy. At the C-terminus of α GFPNB containing c-myc-tags (1.2 kDa), the BirA* was fused to be endogenously expressed in *unc-50 (x47)* mutants. The α GFPNB::BirA* containing c-myc-tags was detected to be approximately ~ 50 kDa with α -c-myc by Western blots (Fig. VII.8C),

indicating that all the BirA* fusion proteins function properly in *C. elegans*. Any defects were not found in the strains of the BirA* fusion proteins integrated into *unc-50* (*x47*) mutants.

Figure VII.7 Schematic representation of BirA*-fusion proteins to be integrated into *unc-50* mutants. As proteins of interest UNC-50, GFP and anti-GFP nanobody (αGFPNB) that contains c-myc tags were subcloned into the expression vector pPD115.62. Transgenic worm lines induced by extrachromosomal arrays were subsequently integrated by gamma irradiation to be expressed endogenously in the worm strains *gls20*, *gls21* and *gls22*. Each clone of the plasmid constructs was confirmed by sequencing.

Figure VII.8 Functional expression of BirA*-fusion proteins in *C. elegans* muscles. Integrated worm strains *unc-50* (*x47*) III; *gls20* [*pmyo-3::unc-50::BirA**], *unc-50* (*x47*) III; *gls21* [*pmyo-3::GFP::BirA**] and *unc-50* (*x47*) III; *gls22* [*pmyo-3::αGFPNBmyc::BirA**] were

used in this study that were three-time outcrossed with N2 wild types. The *gzIs60* strains were tested on the 1 mM levalmisole plates for two hours. Thirty worms in total were 100% rescued by three independent experiments (A). The functional expression of GFP::BirA* into *unc-50* mutants was evaluated by microscopy (BZ-9000 BIOREVO by Keyence). The dashed boxes in order (B and B') indicate magnified images of the *gzIs21* expressed in the body wall muscles. Scale bars, 10 μ m. By using the α -c-MYC (200 ng/ml) antibodies, the protein size of the BirA* fused to the anti-GFP nanobodies containing c-myc tags (α GFPNB::BirA*, approx. ~ 50 kDa) was confirmed by Western blots. (BirA*, 35.5 kDa; α GFPNB, 12-15 kDa and c-MYC tags, 1.2 kDa) (C).

7.5 Workflow strategy of SILAC-BioID in *C. elegans*

UNC-50/GMH1 is a physical binding partner of ARF-GEFs GEA1/2 containing a conserved SEC7 domain in yeast, nematode and human (Chantalat et al., 2003; Eimer et al., 2007), indicating UNC-50/GMH1 is likely to be involved in the intracellular trafficking as ARF-GEF-dependent manners that may undergo highly dynamic and transient protein interactions in the intracellular compartments.

To identify the interaction proteins of UNC-50, it was initially labeled by BirA* (Fig. VII.7). Since all the BirA*-fusion proteins were functional *in vivo* as shown in the section of VII.5, we designed the proteomic experiments MS-based in *C. elegans* for finding specific interaction proteins of UNC-50 by using metabolic heavy/light-isotope labeled essential amino acids (SILAC) combined with BioID. As described previously, the BioID is based on promiscuous biotinylated-protein identification to be subsequently purified in affinity isolation of specific proteins with streptavidin-coated matrixes prior to be identified by MS analysis (Fig. VII.9). In the process, the lines expressing BirA* fusion proteins that have been confirmed to be endogenously functional *in vivo* (Fig. VII.8) *gzIs20* (UNC-50::BirA*), *gzIs21* (GFP::BirA*) and *gzIs22* (α GFPNB::BirA*) were SILAC labeling via feeding SLE1 bacteria (50-fold, OD_{600nm} of 1) containing 50 μ M biotin (final conc.) in each light-(Lys0, Arg0) and heavy-(Lys8, Arg10) SILAC plate (Fig. VIII.9).

To validate whether endogenous biotinylated proteins are detected in *C. elegans*, whole worm lysates were analyzed by streptavidin-conjugated horseradish peroxidase (HRP) blots (10 minutes exposure, 250 ng/ml, Thermo scientific) (Fig. VII.10). As shown in the

controls (lanes 1 and 2), endogenously biotinylated proteins that do not have any BirA*-fusion proteins in worm strains were revealed to be approximately 80 kDa in size in *C. elegans*, which is similar to the endogenously biotinylated proteins in humans (80.8 kDa) and yeast (76.4 kDa) as a monomeric size (Chapman-Smith and Cronan, 1999; Rhee et al., 2013). The worm lysates of SILAC labeling with biotin supplements during the growth were shown to be strongly biotinylated by streptavidin-HRP blotting, suggesting that the SILAC strains containing BirA*-fusion proteins with 50 μ M biotin were endogenously biotinylated in the interacting proteins of the proteins of interest (UNC-50, GFP and α GFPNB) as a proximity-dependent manner. Taken together, the results suggest that UNC-50::BirA fusion protein biotinylates *in vivo* and can then be used for BioID experiments.

Figure VII.9 Schematic workflow of SILAC-BioID in *C. elegans*. Experimental processes of SILAC-combined affinity-pulldown experiments are to identify novel UNC-50-binding proteins by high-affinity biotinylated proteins captures using streptavidin-coated magnetic beads in pulling conditions, which can be divided into five steps. In STEP1, the worm strains *gzIs20* (UNC-50::BirA*), *gzIs21* (GFP::BirA*) and *gzIs22* (αGFPNB::BirA*) used in this study were labeled by SILAC via feeding SLE1 bacteria to be fully incorporated isotopes into the proteomes. Based on the metabolic incorporation experiments in the section of VII.2, the worms are subjected to maintain in the SILAC plates at least until F5-generation or more. Harvesting worm populations

each by each before frozen worms were done with ice-cold-PBS containing protease inhibitors (Appendix) and extracted proteins according to the described previously were combined to be equal amounts of each in STEP2 (Roux et al., 2012). The test-SILAC [UNC-50(L):GFP(H)] and control-SILAC [α GFPNB(L):GFP(H)] in STEP3 were immunoprecipitated by affinity-pulldown isolation steps using streptavidin-coated magnetic beads (Dynabeads MyOne C1, Invitrogen). Triplicated test-SILAC samples and control-SILAC were subjected to 10% SDS-PAGE in STEP4 and then samples were treated to be MS analysis that was conducted by collaboration in this study. STEP1-4 were done by the author and STEP5* is ongoing in collaboration with Sebastian Wiese, Laboratory of proteomics, Uni-Ulm.

Figure VII.10 Analysis of the endogenously biotinylated proteins in *C. elegans*. Whole cell worm lysates were analyzed to confirm endogenous biotinylated proteins in controls (lane 1, wild type; lane 2, *unc-50* (x47) mutant), endogenous BirA*-fusion protein-containing worms (lane 3, *gzIs21* (*unc-50* (x47) III; *gzIs21* [*pmyo-3::GFP::BirA**]); lane 4, *gzIs22* (*unc-50* (x47) III; *gzIs22* [*pmyo-3:: α GFPNBmyc::BirA**]) and SILAC labeling worms of endogenous BirA*-fusion proteins in 50 μ M biotin plates (lane 5, *gzIs20* (*unc-50* (x47) III; *gzIs20* [*pmyo-3::unc-50::BirA**]); lane 6, (*unc-50* (x47) III; *gzIs22* [*pmyo-3:: α GFPNBmyc::BirA**])). Arrowheads indicate endogenously biotinylated proteins (approx. 80 kDa and 120 kDa) present in all worm lysates. The worm lysates (lane 3) containing BirA*-fusion proteins without treatment of 50 μ M biotin are shown to be similar blots compared to the controls (lanes 1 and 2) and detected in the extensively modified molecular sizes that seems to be endogenously biotinylated in lane 4. In the

SILAC labeling samples (lanes 5 and 6) with 50 μ m biotin, lots of biotinylated proteins are shown in the streptavidin-HRP blots, 10 minutes (min) exposures.

7.6 Functional specificity of the GFP nanobodies in the intracellular compartment via spatially labeling of the lumen and cytosol in *C. elegans* muscles

Based on the matters that are associated with the generation of fusion protein, we wanted to determine whether an anti-GFP nanobody (α GFPNB)::BirA fusion protein could be used as a universal *in vivo* affinity reagent to be used with GFP-tagged target protein for BioID applications. First, we tested the functionality of the intracellular detection of GFP fusion protein by the α GFPNB *in vivo*. For this purpose, we coexpressed GFP-fusion protein and α GFPNB::tagRFPT fusion construct in order to confirm whether the GFP fusion protein could be detected with α GFPNB::tagRFPT fusion construct in *C. elegans* (VII.11). The GFP-fusion proteins were targeted to different subcellular compartment such as the ER, Golgi (Fig. VII.12), Mitochondria (outer and matrix) (Fig. VII.13), Nucleus and Synapse at NMJs (Fig. VII.14). Furthermore, we also tested GFP-fusion proteins where the GFP-tag was placed within the lumen at the ER or Golgi.

The anti-GFP Nanobodies (12 – 15 kDa) are a small antibody fragment (2.5 nm in diameter and 4.2 nm in length) as a single variable domain known as heavy chain-only antibodies of *Camelidae* family (Chakravarty et al., 2014; Kubala et al., 2010; Rothbauer et al., 2008; Rothbauer et al., 2006). The antigen GFP binding specificity of the single-domain nanobodies (α GFPNB) was reported as chromobodies and/or GFP-nanotrap complexes that can be stably expressed in living cells (Rothbauer et al., 2008; Rothbauer et al., 2006). With this, studying the α GFPNB complexes based on the structural binding analysis revealed that the conformation/manipulation of the complexes can be monitored in protein expression of subcellular localization as well as translocation of proteins in living cells, that led us to study the functional-spatial restriction of the α GFPNB in the complex with the antigen GFP in the intracellular compartments in the living organism, *C. elegans* (Kirchhofer et al., 2010; Rothbauer et al., 2008; Rothbauer et al., 2006; Schornack et al., 2009).

For this purpose, the constructs of the α GFPNB were subcloned into the expression vector pPD115.62 (Appendix). The α GFPNB was fused to the tagRFpT, a photostable red-fluorescent protein (Fig. VII.10), to be tested with the GFP/YFP/CFP fusion proteins labeled at the N- or C-terminus of the organelle specific proteins. We tested whether the complex conformation of GFP- α GFPNB are affected in functional-spatial restriction by the membrane-barrier of the subcellular compartments (Fig. VII.12, 13 and 14).

Figure VII.11 Schematic representation of soluble α GFPNB::tagRFpT and GFP-fusion organelle specific proteins. In order to be co-expressed in *C. elegans* muscles, we constructed α GFPNB::tagRFpT and GFP-fusion organelle specific proteins to test whether the complexes are functional to be colocalized through the organelle membrane barriers (A). In this study, we used the fluorescent-fusion organelle markers in either cytosolic- and/or lumen/cellular-faced domain structures. For example, an organelle marker as a type II of transmembrane shows GFP-labeled at the end of N-terminus targeted to the cytosol while the C-terminus of the protein faces to the ER lumen (B). Source: crystallization structures of the GFP- α GFPNB complexes were taken from the reference (Kubala et al., 2010).

Figure VII.12 Specificity of the α GFPNB to the GFP fusion proteins in the Golgi and ER. Schematic topologies of the organelle proteins which were fused to GFP or YFP in either cytosolic and/or luminal compartments are shown. The confocal images of the fusion proteins and α GFPNB are indicated to be functional, but spatially restricted in the lumen. The C-terminal lumen fusion proteins UNC-50, α -Mannosidase II and Cytochrome b5 (cb5) did not colocalize with α GFPNB::tagRFPT (A,B and D), whereas the N-terminal cytosolic-fusion protein TRAM1 TRAM1 was shown to be colocalized as the complex GFP:: α GFPNB::tagRFPT (C). White boxes were magnified at the bottom of each image. Scale bars, 10 μ m.

Figure VII.13 Specificity of the α GFPNB to the GFP/CFP fusion proteins in the mitochondria. As a control, α GFPNB::tagRFpt was expressed alone in *C. elegans* muscles, which appeared weak signal-intensities due to no antigen fusion proteins in cytosol (A). Schematic topologies of the mitochondria proteins TOM-70 and matrix-targeting signals using the stable transgenic line *zcls14* [*pmyo-3::GFP (mito-matrix)*] are depicted in (B) and (C). As shown in confocal images, the α GFPNB::tagRFpt was shown to be the same images of the TOM-70::CFP, indicating the α GFPNB::tagRFpt was physically binding to the mitochondria outer-

membrane fusion protein TOM-70::CFP in the cytosol (B). Mitochondria (mito) matrix-targeting import-signals (mito-matrix) are synthesized in cytosol and transferred into the matrix by cytosolic chaperones through the translocase machinery of TOM-TIM in the outer membrane (OM) and the inner membrane (IM), respectively (Tokatlidis et al., 2000; Wiedemann et al., 2004). The translocation of the cytosolic mito-matrix was normally shown in the GFP fusion proteins (GFP::mito-matrix), but not in the α GFPNB::tagRFPt (E) that was mostly cytosolic. In the merged image (C), the mito-matrix was shown clearly not to be colocalized each other. White boxes were magnified at the bottom of each image. Scale bars, 10 μ m.

Figure VII.14 Specificity of the α GFPNB to the GFP fusion proteins in the nucleus and NMJs. Schematic topologies of the fusion proteins nuclear localization signals (NLS) and a loop β -subunit of GABA_A receptors encoded by *unc-49* in synapses at NMJs. In this study, we used the stable transgenic worm lines *ccIs4251 I/ sjEx [N2; InJ64 (pmyo-3:: α GFPNBmyc::tagRFpt)]* (A) and *oxIs22 II; sjEx [N2; InJ64 (pmyo-3:: α GFPNBmyc::tagRFpt)]* (B). The cytosolic NLS fusion proteins were shown to be colocalized with the α GFPNB::tagRFpt in the nucleus, indicating that the translocation of the NLS from the cytosol into the nucleus was functionally processed by nuclear transport such as importins that strongly binds to the NLS signals. The images of the dashed white boxes indicate magnified images of nucleus colocalized in muscles (A). At the synaptic area, a loop β -subunit of GABA_A receptors was not detected by the α GFPNB::tagRFpt that was shown in the entire cytosol of muscle cells and segregated between the muscles (red) and receptors (green) expressed at the NMJs (B). White boxes were magnified at the bottom of each image. Scale bars, 20 μ m (A) and 10 μ m (B).

Taken together, the data indicate that the soluble α GFPNB complexes were functionally recruited to the GFP/CFP/YFP fusion proteins. As long as the GFP-tag is accessible from the cytosol, the α GFPNB-BirA* fusion protein should be able to be used as an affinity reagent for BioID. Except the NLS with the α GFPNB::tagRFpt that was transported into and be colocalized in the nucleus, the α GFPNB::tagRFpt was not able to bind GFP fusion proteins in the lumen of Golgi and ER, the translocation of mitochondria matrix-targeting signals into the matrix and a loop β -subunit of GABA_A receptor at the NMJs in *C. elegans*. This suggests that the highly soluble α GFPNB complexes were spatially restricted to interact the antigen GFP fusion proteins in the lumen by the membrane barriers of the intracellular organelles ER-Golgi-endosomes.

VIII. DISCUSSION

8.1 Metabolic SILAC labeling of proteins relies on feeding periods and concentration of the bacteria SLE1 in *C. elegans*

Intracellular protein trafficking requires highly dynamic interactions among proteins that occur differential abundance of proteome in certain internal/external biological conditions. To reveal the quantification and identification of the proteins in living cells, the metabolic isotope labeling by SILAC into the proteome is required for accurate quantitative analysis of differential proteomes based on mass spectrometry. *In vivo* metabolic labeling had held a new era in studying proteomics in versatile research fields in living cells and have been extended to apply complex living organisms (Findlay et al., 2008; Fredens et al., 2011; Gruhler et al., 2005; Krijgsveld et al., 2003; Kruger et al., 2008; Lahm and Langen, 2000; Larance et al., 2011; Oda et al., 1999; Ong et al., 2002).

Applying SILAC labeling ($^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine and $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine) into the nematodes can be difficult due to incomplete incorporation of heavy amino acids into the whole proteomes in living organisms and arginine-to-proline conversion as well by proline biosynthetic pathways in *C. elegans*. By using a stable SILAC labeling method for *C. elegans* developed by the group of Lamond (Larance et al., 2011), the proline conversion can be negated by feeding *E. coli* SLE1 the ornithine transaminase encoded by *orn-1* targeted RNAi feeding vector-containing strains during the growth in SILAC *in vivo* labeling processes (Larance et al., 2011). In this study, the metabolic isotope labeling by SILAC for accurate quantitative proteomics by referring the two experiments was established and the rates of metabolic incorporation were evaluated in several phased generations of *C. elegans* via feeding bacteria SLE1 (Fredens et al., 2011; Larance et al., 2011). As the results, we found that fully stable incorporation of the isotopes into proteomes was affected in feeding periods to be near-complete incorporation ($\pm 93\%$) that should be fed at least until F5-generation or more and be considered about the bacteria concentration (50-fold, OD₆₀₀ of 1) for efficient *in vivo* labeling in *C. elegans* (Fig. VII.2). Why is the labeling efficiency not 100%? In principle, it should be labeled with heavy isotopes to 100% but in practice the commercial sources ($^{13}\text{C}_6$, $^{15}\text{N}_2$,

Cambridge Isotope Laboratories) assure isotope enrichment between 95% and 98% in cell cultures according to the group of Matthias Mann. In the case of *C. elegans* as a living organism, the labeling efficiency of SILAC was reported to ~93% (Lys8, Arg10, 200) and ~98% (Lys8) by the commercial isotope (Fredens et al., 2011; Larance et al., 2011). Based on the two references, we found the difference in molar amounts of both light and heavy-isotope (labeling efficiency of ~93% from 0.2 mM and ~98% from 0.4 mM), suggesting that in order to complete incorporation in *C. elegans* the isotope enrichment may affect the labeling efficiency in practice. In addition, Arg-to-Pro conversion through the biosynthetic pathway occurs in *C. elegans* unlike bacteria. As such, metabolic labeling of proteins in *C. elegans* should be applied in both light- and heavy-isotope to be normalized prior to mass ratio analysis on the peptide levels (Larance et al., 2011).

As an alternative way to incorporate isotope labeling instead of SILAC, the heavy nitrogen (^{15}N) can be used in metabolic labeling to avoid the arginine-to-proline conversion in *C. elegans* (Gouw et al., 2010; Gouw et al., 2011; Krijgsveld et al., 2003). However, different amino acids have various numbers of the nitrogen atoms, unlike SILAC that labels the isotopes by essential amino acids as defined numbers in living cells, which makes ambiguous interpretation in the results of MS spectra that would miss identification of crucial peptides in the end (Ong et al., 2002).

In the case of the SILAC via the RNAi feeding vector-containing bacteria that could be reduced efficiency in knockdown by incomplete RNAi in the neurons of *C. elegans* as reported previously (Calixto et al., 2010), the gene targeted-RNAi would be affected in other protein expression compared to the control RNAi samples even though it appeared to be minimized (Larance et al., 2011). However, in SILAC labeling, the treatment of the RNAi via feeding bacteria should be applied in both heavy- and light-isotopes for executing control-SILAC and test-SILAC. Equal treatment of samples could consequently be negated in ratios and be considered as experimental contaminants in peptide pools identified, in which the proteome profiling results to be verified would also be normalized by sample replicates and reciprocal labeling arrangements (Larance et al., 2011).

Therefore, using SILAC labeling, quantifying and identifying protein-protein interactions of transient proteins involved in intracellular dynamic trafficking would be a proper application in studying proteomics as well as our findings in metabolic incorporates of isotopes into the proteins would be fundamentally guide to the further applications based on various manipulations of neurogenetics in *C. elegans*.

8.2 *unc-50* mutants lead to protein abundance involved in the intracellular transport

We have optimized MS-based quantitative proteomics by SILAC-isotope labeling into proteomes to investigate accurate protein quantification and identification in the functional organism *C. elegans*, in which *unc-50* mutants lead to quantitative protein abundance in anterograde-promoting trafficking factors and actin-myosin cytoskeleton-related scaffold factors that were significantly shown to be up-regulated at least in one replicate (Table VII.1B).

It is likely that the absence of the retrograde trafficking factor UNC-50 may cause disturbance in tight regulation that results in promoting endosome-mediated membrane dynamic factors that include GPB-1, UNC-18 and VPS-32.1 shown to be up-regulated in this study (McEwen and Kaplan, 2008; Thyagarajan et al., 2011), supporting that UNC-50 localized at the Golgi-endosomal interface may be right place to regulate as a trafficking factor.

In addition, quantitative protein abundance of ACT-2 and NMY-2 in *unc-50* mutants would be critical evidence to be involved in actin-myosin cytoskeleton motility as a trafficking regulator, supporting that the ARF-GEFs GEA1/2, a physical interacting partner of UNC-50/GMH1, are required to maintain cell polarity and the organization of the actin cytoskeleton in previous studies (Zakrzewska et al., 2003). Moreover, a weak physical interaction between GMH1 and Rho4 GTPase known as a regulator in the interaction of BNR1-HOF1 involved in cytoskeleton organization was reported by the group of Peyroche (Chantalat et al., 2003). Consequently, UNC-50 is likely to be involved in actin-myosin cytoskeleton motility through the ARF-GEFs-dependent trafficking at the Golgi-endosomal interface.

As shown to be up regulated proteins in *unc-50* mutants, a highly conserved histone transport protein NASP-2 in both replicates (Nabeel-Shah et al., 2014), a component of chromatin H2B1, and PBRM-1, a transcriptional regulator by chromatin remodeling, are also consistent with the study of the mammalian UNC-50 called UNCL that has been suggested as an inner nuclear membrane protein (INM) such as lamina-associated polypeptide-2 (LAP-2) that binds directly to chromatin, but not involving direct interaction with UNCL (Fitzgerald et al., 2000).

Based on the fold changes by SILAC ratios in *unc-50* mutants, there are two-pair quantification results in transport factors. The anterograde and retrograde promoting factors were detected to be up and/or down-regulated. At the same time, the cytoskeleton-related motility actin-myosin factors *versus* dynein-mediated microtubule factors appeared to be up and down-regulated (Table VII.1). In particular, the BICD-1, which is required for the retrograde migration of Rab6-positive vesicles to the pericentrosomes and the Golgi-to-ER as a dynein-mediated transport along microtubules, was shown in both replicates to be down-regulated, suggesting that the retrograde secretory vesicles could be altered by the lack of the retrograde factors such as BICD-1 and UNC-50 (Matanis et al., 2002; Schlager et al., 2010; Terenzio and Schiavo, 2010).

As the other findings, the secretory yolk proteins VIT-3 and VIT-5 as lipid transport proteins that are secreted into the body cavity from the intestine and then taken up by oocytes of *C. elegans* gonad were quantitatively down-regulated in *unc-50* mutants (Table VII.1D) (Kamath et al., 2003; Spieth and Blumenthal, 1985). As the corresponding results as initially observed, *unc-50* mutants may cause secretion defects of MIG-17 from the muscles into the body cavity for distal tip cell migrations of gonads as well as a secreted signal sequence tagged-GFP (ssGFP) was observed to be trapped in the body wall muscles in the absence of UNC-50. In addition, the morphological analysis of the body wall muscles by HPF-EM in Chapter I revealed that the absence of UNC-50 causes unusual large vesicles (lipid-like) near mitochondria in the muscle belly. These results suggest that the lipid metabolisms in the *unc-50* mutants might be unbalanced by the lipid binding precursors VIT-3 and VIT-5 that were shown to be down-regulated.

Based on the differential quantification of proteins, the fundamental role of UNC-50 is likely to be largely involved in transport, which could support all the results of Chapter I.

As a conclusion, *unc-50* mutants lead to enhanced forward trafficking dynamic by differential quantitative proteins up- and down-regulated in the intracellular transport.

8.3 Specificity of the anti-GFP nanobodies based on the protein structure

To identify binding partners of the transient protein UNC-50, we adapted rising proteomic technologies that provide accurate, stable and *in vivo* functional information. We found that the biotin protein ligase (BirA*, R118G) to apply SILAC-BioID for affinity purification prior to MS was functional for promiscuous *in vivo* labeling of UNC-50 in all three strains *gzIs20* (UNC-50::BirA*), *gzIs21* (GFP::BirA*) and *gzIs22* (α GFPNB::BirA*) in *C. elegans*. From this, we revealed that endogenously biotinylated proteins in *C. elegans* were about 80 kDa in size to be similar in yeast and humans, which would be informed and applied in functional studies including SILAC and affinity purifications in *C. elegans*.

As the strains α GFPNB and GFP that can be used in control-SILAC (Fig. VII.12), the α GFPNB complexes were evaluated in organelle specific markers (ER, Golgi, mitochondria, nucleus, and synapses of neuromuscular junctions) with GFP/YFP/CFP fusion proteins in *C. elegans*. We showed that highly soluble α GFPNB was specifically targeted into the organelle fusion proteins GFP/YFP/CFP using the form of α GFPNB::tagRFpt (2.5 nm, < 40 kDa) in the cytoplasm (Rothbauer et al., 2008; Rothbauer et al., 2006), however the α GFPNB complexes was spatially restricted in the lumen by membrane barriers between the fusion proteins and α GFPNB::tagRFpt (Fig. VIII.1).

Figure VIII.1 Schematic specificity of α GFPNB complexes in the lumen and cytosol. The α GFPNB complexes can be functionally used in *C. elegans*. According to the labeling structure of the fusion proteins, it can be binding to the antigen GFP in the cytoplasm (A), whereas the GFP fusion proteins buried in the lumen are not conjugated with α GFPNB complexes by the membrane barrier (B).

In order to test whether translocation of mitochondria (mito) matrix-targeting import-signals (mito-matrix) from the cytosol into the matrix of mitochondria can be through to be functional by using the α GFPNB complexes, the GFP fusion protein of the mito-matrix signals was investigated. In this case, only the GFP fusion-mito signals were normally imported into the matrix but not with the α GFPNB complexes. Whereas the α GFPNB complexes with the TOM70 fusion protein as a cytosolic targeting tail in the outer membrane of mitochondria and NLS into the nucleus from the cytosol as the complexes with α GFPNB were shown to be functional and localized in *C. elegans*. In the NMJs, the α GFPNB complexes were mostly segregated into the muscle and a loop β -subunit of GABA_A receptors at synapses (Fig. VII.11), indicating that the GFP fusion proteins and α GFPNB complexes may be spatially and structurally restricted by intracellular membrane barriers.

These practical examples for functional labeling with the intracellular organelle proteins, translocation and synapses in *C. elegans* will provide possibilities of the structural manipulations to be functional and the fusion proteins with the α GFPNB complexes can also be used versatile applications in combination with biochemical, proteomic and functional studies as an endogenous epitope in living cells.

IX. MATERIALS AND METHODS

9.1 Materials and Reagents

All materials, reagents, oligonucleotides, plasmids, research equipments and strains (bacterial *E. coli*, yeast *S. cerevisiae* and nematode *C. elegans* strains) are listed and described in XI. Appendix.

9.2 Methods in Molecular biology

9.2.1 Maintenance of bacterial strains

All *E. coli* strains used in this study are listed in Table XI.1.1 with description. A single colony grown on a fresh LB-agar solid medium containing antibiotics if necessary was selected and overnight cultured in sterile LB medium with antibiotics (Appendix) at 37°C on a shaking incubator. A colony was streaked out on a fresh plate every other week.

9.2.2 Molecular biology: PCR, Cloning and Transformation

All constructs, primers for Polymerase Chain Reaction (PCR) and restriction enzymes used were described in the Appendix. Interested genes in *C. elegans* were amplified in genomic DNA and cDNA library (ProQuest, Invitrogen and/or RB1/2/3). PCR was conducted with a standard protocol as follows: if total volume is 25 µl, the PCR mixture contains 2.5 µl of 10x PCR buffer (Stratagene, Invitrogen or Roche), 1 µl of each primer [10 µM], 0.5 µl dNTPs [12.5 mM], 0.5 µl of Polymerase (Stratagene, Invitrogen or Roche for cloning and pTC for genotyping), DNA conc. 20 - 70 ng as a template and double distilled H₂O (ddH₂O) for the rest up to the total volume. A standard parameter of thermocycler was used for reactions as follows: 95°C for 2 minutes; 35 cycles [95°C for 20 seconds to (x)°C for 20 seconds to 72°C for (x) minutes]; 72°C for 3 minutes; 4°C. The value of x was dependent on each primer pair used for PCR reaction. Otherwise the conditions used in this study were shown in Table XI.2.2 and 2.3 (Appendix). PCR

products were confirmed on 1% agarose gels (Table XI.5.1).

Molecular cloning was performed using classical techniques (Sambrook and Russell, 2001). In this study, restricted digestion and ligation of constructs as a typical cloning strategy were performed with restriction enzymes, T4 DNA ligase and proper buffers as manufacturer's instructions (Fermentas and New England Biolabs). As for the enzyme digestion, total 20 µl of volume contains 2 µl of 10x digestion buffer, 0.5 µl of each restriction enzyme, 1 µg of DNA and ddH₂O for the rest volume. The reaction of digestion was kept at 37°C for 3 - 4 hours. Then after, the reaction was confirmed on 1% agarose gels with 1x TBE buffer (Appendix). The ligation reaction of the fragments digested by restriction enzymes was carried out in total 20 µl volume containing 2 µl of 10x ligation buffer, 1 µl of T4 ligase, 40 ng DNA (1:3 ratios of vector and insert) and ddH₂O for the rest volume. It was reacted at 4°C for overnight and transformed into 100 µl of DH5α competent cells by using heat-shock transformation on a heat-block at 42°C for 45 seconds and put it back on ice for 5 minutes. The transformed cells were recovered in 900 µl of LB medium (Appendix) and incubated on a shaker at 37°C. After 45 - 60 minutes, the cells were collected and plated on LB-agar plates containing ampicillin (Appendix). A single colony of the plate overnight-incubated at 37°C was selected and inoculated in 5 ml LB media containing ampicillin for overnight. Plasmid DNAs were isolated by Mini-prep kit (Invitrogen) as manufacturer's instructions. Positive clones confirmed by restriction enzymes were verified by sequencing.

9.2.3 Agarose gel electrophoresis

Amplified PCR reactions and digested plasmids to be verified were analyzed in agarose gel electrophoresis. 1 % of ethidium bromide was added 0.1 µl/ml into the melted agarose in 1x TBE buffer (Appendix) by microwave and poured on the gel caster for electrophoresis. PCR products and the reaction samples after enzyme digestion were size-analyzed on 1% agarose gels with TBE buffer. Before loading on gel pockets, the samples were mixed with 6x DNA loading buffer (Fermentas) and separated on gel in 1x TBE buffer with 1 kb DNA ladder (Fermentas) at 130 V for 40 minutes. In order to do restriction digestion and ligation as a cloning strategy, corresponding bands were

extracted by cut under ultraviolet light and purified using commercial gel extraction kits (Appendix).

9.2.4 Sequencing

All genes in plasmid constructs whether they have correctly sequenced as designed in this study were confirmed by sequencing company (GATC Biotech) before use or after the cloning. Sequencing primers and oligonucleotides used were placed in the Appendix.

9.3 Methods used in the nematode *Caenorhabditis elegans*

9.3.1 Maintenance of strains

All strains used in *C. elegans* were maintained at 20°C on standard nematode growth medium (NGM) plates seeded with *E. coli* OP50 as described previously (Brenner, 1974). All mutant strains were outcrossed at least three times before use. Some nematode strains used in this study were provided by the CGC, which is funded by the NIH NCRR. *arfrp1* (*tm2779*) was supplied by the NBRP (Tokyo Women's Medical University School of Medicine). *unc-50* (*ok1847*) was provided by Gene Knockout Project at Oklahoma Medical Research Foundation (OMRF), which is part of the international *C. elegans* Gene Knockout Consortium.

9.3.2 Crossing worms

To select homozygous mutants [e.g. *unc-50* (*x47*)], the worms were outcrossed with males of a marker strain *ccIs4251* [*pmyo-3::nls-gfp*; *pmyo-3::mt-gfp*]; *him-8* (*e1489*) (Fire et al., 1998) and creating deletion mutants of stable integration lines [e.g. *pWIs50* [*lmp-1::GFP*] (Treusch et al., 2004) and *arIs37* [*pmyo-3::ssGFP*] (Fares and Greenwald, 2001)] was used by crossing steps between the worms as follows. The males of 10 – 15 worms were put together with hermaphrodites of 3 – 4 young adults on bacteria OP50 seeded NGM plates. After 48 hours, all adults were removed out of the plate and their F1 progeny were isolated on a single plate for laying eggs of F2 progeny. Corresponding markers, fluorescent-expressed or drug resistance phenotypes, of the progeny were tested

to isolate homozygous F2 generation, which were confirmed by microscopy, worm PCR and chemical compound sensitivity for isolating homozygous worms.

9.3.3 Worm PCR templates

From a single to tens animals, worm strains were lysed in worm lysis buffer containing proteinase K (Appendix) before freezing at -80°C for 20 minutes. The frozen worm body was initially ice crystalized in the lysis buffer that allows to be lysate and digest proteins by proteinase K at 65°C for 1 hour; 95°C for 20 minutes to inactivate proteinase K for PCR reaction with DNA polymerase. The final worm lysate was used as a template for Worm PCR to confirm the gene-depleted homozygous or heterozygous strains through the PCR steps that has been previously described in IX.2.2. The reaction of worm PCR was verified on 1% agarose gels. All the primers deletion alleles used in this study were listed in the Appendix.

9.3.4 Molecular Cloning Strategy

In this study, all cloned genes and constructs that were amplified from a cDNA library were tabulated in the Appendix. The amplified genes by PCR were cloned by classical cloning methods as previously described in IX.2.2 into *pBluescript SKII* (Stratagene) and/or *pGEMT* cloning vector (Promega). To exam the expression pattern of organelle specific markers (e.g. APT-9 at *trans*-Golgi networks) in body wall muscle cells, the genes were subcloned with a corresponding construct by matching restriction enzyme sites. As a backbone plasmid in this study, *pPD115.62* (*Pmyo-3::gfp*) was used for expression of muscle cells. Fluorescent-labeled genes used in this study were subcloned as pairs of *AgeI/NotI* fragment, *AgeI/XhoI* fragment and *NotI/BamHI* fragment with GFP, mCherry, mYFP, TagRFpt and TagRFP into *pPD115.62* vector bone containing the *myo-3* promoter. All genes cloned and subcloned into the destined vectors in this study were described in the Appendix.

9.3.5 Transgenic lines and chromosomal integrated strains

The transgenic worm lines performed by microinjection methods were typically generated as previously described (Mello and Fire, 1995). Specific cells and tissues in

genetic mosaics can be observed to get insight the cellular specificity of induced genes by microinjection into gonads in *C. elegans*. The mutants of *unc-50* that have a levamisole-resistant phenotype were tested to rescue worms by inducing *unc-50* that has been fused with a fluorescent or enzymatic indicator in muscle cells. Because, in genetic mosaics, a single fertilized egg after microinjection also goes through typical cell division such as meiosis and mitosis by which different cells carrying extrachromosomal precursors have different genotypes in an individual worm (Sulston et al., 1983). Thus, targeted muscle cells for studying the intracellular phenotypes of gene function can be analyzed by microscopy and functional confirmation (e.g. levamisole resistance) of the interest protein through transgenic worms. In order to generate transgenic lines, young adult hermaphrodites were used for microinjection, and heritable transgenic lines were obtained from F2 offspring that contains visual positive markers. In this study, *pttx-3::gfp* and *pRF4 (rol-6 (su1006))* were used as co-injection markers in 20 ng/μl and 40 ng/μl, respectively and the total concentration of DNA mixtures were added up to 100 ng/μl by implementing *pBlueScript SKII*. All the transgenic lines by microinjection in this study were also listed in the Appendix. The *unc-50::birA* [InJ60]*, *GFP::birA* [InJ61]* and *GFPNBmyc::birA* [InJ62]* extrachromosomal arrays, respective *gzIs20*, *gzIs21* and *gzIs22* were chromosomally integrated by gamma irradiation using a ¹³⁷Cs source (RX30/50, Gravatorm Industries). The integrated strains were outcrossed three times with N2 males.

9.3.6 Movement assays

In order to confirm functional rescue in transgenic animals, the locomotion behavior of worms were observed on the non-seeded *E. coli* OP50 NGM plate. For this assay, young worms non-starved were transferred and initially adjusted on the plate for 60 minutes. The sinusoidal wave along the worm body (corresponding to a whole 360° sine wave) was counted over a 1 or 3-minute time interval and twenty to forty worms were analyzed.

9.3.7 Pharmacological assays

Cholinergic neurotransmission in *C. elegans* can be analyzed with pharmacological materials, levamisole and aldicarb (Sigma Aldrich). In order to identify homozygous *unc-50* mutants, worms were analyzed on bacteria OP50 seeded-plates containing 1 mM levamisole as described previously (Lewis et al., 1980). Ten to thirty worms per strain were transferred onto the levamisole (Lev) plates and assessed by knocking the plate or touching the head to confirm paralyzed worms. After one hour of exposure, the percentage of paralyzed worms was quantified or just confirmed by their paralyzed phenotypes on the Lev plate. Drug aldicarb resistance was also observed to test cholinergic transmission in *unc-50* mutants. The plates containing 2 mM aldicarb were prepared and conducted as previously described (Mahoney et al., 2006). The worm response on the plate of aldicarb was analyzed and quantified at the indicated time points.

9.3.8 TR-BSA endocytosis assays

Texas-red conjugated to bovine serum albumin (TR-BSA, 1 mg/ml) was used for analyzing endocytosis dynamics as a bulk fluid-phase internalization marker within coelomocytes of young adults which were crossed with the integrated strain *bIs34 [prme-8::rme-8-GFP; rol-6 (su1006)]* expressing GFP fusions of RME-8 as a positive endosome marker in both wild type and *unc-50 (x47)* mutants. In order to see the liquid flowing from pseudocoelomic body cavity into coelomocytes, it was injected into the body cavity in the pharyngeal region as described previously (Zhang et al., 2001). As the time points 10, 30 and 50 minutes after injecting at least into five worms per each time, the uptake of TR-BSA was observed by confocal microscopy and the images taken by line-averaged at the middle of the cells were analyzed using ImageJ software (National Institute of Health, USA).

9.3.9 Confocal microscopy and imaging

All microscope models used in this study are listed in Table XI.6.1. To analyze the expression of various proteins on body wall muscles of young adults *C. elegans*, inverted confocal microscopy were applied for analysis of immobilized worms with 50 mM Sodium azide on 2% agarose pads. The images were observed and taken using 63x objective with oil immersion at 20°C. Stack images were obtained for maximum intensity

projections by operating the integrated confocal softwares (SP2-Leica in ENI, Göttingen, SP8-Leica in ZBSA, Freiburg and LSM510-Zeiss in ZBSA, Freiburg). By using confocal microscopy, sub-cellular localization and co-localization of proteins were assessed with intracellular organelle specific markers labeled-fluorescence co-expressed in body wall muscles. In order to analyze the relative quantification, the maximum intensity projections were taken by the confocal software (SP series, Leica) and adjusted by threshold to be quantified using ImageJ software (National Institute of Health, USA). Measured values were normalized to wild type and the relative quantification difference was decided by p-value less than 0.05, Student's *t* test. The imaging of bright-field observation was done with objective lenses (2x to 100x) of inverted fluorescence phase-contrast microscope BZ-9000 (Keyence).

9.3.10 Western blots

Worms washed in M9 buffer or PBS (Appendix) were pelleted prior to be frozen and lysed by sonicator in homogenization buffer (20 mM HEPES pH 7.4; 10 mM KCl; 1 mM EDTA; 400 μ M Pefabloc protease, Roche) or protease/phosphatase inhibitor cocktails (Complete protease inhibitor mini-tablets and PhosStop, Roche) with 5 mM N-Ethylmaleimide (NEM, Sigma) in ice-cold PBS before freezing in liquid nitrogen. Worm lysates were boiled at 95°C for 5 – 10 minutes in 4x SDS loading buffer (Appendix) and separated on 10 – 15% SDS-PAGE gels (Appendix) in SDS running buffer (Appendix) at 150 V for 60 – 90 minutes using BioRad Mini-PROTEAN. By using Semi-Dry-Blotter, the proteins were transferred to a nitrocellulose membrane (Protran, Whatman) with the blot-buffer (Appendix) at 100 mA for 60 minutes and the membrane was 30-minute blocked on a shaking platform in the blocking solution (Appendix) before treating the primary antibody (Appendix) overnight at 4°C. The membrane was then washed three times with TBST washing solution (Tris-buffered saline containing 0.1% (v/v) Tween 20) and horseradish peroxidase (HRP)-conjugated secondary antibody (Jacksons Laboratory, Bar Harbor, Maine, USA) was incubated for 50 minutes at RT. The blot-membrane was developed using the developing solution (Table XI.5.1, Appendix) and the corresponding protein bands against the antibodies were detected using FujiFilm LAS 3000. The quantification of bands was analyzed by ImageJ software (National Institutes of Health).

To analyze the quantification of whole proteins or protein-protein interactions (PPI) by mass spectrometry, the solubilized proteins with 4x SDS loading buffer were fragmented on 10% SDS or 4 – 15% Mini-PROTEAN® TGX™ Gel (Bio-rad) after the boiling steps. The gels were stained with Coomassie Brilliant Blue R250 stain solutions (Appendix) for 30 minutes and then destained with Coomassie destaining buffer (Appendix) until protein bands showed up on the gels.

9.3.11 High-pressure freeze electron microscopy (HPF-EM)

Bacteria OP50 were filled onto a 100 µm deep aluminium platelet and twenty young adults were put into the chamber to be frozen immediately using a BalTec HPM 10. Then after, freezing substitution was performed with a Leica AFS2 as follows: Incubating at -90 °C for 100 hours in 0.1 % tannic acid, 7 h in 2 % Osmium tetroxide (OsO₄), and keeping at -20 °C for 16 hours in 2 % OsO₄. Embedding in EPON at room temperature (Rostaing et al., 2004). 50 nm sections were mounted on copper grid-slots and placed for ten minutes on drops of 4% (w/v) uranyl acetate in 75% methanol and washed in ddH₂O. After drying, the grid-slots were placed on lead citrate (Reynolds, 1963) in a CO₂-free chamber for two minutes before rinsed in ddH₂O. For the morphological analysis of the body wall muscles, young adults worms in wild type were compared with *unc-50 (x47)* mutant animals. In this study, HPF-EM was conducted by Oliver Kretz.

9.3.12 Stable isotope labeling by amino acids in *C. elegans* (SILAC)

In order to be isotopically labeled amino acids in *C. elegans*, wild type and *unc-50* mutants (*x47*) were maintained at 20°C as previously described (Brenner, 1974). For metabolically labeling amino acids into the worm, hermaphrodites having eggs can be egg-released by egg preparations (egg prep.) in the solution 500 µl 5 M NaOH and 1 ml bleach solution (5% solution of sodium hypochlorite) in 3 ml M9 buffer (Appendix) by vortexing, centrifugation and washing in ddH₂O (<http://wormbook.org/>), otherwise the worms were grown on nematode growth medium without nitrogen source (NGM-N) agarose plates (Appendix) after removing bacteria contaminants with the bleaching solutions. The worm strains were then egg-laying on the NGM-N and the larval stage (L1) 5 – 10 worms were transferred to the seeded plates of *E. coli* SLE1 strains,

expressing the *orn-1* targeted RNAi feeding construct (Larance et al., 2011), which were grown in M9 minimal medium (M9MM) containing each 40 µg/ml (220 nM) of light (L-KR; $^{14}\text{N}_2$ - $^{12}\text{C}_6$ -lysine and $^{14}\text{N}_4$ - $^{12}\text{C}_6$ -arginine) or heavy (H-KR; $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine and $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine) amino acids (L-lysine and L-arginine), respectively. To prepare the bacteria strains for seeding on NGM-N, the SLE1 strains were streaked out on LB plates from a frozen stock and one colony was grown on the LB containing Amp^r (100 µg/ml) and Tet^r (12.5 µg/ml) plates at 37 °C overnight. A single colony was incubated into L-KR or H-KR M9MM containing 1 µg/ml carbenicillin and 1 mM IPTG for expressing the ornithine transaminase RNAi feeding vector to prevent arginine to proline conversion through biosynthetic urea cycle (Bicho et al., 2010). The worms to apply SILAC labeling for proteomics were then maintained on the SLE1 lawn pads which were concentrated to be 10 to 50-fold in OD₆₀₀ = 1 seeded on NGM-N agarose plates.

9.3.13 Worm stocks

To prepare *C. elegans* stocks, the freezing and recovery steps were performed as described previously (Brenner, 1974). Six worm plates (diameter of 9 mm) were used to collect worms by flushing the plate using S buffer (Appendix) by centrifugation at 800 rpm for 10 minutes. 3 ml worm freezing solutions (S buffer + 30% glycerol) were added into the collected worms 3 ml in S buffer and mixed to be aliquoted into cryovials (1 ml per one cryovial). All six cryovials as worm stocks were kept at -80°C and one of the worm stocks was used to test whether the freezing worms would be able to recover properly on a seeded NGM plate after thawing at RT.

9.3.14 Integration lines by gamma irradiation of *C. elegans*

L4-stage worms of extrachromosomal transgenic-lines Ex:InJ60, Ex:InJ61 and Ex:InJ62 (Table XII.3.4) were prepared to integrate extrachromosomal transgenes by gamma irradiation in *C. elegans*. The integration protocols used in this study were accepted and modified from Peg MacMorris and Ji Ying Sze (<http://wormbook.org/>). Total forty rolling worms per independent extrachromosomal line for the integration were transferred onto NGM OP50-seeded plates and irradiated with 4000 Rads of gamma rays for 10 minutes from a $^{137}\text{Cesium}$ source. The four to five irradiated L4 transgenic worms were

transferred onto 6 cm plates and incubated at 20°C for two weeks. Among the plates after 48 – 72 hours, one was used for quick isolation of the irradiated lines while other plates were being starved. If there was no successful finding, the starved worm plates were chunk and the picking worms after 24 – 36 hours. In this study, *gzIs20/01*, *gzIs21/01*, *gzIs21/02* and *gzIs21/03* and *gzIs22/01* were successfully integrated by gamma irradiation. In order to label amino acids by SILAC, the integrated lines were outcrossed three times with N2 wild type worms. Each location on chromosomes of the integrated lines and whole genome sequences need to be confirmed by chromosomal mapping and sequencing information.

9.4. Methods used in the yeast *Saccharomyces cerevisiae*

9.4.1 Molecular biology and strains used in yeast

General molecular biology and basic methodology for yeast, *Saccharomyces cerevisiae*, strains used in this study were performed as described by *Current Protocols in Molecular Biology* (Ausubel et al., 1997). All strains and plasmids used in this study are listed in the Appendix.

9.4.2 Yeast growth phenotypes

Yeast cells were grown in synthetic complete (SC) or selective media lacking uracil (SC-URA) for overnight at 30°C and normalized to an OD₆₀₀ of 0.2 for six serial dilutions on testing plates. As for yeast growth phenotypes, Ferrozine (3 mM and 1 mM to 0.25 mM), Batho Phenantroline diSulfate (BPS) (40 µM), 2% glycerol/lactate and rapamycin (4 to 0.5 nM) plates were used on starvation and iron-limited conditions. Cells were then allowed to grow on testing plates containing chemical elements after spotting for incubation at 30°C for 3 days. Yeast growth phenotypes were analyzed after images were acquired. All chemicals, media and plates used in this study are shown in the supplementary materials (Appendix)

9.4.3 Transformation of yeast cells

Yeast cells were transformed using the standard lithium acetate (LiAc) protocol as previously described (Gietz et al., 1992). Cells cultured for overnight were inoculated 1:10 to an OD₆₀₀ of 0.2 in fresh media and grown at 30°C on a shaking incubator for log phase yeast cultures. Cell pellets were then harvested and washed in 1 ml ddH₂O for 5 minutes at 2000 rpm before resuspended in 200 µl of a lithium acetate mix solution (1:1:8) with 1.2 ml of a polyethylene glycol (PEG) mix (8:1:1) using the following protocol: 10x TE (0.5 M Tris-HCl pH 7.5, 10 mM EDTA), 10x LiAc (1 M LiAc pH 7.5), 50% PEG (w/v) 3350 in ddH₂O, a LiAc mix solution (1:1:8, e.g. 1 ml 10x TE, 1 ml 10x LiAc, 8 ml ddH₂O) and a PEG mix solution (8:1:1, e.g. 8 ml 50% PEG, 1 ml 10x TE, 1 ml 10x LiAc). 18 µl of carrier DNA (salmon herring sperm DNA, 10 mg/ml in Tris-HCl pH 8.0, 1 mM EDTA) heat-denatured in boiled water using microwave were added to each type of plasmid DNA (0.5 µg) while preparing the mix solutions as described above. Yeast cells treated in the LiAc solution were together in 1.5 ml tube with carrier DNA, each plasmid and the PEG mix solution before heat shock for one hour at 42°C. Then the treated cells were harvested by centrifugation for 3 minutes at 4,000 rpm and resolved in 100 µl ddH₂O. In case of the strains of the knockout-*gmh1*, they were inoculated in YPAD media with clonat (nourseothricin, final conc. 100 µg/ml) for 30 minutes at 30°C before spreading them onto selective solid media. Testing plates were incubated for 3 – 4 days at 30°C until colonies appear.

9.4.4 Creating of knockout-*gmh1* strains by PCR-based deletion cassettes

Yeast strains used in this study were described in XI. Appendix and provided from the groups of Stefan Eimer, Blanche Schwappach and the EUROSCARF collection. *Gmh1* deletion strain, $\Delta gmh1::Nat^R$, was used to generate the strain lacking GMH1 in the following strains, $Anp1-RFP::Kan^R$, $Chc1-RFP::Kan^R$, $Snf7-RFP::Kan^R$ and $\Delta pep4::Kan^R$, by a PCR-mediated homologous recombination approach (Goldstein and McCusker, 1999; Wach et al., 1994). PCR was carried out in a solution containing 5 µl Expand 10x PCR buffer (Roche), 2.5 units Expand DNA polymerase (Roche), 0.2 mM dNTPs, 0.4 µM of the appropriate forward oGQ2785 (GTG AAG TAC GAT CTA AAA CTG TTG) and reverse oGQ2786 (ACG ACC AGG AAC ACA CCA TTA) primer and DNA templates which genomic DNAs of $\Delta gmh1::Nat^R$ were used. The reaction

conditions were as follows: initial denaturation at 95°C for 3 minutes, 35 cycles at 95°C for 20 s, 51°C for 1 minute 30 sec and 72°C for 1 minute with a final extension at 72°C for 5 minutes. PCR products were separated and analyzed on a 1% agarose gel with SafeView™ Nucleic acid stain (Applied Biological Materials Inc.). Fragments of the $\Delta gmh1$ were cut from the gel for purification with High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions and transformed into yeast using the lithium acetate protocol described as above. Colonies in which the destined one had been knockout-Gmh1 were selected on solid YPAD medium containing clonate (nourseothricin) as a resistant marker and confirmed by PCR using another primer forward oGQ617 (GGG ACT AGT TCT AGA ATG TCA TAT TTG CCG ACT TAT TCC) and reverse oGQ618 (GGG GTC GAC GGA TC C TTA ATA ATT GTA AAA GCT TAA TTT CTT GGA) for checking deletion of *gmh1* in the strain after transformation.

9.4.5 Purification of genomic DNA from yeast cells

1.5 ml of yeast culture was incubated for overnight in the appropriate selective yeast medium. Plasmid DNA was purified using the YeaStar™ Genomic DNA Kit (Zymo Research) according to the manufacturer's instructions. Cell lysis is based on digestion of the cell wall using 5 µl of R-Zymolyase (Zymo Research). DNA was then purified on a affinity column of the kit.

9.4.6 Western blotting of yeast cellular lysates

Yeast strains were overnight cultured in 2 ml selective media at 30°C and measured cell growth at OD₆₀₀ for equal loading samples. Each 500 µl of the cultured cells was harvested by low speed centrifugation and then resuspended by 200 µl of 0.1 M NaOH at room temperature for total cellular lysates (Yaffe and Schatz, 1984). After 10 minutes, samples were harvested and resolved by 1x loading buffer of SDS-PAGE containing 100 mM DTT as equivalent to OD₆₀₀ of 0.01 unit per sample. Before loading on the pocket of 10% gel of SDS-PAGE, samples were spun down simply by centrifugation and then 10 µl of each sample was used for running on the gel in 1x SDS running buffer and transferred to nitrocellulose membranes in 1x transfer buffer for one and half an hour at

60 voltage. Membranes were washed in blocking solution (1x TBS, 5% milk powder, 0.02% IGEPAL CA-630) for 30 minutes after checking the total protein on the membrane by 1x Ponceau S solution. For more details for buffers and solutions were described in the supplementary materials (S10). Primary antibodies used in this study were displayed as follows: anti-GFP mouse monoclonal B-2 (Santa Cruz), Living Colors[®] DsRed polyclonal antibody (Clontech Laboratories, Inc) for fluorescent-tagged proteins and anti-PGK1 mouse monoclonal 22C5 (Molecular Probes, Invitrogen), anti-Bmh1p rabbit (Michelsen et al., 2006) as loading controls. After blocking the membrane, each primary antibody diluted to 1:1000 was treated for overnight on a shaking incubator at 4°C and washed thrice by blocking solution. As secondary antibodies for Odyssey[®] Sa Infrared Imaging System (LI-COR), IRDye[®] 800CW and 680LT Infrared Dye-conjugated secondary anti-rabbit or anti-mouse antibodies (LI-COR) were used to 1:10,000 dilution in blocking solution for incubation of one hour at room temperature and then washed three times using TBS (0.02% IGEPAL CA-630). For detection of proteins, membranes were placed on crystal plates and scanned by Odyssey system. Data were quantified and normalized to the control and analyzed by ImageJ software (National Institutes of Health).

9.4.7 Live cell Imaging

Live cell imaging for yeast cells were performed by a Delta Vision RT (Applied Precision) microscopy using a 100x/0.35-1.5 Uplan Apo objective with filter sets of the specific band pass of GFP, RFP or TRITC for imaging of living yeast cells, which were taken by a Coolsnap HQ camera (Photometrics) and at least three independent experiments were done under the same conditions in wild type and mutants at room temperature. As for a pulse-chase assay and the colocalization, the samples were grown to log-phase cells to OD₆₀₀ of between 0.5 and 1.0 after overnight cultures at 30°C and, in case of testing endocytosis, the samples were washed twice by fresh media before adding FM[®] 4-64 (Molecular Probes, Invitrogen), a lipophilic styryl dye for visualizing membranes as a marker of yeast membrane traffic (Vida and Emr, 1995). The samples were then incubated with the dye of a final concentration of 40 µM from a stock solution of 16 mM in Dimethyl sulfoxide (DMSO) on ice for 30 minutes as a labeling step and

spun down gently without washing steps. For imaging, cells were placed back to room temperature for 1 minute and immediately mounted on a microscope slide with a cover slip. Multichannel images of colocalization experiments were analyzed using ImageJ software (National Institutes of Health) and statistical analysis was used by a Student's *t* test.

9.4.8 Glycerol Stocks

Yeast cells overnight inoculated in SC or YPAD media were stocked in glycerol solution at -80°C and liquid nitrogen. For the stock solutions, 400 μ l of cultured cells and 400 μ l of 30% glycerol were used and mixed together and kept in cryovials for frozen conditions.

X. REFERENCES

- Abeliovich, H., Grote, E., Novick, P., and Ferro-Novick, S. (1998). Tlg2p, a yeast syntaxin homolog that resides on the Golgi and endocytic structures. *J Biol Chem* 273, 11719-11727.
- Altschuler, Y., Barbas, S.M., Terlecky, L.J., Tang, K., Hardy, S., Mostov, K.E., and Schmid, S.L. (1998). Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *The Journal of cell biology* 143, 1871-1881.
- Altun, Z.F., Herndon, L.A., Crocker, C., Lints, R. and Hall, D.H. (ed.s) (2002-2012). *WormAtlas*.
- Appenzeller-Herzog, C., and Hauri, H.P. (2006). The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. *J Cell Sci* 119, 2173-2183.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds. (1997). *Current Protocols in Molecular Biology* (New York: Greene Publishing Associates and Wiley-Interscience).
- Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M.F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* 77, 895-907.
- Baugh, L.R., and Hunter, C.P. (2006). MyoD, modularity, and myogenesis: conservation of regulators and redundancy in *C. elegans*. *Genes & development* 20, 3342-3346.
- Baumert, M., Maycox, P.R., Navone, F., De Camilli, P., and Jahn, R. (1989). Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO J* 8, 379-384.
- Bertrand, D., Ballivet, M., Gomez, M., Bertrand, S., Phannavong, B., and Gundelfinger, E.D. (1994). Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate beta 2 subunit and *Drosophila* alpha subunits. *Eur J Neurosci* 6, 869-875.
- Bicho, C.C., de Lima Alves, F., Chen, Z.A., Rappsilber, J., and Sawin, K.E. (2010). A genetic engineering solution to the "arginine conversion problem" in stable isotope labeling by amino acids in cell culture (SILAC). *Molecular & cellular proteomics : MCP* 9, 1567-1577.
- Bird, A.F., and Bird, J. (1991). *The structure of nematodes*, 2nd edn (San Diego: Academic Press).
- Boehm, M., and Bonifacino, J.S. (2001). Adaptins: the final recount. *Mol Biol Cell* 12, 2907-2920.
- Boman, A.L., Zhang, C., Zhu, X., and Kahn, R.A. (2000). A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Mol Biol Cell* 11, 1241-1255.
- Bonifacino, J.S., and Glick, B.S. (2004). The mechanisms of vesicle budding and fusion. *Cell* 116, 153-166.
- Bonifacino, J.S., and Hierro, A. (2011). Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. *Trends Cell Biol* 21, 159-167.
- Bonifacino, J.S., and Rojas, R. (2006). Retrograde transport from endosomes to the trans-Golgi network. *Nature reviews Molecular cell biology* 7, 568-579.

- Boulin, T., Gielen, M., Richmond, J.E., Williams, D.C., Paoletti, P., and Bessereau, J.L. (2008). Eight genes are required for functional reconstitution of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor. *Proc Natl Acad Sci U S A* *105*, 18590-18595.
- Boulin, T., Rapti, G., Briseno-Roa, L., Stigloher, C., Richmond, J.E., Paoletti, P., and Bessereau, J.L. (2012). Positive modulation of a Cys-loop acetylcholine receptor by an auxiliary transmembrane subunit. *Nat Neurosci* *15*, 1374-1381.
- Boulon, S., Ahmad, Y., Trinkle-Mulcahy, L., Verheggen, C., Cobley, A., Gregor, P., Bertrand, E., Whitehorn, M., and Lamond, A.I. (2010). Establishment of a protein frequency library and its application in the reliable identification of specific protein interaction partners. *Molecular & cellular proteomics : MCP* *9*, 861-879.
- Bowers, K., Levi, B.P., Patel, F.I., and Stevens, T.H. (2000). The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* *11*, 4277-4294.
- Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* *14*, 115-132.
- Braeckman, B.P., Houthoofd, K., and Vanfleteren, J.R. (2009). Intermediary metabolism. *WormBook : the online review of C elegans biology*, 1-24.
- Braun, N.A., Morgan, B., Dick, T.P., and Schwappach, B. (2010). The yeast CLC protein counteracts vesicular acidification during iron starvation. *J Cell Sci* *123*, 2342-2350.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* *77*, 71-94.
- Briseno-Roa, L., and Bessereau, J.L. (2014). Proteolytic processing of the extracellular scaffolding protein LEV-9 is required for clustering acetylcholine receptors. *J Biol Chem* *289*, 10967-10974.
- Brown, L.A., Jones, A.K., Buckingham, S.D., Mee, C.J., and Sattelle, D.B. (2006). Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. *Int J Parasitol* *36*, 617-624.
- Bruneau, E., Sutter, D., Hume, R.I., and Akaaboune, M. (2005). Identification of nicotinic acetylcholine receptor recycling and its role in maintaining receptor density at the neuromuscular junction in vivo. *J Neurosci* *25*, 9949-9959.
- Calixto, A., Chelur, D., Topalidou, I., Chen, X., and Chalfie, M. (2010). Enhanced neuronal RNAi in *C. elegans* using SID-1. *Nature methods* *7*, 554-559.
- Camus, G., Jasmin, B.J., and Cartaud, J. (1998). Polarized sorting of nicotinic acetylcholine receptors to the postsynaptic membrane in *Torpedo* electrocyte. *Eur J Neurosci* *10*, 839-852.
- Carroll, K.S., Hanna, J., Simon, I., Krise, J., Barbero, P., and Pfeffer, S.R. (2001). Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science* *292*, 1373-1376.
- Chakravarty, R., Goel, S., and Cai, W. (2014). Nanobody: the "magic bullet" for molecular imaging? *Theranostics* *4*, 386-398.
- Chantalat, S., Courbeyrette, R., Senic-Matuglia, F., Jackson, C.L., Goud, B., and Peyroche, A. (2003). A novel Golgi membrane protein is a partner of the ARF exchange factors Gea1p and Gea2p. *Mol Biol Cell* *14*, 2357-2371.

- Chapman-Smith, A., and Cronan, J.E., Jr. (1999). In vivo enzymatic protein biotinylation. *Biomolecular engineering* 16, 119-125.
- Chardin, P., Paris, S., Antonny, B., Robineau, S., Beraud-Dufour, S., Jackson, C.L., and Chabre, M. (1996). A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature* 384, 481-484.
- Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* 62, 317-329.
- Chen, N., Harris, T.W., Antoshechkin, I., Bastiani, C., Bieri, T., Blasiar, D., Bradnam, K., Canaran, P., Chan, J., Chen, C.K., *et al.* (2005a). WormBase: a comprehensive data resource for *Caenorhabditis* biology and genomics. *Nucleic Acids Res* 33, D383-389.
- Chen, S.H., Chen, S., Tokarev, A.A., Liu, F., Jedd, G., and Segev, N. (2005b). Ypt31/32 GTPases and their novel F-box effector protein Rcy1 regulate protein recycling. *Mol Biol Cell* 16, 178-192.
- Choi-Rhee, E., Schulman, H., and Cronan, J.E. (2004). Promiscuous protein biotinylation by *Escherichia coli* biotin protein ligase. *Protein science : a publication of the Protein Society* 13, 3043-3050.
- Christianson, J.C., and Green, W.N. (2004). Regulation of nicotinic receptor expression by the ubiquitin-proteasome system. *EMBO J* 23, 4156-4165.
- Confalonieri, S., Salcini, A.E., Puri, C., Tacchetti, C., and Di Fiore, P.P. (2000). Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. *The Journal of cell biology* 150, 905-912.
- Conibear, E., Cleck, J.N., and Stevens, T.H. (2003). Vps51p mediates the association of the GARP (Vps52/53/54) complex with the late Golgi t-SNARE Tlg1p. *Mol Biol Cell* 14, 1610-1623.
- Conibear, E., and Stevens, T.H. (2000). Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Mol Biol Cell* 11, 305-323.
- Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. *Nature* 422, 37-44.
- Cosson, P., and Letourneur, F. (1997). Coatamer (COPI)-coated vesicles: role in intracellular transport and protein sorting. *Current opinion in cell biology* 9, 484-487.
- Couve, A., Protopopov, V., and Gerst, J.E. (1995). Yeast synaptobrevin homologs are modified posttranslationally by the addition of palmitate. *Proc Natl Acad Sci U S A* 92, 5987-5991.
- Cowart, R.E., Singleton, F.L., and Hind, J.S. (1993). A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron(II) in reduction reactions. *Analytical biochemistry* 211, 151-155.
- Craft, G.E., Chen, A., and Nairn, A.C. (2013). Recent advances in quantitative neuroproteomics. *Methods* 61, 186-218.
- Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K., and Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450, 736-740.
- Cyert, M.S., and Philpott, C.C. (2013). Regulation of cation balance in *Saccharomyces cerevisiae*. *Genetics* 193, 677-713.

- Davis-Kaplan, S.R., Askwith, C.C., Bengtzen, A.C., Radisky, D., and Kaplan, J. (1998). Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: an unexpected role for intracellular chloride channels. *Proc Natl Acad Sci U S A* 95, 13641-13645.
- de Bono, M., and Maricq, A.V. (2005). Neuronal substrates of complex behaviors in *C. elegans*. *Annual review of neuroscience* 28, 451-501.
- De Meyer, T., Muyldermans, S., and Depicker, A. (2014). Nanobody-based products as research and diagnostic tools. *Trends in biotechnology* 32, 263-270.
- de Renzis, S., Sonnichsen, B., and Zerial, M. (2002). Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. *Nature cell biology* 4, 124-133.
- Deeb, S.J., D'Souza, R.C., Cox, J., Schmidt-Supprian, M., and Mann, M. (2012). Super-SILAC allows classification of diffuse large B-cell lymphoma subtypes by their protein expression profiles. *Molecular & cellular proteomics : MCP* 11, 77-89.
- Dell'Angelica, E.C., Puertollano, R., Mullins, C., Aguilar, R.C., Vargas, J.D., Hartnell, L.M., and Bonifacino, J.S. (2000). GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *The Journal of cell biology* 149, 81-94.
- Dobreva, I., Fielding, A., Foster, L.J., and Dedhar, S. (2008). Mapping the integrin-linked kinase interactome using SILAC. *Journal of proteome research* 7, 1740-1749.
- Donaldson, J., and Segev, N. (2010). Regulation and coordination of intracellular trafficking. In *Trafficking Inside Cells: Pathways, Mechanisms and Regulation*, N. Segev, ed. (Springer).
- Donaldson, J.G., and Jackson, C.L. (2000). Regulators and effectors of the ARF GTPases. *Current opinion in cell biology* 12, 475-482.
- Donaldson, J.G., and Lippincott-Schwartz, J. (2000). Sorting and signaling at the Golgi complex. *Cell* 101, 693-696.
- Eddy, J.A., Hood, L., Price, N.D., and Geman, D. (2010). Identifying tightly regulated and variably expressed networks by Differential Rank Conservation (DIRAC). *PLoS computational biology* 6, e1000792.
- Eimer, S., Gottschalk, A., Hengartner, M., Horvitz, H.R., Richmond, J., Schafer, W.R., and Bessereau, J.L. (2007). Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50. *EMBO J* 26, 4313-4323.
- Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. *Science* 286, 1882-1888.
- Emmott, E., and Goodfellow, I. (2014). Identification of Protein Interaction Partners in Mammalian Cells Using SILAC-immunoprecipitation Quantitative Proteomics. *Journal of visualized experiments : JoVE*.
- Emmott, E., Munday, D., Bickerton, E., Britton, P., Rodgers, M.A., Whitehouse, A., Zhou, E.M., and Hiscox, J.A. (2013). The cellular interactome of the coronavirus infectious bronchitis virus nucleocapsid protein and functional implications for virus biology. *Journal of virology* 87, 9486-9500.
- Eugster, A., Frigerio, G., Dale, M., and Duden, R. (2000). COP I domains required for coatamer integrity, and novel interactions with ARF and ARF-GAP. *EMBO J* 19, 3905-3917.

- Fares, H., and Greenwald, I. (2001). Genetic analysis of endocytosis in *Caenorhabditis elegans*: coelomocyte uptake defective mutants. *Genetics* 159, 133-145.
- Fath, S., Mancias, J.D., Bi, X., and Goldberg, J. (2007). Structure and organization of coat proteins in the COPII cage. *Cell* 129, 1325-1336.
- Findlay, G.D., Yi, X., Maccoss, M.J., and Swanson, W.J. (2008). Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS biology* 6, e178.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.
- Fitzgerald, J., Kennedy, D., Viseshakul, N., Cohen, B.N., Mattick, J., Bateman, J.F., and Forsayeth, J.R. (2000). UNCL, the mammalian homologue of UNC-50, is an inner nuclear membrane RNA-binding protein. *Brain Res* 877, 110-123.
- Fleming, J.T., Squire, M.D., Barnes, T.M., Tornoe, C., Matsuda, K., Ahnn, J., Fire, A., Sulston, J.E., Barnard, E.A., Sattelle, D.B., *et al.* (1997). *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J Neurosci* 17, 5843-5857.
- Forsayeth, J.R., Gu, Y., and Hall, Z.W. (1992). BiP forms stable complexes with unassembled subunits of the acetylcholine receptor in transfected COS cells and in C2 muscle cells. *The Journal of cell biology* 117, 841-847.
- Fotin, A., Cheng, Y., Sliz, P., Grigorieff, N., Harrison, S.C., Kirchhausen, T., and Walz, T. (2004). Molecular model for a complete clathrin lattice from electron cryomicroscopy. *Nature* 432, 573-579.
- Fredens, J., Engholm-Keller, K., Giessing, A., Pultz, D., Larsen, M.R., Hojrup, P., Moller-Jensen, J., and Faergeman, N.J. (2011). Quantitative proteomics by amino acid labeling in *C. elegans*. *Nature methods* 8, 845-847.
- Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000). A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nature cell biology* 2, 379-384.
- Gabriely, G., Kama, R., and Gerst, J.E. (2007). Involvement of specific COPI subunits in protein sorting from the late endosome to the vacuole in yeast. *Mol Cell Biol* 27, 526-540.
- Galan, J.M., Wiederkehr, A., Seol, J.H., Haguenaue-Tsapis, R., Deshaies, R.J., Riezman, H., and Peter, M. (2001). Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. *Mol Cell Biol* 21, 3105-3117.
- Gallagher, M.J., Ding, L., Maheshwari, A., and Macdonald, R.L. (2007). The GABAA receptor alpha1 subunit epilepsy mutation A322D inhibits transmembrane helix formation and causes proteasomal degradation. *Proc Natl Acad Sci U S A* 104, 12999-13004.
- Gally, C., Eimer, S., Richmond, J.E., and Bessereau, J.L. (2004). A transmembrane protein required for acetylcholine receptor clustering in *Caenorhabditis elegans*. *Nature* 431, 578-582.
- Garcia, L.R., Mehta, P., and Sternberg, P.W. (2001). Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating. *Cell* 107, 777-788.

- Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L., and Fink, G.R. (1999). The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc Natl Acad Sci U S A* 96, 1480-1485.
- Gaxiola, R.A., Yuan, D.S., Klausner, R.D., and Fink, G.R. (1998). The yeast CLC chloride channel functions in cation homeostasis. *Proc Natl Acad Sci U S A* 95, 4046-4050.
- Geiger, T., Cox, J., Ostasiewicz, P., Wisniewski, J.R., and Mann, M. (2010). Super-SILAC mix for quantitative proteomics of human tumor tissue. *Nature methods* 7, 383-385.
- Geiger, T., Wisniewski, J.R., Cox, J., Zanivan, S., Kruger, M., Ishihama, Y., and Mann, M. (2011). Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. *Nature protocols* 6, 147-157.
- Gelman, M.S., Chang, W., Thomas, D.Y., Bergeron, J.J., and Prives, J.M. (1995). Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptors. *J Biol Chem* 270, 15085-15092.
- Gendrel, M., Rapti, G., Richmond, J.E., and Bessereau, J.L. (2009). A secreted complement-control-related protein ensures acetylcholine receptor clustering. *Nature* 461, 992-996.
- Gerst, J.E., Rodgers, L., Riggs, M., and Wigler, M. (1992). SNC1, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the RAS and CAP genes. *Proc Natl Acad Sci U S A* 89, 4338-4342.
- Ghosh, P., Dahms, N.M., and Kornfeld, S. (2003). Mannose 6-phosphate receptors: new twists in the tale. *Nature reviews Molecular cell biology* 4, 202-212.
- Gietz, D., St Jean, A., Woods, R.A., and Schiestl, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20, 1425.
- Gillingham, A.K., and Munro, S. (2007). The small G proteins of the Arf family and their regulators. *Annual review of cell and developmental biology* 23, 579-611.
- Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.M., Parton, R.G., and Stenmark, H. (2000). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J* 19, 4577-4588.
- Girard, L.R., Fiedler, T.J., Harris, T.W., Carvalho, F., Antoshechkin, I., Han, M., Sternberg, P.W., Stein, L.D., and Chalfie, M. (2007). WormBook: the online review of *Caenorhabditis elegans* biology. *Nucleic Acids Res* 35, D472-475.
- Goldberg, J. (1998). Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. *Cell* 95, 237-248.
- Goldstein, A.L., and McCusker, J.H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541-1553.
- Gotti, C., and Clementi, F. (2004). Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* 74, 363-396.
- Gould, G.W., and Lippincott-Schwartz, J. (2009). New roles for endosomes: from vesicular carriers to multi-purpose platforms. *Nature reviews Molecular cell biology* 10, 287-292.
- Gouw, J.W., Krijgsveld, J., and Heck, A.J. (2010). Quantitative proteomics by metabolic labeling of model organisms. *Molecular & cellular proteomics : MCP* 9, 11-24.
- Gouw, J.W., Tops, B.B., and Krijgsveld, J. (2011). Metabolic labeling of model organisms using heavy nitrogen (¹⁵N). *Methods Mol Biol* 753, 29-42.

- Grant, B., and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol Biol Cell* 10, 4311-4326.
- Grant, B.D., and Donaldson, J.G. (2009). Pathways and mechanisms of endocytic recycling. *Nature reviews Molecular cell biology* 10, 597-608.
- Grant, B.D., and Sato, M. (2006). Intracellular trafficking. *WormBook : the online review of C elegans biology*, 1-9.
- Green, W.N., and Millar, N.S. (1995). Ion-channel assembly. *Trends in neurosciences* 18, 280-287.
- Greene, J.R., Brown, N.H., DiDomenico, B.J., Kaplan, J., and Eide, D.J. (1993). The GEF1 gene of *Saccharomyces cerevisiae* encodes an integral membrane protein; mutations in which have effects on respiration and iron-limited growth. *Molecular & general genetics : MGG* 241, 542-553.
- Groot, A.J., Verheesen, P., Westerlaken, E.J., Gort, E.H., van der Groep, P., Bovenschen, N., van der Wall, E., van Diest, P.J., and Shvarts, A. (2006). Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha. *Laboratory investigation; a journal of technical methods and pathology* 86, 345-356.
- Gruhler, A., Schulze, W.X., Matthiesen, R., Mann, M., and Jensen, O.N. (2005). Stable isotope labeling of *Arabidopsis thaliana* cells and quantitative proteomics by mass spectrometry. *Molecular & cellular proteomics : MCP* 4, 1697-1709.
- Gu, F., Crump, C.M., and Thomas, G. (2001). Trans-Golgi network sorting. *Cell Mol Life Sci* 58, 1067-1084.
- Gurunathan, S., Chapman-Shimshoni, D., Trajkovic, S., and Gerst, J.E. (2000). Yeast exocytic v-SNAREs confer endocytosis. *Mol Biol Cell* 11, 3629-3643.
- Habibi-Babadi, N., Su, A., de Carvalho, C.E., and Colavita, A. (2010). The N-glycanase png-1 acts to limit axon branching during organ formation in *Caenorhabditis elegans*. *J Neurosci* 30, 1766-1776.
- Halevi, S., McKay, J., Palfreyman, M., Yassin, L., Eshel, M., Jorgensen, E., and Treinin, M. (2002). The *C. elegans* ric-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J* 21, 1012-1020.
- Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M., and Treinin, M. (2003). Conservation within the RIC-3 gene family. Effectors of mammalian nicotinic acetylcholine receptor expression. *J Biol Chem* 278, 34411-34417.
- Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., and Hamers, R. (1993). Naturally occurring antibodies devoid of light chains. *Nature* 363, 446-448.
- Hauri, H.P., Kappeler, F., Andersson, H., and Appenzeller, C. (2000). ERGIC-53 and traffic in the secretory pathway. *J Cell Sci* 113 (Pt 4), 587-596.
- Hayes, G.L., Brown, F.C., Haas, A.K., Nottingham, R.M., Barr, F.A., and Pfeffer, S.R. (2009). Multiple Rab GTPase binding sites in GCC185 suggest a model for vesicle tethering at the trans-Golgi. *Mol Biol Cell* 20, 209-217.
- Helsens, K., Martens, L., Vandekerckhove, J., and Gevaert, K. (2011). Mass spectrometry-driven proteomics: an introduction. *Methods Mol Biol* 753, 1-27.
- Hettema, E.H., Lewis, M.J., Black, M.W., and Pelham, H.R. (2003). Retromer and the sorting nexins Snx4/41/42 mediate distinct retrieval pathways from yeast endosomes. *EMBO J* 22, 548-557.

- Hobert, O., Mori, I., Yamashita, Y., Honda, H., Ohshima, Y., Liu, Y., and Ruvkun, G. (1997). Regulation of interneuron function in the *C. elegans* thermoregulatory pathway by the *ttx-3* LIM homeobox gene. *Neuron* *19*, 345-357.
- Hoffman, G.R., Rahl, P.B., Collins, R.N., and Cerione, R.A. (2003). Conserved structural motifs in intracellular trafficking pathways: structure of the gammaCOP appendage domain. *Molecular cell* *12*, 615-625.
- Huang, K.M., D'Hondt, K., Riezman, H., and Lemmon, S.K. (1999). Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO J* *18*, 3897-3908.
- Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. *Nature* *425*, 686-691.
- Hulme, S.E., and Whitesides, G.M. (2011). Chemistry and the worm: *Caenorhabditis elegans* as a platform for integrating chemical and biological research. *Angew Chem Int Ed Engl* *50*, 4774-4807.
- Husson, S.J., Mertens, I., Janssen, T., Lindemans, M., and Schoofs, L. (2007). Neuropeptidergic signaling in the nematode *Caenorhabditis elegans*. *Prog Neurobiol* *82*, 33-55.
- Ihara, M., Matsuda, K., Otake, M., Kuwamura, M., Shimomura, M., Komai, K., Akamatsu, M., Raymond, V., and Sattelle, D.B. (2003). Diverse actions of neonicotinoids on chicken alpha7, alpha4beta2 and *Drosophila*-chicken SADbeta2 and ALSbeta2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Neuropharmacology* *45*, 133-144.
- Ihara, S., and Nishiwaki, K. (2007). Prodomain-dependent tissue targeting of an ADAMTS protease controls cell migration in *Caenorhabditis elegans*. *EMBO J* *26*, 2607-2620.
- Ishizaki, R., Shin, H.W., Mitsuhashi, H., and Nakayama, K. (2008). Redundant roles of BIG2 and BIG1, guanine-nucleotide exchange factors for ADP-ribosylation factors in membrane traffic between the trans-Golgi network and endosomes. *Mol Biol Cell* *19*, 2650-2660.
- Jackson, C.L., and Casanova, J.E. (2000). Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol* *10*, 60-67.
- Jahn, R., and Scheller, R.H. (2006). SNAREs--engines for membrane fusion. *Nature reviews Molecular cell biology* *7*, 631-643.
- Jarosch, E., Lenk, U., and Sommer, T. (2003). Endoplasmic reticulum-associated protein degradation. *International review of cytology* *223*, 39-81.
- Jobling, S.A., Jarman, C., Teh, M.M., Holmberg, N., Blake, C., and Verhoeyen, M.E. (2003). Immunomodulation of enzyme function in plants by single-domain antibody fragments. *Nature biotechnology* *21*, 77-80.
- Kalatzis, V., Cherqui, S., Antignac, C., and Gasnier, B. (2001). Cystinosin, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter. *EMBO J* *20*, 5940-5949.
- Kaletta, T., and Hengartner, M.O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nature reviews Drug discovery* *5*, 387-398.

- Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., *et al.* (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* *421*, 231-237.
- Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G., and Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome biology* *2*, RESEARCH0002.
- Keller, S.H., Lindstrom, J., Ellisman, M., and Taylor, P. (2001). Adjacent basic amino acid residues recognized by the COP I complex and ubiquitination govern endoplasmic reticulum to cell surface trafficking of the nicotinic acetylcholine receptor alpha-Subunit. *J Biol Chem* *276*, 18384-18391.
- Kim, H.J., Choi, Y.S., Jeong, M.J., Kim, B.O., Lim, S.H., Kim do, K., Kim, C.K., and Park, J.C. (2007). Expression of UNCL during development of periodontal tissue and response of periodontal ligament fibroblasts to mechanical stress in vivo and in vitro. *Cell and tissue research* *327*, 25-31.
- Kim, J., Poole, D.S., Waggoner, L.E., Kempf, A., Ramirez, D.S., Treschow, P.A., and Schafer, W.R. (2001). Genes affecting the activity of nicotinic receptors involved in *Caenorhabditis elegans* egg-laying behavior. *Genetics* *157*, 1599-1610.
- Kirchhausen, T. (2000). Three ways to make a vesicle. *Nature reviews Molecular cell biology* *1*, 187-198.
- Kirchhofer, A., Helma, J., Schmidthals, K., Frauer, C., Cui, S., Karcher, A., Pellis, M., Muyldermans, S., Casas-Delucchi, C.S., Cardoso, M.C., *et al.* (2010). Modulation of protein properties in living cells using nanobodies. *Nature structural & molecular biology* *17*, 133-138.
- Knop, M., Barr, F., Riedel, C.G., Heckel, T., and Reichel, C. (2002). Improved version of the red fluorescent protein (drFP583/DsRed/RFP). *BioTechniques* *33*, 592, 594, 596-598 passim.
- Krijgsveld, J., Ketting, R.F., Mahmoudi, T., Johansen, J., Artal-Sanz, M., Verrijzer, C.P., Plasterk, R.H., and Heck, A.J. (2003). Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nature biotechnology* *21*, 927-931.
- Kruger, M., Moser, M., Ussar, S., Thievensen, I., Lubner, C.A., Forner, F., Schmidt, S., Zanivan, S., Fassler, R., and Mann, M. (2008). SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* *134*, 353-364.
- Kubala, M.H., Kovtun, O., Alexandrov, K., and Collins, B.M. (2010). Structural and thermodynamic analysis of the GFP:GFP-nanobody complex. *Protein science : a publication of the Protein Society* *19*, 2389-2401.
- Kwon, K., and Beckett, D. (2000). Function of a conserved sequence motif in biotin holoenzyme synthetases. *Protein science : a publication of the Protein Society* *9*, 1530-1539.
- Lafourcade, C., Galan, J.M., Gloor, Y., Haguenaer-Tsapis, R., and Peter, M. (2004). The GTPase-activating enzyme Gyp1p is required for recycling of internalized membrane material by inactivation of the Rab/Ypt GTPase Ypt1p. *Mol Cell Biol* *24*, 3815-3826.
- Lahm, H.W., and Langen, H. (2000). Mass spectrometry: a tool for the identification of proteins separated by gels. *Electrophoresis* *21*, 2105-2114.
- Lang, T., and Jahn, R. (2008). Core proteins of the secretory machinery. *Handbook of experimental pharmacology*, 107-127.

- Larance, M., Bailly, A.P., Pourkarimi, E., Hay, R.T., Buchanan, G., Coulthurst, S., Xirodimas, D.P., Gartner, A., and Lamond, A.I. (2011). Stable-isotope labeling with amino acids in nematodes. *Nature methods* 8, 849-851.
- Le Novère, N., Corringer, P.J., and Changeux, J.P. (2002). The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. *Journal of neurobiology* 53, 447-456.
- Lee, C., and Goldberg, J. (2010). Structure of coatamer cage proteins and the relationship among COPI, COPII, and clathrin vesicle coats. *Cell* 142, 123-132.
- Lee, M.C., Miller, E.A., Goldberg, J., Orci, L., and Schekman, R. (2004). Bi-directional protein transport between the ER and Golgi. *Annual review of cell and developmental biology* 20, 87-123.
- Letourneur, F., Gaynor, E.C., Hennecke, S., Demolliere, C., Duden, R., Emr, S.D., Riezman, H., and Cosson, P. (1994). Coatamer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* 79, 1199-1207.
- Lewis, J.A., Wu, C.H., Berg, H., and Levine, J.H. (1980). The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. *Genetics* 95, 905-928.
- Lewis, M.J., Nichols, B.J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H.R. (2000). Specific retrieval of the exocytic SNARE Snclp from early yeast endosomes. *Mol Biol Cell* 11, 23-38.
- Li, H., Li, H.F., Felder, R.A., Periasamy, A., and Jose, P.A. (2008). Rab4 and Rab11 coordinately regulate the recycling of angiotensin II type I receptor as demonstrated by fluorescence resonance energy transfer microscopy. *Journal of biomedical optics* 13, 031206.
- Li, S., Armstrong, C.M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P.O., Han, J.D., Chesneau, A., Hao, T., *et al.* (2004). A map of the interactome network of the metazoan *C. elegans*. *Science* 303, 540-543.
- Lode, A., Paret, C., and Rodel, G. (2002). Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p. *Yeast* 19, 909-922.
- Lowery, J., Szul, T., Styers, M., Holloway, Z., Oorschot, V., Klumperman, J., and Sztul, E. (2013). The Sec7 guanine nucleotide exchange factor GBF1 regulates membrane recruitment of BIG1 and BIG2 guanine nucleotide exchange factors to the trans-Golgi network (TGN). *J Biol Chem* 288, 11532-11545.
- Luo, L., Hannemann, M., Koenig, S., Hegermann, J., Ailion, M., Cho, M.K., Sasidharan, N., Zweckstetter, M., Rensing, S.A., and Eimer, S. (2011). The *Caenorhabditis elegans* GARP complex contains the conserved Vps51 subunit and is required to maintain lysosomal morphology. *Mol Biol Cell* 22, 2564-2578.
- Mahoney, T.R., Luo, S., and Nonet, M.L. (2006). Analysis of synaptic transmission in *Caenorhabditis elegans* using an aldicarb-sensitivity assay. *Nature protocols* 1, 1772-1777.
- Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (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., Tang, B.L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B., and Johannes, L. (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 Maxfield, F.R. (1999). Chimeric forms of furin and TGN38 are transported with the plasma membrane in the trans-Golgi network via distinct endosomal pathways. *The Journal of cell biology* 146, 345-359.
- Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nature reviews Molecular cell biology* 7, 952-958.
- Matanis, T., Akhmanova, A., Wulf, P., Del Nery, E., Weide, T., Stepanova, T., Galjart, N., Grosveld, F., Goud, B., De Zeeuw, C.I., *et al.* (2002). Bicaudal-D regulates COPI-independent Golgi-ER transport by recruiting the dynein-dynactin motor complex. *Nature cell biology* 4, 986-992.
- Mayor, S., and Pagano, R.E. (2007). Pathways of clathrin-independent endocytosis. *Nature reviews Molecular cell biology* 8, 603-612.
- McEwen, J.M., and Kaplan, J.M. (2008). UNC-18 promotes both the anterograde trafficking and synaptic function of syntaxin. *Mol Biol Cell* 19, 3836-3846.
- McKay, J.P., Raizen, D.M., Gottschalk, A., Schafer, W.R., and Avery, L. (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. *Genetics* 166, 161-169.
- McMahon, H.T., and Mills, I.G. (2004). COP and clathrin-coated vesicle budding: different pathways, common approaches. *Current opinion in cell biology* 16, 379-391.
- Mello, C., and Fire, A. (1995). DNA transformation. *Methods in cell biology* 48, 451-482.
- Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* 10, 3959-3970.
- Merlie, J.P., and Lindstrom, J. (1983). Assembly in vivo of mouse muscle acetylcholine receptor: identification of an alpha subunit species that may be an assembly intermediate. *Cell* 34, 747-757.
- Merrifield, C.J., Perrais, D., and Zenisek, D. (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell* 121, 593-606.
- Meyer, S., Savaresi, S., Forster, I.C., and Dutzler, R. (2007). Nucleotide recognition by the cytoplasmic domain of the human chloride transporter ClC-5. *Nature structural & molecular biology* 14, 60-67.
- Michelsen, K., Mrowiec, T., Duderstadt, K.E., Frey, S., Minor, D.L., Mayer, M.P., and Schwappach, B. (2006). A multimeric membrane protein reveals 14-3-3 isoform specificity in forward transport in yeast. *Traffic* 7, 903-916.
- Mittler, G., Butter, F., and Mann, M. (2009). A SILAC-based DNA protein interaction screen that identifies candidate binding proteins to functional DNA elements. *Genome research* 19, 284-293.
- Mouratou, B., Biou, V., Joubert, A., Cohen, J., Shields, D.J., Geldner, N., Jurgens, G., Melancon, P., and Cherfils, J. (2005). The domain architecture of large guanine nucleotide exchange factors for the small GTP-binding protein Arf. *BMC genomics* 6, 20.
- Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* 22, 5767-5768.

- Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* *424*, 277-283.
- Muyldermans, S., Baral, T.N., Retamozzo, V.C., De Baetselier, P., De Genst, E., Kinne, J., Leonhardt, H., Magez, S., Nguyen, V.K., Revets, H., *et al.* (2009). Camelid immunoglobulins and nanobody technology. *Veterinary immunology and immunopathology* *128*, 178-183.
- Nabeel-Shah, S., Ashraf, K., Pearlman, R.E., and Fillingham, J. (2014). Molecular evolution of NASP and conserved histone H3/H4 transport pathway. *BMC evolutionary biology* *14*, 139.
- Naor, D., Nedvetzki, S., Assayag, N., Thurmond, R.L., Huang, J.F., and Turley, E.A. (2005). The mechanism of molecular redundancy in autoimmune inflammation in the context of CD44 deficiency. *Annals of the New York Academy of Sciences* *1050*, 52-63.
- Ni, X., and Morales, C.R. (2006). The lysosomal trafficking of acid sphingomyelinase is mediated by sortilin and mannose 6-phosphate receptor. *Traffic* *7*, 889-902.
- Nichols, B.J., Holthuis, J.C., and Pelham, H.R. (1998). The Sec1p homologue Vps45p binds to the syntaxin Tlg2p. *European journal of cell biology* *77*, 263-268.
- Nielsen, M.S., Madsen, P., Christensen, E.I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R., and Petersen, C.M. (2001). The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *EMBO J* *20*, 2180-2190.
- Nishimoto-Morita, K., Shin, H.W., Mitsuhashi, H., Kitamura, M., Zhang, Q., Johannes, L., and Nakayama, K. (2009). Differential effects of depletion of ARL1 and ARFRP1 on membrane trafficking between the trans-Golgi network and endosomes. *J Biol Chem* *284*, 10583-10592.
- Oda, Y., Huang, K., Cross, F.R., Cowburn, D., and Chait, B.T. (1999). Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci U S A* *96*, 6591-6596.
- Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular & cellular proteomics* : MCP *1*, 376-386.
- Ong, S.E., and Mann, M. (2006). A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nature protocols* *1*, 2650-2660.
- Panic, B., Whyte, J.R., and Munro, S. (2003). The ARF-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Current biology : CB* *13*, 405-410.
- Payne, G.C.a.G.S. (2009). *Trafficking Inside Cells: Pathways, Mechanisms and Regulation_Overview of Protein Trafficking Mechanisms*. Landes Bioscience and Springer Science+Business Media.
- Pinan-Lucarre, B., Tu, H., Pierron, M., Cruceyra, P.I., Zhan, H., Stigloher, C., Richmond, J.E., and Bessereau, J.L. (2014). *C. elegans* Punctin specifies cholinergic versus GABAergic identity of postsynaptic domains. *Nature*.
- Protopopov, V., Govindan, B., Novick, P., and Gerst, J.E. (1993). Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell* *74*, 855-861.

- Puertollano, R., Aguilar, R.C., Gorshkova, I., Crouch, R.J., and Bonifacino, J.S. (2001a). Sorting of mannose 6-phosphate receptors mediated by the GGAs. *Science* 292, 1712-1716.
- Puertollano, R., Randazzo, P.A., Presley, J.F., Hartnell, L.M., and Bonifacino, J.S. (2001b). The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell* 105, 93-102.
- Quenneville, N.R., Chao, T.Y., McCaffery, J.M., and Conibear, E. (2006). Domains within the GARP subunit Vps54 confer separate functions in complex assembly and early endosome recognition. *Mol Biol Cell* 17, 1859-1870.
- Rapti, G., Richmond, J., and Bessereau, J.L. (2011). A single immunoglobulin-domain protein required for clustering acetylcholine receptors in *C. elegans*. *EMBO J* 30, 706-718.
- Ren, X.Q., Cheng, S.B., Treuil, M.W., Mukherjee, J., Rao, J., Braunewell, K.H., Lindstrom, J.M., and Anand, R. (2005). Structural determinants of alpha4beta2 nicotinic acetylcholine receptor trafficking. *J Neurosci* 25, 6676-6686.
- Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *The Journal of cell biology* 17, 208-212.
- Rhee, H.W., Zou, P., Udeshi, N.D., Martell, J.D., Mootha, V.K., Carr, S.A., and Ting, A.Y. (2013). Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science* 339, 1328-1331.
- Richard, M., Boulon, T., Robert, V.J., Richmond, J.E., and Bessereau, J.L. (2013). Biosynthesis of ionotropic acetylcholine receptors requires the evolutionarily conserved ER membrane complex. *Proc Natl Acad Sci U S A* 110, E1055-1063.
- Richardson, B.C., McDonold, C.M., and Fromme, J.C. (2012). The Sec7 Arf-GEF is recruited to the trans-Golgi network by positive feedback. *Developmental cell* 22, 799-810.
- Richmond, J.E., and Jorgensen, E.M. (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat Neurosci* 2, 791-797.
- Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 122, 735-749.
- Robinson, M., Poon, P.P., Schindler, C., Murray, L.E., Kama, R., Gabriely, G., Singer, R.A., Spang, A., Johnston, G.C., and Gerst, J.E. (2006). The Gcs1 Arf-GAP mediates Snc1,2 v-SNARE retrieval to the Golgi in yeast. *Mol Biol Cell* 17, 1845-1858.
- Robinson, M.S. (2004). Adaptable adaptors for coated vesicles. *Trends Cell Biol* 14, 167-174.
- Rohn, W.M., Rouille, Y., Waguri, S., and Hoflack, B. (2000). Bi-directional trafficking between the trans-Golgi network and the endosomal/lysosomal system. *J Cell Sci* 113 (Pt 12), 2093-2101.
- Rojas, R., van Vlijmen, T., Mardones, G.A., Prabhu, Y., Rojas, A.L., Mohammed, S., Heck, A.J., Raposo, G., van der Sluijs, P., and Bonifacino, J.S. (2008). Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. *The Journal of cell biology* 183, 513-526.
- Rostaing, P., Weimer, R.M., Jorgensen, E.M., Triller, A., and Bessereau, J.L. (2004). Preservation of immunoreactivity and fine structure of adult *C. elegans* tissues using high-pressure freezing. *J Histochem Cytochem* 52, 1-12.

- Rothbauer, U., Zolghadr, K., Muyldermans, S., Schepers, A., Cardoso, M.C., and Leonhardt, H. (2008). A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Molecular & cellular proteomics* : MCP 7, 282-289.
- Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M.C., *et al.* (2006). Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nature methods* 3, 887-889.
- Rothman, J.E., and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Current biology* : CB 4, 220-233.
- Rouille, Y., Rohn, W., and Hoflack, B. (2000). Targeting of lysosomal proteins. *Seminars in cell & developmental biology* 11, 165-171.
- Roux, K.J., Kim, D.I., and Burke, B. (2013). BioID: a screen for protein-protein interactions. *Current protocols in protein science / editorial board, John E Coligan [et al]* 74, Unit 19 23.
- Roux, K.J., Kim, D.I., Raida, M., and Burke, B. (2012). A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of cell biology* 196, 801-810.
- Sambrook, J.J., and Russell, D.W. (2001). *Molecular cloning: A Laboratory Manual*.
- Scheel, O., Zdebik, A.A., Lourdel, S., and Jentsch, T.J. (2005). Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature* 436, 424-427.
- Schekman, R., and Orci, L. (1996). Coat proteins and vesicle budding. *Science* 271, 1526-1533.
- Schlager, M.A., Kapitein, L.C., Grigoriev, I., Burzynski, G.M., Wulf, P.S., Keijzer, N., de Graaff, E., Fukuda, M., Shepherd, I.T., Akhmanova, A., *et al.* (2010). Pericentrosomal targeting of Rab6 secretory vesicles by Bicaudal-D-related protein 1 (BICDR-1) regulates neuriteogenesis. *EMBO J* 29, 1637-1651.
- Schmollinger, S., Strenkert, D., Offeddu, V., Nordhues, A., Sommer, F., and Schroda, M. (2012). A protocol for the identification of protein-protein interactions based on 15N metabolic labeling, immunoprecipitation, quantitative mass spectrometry and affinity modulation. *Journal of visualized experiments* : JoVE.
- Schornack, S., Fuchs, R., Huitema, E., Rothbauer, U., Lipka, V., and Kamoun, S. (2009). Protein mislocalization in plant cells using a GFP-binding chromobody. *The Plant journal : for cell and molecular biology* 60, 744-754.
- Schuldiner, M., Metz, J., Schmid, V., Denic, V., Rakwalska, M., Schmitt, H.D., Schwappach, B., and Weissman, J.S. (2008). The GET complex mediates insertion of tail-anchored proteins into the ER membrane. *Cell* 134, 634-645.
- Schuske, K., Beg, A.A., and Jorgensen, E.M. (2004). The GABA nervous system in *C. elegans*. *Trends in neurosciences* 27, 407-414.
- Schwappach, B., Stobrawa, S., Hechenberger, M., Steinmeyer, K., and Jentsch, T.J. (1998). Golgi localization and functionally important domains in the NH2 and COOH terminus of the yeast CLC putative chloride channel Gef1p. *J Biol Chem* 273, 15110-15118.
- Schweizer, A., Fransen, J.A., Bachi, T., Ginsel, L., and Hauri, H.P. (1988). Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *The Journal of cell biology* 107, 1643-1653.

- Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G., and Hardie, D.G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *The Journal of clinical investigation* *113*, 274-284.
- Seaman, M.N. (2005). Recycle your receptors with retromer. *Trends Cell Biol* *15*, 68-75.
- Selbach, M., and Mann, M. (2006). Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK). *Nature methods* *3*, 981-983.
- Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R.A., and Rothman, J.E. (1991). ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* *67*, 239-253.
- Setty, S.R., Shin, M.E., Yoshino, A., Marks, M.S., and Burd, C.G. (2003). Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 is regulated by Arf-like GTPase 3. *Current biology : CB* *13*, 401-404.
- Shanks, S.G., Carpp, L.N., Struthers, M.S., McCann, R.K., and Bryant, N.J. (2012). The Sec1/Munc18 protein Vps45 regulates cellular levels of its SNARE binding partners Tlg2 and Snc2 in *Saccharomyces cerevisiae*. *PLoS One* *7*, e49628.
- Shin, H.W., Kobayashi, H., Kitamura, M., Waguri, S., Suganuma, T., Uchiyama, Y., and Nakayama, K. (2005). Roles of ARFRP1 (ADP-ribosylation factor-related protein 1) in post-Golgi membrane trafficking. *J Cell Sci* *118*, 4039-4048.
- Short, B., Haas, A., and Barr, F.A. (2005). Golgins and GTPases, giving identity and structure to the Golgi apparatus. *Biochimica et biophysica acta* *1744*, 383-395.
- Simon, S.M. (2008). Golgi governance: the third way. *Cell* *133*, 951-953.
- Siniosoglou, S., and Pelham, H.R. (2001). An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. *EMBO J* *20*, 5991-5998.
- Smith, M.M., Lindstrom, J., and Merlie, J.P. (1987). Formation of the alpha-bungarotoxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. *J Biol Chem* *262*, 4367-4376.
- Sohn, K., Orci, L., Ravazzola, M., Amherdt, M., Bremser, M., Lottspeich, F., Fiedler, K., Helms, J.B., and Wieland, F.T. (1996). A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatamer binding. *The Journal of cell biology* *135*, 1239-1248.
- Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *The Journal of cell biology* *149*, 901-914.
- Spieth, J., and Blumenthal, T. (1985). The *Caenorhabditis elegans* vitellogenin gene family includes a gene encoding a distantly related protein. *Mol Cell Biol* *5*, 2495-2501.
- Stagg, S.M., LaPointe, P., and Balch, W.E. (2007). Structural design of cage and coat scaffolds that direct membrane traffic. *Current opinion in structural biology* *17*, 221-228.
- Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nature reviews Molecular cell biology* *10*, 513-525.
- Sulston, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* *100*, 64-119.
- Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* *395*, 347-353.

- Szewczyk, N.J., Kozak, E., and Conley, C.A. (2003). Chemically defined medium and *Caenorhabditis elegans*. *BMC biotechnology* 3, 19.
- Takahashi, S., Kubo, K., Waguri, S., Yabashi, A., Shin, H.W., Katoh, Y., and Nakayama, K. (2012). Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. *J Cell Sci* 125, 4049-4057.
- Takatsu, H., Katoh, Y., Shiba, Y., and Nakayama, K. (2001). Golgi-localizing, gamma-adaptin ear homology domain, ADP-ribosylation factor-binding (GGA) proteins interact with acidic dileucine sequences within the cytoplasmic domains of sorting receptors through their Vps27p/Hrs/STAM (VHS) domains. *J Biol Chem* 276, 28541-28545.
- Takizawa, P.A., and Malhotra, V. (1993). Coatomers and SNAREs in promoting membrane traffic. *Cell* 75, 593-596.
- Tam, E.C.a.Y.Y.C. (2009). The endocytic pathway. *Trafficking Inside Cells: Pathways, Mechanisms and Regulation*.
- Teasdale, R.D., and Jackson, M.R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annual review of cell and developmental biology* 12, 27-54.
- Teichert, U., Mechler, B., Muller, H., and Wolf, D.H. (1989). Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J Biol Chem* 264, 16037-16045.
- Terenzio, M., and Schiavo, G. (2010). The more, the better: the BICD family gets bigger. *EMBO J* 29, 1625-1626.
- Thany, S.H., Lenaers, G., Raymond-Delpech, V., Sattelle, D.B., and Lapied, B. (2007). Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. *Trends in pharmacological sciences* 28, 14-22.
- Thyagarajan, K., Afshar, K., and Gonczy, P. (2011). Polarity mediates asymmetric trafficking of the Gbeta heterotrimeric G-protein subunit GPB-1 in *C. elegans* embryos. *Development* 138, 2773-2782.
- Tokatlidis, K., Vial, S., Luciano, P., Vergnolle, M., and Clemence, S. (2000). Membrane protein import in yeast mitochondria. *Biochemical Society transactions* 28, 495-499.
- Touroutine, D., Fox, R.M., Von Stetina, S.E., Burdina, A., Miller, D.M., 3rd, and Richmond, J.E. (2005). *acr-16* encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. *J Biol Chem* 280, 27013-27021.
- Town, M., Jean, G., Cherqui, S., Attard, M., Forestier, L., Whitmore, S.A., Callen, D.F., Gribouval, O., Broyer, M., Bates, G.P., *et al.* (1998). A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. *Nature genetics* 18, 319-324.
- Traub, L.M. (2003). Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *The Journal of cell biology* 163, 203-208.
- Treusch, S., Knuth, S., Slaugenhaupt, S.A., Goldin, E., Grant, B.D., and Fares, H. (2004). *Caenorhabditis elegans* functional orthologue of human protein h-mucolipin-1 is required for lysosome biogenesis. *Proc Natl Acad Sci U S A* 101, 4483-4488.
- Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci U S A* 85, 4538-4542.
- Trinkle-Mulcahy, L., Boulon, S., Lam, Y.W., Urcia, R., Boisvert, F.M., Vandermoere, F., Morrice, N.A., Swift, S., Rothbauer, U., Leonhardt, H., *et al.* (2008). Identifying specific

- protein interaction partners using quantitative mass spectrometry and bead proteomes. *The Journal of cell biology* 183, 223-239.
- van den Elsen, J.M., Kuntz, D.A., and Rose, D.R. (2001). Structure of Golgi alpha-mannosidase II: a target for inhibition of growth and metastasis of cancer cells. *EMBO J* 20, 3008-3017.
- van Ijzendoorn, S.C. (2006). Recycling endosomes. *J Cell Sci* 119, 1679-1681.
- van Koningsbruggen, S., de Haard, H., de Kievit, P., Dirks, R.W., van Remoortere, A., Groot, A.J., van Engelen, B.G., den Dunnen, J.T., Verrips, C.T., Frants, R.R., *et al.* (2003). Llama-derived phage display antibodies in the dissection of the human disease oculopharyngeal muscular dystrophy. *Journal of immunological methods* 279, 149-161.
- Vida, T.A., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *The Journal of cell biology* 128, 779-792.
- Von Stetina, S.E., Treinin, M., and Miller, D.M., 3rd (2006). The motor circuit. *International review of neurobiology* 69, 125-167.
- Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 10, 1793-1808.
- Wachter, A., and Schwappach, B. (2005). The yeast CLC chloride channel is proteolytically processed by the furin-like protease Kex2p in the first extracellular loop. *FEBS Lett* 579, 1149-1153.
- Wanamaker, C.P., Christianson, J.C., and Green, W.N. (2003). Regulation of nicotinic acetylcholine receptor assembly. *Annals of the New York Academy of Sciences* 998, 66-80.
- Wang, J.M., Zhang, L., Yao, Y., Viroonchatapan, N., Rothe, E., and Wang, Z.Z. (2002). A transmembrane motif governs the surface trafficking of nicotinic acetylcholine receptors. *Nat Neurosci* 5, 963-970.
- Wang, X., Matteson, J., An, Y., Moyer, B., Yoo, J.S., Bannykh, S., Wilson, I.A., Riordan, J.R., and Balch, W.E. (2004). COPII-dependent export of cystic fibrosis transmembrane conductance regulator from the ER uses a di-acidic exit code. *The Journal of cell biology* 167, 65-74.
- Wei, N., Serino, G., and Deng, X.W. (2008). The COP9 signalosome: more than a protease. *Trends in biochemical sciences* 33, 592-600.
- Wessler, I., Kilbinger, H., Bittinger, F., Unger, R., and Kirkpatrick, C.J. (2003). The non-neuronal cholinergic system in humans: expression, function and pathophysiology. *Life Sci* 72, 2055-2061.
- Westermann, B., and Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast* 16, 1421-1427.
- Wiedemann, N., Frazier, A.E., and Pfanner, N. (2004). The protein import machinery of mitochondria. *J Biol Chem* 279, 14473-14476.
- Wilcox, C.A., Redding, K., Wright, R., and Fuller, R.S. (1992). Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol Biol Cell* 3, 1353-1371.
- Wu, Y., Yeh, F.L., Mao, F., and Chapman, E.R. (2009). Biophysical characterization of styryl dye-membrane interactions. *Biophysical journal* 97, 101-109.

- Yaffe, M.P., and Schatz, G. (1984). Two nuclear mutations that block mitochondrial protein import in yeast. *Proc Natl Acad Sci U S A* 81, 4819-4823.
- Yeung, B.G., Phan, H.L., and Payne, G.S. (1999). Adaptor complex-independent clathrin function in yeast. *Mol Biol Cell* 10, 3643-3659.
- Yuan, D.S., Dancis, A., and Klausner, R.D. (1997). Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J Biol Chem* 272, 25787-25793.
- Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R.D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc Natl Acad Sci U S A* 92, 2632-2636.
- Zahn, C., Hommel, A., Lu, L., Hong, W., Walther, D.J., Florian, S., Joost, H.G., and Schurmann, A. (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.
- Zakrzewska, E., Perron, M., Laroche, A., and Pallotta, D. (2003). A role for GEA1 and GEA2 in the organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. *Genetics* 165, 985-995.
- Zeiler, M., Straube, W.L., Lundberg, E., Uhlen, M., and Mann, M. (2012). A Protein Epitope Signature Tag (PrEST) library allows SILAC-based absolute quantification and multiplexed determination of protein copy numbers in cell lines. *Molecular & cellular proteomics : MCP* 11, O111 009613.
- Zerangue, N., Schwappach, B., Jan, Y.N., and Jan, L.Y. (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* 22, 537-548.
- Zhang, B., and Zehhof, A.C. (2002). Amphiphysins: raising the BAR for synaptic vesicle recycling and membrane dynamics. *Bin-Amphiphysin-Rvsp. Traffic* 3, 452-460.
- Zhang, Y., Grant, B., and Hirsh, D. (2001). RME-8, a conserved J-domain protein, is required for endocytosis in *Caenorhabditis elegans*. *Mol Biol Cell* 12, 2011-2021.

XI. APPENDIX

All used strains (bacteria *E. coli*, nematode *C. elegans* and yeast *S. cerevisiae*), oligonucleotides, plasmids, media, buffer solutions, materials, reagents and research equipment in this study are listed in the table XI.1.1 - XI.6.4 with description, source and manufacturer information provided.

11.1 Strains of bacteria/yeast and *C. elegans*

Table XI.1.1 Bacterial *E. coli* strains used in this study

Strain	Description	Source
<i>E. coli</i> OP50	Feeding strains for <i>C. elegans</i> (uracil auxotroph)	(Brenner, 1974)
<i>E. coli</i> DH5 α	Competent cells for molecular cloning	Invitrogen
<i>E. coli</i> SLE1	Strains carrying pAG608 (<i>orn-1</i> RNAi feeding vector) for SILAC, [<i>argA</i> , <i>lysA</i> , <i>mcrA</i> , <i>mcrB</i> , <i>IN</i> (<i>rrnD-rrnE</i>) 1, <i>lambda</i> -, <i>rcn14::Tn10</i> (<i>DE3</i> lysogen:: <i>lavUV5 promoter</i> - <i>T7 polymerase</i>)]	(Larance et al., 2011)

Table XI.1.2 Yeast *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Background	Reference
BY4741	MATa <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	S288C	(Brachmann et al., 1998)
<i>Δgmh1a</i>	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>lys2Δ0</i> <i>ura3Δ0</i> <i>gmh1::KanMX4</i>	S288C	Stefan Eimer
<i>Δgmh1::Nat^R</i>	MATa <i>Δgmh1::Nat^R</i>	Y5563	Stefan Eimer
<i>Δgef1::Nat^R</i>	BY4741; <i>Δgef1::Nat^R</i>	BY4741	Matthieu S. Peters ^{\$}
<i>Δgmh1;Δgef1</i>	S288C MATa <i>Δgmh1::KAN</i> ; BY4741 MATa <i>Δgef1::Nat^R</i>		Matthieu S. Peters ^{\$}
<i>Δpep4</i>	BY4741; <i>Δpep4::KanMX4</i>	BY4741	EUROSCARF*
<i>Δfet3</i>	BY4741; <i>Δfet3::KanMX4</i>	BY4741	(Braun et al., 2010)
<i>Δgmh1;Δpep4</i>	Y5563 MATa <i>Δgmh1::NAT</i> ; BY4741 MATa <i>Δpep4::KanMX4</i>		This study
Anp1-RFP	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>lys2Δ0</i> <i>ura3Δ0</i> <i>YEL036c-mRFP::Kan^R</i>	BY4742	(Huh et al., 2003)

Anp1-RFP; <i>Δgmh1::Nat^R</i>	MATα <i>his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i> <i>YEL036c-mRFP::Kan^R</i> ; <i>Δgmh1::Nat^R</i>	Anp1-RFP	This study
Chc1-RFP	MATα <i>his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i> <i>YGL206c-mRFP::Kan^R</i>	BY4742	(Huh et al., 2003)
Chc1-RFP; <i>Δgmh1::Nat^R</i>	MATα <i>his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i> <i>YGL206c-mRFP::Kan^R</i> ; <i>Δgmh1::Nat^R</i>	Chc1-RFP	This study
Snf7-RFP	MATα <i>his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i> <i>YLR025w-mRFP::Kan^R</i>	BY4742	(Huh et al., 2003)
Snf7-RFP; <i>Δgmh1::Nat^R</i>	MATα <i>his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i> <i>YLR025w-mRFP::Kan^R</i> ; <i>Δgmh1::Nat^R</i>	Snf7RFP	This study

EUROSCARF*: <http://web.uni-frankfurt.de/fb15/mikro/euroscarf>

Matthieu S. Peters[§]: this stain was made by Matthieu S. Peters in collaboration with Blanche Schwappach and Stefan Eimer, 2011, Göttingen, Germany

Table XI.1.3 *C. elegans* strains used in this study

Strain	Genotype
<i>Bristol</i>	<i>N2 (wild type)</i>
X47	<i>unc-50 (x47) III</i>
RB1539	<i>unc-50 (ok1847) III</i>
MF200	<i>ric-3 (hm9) IV</i>
CB1072	<i>unc-29 (e1072) I</i>
DH1336	<i>bIs34 [rme-8::GFP + rol-6 (su1006)]</i>
This study	<i>unc-50 (x47) III; bIs34 [rme-8::GFP + rol-6 (su1006)]</i>
RT258	<i>unc-119 (ed3) III; pwIs50 [lmp-1::GFP + Cbr-unc-119 (+)]</i>
This study	<i>unc-50 (x47) III; unc-119 (ed3) III; pwIs50 [lmp-1::GFP + Cbr-unc-119 (+)]</i>
CB5600	<i>him-8 (e1489) IV; ccls4251 [pmyo-3::nls-gfp-lacZ; pmyo-3::mt-gfp] I</i>
This study	<i>unc-50 (x47) III; ccls4251 I; him-8 (e1489) IV</i>
GS1912	<i>arIs37 [pmyo-3::ssGFP] I; dpy-20 (e1282) IV</i>
This study	<i>N2; arIs37 [pmyo-3::ssGFP] I; dpy-20 (e1282) IV; rol-6 (su1006)</i>
This study	<i>unc-50 (x47) III; arIs37 [pmyo-3::ssGFP] I; dpy-20 (e1282) IV</i>
This study	<i>unc-50 (x47) III; arIs37 [pmyo-3::ssGFP] I; dpy-20 (e1282) IV; rol-6 (su1006)</i>
Y54E10BR.2	<i>Arfrp1 (tm2779) I</i>

KN555	<i>vps-35 (hu68) II</i>
VC2202	<i>vps-53 (ok2864) III</i>
NF1684	<i>cogc-3 (k181) I</i>
This study	<i>mIn1[dpy-10 (e128) mIs14 (myo-2::GFP)] II/ vps-35 (hu68) II;</i> <i>Arfrp1 (tm2779) I</i>
This study	<i>ccls4251 I/ cogc-3 (k181) I; vps-35 (hu68) II</i>
This study	<i>ccls4251 I/ Arfrp1 (tm2779) I; vps-53 (ok2864) III</i>
This study	<i>unc-50 (x47) III; gZIs20 [pmyo-3::unc-50::BirA*]</i>
This study	<i>unc-50 (x47) III; gZIs21 [pmyo-3::GFP::BirA*]</i>
This study	<i>unc-50 (x47) III; gZIs22 [pmyo-3::αGFPNBmyc::BirA*]</i>
EG1653	<i>oxIs22 II [punc-49::unc-49::GFP + lin-15 (+)]</i>
SJ4103	<i>zcls14 [pmyo-3::GFP (mito-matrix)]</i>
This study	<i>oxIs22 II; sjEx [N2; InJ64 (pmyo-3::αGFPNBmyc::tagRFPT)]</i>
This study	<i>zcls14; sjEx [N2; InJ64 (pmyo-3::αGFPNBmyc::tagRFPT)]</i>
This study	<i>ccls4251 I/ sjEx [N2; InJ64 (pmyo-3::αGFPNBmyc::tagRFPT)]</i>

BirA, biotin protein ligase in *E. coli*; BirA*, BirA (R118G); αGFPNB, anti-GFP-nanobody that contains the peptide sequences of a myc-tag. All mutant strains used in this study were outcrossed at least three times before use. Some nematode strains used in this study were provided by the CGC, which is funded by the NIH NCCR. *arfrp1 (tm2779)* was supplied by the NBRP (Tokyo Women's Medical University School of Medicine). *unc-50 (ok1847)* was provided by Gene Knockout Project at Oklahoma Medical Research Foundation (OMRF), which is part of the international *C. elegans* Gene Knockout Consortium.

11.2 Oligonucleotides and deletion alleles

Table XI.2.1 DNA sequences of primers used in this study

Primer	Sequence	Description
oGQ2785	GTGAAGTACGATCTAAACTGTTG	Forward primer for <i>gmh1</i> , that binds before the promotor, for creating <i>gmh1;pep4</i> double-deleted mutants using $\Delta gmh1$ strains without restriction enzyme sites
oGQ2786	ACGACCAGGAACACACCATTA	Reverse primer for <i>gmh1</i> , that binds after the terminator, for creating <i>gmh1;pep4</i> double-deleted mutants using $\Delta gmh1$ strains without restriction enzyme sites

oGQ617	GGGACTAGTTCTAGAAATGTCATAT TTGCCGACTTATTCC	Forward primer of <i>gmh1</i> without promoter that includes SpeI and XbaI sites
oGQ618	GGGGTCGACGGATCCTTAATAATT GTAAAAGCTTAATTTCTTGGA	Reverse primer of <i>gmh1</i> without promoter that includes Sall and BamHI sites
GefE287QF	TTGGAACAGATTGCTTCTG	Forward primer for checking deletions of <i>gef1</i> in yeast
S618AR	ACCATGCACAGCATATTCAGACGC TGAGTC	Reverse primer for checking deletions of <i>gef1</i> in yeast
oGQ1974	TACACACTTTCGGCGGGTAT	External forward primer for <i>arfrp1</i> (<i>tm2779</i>)
oGQ1975	ACAATCGGCAAGAAGCGGTG	External reverse primer for <i>arfrp1</i> (<i>tm2779</i>)
oGQ1976	TTCGGCGGGTATCTGGGAGA	Internal forward primer for <i>arfrp1</i> (<i>tm2779</i>)
oGQ1977	GGTTAGGAAATCATCACAGG	Internal reverse primer for <i>arfrp1</i> (<i>tm2779</i>)
oGQ415	ATGTACGAAAATTCGGGGAATAC GACCGACCAGGAGAAGTTTTTG	Forward primer for <i>vps-35</i> (<i>hu68</i>) (outside deletion)
oGQ416	TTAAGACGGAGGTTGCGGAAGTT CAGGTTCTGAACGAATTGTTGT	Reverse primer for <i>vps-35</i> (<i>hu68</i>) (outside deletion)
oGQ417	GTGAGTTCCGACACATTTTTTTTA ATTAGGGATGATTCTCAAAAA	Forward primer for <i>vps-35</i> (<i>hu68</i>) (inside deletion)
oGQ418	ATTAAGATACAACCTTCATTGGAAG TTCTGCTAATTCAGCTGTCTGA	Reverse primer for <i>vps-35</i> (<i>hu68</i>) (inside deletion)
oGQ1654	CCAAGAAAGTATCAAAAACACTC CAAATAG	Forward primer <i>vps-53</i> (<i>ok2864</i>) (inside deletion)
oGQ1655	GCTGATAATGAAGAAGGCGCATG GTTGGAT	Reverse primer <i>vps-53</i> (<i>ok2864</i>) (inside deletion)
oGQ1656	ATCAATGCATCAGGTTCACTGCTC AACTTG	Forward primer <i>vps-53</i> (<i>ok2864</i>) (outside deletion)
oGQ1657	CGTATCGTAGATACCTTCATAATA GAGTTA	Reverse primer <i>vps-53</i> (<i>ok2864</i>) (outside deletion)
oSE014	GGGGATCCACCGGTAGAAAAAAT GTTACCGAGTACTTCAGTG	Human <i>gmh1</i> forward primer that introduces a BamHI and AgeI site upstream of the ATG
oGQ2649	GGGCGGCCGCTTTCACTCTGTACT TATAGAAAGAACAGAGAGT	Reverse primer for h <i>Gmh1</i> ^a that removes the stop codon and introduces a NotI site afterwards
oSE016	GGGGATCCACCGGTAGAAAAAAT GTCATATTTGCCGACTTATTCC	Forward primer for <i>gmh1</i> that introduces a BamHI and AgeI site upstream of the ATG
oGQ2648	GGGCGGCCGCATAATTGTAAAAG CTTAATTTCTTGATAAGTCG	Reverse primer for <i>gmh1</i> that removes the stop codon and introduces a NotI site afterwards
oSE004	CTTCTTCTTCCACTTTTACCG	Forward primer for <i>myo-3</i> promoter

oGQ1395	AACAGGTAGTTTTCCAGTAGTGCA	Reverse primer matching the first exon in GFP/YFP
oGQ849	GGATCCAGGGTACCGGTAGCCAT GGTTAAGCCGCAAGGAGGG	Forward primer for <i>tram-1</i> that starts at the start ATG and introduces a BamHI, KpnI, AgeI and NcoI site upstream
oGQ850	CTCGAGAATTCAATTTTTCTTCTTC GAATCGCTCTC	Reverse primer for <i>tram-1</i> that starts at the stop codon and introduces a EcoRI and XhoI site afterwards
oGQ440	AATTGCCAGATGAACAGCAGC	Reverse sequencing primer for <i>apt-9</i> (GGA adaptor arf-binding)
oGQ2238	GGCTTGCCTTCGCCCTCGGATGTG C	Reverse sequencing primer for TagRFP_T binding at N-terminus
oGQ2912	GGGGATCCGCGGTACCGCATATG AAGGATAACACC GTGCCA	Forward primer for <i>E. coli birA</i> that start at the start ATG and introduces a BamHI, KpnI, and NdeI site upstream
oGQ2913	GAATTCTACCGGTGAGCGGCCGCC TGCAGAACCACCTTTTTCTGCACT ACGCAGGGA	Reverse primer for <i>E. coli birA</i> that removes the stop codon and introduces GGSA linker and a NotI and AgeI site afterwards
oGQ2914	CAGGCTGGCCGTGGTGGTTCGGGG TCGGAAATGGTTTTTCG	Forward mutagenesis primer for <i>E. coli birA</i> that introduces the R118G mutation
oGQ2915	CCATTTCCGACCCCGACCACCACG GCCAGCCTGCTGATG	Reverse mutagenesis primer for <i>E. coli birA</i> that introduces the R118G mutation
oGQ2943	GCGGTCTTGGTGGCGGCCGCGCG GAGGCGGCTAGCGTGAAGGATAA CACCGTGCCACT	Forward primer for <i>birA</i> (R118G) pGEMT that contains overlapped seq. of C-terminal UNC-50 and introduces a NotI with linker (GSGAS, 15nt) without the start codon of <i>birA</i> for fusion at the downstream of UNC-50
oGQ2944	CCGAATTCTCATTTTTCTGCACTA CGCAGGGA	Reverse primer for <i>birA</i> (R118G) pGEMT that introduces a EcoRI and the stop codon of <i>birA</i> for fusion at the downstream of UNC-50
oGQ3032	GCAAGCTTGGTACCGGATCCATGG CC	Forward primer for antiGFP-nanobody-myc-tag that contains Met, Ala of the construct GFPNB-Myc_pMAT
oGQ3033	GGGAATTCGCGGCCGCCAGGTC	Reverse primer for antiGFP-nanobody-myc-

CTCTTCGGA	tag that introduces a NotI site behind the myc-tag
-----------	--

^a : *hGmh1* represents human *gmh1* which is *unc-50* like.

Table XI.2.2 List of deletion alleles used in *C. elegans*

Deletion strain	Size of deletion (bp) or approx.	Outside deletion primers	PCR band sizes in WT or mutant (m) (bp)	Inside deletion primers	PCR band sizes in WT (bp)	Anneal. temp./ Extens. time
<i>Arfrp1</i> (<i>tm2779</i>)	404	oGQ1974	493 (m)	oGQ1977	897	50°C/ 1:00 min
<i>vps-53</i> (<i>ok2864</i>)	373	oGQ1656/oGQ1657	1060	oGQ1654/oGQ1657	855	51°C/ 1:30 min
<i>vps-53</i> (<i>ok2864</i>)	314	oGQ1657	541 (m)	oGQ1654	855	51°C/ 1:30 min
<i>vps-53</i> (<i>ok2864</i>)	–	–	no bands (m)	oGQ1654/oGQ1655	334	59°C/ 2:00 min
<i>vps-35</i> (<i>hu68</i>)	–	oGQ415	380 (m)	oGQ416	2889	68°C/ 2:30 min
<i>vps-35</i> (<i>hu68</i>)	–	oGQ415	no bands (m)	oGQ418	2137	64°C/ 2:30 min

Table XI.2.3 Polymerase chain reaction conditions in yeast cells

Reagents for master mix	(μl) vol. in 50 μl per reaction	Program of PCR (cycle in order)	Amplified with the primer pairs (Forward / Reverse)	Predicted sizes (bp) approx.
10X PCR Buffer	5	(1) 3 m, 95°C	oGQ617/oGQ618	850
2.5 mM dNTP	4	(2) 20 s, 95°C	GefE287QF/S618AR	1100
10 μM (F) primer	2	(3) 1 m 30 s, 51°C	<i>for checking deletions (gmh1, gef1)</i>	
10 μM (R) primer	2	(4) 1 m, 72°C	oGQ2785/oGQ2786	1800
Taq-Polymerase (2.5 unit, Roche)	1	(5) 35 cycles	Template*: $\Delta gmh1::Nat^R$	
ddH ₂ O	35	from (2) to (4)	<i>for making $\Delta gmh1;\Delta pep4$</i>	
Template* (Chen et al.)	1 (20-80 ng)	(6) 5 m, 72°C	oGQ617/oGQ618	850
		(7) keep at 4°C	<i>for double-checking gmh1 mutants</i>	

Template*, genomic DNAs purified were used for checking deletion mutants and also creating $\Delta gmh1::Nat^R$ knockout strains in order to be transformed in various strains which were $\Delta pep4::Kan^R$, Anp1-RFP::Kan^R, Chc1-RFP::Kan^R and Snf7-RFP::Kan^R by PCR-based drug resistance cassettes for gene deletions (Goldstein and McCusker, 1999; Wach et al., 1994).

11.3 Plasmids and injection constructs

Table XI.3.1 List of backbone vectors used in this study

Backbone vectors	Resistance Gene	Description	Source
pBluescript II SK(+)	Amp ^r	Entry vector for cloning and used for injection mixtures	Stratagene
pGEMT	Amp ^r	Entry vector for TA cloning	Promega
pPD115.62 (<i>pmyo-3::gfp</i>)	Amp ^r	Muscle expression vector for <i>C. elegans</i> contains <i>unc-54</i> 3'UTR	Addgene, Andrew Fire vector kit

Table XI.3.2 List of genes of interest in entry vectors

Genes of interest in entry vectors	Resistance Gene	Cloning Strategy	Source
pGEMT <i>gmh1</i>	Amp ^r	PCR with oSE016/oGQ2648 and digested with AgeI/NotI	Cloned in this study
pGEMT <i>hGmh1</i>	Amp ^r	PCR with oSE014/oGQ2649 and digested with AgeI/NotI	Cloned in this study
pGEMT <i>tram-1</i>	Amp ^r	–	Kindly provided by Sabine Koenig
pGEMT <i>BirA</i>	Amp ^r	Mutagenesis PCR with oGQ2914/oGQ2915 for <i>E. coli birA</i> (R118G) pGEMT	Kindly provided by Sabine Koenig
pGEMT <i>BirA</i> *	Amp ^r	PCR with oGQ2943/oGQ2944 and digested with NotI/EcoRI	Cloned in this study
pMAT <i>αGFPNBmyc</i>	Amp ^r	PCR with oGQ3032/oGQ3033 and digested with KpnI/NotI	Kindly provided by Nora Wender

αGFPNB-myc, anti-GFP nanobodies that contain a polypeptide protein myc-tag.

Table XI.3.3 List of genes of interest in injection vectors

Genes of interest in injection vectors	Resistance Gene	Description	Source
<i>pRF4-rol-6</i>	Amp ^r	Co-injection marker, <i>rol-6</i> (<i>su1006</i>)	(Mello and Fire, 1995)

<i>pttx-3::gfp</i>	Amp ^r	Co-injection marker, <i>gfp</i> expression in head as a pair of AIY inter-neuron	(Hobert et al., 1997)
<i>pttx-3::rfp</i>	Amp ^r	–	Kindly provided by Sabine Koenig
<i>pmyo-3::tom70-cfp</i>	Amp ^r	Mitochondrial outer membrane marker in <i>C. elegans</i> muscle cells	Kindly provided by Alexander van der Blik
<i>pmyo-3::unc-50-yfp</i>	Amp ^r	–	Kindly provided by Stefan Eimer
<i>pmyo-3::hGmh1-yfp</i>	Amp ^r	PCR with oSE014/oGQ2649; digested with AgeI/NotI	Cloned in this study
<i>pmyo-3::gmh1-yfp</i>	Amp ^r	PCR with oSE016/oGQ2648; digested with AgeI/NotI	Cloned in this study
<i>pmyo-3::mans-tagRFP</i>	Amp ^r	–	Kindly provided by Ling Luo
<i>pmyo-3::ctns-1-yfp</i>	Amp ^r	–	Kindly provided by Ling Luo
<i>pmyo-3::gfp-2xfyve</i>	Amp ^r	–	(Luo et al., 2011)
<i>pmyo-3::yfp-rab-5</i>	Amp ^r	–	Kindly provided by Marija Sumakovic
<i>pmyo-3::mcherry-rab-7</i>	Amp ^r	–	Kindly provided by Sabine Koenig
<i>pmyo-3::mcherry- εCop</i>	Amp ^r	–	Kindly provided by Ling Luo
<i>prab-3::tagRFP-rab-11.1</i>	Amp ^r	–	Kindly provided by Nikhil Sasidharan
<i>pmyo-3::tagRFP-rab-11.1</i>	Amp ^r	Subcloned from <i>prab-3::tagRFP-rab-11.1</i> ; digested with KpnI/HindIII	Cloned in this study
<i>prab-3::mcherry-apt-9</i>	Amp ^r	–	Kindly provided by Mandy Hannemann
<i>pmyo-3::tagRFPt-apt-9</i>	Amp ^r	Subcloned from <i>prab-3::mcherry-apt-9</i> ; digested with NotI/XhoI	Cloned in this study

<i>pmyo-3::gfp-tram-1</i>	Amp ^r	Subcloned from pGEMT <i>tram-1</i> with oGQ849/oGQ850; digested with AgeI/EcoRI	Cloned in this study
<i>pmyo-3::unc-50-BirA*</i>	Amp ^r	Subcloned from pGEMT <i>BirA*</i> with NotI/EcoRI	Cloned in this study
<i>pmyo-3::gfp-BirA*</i>	Amp ^r	Subcloned from <i>pmyo-3::gfp-rab-10</i> ; digested with KpnI/NotI	Cloned in this study
<i>pmyo-3::αGFPNBmyc-BirA*</i>	Amp ^r	Subcloned from <i>αGFPNB-myc-pMAT</i> with oGQ3032/oGQ3033; digested with KpnI/NotI	Cloned in this study
<i>pmyo-3::αGFPNBmyc-tagRFPT</i>	Amp ^r	Subcloned from <i>pmyo-3::moesin-tagRFPT</i> ; digested with NotI/EcoRI	Cloned in this study

Amp^r, ampicillin; BirA, biotin protein ligase in *E. coli*; BirA*, BirA(R118G); αGFPNB, anti-GFP nanobody that contains the peptide sequences of c-myc-tag.

Table XI.3.4 List of transgenic lines of extrachromosomal arrays (Ex.)

Ex. Name	Plasmids of interest	Injection markers
InJ13	3 ng/μl <i>pmyo-3::unc-50-yfp</i>	40 ng/μl <i>prab-3::gfp</i>
InJ14	2 ng/μl <i>pmyo-3::unc-50-yfp</i>	40 ng/μl <i>pRF4 (rol-6 (su1006))</i>
InJ16	2 ng/μl <i>pmyo-3::unc-50-yfp</i>	20 ng/μl <i>pttx-3::gfp</i> ; 40 ng/μl <i>pRF4 (rol-6 (su1006))</i>
InJ18	3 ng/μl <i>pmyo-3::gmh1 (human)-yfp</i> ; 1 ng/μl <i>pmyo-3::mans-tagRFP</i>	20 ng/μl <i>pttx-3::gfp</i> ; 40 ng/μl <i>pRF4 (rol-6 (su1006))</i>
InJ19	3 ng/μl <i>pmyo-3::gmh1 (yeast)-yfp</i> ; 1 ng/μl <i>pmyo-3::mans-tagRFP</i>	20 ng/μl <i>pttx-3::gfp</i> ; 40 ng/μl <i>pRF4 (rol-6 (su1006))</i>
InJ20	3 ng/μl <i>pmyo-3::unc-50-yfp</i> ; 1 ng/μl <i>pmyo-3::mans-tagRFP</i>	20 ng/μl <i>pttx-3::gfp</i> ; 40 ng/μl <i>pRF4 (rol-6 (su1006))</i>
InJ40	2 ng/μl <i>pmyo-3::ctns-1-yfp</i>	40 ng/μl <i>pRF4 (rol-6 (su1006))</i> ; 5 ng/μl <i>pmyo-3::tom-70-CFP</i>

InJ42	<i>2 ng/μl pmyo-3::gfp-2xfyve</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP</i>
InJ43	<i>2 ng/μl pmyo-3::yfp-rab-5</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP</i>
InJ47	<i>10 ng/μl pmyo-3::mcherry-eCop</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP</i>
InJ49	<i>5 ng/μl pmyo-3::tagRFP-rab-11.1</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP;</i> <i>20 ng/μl pttx-3::gfp</i>
SK48	<i>2 ng/μl pmyo-3:: mcherry-rab-7</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP</i>
InJ51	<i>5 ng/μl pmyo-3::tagRFP-apt-9</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP;</i> <i>20 ng/μl pttx-3::gfp</i>
InJ53	<i>3 ng/μl pmyo-3::mans-tagRFP</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP;</i> <i>20 ng/μl pttx-3::gfp</i>
InJ54	<i>5 ng/μl pmyo-3::gfp-tram-1</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>20 ng/μl pttx-3::gfp</i>
InJ55	<i>3 ng/μl pmyo-3::unc-50-yfp;</i> <i>5 ng/μl pmyo-3::tagRFP-apt-9</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>5 ng/μl pmyo-3::tom-70-CFP;</i> <i>20 ng/μl pttx-3::gfp</i>
InJ60	<i>10 ng/μl pmyo-3::unc-50-BirA*</i> <i>unc-50 (x47)-background</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>20 ng/μl pttx-3::gfp</i>
InJ61	<i>10 ng/μl pmyo-3::gfp-BirA*</i> <i>unc-50 (x47)-background</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>20 ng/μl pttx-3::gfp</i>
InJ62	<i>10 ng/μl pmyo-3::αGFPNBmyc-BirA*</i> <i>unc-50 (x47)-background</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>20 ng/μl pttx-3::gfp</i>
InJ63	<i>8 ng/μl pmyo-3::αGFPNBmyc-tagRFPt</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>8 ng/μl pmyo-3::tom-70-CFP;</i> <i>20 ng/μl pttx-3::gfp</i>
InJ64	<i>8 ng/μl pmyo-3::αGFPNBmyc-tagRFPt</i>	<i>40 ng/μl pRF4 (rol-6 (su1006));</i> <i>20 ng/μl pttx-3::gfp</i>

InJ65	5 ng/ μ l <i>pmyo-3::mans::gfp</i>	40 ng/ μ l <i>pRF4 (rol-6 (su1006))</i> ; 20 ng/ μ l <i>pttx-3::rfp</i>
InJ66	15 ng/ μ l <i>pmyo-3::mans::gfp</i> 4 ng/ μ l <i>pmyo-3::αGFPNBmyc-tagRFp</i>	40 ng/ μ l <i>pRF4 (rol-6 (su1006))</i> ; 20 ng/ μ l <i>pttx-3::rfp</i>
InJ67	10 ng/ μ l <i>pmyo-3::gfp-tram-1</i> 4 ng/ μ l <i>pmyo-3::αGFPNBmyc-tagRFp</i>	40 ng/ μ l <i>pRF4 (rol-6 (su1006))</i> ; 20 ng/ μ l <i>pttx-3::rfp</i>
InJ68	7 ng/ μ l <i>pmyo-3::gfp (L221K)-unc-50</i> 4 ng/ μ l <i>pmyo-3::αGFPNBmyc-tagRFp</i>	40 ng/ μ l <i>pRF4 (rol-6 (su1006))</i> ; 20 ng/ μ l <i>pttx-3::rfp</i>

Mans, alpha-mannosidase II; BirA, biotin protein ligase in *E. coli*; BirA*, BirA (R118G).

Table XI.3.5 List of plasmids used in yeast experiments

Plasmid	Selective Marker	Description	Reference
pAG25	Nat ^R (natMX4)	Template for gene deletions by drug resistance cassettes	(Goldstein and McCusker, 1999; Wach et al., 1994)
p416MET25	URA3	Expression vector in yeast	(Mumberg et al., 1994)
p416MET25-GFP-GEF1	URA3	N-terminally tagged-GFP	(Braun et al., 2010)
GEF1-GFP-p416MET25	URA3	C-terminally tagged-GFP	(Schwappach et al., 1998)
p416 NHX1-GFP	URA3	Sodium/proton exchanger	(Bowers et al., 2000)
p415 Redstar-GEF1	LEU2	Tandem dimer-tagged	(Knop et al., 2002)
pYESmt-GFP	URA3	pre Su9 as Mitochondria markers (2% galactose)	(Westermann and Neupert, 2000)
pRS315 GFP-SNC2	URA3	ν -SNARE	(Protopopov et al., 1993)
pRS315 GFP-TLG2	URA3	t -SNARE	(Schuldiner et al., 2008)
TLG2-mCherry	URA3	t -SNARE	Schwappach lab (Schuldiner et al., 2008)

11.4 Media

Table XI.4.1 List of media used in this study

Synthetic complete (SC) medium
6.7 g Yeast Nitrogen Base w/o Amino Acids
0.65 g Amino acid powders w/o URA
50 ml 40% Glucose
10 ml 100x Uracil (2 g/L)
Up to 1 Liter ddH ₂ O and use sterile filter
Yeast extract peptone-dextrose (YPAD) medium
10 g Yeast Extract
20 g Bacto-Peptone
Autoclave and add below ones after cooling down
10 ml 100x Adenine (4 g/L)
50 ml 40% Glucose
Up to 1 Liter ddH ₂ O
SC-URA medium
6.7 g Yeast Nitrogen Base w/o Amino Acids
0.65 g CSM amino acid powders w/o URA
50 ml 40% Glucose or 40% Galactose
Up to 1 Liter ddH ₂ O and use sterile filter
YPAD plate
10 g Yeast Extract
20 g Bacto-Peptone
15 g Bacto-Agar
Autoclave and add below ones after cooling down
10 ml 100x Adenine (4 g/L)
50 ml 40% Glucose
Up to 1 Liter ddH ₂ O
SC-URA or SC-URA/LEU plate
6.7 g Yeast Nitrogen Base w/o Amino Acids
15 g Bacto-Agar
Autoclave and add below ones after cooling down
0.65 g CSM-ADE-HIS-LEU-MET-TRP-URA
4 ml 100x Adenine (4 g/L)
2 ml 100x Histidine (2 g/L)
2 ml 100x Methionine (2 g/L)
5 ml 100x Tryptophan (5 g/L)
50 ml 40% Glucose or 40% Galactose
Up to 1 Liter ddH ₂ O and use sterile filter
YPAD plate with Antibiotics (Clonat, 100 mg/ml)
10 g Yeast Extract

20 g Bacto-Peptone
15 g Bacto-Agar
Autoclave and add below ones after cooling down
10 ml 100x Adenine (4 g/L)
50 ml 40% Glucose
1 ml Clonat (final conc. 100 µg/ml)
Up to 1 Liter ddH₂O

YP.Gly/Lac. plate with iron chelators (Ferrozine, BPS)

10 g Yeast Extract
20 g Bacto-Peptone
15 g Bacto-Agar
37 ml DL-Lactic acid (85%)
20 ml Glycerol (final. 2%)
pH 5.5 with approx. 30 ml NaOH
Autoclave and up to 1 Liter ddH₂O
As for one plate with Ferrozine (final conc. 3, 1, 0.5 and 0.25 mM)
Add 1 ml to 0.25 ml 1 M Ferrozine as each final conc. for 1 Liter
BPS (40 µM) SC plates were kindly provided by Kirsten Unthan-Fechner.

YP.Gly/Lac. plate with antibiotics (Rapamycin, 1 mg/ml)

10 g Yeast Extract
20 g Bacto-Peptone
15 g Bacto-Agar
37 ml DL-Lactic acid
20 ml Glycerol (final. 2%)
pH 5.5 with approx. 30 ml NaOH
Autoclave and up to 1 Liter ddH₂O
As for one plate with Rapamycin (final conc. 4, 2, 1 and 0.5 nM)
3.7 µl to 0.45 µl per 1ml (total 12 ml YP.Gly/Lac for 1 plate)

Luria-Bertani (LB) Medium

5 g/l Peptone (Roth)
2.5g/l Yeast extract (Roth)
5 g/l NaCl (Roth)
7.5 g/l Agar (Roth), for plates
100 µg/ml Ampicillin (Roth)
15 µg/ml Kanamycin (Roth)

Nematode Growth Medium (NGM)

16 g/l Agar (Roth)
3 g/l Peptone (Roth)
3 g/l NaCl (Roth)
Add up to 1 Liter ddH₂O
Autoclave and add below ones after cooling down

25 ml/l 1 M KPO ₄ Solution (Roth)
1 ml/l 1 M MgSO ₄ Solution (Roth)
1 ml/l 1 M CaCl ₂ Solution (Roth)
1ml/l 10 mg/ml Nystatin (Sigma)
1 ml/l 5 mg/ml Cholesterol (Roth)
NGM-N medium (nitrogen-free)
3 g/l NaCl (Roth)
12 g/l Agarose (Roth or Invitrogen)
Add up to 1 Liter ddH ₂ O and autoclave
Add below ones after cooling down
25 ml/l 1M KPO ₄ Solution (Roth)
1 ml/l 1M MgSO ₄ Solution (Roth)
1 ml/l 1M CaCl ₂ Solution (Roth)
1ml/l 10 mg/ml Nystatin (Sigma)
1 ml/l 5 mg/ml Cholesterol (Roth)
M9 minimal growth medium (M9MM complete)
100 ml/l 10x M9 salts
5 ml /l 40% glucose
1 ml/l 1 M MgSO ₄
1 ml/l 1% Thiamine (wt/vol, final 0.01g/L)
Up to 1 Liter ddH ₂ O
Heavy-M9MM: Heavy amino acids (13C15N)
1 ml/l of stock 0.2 M heavy-Lysine in PBS
1 ml/l of stock 0.2 M heavy-Arginine in PBS
1 ml/l 1 M IPTG
0.1 ml/l 10 mg/ml Carbenicillin
If needed, Biotine final conc. 50 µM in solution (10 mM stock filter-sterilized)
Up to 1 Liter M9MM complete
Light-M9MM: Light amino acids (12C14N)
1 ml/l of stock 0.2 M light-Lysine in PBS
1 ml/l of stock 0.2 M light-Arginine in PBS
1 ml/l 1 M IPTG
0.1 ml/l 10 mg/ml Carbenicillin
If needed, Biotine final conc. 50 µM in solution (10 mM stock filter-sterilized)
Up to 1 Liter M9MM complete
D-Biotin Stock (10 mM, pH 7 ~ 9)
Use 50 µM as a final conc. in M9MM for SILAC
Stock 10 mM solution in ddH ₂ O
With constant stirring, add 1 M NaOH adjusted to pH 7 ~ 9
Filter-sterilize into sterile bottle
4°C and/or Store at -20°

Table XI.4.2 Amino acids of selective media

Amino acid	Resulting conc. per 1L (mg/L)	mL of 100x Stock (g/L)
Adenine	40	4
Histidine	20	2
Leucine	100	10
Uracil	20	2
Methionine	20	2
Tryptophan	50	5

11.5 Buffers and solutions

Table XI.5.1 List of buffers and solutions

10x M9 salts (pH 7.4)
60 g/l Na ₂ HPO ₄
30 g/l KH ₂ PO ₄
5 g/l NaCl
10 g/l NH ₄ Cl
M9 buffer
3 g/l KH ₂ PO ₄ (Roth)
6 g/l Na ₂ HPO ₄ (Roth)
5 g/l NaCl (Roth)
1 ml 1 M MgSO ₄ (Roth)
PBS buffer (1x)
8 g/l NaCl
0.2 g/l KCl
1.44 g/l Na ₂ HPO ₄
0.24 g/l KH ₂ PO ₄
Blot-buffer, 2 liter
11.6 g Tris
5.8 g Glycine
0.74 g SDS
400 ml Methanol
pH 8.9
Coomassie Blue Staining buffer
0.25% Coomassie Brilliant Blue R250 (Bio-Rad)
50% Methanol (Roth)
10% Acetic acid (Roth)
Coomassie destaining buffer
5% Methanol (Roth)

7.5% Acetic acid (Roth)

Sample buffer for agarose gel loading (10x)

30 ml 100 % Glycerol

20 ml EDTA pH 8.0

0.5 ml 1 M Tris pH 7.5

A bit of bromphenol blue

H₂O up to 50 ml

S buffer

129 ml 0.05 M K₂HPO₄

871 ml 0.05 M KH₂PO₄

5.85 g NaCl

pH 6.0

Autoclave or filtrate

SDS running buffer (10x)

30.3 g/l Tris

144 g/l Glycine (Roth)

10 g/l SDS (Roth)

pH 8.3

2x SDS sample buffer

121.1 g/l Tris pH 6.8

20 % Glycerol (v/v)

100 g/l SDS

0.002 % Bromophenol blue (w/v)

200 ml 2-Mercaptoethanol

Solutions for developing Western blots

Solution A (store in refrigerator, 4°C)

200 ml 0.1M Tris HCl (pH 8.6)

50 mg Luminol (Sigma)

Solution B (store in the dark)

11 mg para-hydroxycoumarin acid (dilute in 10 ml DMSO)

35% H₂O₂

Prepare the mixture before use: 1 ml Solution A, 100 µl Solution B and 3 µl H₂O₂ (5 x 8 cm for one nitrocellulose membrane, Protran[®]. Whatman[®]). Vortex the solution and distribute it onto the blot-membrane. Incubate for 2 min in the dark before developing.

TBE buffer (10x)

108 g/l Tris NaOH

55 g/l Boric acid (Roth)

9.3 g/l EDTA (pH 8.0)

TE buffer

10 mM Tris-HCl pH 8.0

1 mM EDTA (Roth)
Transformation and Storage Solution for chemical transformation (TSS)
85% LB medium (42.5 ml)
10% PEG (wt/vol, MW 8000) (5 g)
5% DMSO (vol/vol) (2.5 ml)
50 mM MgCl ₂ (pH 6.5) (0.51 g)
Autoclave or filtrate and store at 4°C.
Always prepare a fresh solution before preparing competent cells.
Worm freezing solution
S buffer + 30% glycerol
Worm lysis/PCR buffer (1x)
100 mM Tris pH 8.3
500 mM KCl (Roth)
20 mM MgCl ₂ (Roth)
20 mg/ml Proteinase K added fresh prior to lysis (Roth)
Worm lysis/PCR buffer (10x)
1 M Tris pH 8.3
5 M KCl
200 mM MgCl ₂
1% Agarose gel
1 g Agarose
100 ml ddH ₂ O
50% PEG
500 g Polyethylglycol 3350
per 1 Liter ddH ₂ O
10% SDS (v/v)
10 ml SDS (sodium dodecyl sulfate)
per 100 ml ddH ₂ O
10% APS (ammonium persulfate)
1 g APS
10 ml ddH ₂ O
DTT (dithiothreitol)
3.86 g DTT in ddH ₂ O, 25 ml final volume for 1 M stock
1.5 M Tris-HCl for separating gel
118.2 g Tris-HCl, pH 8.8 for 500 ml solution
1 M Tris-HCl for stacking gel
78.8 g Tris-HCl, pH 6.8 for 500 ml solution

10x TBS
80 g NaCl
2 g KCl
30 g Tris
at pH 7.4 (with 37% HCl, ca. 12 ml)
Blocking solution
1x TBS
5% (w/v) milk powder
0.02 % IGEPAL CA-630
10% SDS-PAGE separation gel, 10 ml
4 ml ddH ₂ O
3.3 ml 30% acrylamide mix
2.5 ml 1.5 M Tris (pH 8.8)
0.1 ml 10% SDS
0.1 ml 10% ammonium persulfate
4 µl TEMED
15% SDS-PAGE separation gel, 10 ml
2.3 ml ddH ₂ O
5.0 ml 30% acrylamide mix
2.5 ml 1.5 M Tris (pH 8.8)
0.1 ml 10% SDS
0.1 ml 10% ammonium persulfate
4 µl TEMED
Stacking gel solution for 2 ml
1.4 ml ddH ₂ O
0.33 ml 30% acrylamide mix
0.25 ml 1.0 M Tris (pH 6.8)
0.02 ml 10% SDS
0.02 ml 10% ammonium persulfate
2 µl TEMED
10x Transfer buffer
390 mM Glycin, 480 mM Tris pH 8.3, 3.7 g SDS
(add 20% (v/v) Methanol in 1x buffer)
5x SDS running buffer
250 mM Glycin , 125 mM Tris, 0.1% (v/v) 10% SDS, pH 8.3
5x SDS loading buffer
250 mM Tris-HCl pH 6.8, 5% (v/v) SDS, 0.5% (w/v) BromphenolBlue, 50% (v/v) Glycerol, 250 mM DTT
20x TAE buffer

800 mM Tris-HCl pH 7.5, 200 mM Sodium-Acetate, 20 mM EDTA pH 8.0
10x Ponceau S stock
20 g Ponceau S, 300 g Trichloroacetic acid, 300 g Sulfosalicylic acid per 1 Liter ddH ₂ O

Table XI.5.2 Antibiotic concentrations used in this study

Working concentration	Antibiotics (Stock, -20°C)
100 µg/ml	Clonat (100 mg/ml)
2 – 0.5 nM	Rapamycin (1 mg/ml)
200 µg/ml	G418 (Kan) (200 mg/ml)
1 µg/ml	Carbenicillin (10 mg/ml)
100 µg/ml	Ampicillin (50 mg/ml)
12.5 µg/ml	Tetracycline (12.5 mg/ml)
25 µg/ml	Kanamycin (25 mg/ml)

11.6 Materials and reagents

11.6.1 Research equipment used in this study

Table XI.6.1 Laboratory equipment (Equipment / Manufacturer)

Centrifuges	Company
4K15	SIGMA
5415D	Eppendorf
Biofuge pico	Heraeus
Centrifuge 5424	Eppendorf
Mini-centrifuge	Eppendorf
Microcentrifuge 5417	Eppendorf
5810 R and rotor A-4-62	Eppendorf
Microscopes	
Delta Vision RT microscope	Applied Precision
ZEISS Axioskop	ZEISS
LSM 510 (inverted)	ZEISS
LSM 510 META UV (inverted)	ZEISS
TCS SP8 STED (inverted)	LEICA
TCS SP2 (inverted)	Leica
BZ-9000 (BIOREVO)	Keyence
Stereo Microscope system	Olympus
SZX9 Fluorescence Stereomicroscope	Olympus
MZ16 FA, Light source: KL2500 LCD	Leica

MZ6 Stereo Microscope	Leica
Power suppliers and pouring machines	
PowerPAC™ Basic	Bio-Rad
PowerPAC™ 200	Bio-Rad
Power supply EV231	Consort
Power supply EV213	Consort
Power supply	Invitrogen
Plate pouring pump MCP	ISMATIC
PourMatic MP-1000	New Brunswick Scientific GmbH
Incubators and Shakers	
Innova 4230	New Brunswick Scientific GmbH
Innova 44	New Brunswick Scientific GmbH
Worm incubator MPR-1410	Sanyo
Incubators for 30°C and 37°C	Memmert and Binder
Block heater SBH130	Stuart
Heat Block: DRI-BLOCK® DB-2D	TECHNE
Shaker: ST5	CAT
Waterbath Lauda EcoLine 003 E100	Gemini BV Laboratory
FUNCTION Line	Heraeus
Orbital shaker for 37°C	New Brunswick Scientific GmbH
Orbital shaker Rotamax 120	Heidolph, Germany
Thermomixer comfort	Eppendorf
Measuring machines	
NanoDrop1000 and 2000	Thermo Scientific
Spectrophotometry Biomate5	Thermo Scientific
Odyssey	LI-COR
Precision scale and easy-use scale	Kern&Sohn GmbH
pH-Meter WTW Series	InoLab
pH-Meter 766 calimatic	Knick
BioPhotometer	Eppendorf
UV systems for DNA gel pictures	INTAS
LAS3000 and 4000	Fujifilm
Extend ED224S and ED4202S	Satorious
Libra S22	Biochrom
Injection machine	
Microscope Axiovert 200	Zeiss
Pump Eppendorf FemtoJet	Eppendorf
Micro manipulator	Leica
Electrophoresis systems and others	
Mini-PROTEAN® 3 system	Bio-Rad
Horizontal electrophoresis system unit	PEQLAB Biotechnologie GmbH
Semi-Dry-Blotter	Phase
MasterCycler EP S PCR thermal cyclers	Eppendorf
Sonicator UP200S	Hielscher
SpeedVac 5301 concentrator	Eppendorf
Magnetic Stirrer MR3001	Heidolph
Magnetic Stirrer: IKAMAG® RET	Fisher Scientific
Disruptor Genie	Scientific Industries

11.6.2 Chemicals

Table XI.6.2 Chemicals (Chemicals / Manufacturer) used in this study

Chemicals	Company
20% SDS Solution	AppliChem
30% acrylamide mix	AppliChem
Adenine	SIGMA
Adeninsulfate	AppliChem
Agar Kobe I	AppliChem
Agarose (Biochemistry, Uni-Göttingen)	PEQLAB
Agarose	Invitrogen
Alanine	AppliChem
Ampicillin	Roth
APS (ammonium persulfate)	BIOMOL
Arginine	AppliChem
Asparagine	AppliChem
Bacto-Agar	BD
Bacto-Pepton	BD
Boric acid	Roth
BPS (Batho Phenantroline diSulfate)	SIGMA
Bromphenol blue	Roth
Carbenicillin	Roth
Chloramphenicol	Roth
Cholesterol	Roth
CHX (Cycloheximides)	SIGMA
Clonat (nourseothricin)	WERNER BioAgents
Coomassie Brilliant Blue R250 stain	Bio-Rad
CSM amino acid powders w/o URA	Mpbio
CSM-ADE-HIS-LEU-MET-TRP-URA	Mpbio
D-Lactic Acid	SIGMA
Dithiothreitol (DTT)	Roth
DL-Lactic acid (85%)	SIGMA
DMEM	PAA
DMSO (Dimethylsulfoxide)	SIGMA
DTT (dithiothreitol)	SIGMA
EDTA (Ethylenediaminetetraaceticacid)	AppliChem
Ethanol absolute 100%	Roth
Ethidium bromide 1%	Roth
Ferrozine	SIGMA
G418 (Kanamycin)	SIGMA
Glass beads, acid-washed 710-1,180 µm	SIGMA
Glucose	Roth

Glutamic Acid	AppliChem
Glutamine	AppliChem
Glutathione	Roth
Glycerol	Roth
Glycerol 87%	AppliChem
Glycerol 99.9%	University of Göttingen
Glycine	Roth
Histidine Monohydrochloride	Sigma
Hydrochloric acid 32%	Roth
Hydrogen peroxide	Roth
hydrogenphosphate	Roth
IGEPAL CA-630	SIGMA
Imidazole	Roth
Immersion oil	Roth
IPTG	Roth
Isoleucine	AppliChem
Isopropanol	Roth
Kanamycin	Roth
KCl (potassium chloride)	Merck
L-arginine	AppliChem
L-lysine	AppliChem
L-arginine:HCL (U-13C6, U-15N4)	Cambridge Isotope Laboratories, Inc.
L-lysine:2HCL (U-13C6, U-15N2)	Cambridge Isotope Laboratories, Inc.
LB media	Roth
Leucine	Sigma
Lithium acetate	Roth
Lithium chloride	Roth
Luminol	Sigma
Mercaptoethanol	Sigma
Methionine	AppliChem
Milk powder	Roth
Milk powder low fat	Rewe
N-Ethylmaleimide (NEM)	Sigma
NaCl (sodium chloride)	Roth
NaOH (sodium hydroxide)	Roth
Nystatine	AppliChem
PEG (Polyethylene glycol) 3350	SERVA
PEG (Polyethylene glycol) 4000	SERVA
Peptone	Roth
Pefabloc protease inhibitor cocktail	Roche
Phenylalanine	AppliChem
Polyethylene glycol PEG	Roth
Ponceau S	AppliChem

Potassium chloride	Roth
Powdered guanidine-HCl	Roth
Prefabloc SC-Protease Inhibitor	Roth
Proline	AppliChem
Rapamycin	SIGMA
Rotiphorese gel	Roth
SDS (sodium dodecyl sulfate)	AppliChem
SDS pellet	Roth
Select Agar	Invitrogen
Serine	AppliChem
Sodium azide	Sigma
Sodium chloride	Roth
Sodium dihydrogenphosphate di-sodium	Roth
Sodium hydroxide	Roth
Sodium hydroxide 32%	Roth
Sodium hypochloride 12%	Roth
Sodium sulfate	Roth
TEMED	Roth
Threonine	AppliChem
Tris	Roth
Tris (2-carboxyethyl) phosphine HCl (TCEP)	Sigma
Tris-HCl	AppliChem
Triton X 100	Roth
Tryptophan	Sigma
Tween 20	Roth
Tyrosine	AppliChem
UltraPure Agarose	Invitrogen
Uracil	Fluka
Valine	AppliChem
Yeast Extract	AppliChem
Yeast extract	Roth
Yeast Nitrogen Base	Invitrogen
Yeast Nitrogen Base w/o Amino Acids	Melford

Other chemicals used in this study were manufactured by Sigma, Roth, Roche, Serva, Merck, USB, AppliChem, J.T.Baker, Boehringer, Biomol, Amersham, Molecular Probes and Bio-Rad and supported by Prof. Dr. Stefan Eimer (ENI, University of Göttingen and Bioss, University of Freiburg) and Prof. Dr. Blanche Schwappach, Biochemistry I, Universitätsmedizin Göttingen, Germany.

11.6.3 Enzymes and kits

Table XI.6.3 Enzymes or Kits (Kits / Supplier)

Kits	Supplier
dNTP Set, 100 mM Solutions	Fermentas

YeaStar™ Genomic DNA Kit	Zymo Research
Nucleic Acid and protein purification	MACHEREY-NAGEL
NucleoSpin® Plasmid	MACHEREY-NAGEL
High pure PCR product purification kit	Roche
Restriction Enzymes	Fermentas
T4 DNA Ligase	Fermentas
GeneRuler™ 1kb DNA ladder	Fermentas
PageRuler™ Prestained Protein Ladder	Thermo Scientific
6x DNA loading buffer	Fermentas
Expand High Fidelity (Taq, 10x buffer)	Roche
PfuUltra II Fusion HS DNA Polymerase	Stratagene
TaqA Polymerase	Lab-made, recombinant
pTC Polymerase	Lab-made, recombinant
SafeView™ Nucleic acid stain	Applied Biological Materials Inc.
LysoTracker® Red DND-99	Molecular Probes®
FM® 4-64	Molecular Probes®
Mini-prep Kit	Invitrogen
Gel Extraction Kits	Fermentas
PCR purification Kits	Fermentas
Proteinase K	Roth
Complete protease inhibitor mini-tablets	Roche
PhosStop	Roche
Roti®-Quant	Roth
4 – 15% Mini-PROTEAN® TGX™ Gel	Bio-Rad
Dynabeads MyOne C1	Invitrogen

11.6.4 Antibodies

Table XI.6.4 List of antibodies used in this study

Primary antibodies, 1:1000 dilution
anti-GFP mouse monoclonal clone 3E6 (0.2 mg/ml) / Invitrogen
anti-GFP mouse monoclonal clone 7.1 and 13.1 (0.4 mg/ml) / Roche
anti-GFP mouse monoclonal B-2 (0.2 mg/ml) / Santa Cruz
Living Colors® DsRed polyclonal antibody (1 mg/ml) / Clontech Laboratories, Inc
anti-PGK1 mouse monoclonal 22C5 (1 mg/ml) / Molecular Probes, Invitrogen
anti-Bmh1p rabbit (1:1000) / Schwappach lab (Michelsen et al., 2006)
anti-c-myc mouse (1:2000), 200 ng/ml (provided from Stefan Eimer)
Secondary antibodies, 1:10,000 dilution
IRDye® 680LT Infrared Dye-conjugated secondary anti-rabbit or anti-mouse, LI-COR
IRDye® 800CW Infrared Dye-conjugated secondary anti-rabbit or anti-mouse, LI-COR
Peroxidase-conjugated AffiniPure anti-mouse IgG 0.8 mg/ml anti-goat, Jackson
Peroxidase-conjugated AffiniPure anti-rat IgG 0.8 mg/ml anti-goat, Jackson
Peroxidase-conjugated AffiniPure anti-rabbit IgG 0.8 mg/ml anti-goat, Jackson
Streptavidin-HRP, 1:4000, 1mg/ml, Thermo Scientific

CURRICULUM VITAE

PERSONAL INFORMATION

Name	Suekyoung Jeon
Date of birth	10 October 1977
Place of birth	Seoul, South Korea
Address	Zähringer Str. 361, 79108 Freiburg im Breisgau
Nationality	Republic of Korea (South Korea)
Email	suekyoung.jeon@stud.uni-goettingen.de

EDUCATION

Project of PhD thesis	Feb. 2011 – 2014
Dec. 2013 to date	Centre for Biological Signalling Studies, University of Freiburg, Germany
Feb. 2011 – Nov. 2013	European Neuroscience Institute, University of Göttingen, Germany

Master of Science in Microbiology

Mar. 2000 – Feb. 2002 Microbiology, Konkuk University, South Korea
Master's Thesis: Characteristics of the Molecular Genetics for env (V1 – C5) Gene isolated from Asymptomatics and AIDS Patients with HIV-1 Korean Subtype

Bachelor of Science in Molecular Biology

Mar. 1995 – Feb. 2000 Molecular Biology, Konkuk University, South Korea

SCHOLARSHIPS AND RESEARCH SUPPORTS

Dec. 2013 – At present	Centre for Biological Signalling Studies, Universität Freiburg
Feb. 2011 – Nov. 2013	SyMBaD International Training Program, EU consortium

WORK EXPERIENCE

May 2013 – At present	Center for Biological Systems Analysis, Freiburg, Germany
Nov. 2012 – Apr. 2013	Biochemistry I, Universitätsmedizin Göttingen, Germany
Feb. 2011 – Oct. 2012	European Neuroscience Institute, Göttingen, Germany
Feb. 2003 – Mar. 2010	Beckman Coulter Korea Ltd. and BKI Inc., South Korea
Sep. 2000 – Jan. 2003	National Institute of Health, Seoul, South Korea

LIST OF PUBLICATIONS

Nam JG, Kim KC, **Jeon SK**, Kim SS, Lee SW, An SW, Lee JS. Immunodeficiency Virus Type 1 Infection of H9 Cells. Journal of Bacteriology and Virology, 2004 Mar;34(1):57-66. The Korean Society for Microbiology.