The role of toca-1-N-wasp complex in filopodia formation and endocytosis

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2010

Bu, W. (2010). The role of toca-1-N-wasp complex in filopodia formation and endocytosis.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/41562>

<https://doi.org/10.32657/10356/41562>

THE ROLE OF TOCA-1-N-WASP COMPLEX IN FILOPODIA FORMATION AND ENDOCYTOSIS

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

2010

Acknowledgements

I would like to extend my gratitude to A/P Sohail Ahmed for providing me the opportunity to work and study in the field. I wish to thank him for all along guidance, encouragement and patience in the progress of the research project. In time of difficulty, his understanding and support are always there. I would like to say that all his support has turned the tough journey into a pleasant one. I am grateful to Dr Thirumaran Thanabalu for being my co-supervisor. Thank you for the advice and help in the course of the study.

My thanks also go to all previous and current SA lab members for teaching, sharing and discussions. I would like to thank Amy Chou, Mike Yu and Liz Lim for collaboration on part of the project. I thank Dr Sudhaharan Thankiah for teaching me F-techniques and Srivats Hariharan for his excellent microscopy support. I wish to thank Dr Victor Racine (IMCB) for his help in vesicle tracking. I appreciate the help from Dr Eric Lam and Hwee Goon Tay (IMB-EM) to proofread my thesis.

I thank A-STAR for the financial support through Scientific Staff Development Scheme in the course of the study.

Finally, I wish to thank my family and friends for their care and love.

Table of contents

Acknowledgements	i
Table of contents	ii
List of publications	viii
Summary	ix
List of tables and figures	x
List of movies	xiii
Abbreviations	xiv

CHAPTER 1 Introduction	1
1.1 Cell biology of the central nervous system	1
1.2 Cell migration	2
1.3 Cell cytoskeleton	3
1.3.1 Microtubules	3
1.3.2 Intermediate filaments	4
1.3.3 Microfilaments	5
1.4 Small GTP-binding proteins (G-proteins)	8
1.4.1 Ras GTPases	8
1.4.2 Rho family GTPases	9
1.4.2.1 WASP/N-WASP	9
1.5 Actin-based protrusions at the leading edge of migratory cells	19
1.5.1 Lamellipodia and membrane ruffles	19
1.5.2 Filopodia	22
1.5.2.1 Functions of filopodia	22
1.5.2.2 Molecules involved in filopodia formation	23
1.5.2.3 Mechanisms of filopodia formation	24
1.6 Endocytosis	26
1.6.1 Clathrin-mediated endocytosis	26
1.6.1.1 Steps of clathrin mediated endocytosis	26
1.6.1.2 Clathrin-coat components	27
1.6.1.3 Cdc42 and clathrin-mediated endocytosis	33

1.6.1.4 Intracellular trafficking of the endocytosed vesicles	34
1.6.2 Clathrin-independent pathways	35
1.6.2.1 Caveolae mediated endocytosis	35
1.6.2.2 Macropinocytosis	37
1.6.2.3 GEEC pathway	37
1.6.2.4 Flotilin pathway	38
1.6.3 Endocytosis and actin cytoskeleton	38
1.6.3.1 Mammalian cells	38
1.6.3.2 <i>S. cerevisiae</i>	39
1.6.3.3 Knowledge from time-lapse imaging	40
1.6.3.4 The actin cytoskeleton and endosome motility	40
1.6.3.5 The actin polymerization during endocytosis is possibly through the Arp2/3 mediated pathway	41
1.6.3.6 Linking molecules between the actin cytoskeleton and endocytosis	42
1.7 Aims	52
CHAPTER 2 Materials and methods	54
2.1 Materials	54
2.1.1 General laboratory chemicals	54
2.1.2 Materials for cloning and mutagenesis	54
2.1.3 Materials for cell culture, transfection, manipulation, fixation and staining	54
2.1.4 Materials for GST tagged protein expression, purification and detection	55
2.1.5 Materials for liposome binding assay	55
2.1.6 Bacteria and mammalian cell lines	55
2.1.7 Plasmids	56
2.1.8 Media and solutions	56
2.2 Methods	57
2.2.1 DNA manipulation methods	57

2.2.1.1 Isolation of plasmid DNA	57
2.2.1.2 Agarose gel electrophoresis of DNA	58
2.2.1.3 Digestion of DNA with restriction endonucleases	58
2.2.1.4 Purification of DNA fragments with QIAEX II DNA gel extraction kit	59
2.2.1.5 Purification of PCR mixture using QIAGEN PCR purification kit	59
2.2.1.6 Ligation of DNA insert with the vector	59
2.2.1.7 Transformation of competent cells by heat-shock	60
2.2.1.8 DNA sequencing	60
2.2.1.9 DNA sequence analysis	60
2.2.1.10 Polymerase Chain Reaction (PCR) amplification	60
2.2.2 Methods for cell culture	62
2.2.2.1 Mammalian cell culture	62
2.2.2.2 Transfection	63
2.2.2.3 Microinjection of mammalian cells	65
2.2.2.4 Immunofluorescence	66
2.2.2.5 Blockers of endocytosis	66
2.2.2.6 Transferrin labeling	66
2.2.2.7 Cholera toxin B uptake assay	67
2.2.2.8 Dextran uptake assay	67
2.2.2.9 Determination of endocytosis inhibition using labeled transferrin/cholera toxin B/ dextran	67
2.2.2.10 Filopodia assay	67
2.2.2.11 Scoring of filopodial and neurites	68
2.2.2.12 Intensity profiling	68
2.2.2.13 Colocalization	68
2.2.2.14 Characterization of Rab5 positive vesicles	69
2.2.2.15 Quantification of tubulation, vesicle number and motility	69
2.2.2.16 Statistical analysis	70
2.2.3 Methods for protein work	70

2.2.3.1 Sample preparation for Western blot	70
2.2.3.2 Western blot	70
2.2.3.3 Semi-Dry transfer of proteins	71
2.2.3.4 Western blot	71
2.2.3.5 Coomassie Blue staining of the SDS-PAGE gel	72
2.2.3.6 GST tagged protein expression and purification	72
2.2.3.7 Liposome binding assay	73
2.2.4 Microscopy methods	74
2.2.4.1 Confocal Microscopy and timelapse acquisition	74
2.2.4.2 FRET Measurement	74
2.2.4.3 Fluorescence Lifetime Imaging Microscopy (FLIM)	76
2.2.4.4 Fluorescence Recovery After Photobleaching (FRAP)	79
2.2.4.5 Total Internal Reflection Fluorescence Microscopy (TIRFM)	79
2.2.4.6 Electron microscopy	80
CHAPTER 3 The Toca-1-N-WASP complex links filopodia formation to endocytosis	81
3.1 Toca-1 and N-WASP induce filopodia and neurite formation	81
3.2 Domains of Toca-1 required for filopodia and neurite formation	85
3.3 Toca-1 requires N-WASP for filopodia formation	85
3.4 N-WASP requires Toca-1 for filopodia formation	94
3.5 Localization of the Toca-1 and N-WASP	94
3.6 N-WASP and Toca-1-N-WASP expression affects the distribution, size and number of Rab5 membranes	98
3.7 Toca-1 interacts with N-WASP in filopodia and Rab5 positive membranes	104
3.8 Inhibitors of endocytosis inhibit Toca-1 induced filopodia formation	105
CHAPTER 4 The Toca-1-N-WASP complex induces membrane tubulation and vesicle formation	120
4.1 Toca-1-N-WASP interaction induces the formation of dynamic	

membrane tubules and vesicles	120
4.2 Characteristics of Toca-1-N-WASP induced membrane tubules and vesicles	128
4.3 Toca-1 and N-WASP interact in tubules and vesicles	128
CHAPTER 5 Effect of Toca-1 F-BAR mutants, dynamin, F-actin and microtubules on Toca-1-N-WASP induced tubules and vesicles	150
5.1 F-BAR domain of Toca-1 is essential for membrane tubulation induced by Toca-1 and N-WASP	150
5.2 Dynamin is essential for vesicle formation but not for tubulation	150
5.3 Role of F-actin and microtubules on Toca-1/N-WASP phenotypes and dynamics	157
CHAPTER 6 Cdc42 regulates Toca-1-N-WASP complex formation	163
6.1 Cdc42 forms complex with Toca-1-N-WASP on tubules and vesicles	163
6.2 Cdc42 interaction with Toca-1 and N-WASP regulates membrane tubulation, vesicle formation and motility	164
CHAPTER 7 Discussion	177
7.1 Toca-1-N-WASP complex links filopodia formation to endocytosis	177
7.1.1 Toca-1/N-WASP induces filopodia formation	178
7.1.2 Toca-1 requires N-WASP for filopodia formation	180
7.1.3 Toca-1 directly interacts with N-WASP in vesicles and filopodia	181
7.1.4 Mechanism of the Toca-1-N-WASP complex induced filopodia formation	183
7.1.5 Is there a linkage between filopodia formation/actin cytoskeleton and endocytosis?	184
7.2 Cdc42 interaction with N-WASP and Toca-1 regulates the membrane tubulation, vesicle formation and vesicle motility.	187
7.2.1 Toca-1-N-WASP induces membrane tubules and vesicles formation	187

7.2.2 Actin polymerization and Toca-1-N-WASP induced tubules and vesicles	188
7.2.3 What is responsible for the polarized actin polymerization?	189
7.2.4 Microtubules and Toca-1-N-WASP induced vesicle/tubule phenotype	189
7.2.5 Effect of dynamin on Toca-1-N-WASP induced tubules and vesicles	190
7.2.6 Functions of Cdc42 in regulating Toca-1-N-WASP induced vesicle/tubule phenotype	191
7.2.7 Which endocytosis pathway does the Cdc42-Toca-1-N-WASP complex regulate?	193
7.2.8 Summary of effects of various factors on Toca-1-N-WASP induced tubules and vesicles	194
7.2.9 The proposed model	194
7.3 Cdc42 coordinates cell morphology, endocytosis and polarity	199
Reference list	206
Appendix 1 Media and solutions	235
Appendix 2 Vectors	242
CD-ROM Timelapse movies (inside of back cover)	

List of Publications

Bu W, Lim KB, Yu YH, Chou AM, Sudhaharan T and Ahmed S. Cdc42 interaction with N-WASP and Toca-1 regulates membrane tubulation, vesicle formation and vesicle motility steps of endocytosis. Manuscript in preparation.

Sudhaharan T, Liu P, Foo YH, **Bu W**, Lim KB, Wohland T, Ahmed S. Determination of in vivo dissociation constant, K_D , of Cdc42-effector complexes in live mammalian cells using single wavelength fluorescence cross-correlation spectroscopy. J Biol Chem. 2009 May 15;284(20):13602-9.

Bu W, Chou AM, Lim KB, Sudhaharan T, Ahmed S. The Toca-1-N-WASP complex links filopodial formation to endocytosis. J Biol Chem. 2009 Apr 24;284(17):11622-36.

Yu W, Lee HK, Hariharan S, **Bu W**, Ahmed S. Quantitative neurite outgrowth measurement based on image segmentation with topological dependence. Cytometry A. 2009 Apr;75(4):289-97.

Lim KB, **Bu W**, Goh WI, Koh E, Ong SH, Pawson T, Sudhaharan T, Ahmed S. The Cdc42 effector IRSp53 generates filopodia by coupling membrane protrusion with actin dynamics. J Biol Chem. 2008 Jul 18;283(29):20454-72.

Liao H, **Bu W**, Wang TH, Ahmed S, Xiao ZC. Tenascin-R plays a role in neuroprotection via its distinct domains that coordinate to modulate the microglia function. J Biol Chem. 2005 Mar 4;280(9):8316-23.

Liao H, Duka T, Teng FY, Sun L, **Bu W**, Ahmed S, Tang BL, Xiao ZC. Nogo-66 and myelin-associated glycoprotein (MAG) inhibit the adhesion and migration of Nogo-66 receptor expressing human glioma cells. J Neurochem. 2004 Sep;90(5):1156-62.

Summary

Transducer of Cdc42 mediated actin polymerization (Toca-1) was identified in 2004 as an essential component of Cdc42 mediated actin polymerization using *Xenopus* extract. Toca-1 consists of an F-BAR domain at the N-terminal, an HR1 domain (Cdc42 binding site) in the middle and an SH3 domain (N-WASP binding site) at the C-terminal. N-WASP is an activator of actin nucleation through the Arp2/3 complex. Toca-1 or N-WASP induces neurite outgrowth and filopodia formation in N1E115 cells. Toca-1 requires the F-BAR domain, Cdc42 binding site and SH3 domain to induce filopodia. Toca-1 and N-WASP require each other for filopodia formation and synergize to induce filopodia. Toca-1 does not localize in filopodia but is recruited to filopodia by N-WASP. Toca-1 directly interacts with N-WASP in filopodia and Rab5 positive vesicles. Coexpression of Toca-1 and N-WASP affects the distribution and size of Rab5 positive vesicles. Therefore this Toca-1-N-WASP complex not only localizes in filopodia but also induces the formation of filopodia and endocytic vesicles. Three inhibitors of endocytosis, dynamin-K44A, Eps15 Δ 95/295, and clathrin heavy chain RNAi, block Toca-1 induced filopodia formation. These data suggest that the Toca-1-N-WASP complex can link filopodia formation to endocytosis. The coordinated filopodia formation and endocytosis may be crucial for diverse cellular and developmental processes, such as growth cone guidance and collapse, epithelial cell polarity and border cell migration of *Drosophila*. Therefore the linkage between filopodia formation and endocytosis may be essential for cellular response to the environmental cues or establishing cell polarity, thus providing a mechanism to coordinate cell signaling events and developmental processes.

List of tables and figures

List of tables

CHAPTER 1

Table 1.1 Rho GTPases and their effectors.	17
---	----

Table 1.2 Classification, structures and protein examples of BAR domains.	48
--	----

CHAPTER 3

Table 3.1 Characteristics of endogenous and induced filopodia in N1E115 cells.	84
---	----

List of figures

CHAPTER 1

Figure 1.1 Formins, the Arp2/3 complex and Spire, nucleate actin by different mechanisms.	10
--	----

Figure 1.2 Regulators of G-proteins.	12
---	----

Figure 1.3 Phylogenetic tree of the Rho family GTPases and representatives of other Ras superfamily GTPases.	15
---	----

Figure 1.4 Domain architectures of WASP, N-WASP and WAVE proteins.	20
---	----

Figure 1.5 Endocytic pathways, key regulators and functions.	28
---	----

Figure 1.6 Requirement for F-actin dynamics in multiple steps of clathrin mediated endocytosis.	44
--	----

Figure 1.7 Classification and domain structures of BAR proteins.	49
---	----

CHAPTER 2

Figure 2.1 The excitation intensity is modulated in the frequency domain FLIM.	77
---	----

Figure 2.2 Life time measurement by frequency domain method.	78
---	----

CHAPTER 3

Figure 3.1 Toca-1 and N-WASP induce filopodia and neurites in N1E115 cells.	82
--	----

Figure 3.2 Effect of mutations in Toca-1 on filopodia formation.	86
Figure 3.3 Toca-1 does not generate filopodia in N-WASP KO cells.	89
Figure 3.4 N-WASP induced filopodia formation requires and synergizes with Toca-1.	95
Figure 3.5 Localization of N-WASP and Toca-1 with Rab5.	99
Figure 3.6 Localization of N-WASP and Toca-1 with actin.	102
Figure 3.7 Effect of N-WASP and Toca-1 on the distribution, size and number of Rab5 membranes.	107
Figure 3.8 Toca-1 and N-WASP interact directly in filopodia and vesicles.	111
Figure 3.9 Effect of inhibitors of endocytosis on filopodia formation.	114
 CHAPTER 4	
Figure 4.1 Phenotypes of N-WASP, Toca-1 and domains.	121
Figure 4.2 Characteristics of Toca-1/N-WASP induced membrane tubulation, vesicle scission and motility.	124
Figure 4.3 Vesicles and tubules induced by Toca-1 and N-WASP colocalise with Rab5, Rab7, clathrin, actin and PH domain probes, but not caveolin.	129
Figure 4.4 Uptake assays of endocytic markers by Toca-1 and N-WASP expressing cells.	139
Figure 4.5 FRET and FLIM analysis of the Toca-1-N-WASP interaction.	145
 CHAPTER 5	
Figure 5.1 Effect of Toca-1 F-BAR mutants on Toca-1/N-WASP phenotypes.	151
Figure 5.2 Dynamin function, Toca-1 interaction and phenotype.	155
Figure 5.3 Effect of cytochalasin D and nocodazole on the Toca-1/N-WASP phenotypes.	159
 CHAPTER 6	
Figure 6.1 Cdc42, Toca-1 and N-WASP interactions and phenotypes.	166

Figure 6.2 Effect of Toca-1MGD383-385IST and Cdc42 inhibitor/activation on Toca-1/N-WASP phenotypes. 170

Figure 6.3 Effect of CRIB domain on Toca-1/N-WASP phenotype. 175

CHAPTER 7

Figure 7.1 Effect of various reagents on Toca-1-N-WASP induced tubules and vesicles. 196

Figure 7.2 Model for Cdc42 regulation of Toca-1/N-WASP induced membrane tubulation, vesicle formation and motility. 202

Figure 7.3 Cdc42 is involved in coordinating filopodia formation, endocytosis and polarity. 204

List of Movies

CHAPTER 3

Movie 3.1 Toca-1 induces neurite outgrowth and filopodia formation in N1E115 cells.

Movie 3.2 N-WASP induces neurite outgrowth and filopodia formation in N1E115 cells.

CHAPTER 4

Movie 4.1 Toca-1/N-WASP vesicle dynamics.

Movie 4.2 mRFP-Toca-1/GFP-N-WASP induced tubule/vesicle transitions with TIRF microscopy.

Movie 4.3 mRFP-Toca-1/HA-N-WASP/GFP-actin induced vesicle motility.

Abbreviations

Aa	amino acid
ABP	actin binding protein
ACK	activated Cdc42-associated tyrosine kinase
AP-FRET	acceptor photobleaching-forster resonance energy transfer
ARPC	actin related protein complex
Arf1	ADP-ribosylation factor 1
Arp2/3	actin-related protein 2/3
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
BAR	Bin-Amphiphysin-Rsv domain
CC	coiled-coil domain
CC	correlation coefficient
CCS	clathrin coated structure
Cdc42	cell division cycle 42
cDNA	complementary DNA
<i>C.elegans</i>	<i>Caenorhabditis elegans</i>
CHC	clathrin heavy chain
CME	clathrin mediated endocytosis
CIP4	Cdc42 interacting protein
CLASP	clathrin-associated sorting proteins
Cobl	Cordon-bleu
CR16	corticosteroids and regional expression-16
CRIB	Cdc42/Rac interacting binding region
DRP	dynamin related proteins
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DN	dominant negative
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemiluminescence
<i>E.coli</i>	<i>Escherichia coli</i>
ECV	endosomal carrier vesicle
EDTA	ethylenediamine tetra-acetate
EFC	extended FCH domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EH	Eps15 homology
EtBr	ethidium bromide
Eps15	EGF phosphorylation substrate 15
EVH1	Ena-VASP-homology-1
F-actin	filamentous actin
F-BAR	FCH-BAR
FBP17	formin binding protein 17
FBS	fetal bovine serum
FCH	Fes CIP4 homology
FH2	formin-homology-2
FITC	fluorescein isothiocyanate
FLIM	fluorescence lifetime imaging microscopy

FP	forward primer
FRAP	fluorescence recovery after photobleaching
FRET	Forster Resonance Energy Transfer
G-actin	globular monomeric actin
GAP	GTPase activating protein
GBD	GTPase binding domain
GDI	guanine nucleotide inhibitor protein
GDP	guanosine-5 –diphosphate
GED	GTPase effector domain
GEEC	GPI-AP –enriched early endosomal compartments
GEF	guanine nucleotide exchange factor
GFP	green fluorescence protein
GPI-AP	glycosyl-phosphatidylinositol-anchored protein
G-protein	small GTP-binding protein
GRAF1	GTPase regulator associated with focal adhesion kinase
GRB2	growth factor receptor-bound protein 2
GST	glutathione-S-transferase
GTP	guanosine-5 -triphosphate
HCl	hydrochloric acid
HRP	horseradish peroxidase
I-BAR	inverse-Bin-Amphiphysin-Rsv domain
IF	intermediate filament
IMD	IRSp53-MIM homology domain
IRSp53	insulin receptor substrate protein 53 kDa
IPTG	isopropyl- β -D-thiogalactopyranoside
IQGAP	GAP containing Ile-Glu motif
JNK	c-Jun N-terminal kinase
KD	knock down
kDa	kilodalton
KO	knock out
LB	Luria Bertani medium
mg	microgram
ml	milliliter
Mena	mammalian enabled
MLC	myosin light chain
mRFP	monomeric red fluorescence protein
MTOC	microtubule organizing center
MVB	multi-vesicular bodies
NaOH	sodium hydroxide
Nck	non-catalytic region of tyrosine kinase
NF- κ B	nuclear factor κ B
ng	nanogram
NGF	nerve growth factor
N-WASP	neuronal-Wiskott Aldrich syndrome protein
OD	optical density
PAK	p21 –activated kinase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCH	<i>pombe</i> Cdc15 homology
PE	phosphatidylethanolamine
PH	pleckstrin homology domain

PI(3)P	phosphatidyl-inositol-3-phosphate
PIP2	phosphatidylinositol 4,5,-biphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PI3-K	phosphatidylinositol 3-kinase
PDGF	platelet-derived growth factor
PH	plestrin homology domain
PKC	protein kinase C
PMA	phosphomolybdic acid reagent
PMSF	phenylmethyl-sulfonyl fluoride
PRD	polyproline rich domain
PS	phosphatidylserine
PX	phosphoinositide binding motifs or phox
RhoGAP	Rho GTPase activating protein
RNAi	RNA interference
ROI	region of interest
ROK	Rho kinase
RP	reverse primer
RTK	receptor tyrosine kinase
Rvs	rescue viability upon starvation
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
Scar	suppressor of cyclic AMP receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3	Src homology domain
SH3PX1	SH3 and PX domain containing protein 1
SNX9	sorting nexin 9
<i>S.pombe</i>	<i>Schizosaccharomyces pombe</i>
SRA1	specially Ra1-associated protein 1
Syndapin	synaptic dynamin-associated protein
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	transferrin
TGN	trans-Golgi network
TIRFM	total internal reflection microscopy
Toca-1	transducer of Cdc42 dependent actin polymerization-1
TRITC	tetramethylrhodamine isothiocyanate
μl	microliter
VCA	verprolin homology, cofilin homology and acidic domains
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Veprolin-homologous protein
WH1	WASP homology domain 1
WH2	WASP homology domain 2
WICH	WIP- and CR16-homologous protein
WT	wild type
WIP	WASP-interacting protein
WISH	WASP interacting SH3 protein

CHAPTER 1 Introduction

1.1 Cell biology of the central nervous system

The human brain is highly complex and consists of approximately 100 billion neurons, which perform the function of transmitting nerve impulses. The total length of the nerve fibers in a brain adds up to about four million miles. Besides neurons, there are an even larger number of glia cells which provide supportive functions to neurons. Glia cells include Schwann's cells, satellite cells, microglia, oligodendroglia, and astroglia.

Different types of neurons are present in the brain. Their size varies between 4 microns to 100 microns and their length between a fraction of an inch to several feet. A neuron can be divided into three parts; cell body, dendrites and axon. The cell body is similar to that of other typical eukaryotic cells which contains the nucleus and all the organelles. Dendrites are short and multiple branches from the cell body. Thus dendrites allow one neuron to connect with multiple other neurons and transmit signals to the cell body. The axon is the longest fiber of a neuron and it delivers the signal from the cell body to the connecting neuron. The axon is surrounded with myelin except for periodic breaks at nodes of Ranvier, which propagate the nerve impulses. Myelin insulates the axon and increases the transmission speed of the signal. The distal end of the axon is dynamic and called growth cone, which facilitates axon growth and guidance. The correct pathfinding of growth cones depends on complex cellular machinery which responds to the environmental guidance cues (Kandel et al., 2000).

The communication of two neurons is through the synapse, which consists of three parts; a presynaptic ending, a postsynaptic ending and a synaptic cleft. The electric signal transmits along the axon to the presynaptic ending and causes the release of the neurotransmitters, which then cross the synaptic cleft and are recognized by the neurotransmitter receptors at the postsynaptic ending (Kandel et al., 2000).

N1E115 is a neuroblastoma cell line and the majority of the cells are round under normal culture conditions. These cells can adopt a neuronal morphology under certain conditions such as serum starvation for 12~24 hrs (Govind et al., 2001). Actin reorganization is crucial in the process of neurite outgrowth. Rho GTPases play an important role in neuronal morphology changes. Rac and Cdc42 promote neurite outgrowth and Rho inhibits neuritogenesis. Collapsin response mediator protein, activated by Rho kinase, can response to active Rho and Rac to induce neurite outgrowth or retraction. However, the morphological effect of Rho and Rac on neuritogenesis was reversed in presence of collapsin response mediator protein (Hall, et al., 2001). Diacylglycerol kinase ξ directly interacts with Rac1 (Yakubchik, et al., 2005). Syntrophin, a cytoskeleton interacting protein, acts as a scaffold protein to induce neurite outgrowth through regulating the subcellular localization of diacylglycerol kinase ξ /Rac1 complex (Yakubchik, et al., 2005). PAK5, a member of the mammalian PAK family of serine/threonine protein kinase, induces neurite outgrowth downstream of Cdc42 and Rac and antagonizes Rho (Ishii, et al., 2001). Interaction between cell and extracellular matrix is primarily mediated by integrin, which is activated by integrin-linked kinase. Integrin-linked kinase was shown to induce neurite outgrowth through integrin and the process is associated with the activation of p38 MAP kinase (Dan, et al., 2002). N1E115 has been used to study the morphological roles of Rho GTPases and their effectors (Govind et al., 2001; Kozma et al., 1995; Kozma et al., 1996).

1.2 Cell migration

Cell migration is an important process both in physiological and pathological conditions. Cell migration is used by cells in multiple processes, such as wound healing (Lambrechts et al., 2004), differentiation (Weston, 1971), neural development (Kanatani et al., 2005) and tumor invasion and metastasis (Titus et al., 2005). When cell migrates, the extracellular matrix, cell cytoskeleton and cell-substrate adhesion rearrange coordinately. Cell migration is a multiple-step process. Cells first polarize through asymmetric redistributions of signaling molecules, adhesion

complexes and cytoskeleton through the directed vesicle trafficking. The morphology of a polarized cell typically includes the lamellipodia/membrane ruffling and filopodia at the leading edge responding to migratory stimulants, reorientation of the microtubule-organising center (MTOC) (Xie and Tsai, 2004) and the Golgi towards the migrating direction (Bershadsky and Futerman, 1994). The directed membrane trafficking is facilitated by temporal capture and stabilization of the specific microtubule plus ends near the leading edge, which enable motor proteins such as dynein and kinesin to direct membrane trafficking. At the same time, the rear of cells retracts by disassembling the adhesion (Vicente-Manzanares et al., 2005). This directional movement is stabilized by forming adhesions with the extracellular matrix (Tamura et al., 1998) or the adjacent cells through transmembrane receptors. The adhesions at the rear of the cell are disassembled for the cell to move forward. This process repeatedly happens to facilitate dynamic cell movement (Lauffenburger and Horwitz, 1996).

1.3 Cell cytoskeleton

Cell cytoskeleton is a filamentous network inside the cell and consists of three kinds of filaments, microtubules, intermediate filaments and microfilaments. The cell cytoskeleton undergoes dynamic reorganization during the dynamic cellular process such as cell migration and mitosis (Machesky and Schliwa, 2000). The importance of the cytoskeleton is manifested by the multiple functions performed by the cytoskeleton, including maintaining cell shape, intracellular transport, cell motility, mitosis and meiosis.

1.3.1 Microtubules

The building block of microtubules consists of $\alpha\beta$ -tubulin heterodimer, which forms protofilament through head to tail binding. The microtubule is assembled by parallel alignment of ~13 protofilaments. The end terminated with β -tubulin is called plus end and the end terminated with α tubulin is called minus end (Mitchison, 1993).

Microtubules are one of important components of the eukaryotic cytoskeleton, which play important roles in various cellular processes namely mitosis, meiosis, cell motility, intracellular transport and maintaining cell shape. The assembly and orientation of microtubules have to be controlled in order to perform their functions.

Microtubules play a role in cell polarity during cell migration. In static state, the minus ends of microtubules reside in MTOC and the plus ends undergo dynamic elongation and retraction, which are thought to be important for microtubules to search and capture special sites (Benard et al., 1999). In migrating cells, the plus ends of microtubules are targeted near the leading edge and the MTOCs are oriented to the direction of cell migration (Gundersen and Bulinski, 1988). The microtubules are also shown to be implicated in the directed transport of vesicles or proteins to the leading edge.

1.3.2 Intermediate filaments

Intermediate filaments (IFs) have an average diameter about 10 nm, which is between that of microfilaments (7-8 nm) and microtubules (25 nm) (Herrmann et al., 2007; Kim and Coulombe, 2007). IFs are grouped into 5 major families based on their specific expression profile. The common building block is a central α -helical coiled-coil rod and the exceptional diversity among different IFs is the highly diverse N- and C-termini (Parry et al., 2007). IFs are dynamic structures with a much lower turnover rate compared to actin filaments and microtubules. IFs are bound to the nucleus at one end with the other end extends into the cytoplasm serving as a scaffold for organelles such as Golgi complex, mitochondria and MTOC (Herrmann et al., 2007; Kim and Coulombe, 2007; Green et al., 2005; Toivola et al., 2005). IFs are also associated with the specific structures on the plasma membrane such as desmosomes, hemidesmosomes and focal adhesions. IFs network provides cells with mechanical forces important for tissue functions.

1.3.3 Microfilaments

Actin, an ATP-binding protein, is the building block of the actin cytoskeleton and exists either as a monomer (G-actin or globular-actin) or polymerized filaments (F-actin or filamentous actin). Actin can hydrolyze its bound GTP to GDP and also exchange its bound GDP to GTP. Actin monomers align in the same direction, thus creating two different ends. One end grows faster and is called barbed end or plus (+) end, while the other end grows slowly and called pointed end or minus (-) end. Polymerization occurs primarily at the barbed end and is tightly regulated by actin and filament binding proteins (Pollard et al., 2000). Actin treadmilling happens when the actin monomer is added to the plus end and dissociated from minus end while keeping the length of filament constant. This process is essential for the function of actin in motility (Korn et al., 1987).

Microfilaments are actin-based structures, which are present in multiple sites within a cell. There are a mesh of filaments underlying the plasma membrane, a highly branched actin filament network (lamellipodia) and long unbranched actin bundles (filopodia) at the leading edge of the migratory cell, stress fibers anchoring to the adhesion site and actin-rich structures under invaginated membrane in endocytosis and phagocytosis. Actin filament based structures are highly dynamic and play important roles in cellular responses to the environment.

Actin polymerization requires a free barbed end (Pollard et al., 2000). Free barbed ends may be generated in three ways: uncapping of pre-existing filaments, severing of filaments, or *de novo* filament nucleation. Nucleation means to initiate actin polymerization from free monomers. To date, four actin nucleators have been identified: the Arp2/3 complex (Mullins et al., 1997; Welch et al., 1997), formins (Pruyne et al., 2002; Sagot et al., 2002), spire (Quinlan et al., 2005) and Cordon-bleu (Cobl) (Ahuja et al., 2007) (Figure 1.1).

The Arp2/3 complex is one of the major complexes that nucleate new actin filaments with free barbed ends. The Arp2/3 complex consists of seven subunits including actin-related proteins

Arp2 and Arp3 and five other proteins named ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5 (actin related protein complex). Several proteins function as activators of the Arp2/3 complex in different organisms, including *Listeria monocytogenes* ActA protein (Welch et al., 1998), Wiskott-Aldrich syndrome protein (WASP) (Winter et al., 1999; Yarar et al., 1999), N-WASP (Rohatgi et al., 1999), suppressor of cyclic AMP receptor (Scar)/WASP family verprolin homologous (WAVE) proteins (Machesky et al., 1999), yeast myosin I (Yarar et al., 1999; Lechler et al., 2000; Machesky et al., 1999; Lee et al., 2000), amoeba capping protein, myosin I linker (CARMIL) (Jung et al., 2000), metazoan cortactin (Urano et al., 2001; Weaver et al., 2001; Weed et al., 2000), yeast Abp1p (Goode et al., 2001) and yeast Pan1p (Duncan et al., 2001). All these activators contain a CA region, which binds to the Arp2/3 (Zalevsky et al., 2001b; Zalevsky et al., 2001a). C region which is called connector region is a short stretch of basic amino acids and the A region is called acidic region that consists a short stretch of acidic region. Although the CA domain is sufficient for Arp2/3 binding, it is not sufficient to activate the Arp2/3 complex (Hufner et al., 2001; Lechler et al., 2001; Marchand et al., 2001; Rohatgi et al., 1999; Weaver et al., 2001). To activate the Arp2/3 complex, additional binding sites for either G-actin or F-actin are required. The WH2 domain (WASP homology 2, also called verprolin homology) of WASP, N-WASP and WAVE which binds to G-actin, may facilitate the formation of the actin nucleus for initiating actin polymerization. Full activation of the Arp2/3 complex by WASP/WAVE proteins requires a further interaction between Arp2/3/activator/G-actin complex and a preexisting actin filament (Machesky et al., 1999), which is mediated through the Arp2/3 complex (Gournier et al., 2001; Mullins et al., 1998; Volkmann et al., 2001). The actin network nucleated by the Arp2/3 complex is branched with 70 degrees between filaments. Two models are postulated to generate this branching actin network. One is the “dendritic nucleation” model of the Arp2/3 complex (Pollard et al., 2000), which is believed that the active Arp2/3 generates new filaments by binding to the side of a pre-existing filament. Another model is the “barbed end branching” model, which

proposes that the active Arp2/3 together with its activators copolymerizes at the barbed ends and induces the branching of the filaments (Pantaloni et al., 2000).

The second actin nucleators are formins. The actin filaments nucleated by formins are linear and unbranched (Pruyne et al., 2002; Sagot et al., 2002). Formins are single polypeptides with several domains (Higgs, 2005). Formins dimerize through the formin homology 2 (FH2) domains which are the defining characteristic of formins. Formins nucleate actin filaments by barbed end addition through FH2 domains. The FH2 domains share no similarity to actin in structure and their affinity to actin monomer is very weak. As suggested by kinetic modeling studies (Pring et al., 2003; Shimada et al., 2004; Xu et al., 2004), the FH2 domains directly bind and stabilize the actin dimer/trimers, thus initiating actin nucleation. The FH2 domains still bind to the barbed end after nucleation and move processively with filament elongation. The FH2 domains also prevent the binding of capping proteins, which are the terminators of actin polymerization (Kovar, 2006). One mechanism for regulation of formins is by Rho family GTPases (Waller and Alberts, 2003). The auto-inhibitory conformation of formins is relieved by direct binding with GTP-loaded Rho. The exposed FH2 domain then binds to the barbed ends of actin filaments (Zigmond, 2004).

The third actin nucleator is spire, which uses a totally different mechanism. Multiple WH2 domains bind to actin monomers (up to 4), which serves as a prenucleation platform for the side-growth of the new actin filament. In this way, the new actin filaments are unbranched and spire is localized at the pointed ends (Quinlan et al., 2005). Spire can also crosslink microtubules and actin filaments (Rosales-Nieves et al., 2006).

Cobl is the fourth actin nucleator enriched in the brain and controlling neuronal morphology and development. Cobl has three WH2 domains at the N-terminal, which bind to three actin monomers respectively. The assembly of three actin monomers is believed to initiate the actin nucleation in cross filament direction. Cobl promotes the formation of unbundled and linear actin filaments (Ahuja et al., 2007).

1.4 Small GTP-binding proteins (G-proteins)

Small GTP-binding proteins consist of more than 100 members and are widely expressed from the yeast to the human. This protein super-family has been classified into 5 categories based on structure, the Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf and Ran family. G-proteins exist either in active GTP or inactive GDP binding state. Guanine nucleotide exchange factor (GEF) catalyses the exchange of GTP with GDP bound to the G-protein. The GTP-bound G-protein undergoes a conformational change and can bind to the downstream effectors. The GTP bound G-protein is hydrolyzed to GDP by the intrinsic GTPase activity of GTPase activating protein (GAP), which then releases the downstream effectors to become inactive. Another regulator of the conversion of GTP to GDP for G-proteins is guanine nucleotide dissociation inhibitor (GDI), which inhibits the exchange of GTP from GDP bound to the G-protein stimulated by GEF. G-proteins regulate the signaling pathway both temporally and spatially (Matozaki et al., 2000). G-proteins regulate many cellular functions such as budding process of the yeast and the cytoskeleton in mammalian cells. (Figure 1.2)

1.4.1 Ras GTPases

Ras is the founding member of the Ras superfamily of small GTPases. Besides three Ras proteins encoded by the *H-ras*, *K-ras* and *N-ras* genes, other Ras family members include the R-Ras proteins (R-Ras, R-Ras2/TC21, R-Ras3/M-Ras), the Ral protein A and B, the Raps 1A, 1B, 2A and 2B, Rheb, Rin and Rit (Reuther and Der, 2000). Ras family members participate in the regulation of diverse cellular functions including cell proliferation, survival, differentiation, actin organization, vesicular trafficking and gene expression through various signal transduction pathways (Mitin et al., 2005).

1.4.2 Rho family GTPases

Rho GTPases belong to the Ras-related small GTPases superfamily and are present in all eukaryotic cells. Rho family GTPases play roles in cell morphology, motility and adhesion through regulation of the actin cytoskeleton (Hall, 1998; Ridley, 2001). Typical Rho family GTPases are small (190-250 residues) and contain a Rho-type GTPase domain with short N-terminal and C-terminal segment (Wennerberg and Der, 2004). Based on the similarity in sequence, structure and function, the Rho family can be divided into six subfamilies (Figure 1.3). The human Rho GTPases family consists of at least 23 members and the best studied ones include Rho, Rac and Cdc42 (Bishop and Hall, 2000). Rho, Rac and Cdc42 all promote cell growth and inhibit apoptosis; they also regulate gene expression similarly through activation of signaling molecules such as serum response factor and NF- κ B, the stress activated kinases and cyclin D (Pruitt and Der, 2001; Van and Souza-Schorey, 1997) and regulate actin reorganization but affect different actin-based structures. RhoA is important for the stress fiber and focal adhesion formation; Rac1 is responsible for lamellipodia formation and Cdc42 induces filopodia formation. Two point mutants of Rho have been widely used in the study of their functions. One is the constitutively active form, which loses the GTPase activity and is always GTP-bound. The other one is the dominant negative form, which is deficient in nucleotide-binding and possibly titrates out GEF (Feig, 1999; Self and Hall, 1995).

For Rho, Rac and Cdc42, over 50 effectors have been discovered including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins (Table 1.1). The binding of the effector proteins with Rho GTPases may result in a conformational change from closed to open or may act as a spatial recruiter for the effector proteins.

1.4.2.1 WASP/N-WASP

Rho family small GTPases including Rho, Rac and Cdc42 are important mediators of the signal transduction from cell surface into the cell to regulate actin

Figure 1.1 Formins, the Arp2/3 complex and Spire, nucleate actin by different mechanisms.

The Arp2/3 complex binds to WASp actin and is believed to function as an actin trimer. Formins stabilize actin dimers. Spire binds up to four actin monomers, which serve as a stable prenucleation complex. In each panel, the dotted line (with *arrow*) points to the barbed end of the polymerized filament. The two halves of the formin FH2 dimer are green, connected by flexible linkers (*black*). The two actin-like subunits of Arp2/3 complex (Arp2 and Arp3) are pink. The four WH2 domains of Spire (*purple*) each are capable of binding one actin monomer. (Adapted with permission from (Goode and Eck, 2007))

Figure 1.2 Regulators of G-proteins.

The status change of G-protein between GTP bound and GDP bound is regulated by GAP, GEF and GDI. The GTP bound G-proteins can bind to effectors and activate the downstream signaling pathways. G-proteins associate with membrane through the farnesyl or geranylgeranyl group. (Adapted with permission from (Matozaki et al., 2000)).

polymerization/depolymerization. WAVE family proteins, including WASP, N-WASP, SCAR/WAVE proteins (WAVE1, WAVE2 and WAVE3) (Takenawa, 2005) link Rac/Cdc42 to the Arp2/3 actin nucleation complex in different systems (Machesky et al., 1999; Rohatgi et al., 1999; Derry et al., 1994; Miki et al., 1998b). The actin polymerization activity induced by WAVE family members participates in several cellular processes, such as filopodia and lamellipodia formation during cell migration (Hall, 1998), cell adhesion (Wu et al., 2004), vesicle motility, neurite extension and spine formation (Suetsugu et al., 2002b; Park et al., 2005; Banzai et al., 2000; Wong et al., 2001).

WASP is the first identified member of WAVE family proteins and is encoded by the mutated gene in patients with Wiskott-Aldrich syndrome (Derry et al., 1994). WASP is found predominantly in white blood cells and N-WASP is ubiquitously expressed. N-WASP and WASP proteins share ~ 50% sequence similarity and similar domain structures. The architecture of WASP and N-WASP is illustrated in Figure 1.4. WASP and N-WASP contain a WASP homology 1 domain (WH1 domain, also called Ena-VASP-homology-1 (EVH1) domain) at the N-terminal. The mutations responsible for Wiskott-Aldrich syndrome are in the WH1 domain. The WH1 domain binds to the poline-rich region of the WASP-interacting proteins (WIP) consisting of WIP, corticosteroids and regional expression-16 (CR16) and WIP- and CR16-homologous protein (WICH, also called WIP-related, WIRE) (Volkman et al., 2002; Ramesh et al., 1997; Aspenstrom, 2002; Kato et al., 2002; Ho et al., 2001). WIP proteins form stable complexes with WASP/N-WASP indicating that WIPs contribute to the stability of WASPs (Aspenstrom, 2002; Kato et al., 2002; Ho et al., 2001; Aspenstrom, 2004; Krzewski et al., 2006; Sawa and Takenawa, 2006). WIPs are also thought to inhibit the activity of WASP or N-WASP (Martinez-Quiles et al., 2001; Ho et al., 2004; Hertzog et al., 2004). After isolation, the N-WASP-WIP complex is unable to activate the Arp2/3 complex for actin polymerization in the presence of active Cdc42 due to WIP inhibition. Addition of the transducer of Cdc42-dependent actin-assembly 1 (Toca-1) releases the inhibition of N-WASP activity by WIP and leads to the Cdc42

Figure 1.3 Phylogenetic tree of the Rho family GTPases and representatives of other Ras-superfamily GTPases.

The Rho family GTPases can be roughly grouped into 6 subfamilies: RhoA-related, Rac-related, Cdc42-related, Rnd proteins, RhoBTB proteins and Miro proteins. (Adapted with permission from (Wennerberg and Der, 2004).

Table 1.1 Rho GTPases and their effectors. (Adapted with permission from (Bishop and Hall, 2000)).

Potential effector protein	functions	Selectivity of Rho GTPase binding		
ROK α , ROK β	Actin/myosin	Rho		
PKN/PRK1, PRK2	unknown	Rho		
Citron kinase (citron)	cytokinesis	Rho		
P70 S6 kinase	Translation regulation		Rac	Cdc42?
Mlk2,3	JNK		Rac	Cdc42
MEKK1, 4	JNK		Rac	Cdc42
PAK1, 2, 3	JNK/actin		Rac	Cdc42
PAK4	actin			Cdc42
MRCK α , MRCK β	actin			Cdc42
Ack1, 2	Endocytosis and cancer			Cdc42
MBS	MLC inactivation	Rho		
PI-4-P5K	PIP2 levels/actin	Rho	Rac	
PI3K	PIP3 levels		Rac	Cdc42
DAG kinase	PA levels	Rho	Rac	
PLD	PA levels	Rho	Rac	Cdc42
PLC- β 2	DAG/IP ₃ levels		Rac	Cdc42
Rhopilin	Endocytosis and actin organization	Rho		
Rhotekin	cancer	Rho		
Kinectin	Kinesin binding	Rho		
Dia1, Dia2	Actin organization	Rho		
WASP, N-WASP	Actin organization			Cdc42
WAVE/Scar	Actin organization		Rac	
POSH	Apoptosis and axon outgrowth		Rac	
POR-1	Actin organization		Rac	
p140Sra-1	Actin organization		Rac	
p67 ^{phox}	NADPH oxidase		Rac	
MSE55, BORGs	unknown		Rac	
IQGAP1,2	Actin/cell-cell contacts		Rac	Cdc42
CIP4 and Toca-1	Actin organization			Cdc42

mediated activation of N-WASP. WIPs also play a role in localizing WASP to active actin polymerization areas following antigens/chemokines binding to the receptor (Tsuboi, 2006; Sasahara et al., 2002; Gallego et al., 2006; Anton et al., 2002). A basic region (Miki et al., 1996; Symons et al., 1996; Aspenstrom et al., 1996) follows WH1 domain and the basic region electrostatically interacts with phosphoinositides (Rohatgi et al., 1999; Rohatgi et al., 2000; Higgs and Pollard, 2000). The Cdc42/Rac interactive binding (CRIB) domain (also known as GTPase binding domain (GBD)) next to the basic region binds to Cdc42, which is a small GTPase implicated in the filopodia formation and cell polarity (Symons et al., 1996; Miki et al., 1998a; Hall, 1998). Overexpression of the CRIB domain blocks Cdc42-induced filopodia suggesting that the Cdc42-N-WASP-Arp2/3 complex pathway may be implicated in the filopodia formation (Pellegrin and Mellor, 2005). WASP proteins contain a verprolin-homology domain (V, also called WH2 domain), the cofilin-homology domain (also called a central domain; C) and the acidic domain; A. V, C and A domains are grouped together and named the VCA domain, in which the V domain binds to actin monomer and the CA domain binds to the Arp2/3 complex.

Without activating factors, N-WASP/WASP is in an autoinhibited state, in which the C-terminal VCA domain is masked by the N-terminal region, including the CRIB domain and the region surrounding it (Rohatgi et al., 1999; Miki et al., 1998a; Kim et al., 2000; Prehoda et al., 2000). The basic region also plays a role in the auto-inhibition (Suetsugu et al., 2001). Binding of Cdc42 to the CRIB domain can expose the VCA domain and activate the molecule. PIP2 can bind to the basic region and activates WASP/N-WASP synergistically with Cdc42 (Rohatgi et al., 1999; Miki and Takenawa, 1998; Kim et al., 2000; Prehoda et al., 2000). It is reported that the binding of polyproline rich region of WASP/N-WASP to the SH3 domain containing proteins, such as Nck, GRB2 and WISH (Rohatgi et al., 2000; Carrier et al., 2000) also activates WASP/N-WASP, but the detailed mechanism is unknown (Fukuoka et al., 2001; Carrier et al., 2000; Rohatgi et al., 2001; Tsujita et al., 2006). Ash/Grb2, another SH3 containing protein, can weakly activate N-WASP (Carrier et al., 2000). Another example is Toca-1, which activates N-WASP-

WIP or N-WASP-CR16 through the SH3 domain downstream of Cdc42 (Ho et al., 2004). WASP/N-WASP activation is also regulated by phosphorylation and the phosphorylation sites by the Src tyrosine kinase are near the CRIB domain. The phosphorylation can revert the autoinhibitory effect (Cory et al., 2002; Suetsugu et al., 2002a; Torres and Rosen, 2003), which is enhanced by Cdc42 activation (Sasahara et al., 2002). Furthermore, the phosphorylation and Cdc42 binding can synergistically activate the Arp2/3 complex. In conclusion, Cdc42 and PIP2 might not be the sole upstream activators. Any proteins or lipids that can bind to N-WASP may expose the VCA domain and induce the Arp2/3 activation.

1.5 Actin-based protrusions at the leading edge of migratory cells

Upon stimulation by chemoattractants, the migratory cells protrude specialized cellular extensions in the direction of movement. Such protrusions include lamellipodia/membrane ruffles and filopodia, both of which are driven by actin polymerization.

1.5.1 Lamellipodia and membrane ruffles

Lamellipodia are sheet-like protrusions formed at the leading edge of the moving cell and contain branched actin filaments. Lamellipodia contain dendritic actin filaments and actin polymerization occurs towards the plasma membrane. Daughter branches grow from mother branches at a 70 degree angle and the Arp2/3 is located at the branching points (Korn et al., 1987). The pathway involved in the formation of lamellipodia is the activated Rac through WAVE2. WAVE2 is essential for the formation of lamellipodia based on the observation in WAVE2-knockout cells (Suetsugu et al., 2003; Yan et al., 2003; Yamazaki et al., 2003). SRA1/PIR121, a protein in WAVE complex, interacts with Rac (Kobayashi et al., 1998). Besides Rac, Cdc42 is also active at the leading edge and its inhibition reduces the lamellipodia formation (Kurokawa et al., 2004; Nobes and Hall, 1999). The Cdc42 effector, N-WASP is localized at the leading edge of lamellipodia, but its role in lamellipodia is controversial

Figure 1.4 Domain architectures of WASP, N-WASP and WAVE proteins.

The various protein domains and regions are indicated. B indicates a stretch of basic amino acids mediating F-actin and (for WASP and N-WASP) PIP₂ binding. The GTPase-binding domain (GBD) interacts directly with activated, GTP-loaded Cdc42. Poly-proline indicates a proline-rich region that contains Src-homology-3- and profilin-binding sites. V, C and A form the VCA (verprolin homology, cofilin homology and acidic domains, respectively) modular ‘output’ domain, which is responsible for initiating the growth of new actin filaments by bringing together actin monomers and the actin-nucleating Arp2/3 complex (Adapted with permission from (Stradal et al., 2004).)

(Sukumvanich et al., 2004; Kawamura et al., 2004; Innocenti et al., 2005). Membrane ruffles form when lamellipodia detach from the substrate forming vertical membrane crimples, which are also called “ruffling lamella” (Abercrombie et al., 1970).

1.5.2 Filopodia

Filopodia are thin (less than 200 nm in diameter) and small protrusions from the plasma membrane. Filopodia contain dynamic and tightly bundled parallel actin filaments. The protrusion or retraction of filopodia is determined by the overall activity of the actin polymerization at the barbed ends and the actin retrograde flow at the pointed ends (Mallavarapu and Mitchison, 1999). Filopodia from different cells vary in size. Filopodia found in fibroblast, macrophages or nerve growth cones have a maximum size of 10 μm , while in sea urchin embryos, they can be as long as 40 μm and in *Drosophila* are up to 800 μm (Ramirez-Weber and Kornberg, 2000).

1.5.2.1 Functions of filopodia

Filopodia are implicated in cell-substrate adhesion as well as cell movement (Small et al., 2002; Han et al., 2002; Svitkina et al., 2003; Lebrand et al., 2004). Filopodia either adhere to a substratum or another cell or have receptors for various signaling molecules and extracellular matrix molecules. Filopodia are used by cells as sensors of environmental cues perhaps through enrichment of activated integrins at filopodial tips (Galbraith et al., 2007) and help in guiding the direction of cell movement (Faix and Rottner, 2006). During cell spreading, the integrin-containing filopodia form the primitive adhesion sites prior to the mature focal adhesion sites formation by other focal adhesion complex proteins (Partridge and Marcantonio, 2006). Other cell adhesion molecules such as cadherins also localize at the tips or shafts of filopodia (Steketee and Tosney, 2002; Vasioukhin et al., 2000). Metastasis cancer cells have abundant filopodia facilitating the cell movement (Vignjevic et al., 2007). Filopodia tips are often the binding sites

for viruses, which are transported back to the cell body before internalization (Lehmann et al., 2005; Sherer et al., 2007). Filopodia in macrophages bind and retract to facilitate the internalization by force generated by actin bundles (Niedergang and Chavrier, 2004; Kress et al., 2007; Vonna et al., 2007). The activated epidermal growth factor (EGF) is also internalized into the cell through a similar mechanism in adenocarcinoma cells (Lidke et al., 2005). Other functions of filopodia include wound healing, embryonic development, dendritic spine development, neuritogenesis, chemotaxis, and neuronal growth cone pathfinding (Faix and Rottner, 2006; Mallavarapu and Mitchison, 1999; Gupton and Gertler, 2007). During wound healing and embryonic development, filopodia project at the edge of epithelial cells in opposite directions during fusion process of the epithelial cell sheet, thereby facilitating the alignment and adherence of the two cell sheets (Vasioukhin et al., 2000). Neuronal dendrites are enriched with filopodia, which seem to be important for the development of dendritic spines (Jontes and Smith, 2000). It has been suggested that filopodia act as a precursor of spine undergoing dynamic growth and will either become a mature spine with the correct presynaptic partner or retract back to the dendrite without proper signals (Sekino et al., 2007). Filopodia are also required for neuritogenesis in cortical neurons (Dent et al., 2007; Kwiatkowski et al., 2007). Filopodia are suggested to play important roles in neuronal growth cone pathfinding by acting as chemoattractant sensors and mediating downstream signal transductions (Gallo and Letourneau, 2004), although controversial results have been obtained in different types of neurons (Dwivedy et al., 2007).

1.5.2.2 Molecules involved in filopodia formation

Cdc42 and its effectors such as IRSp53 and N-WASP have been shown to play a role in filopodia formation by coupling membrane deformation and actin polymerization (Miki et al., 1998a; Miki and Takenawa, 1998; Govind et al., 2001; Nobes and Hall, 1995). Ena/VASP-family proteins are multi-functional actin-binding proteins and are implicated in filopodia

formation in several organisms (Krause et al., 2003). One role of Ena/VASP proteins in filopodia formation is to protect the barbed ends of actin filaments from being capped (Bear et al., 2002; Barzik et al., 2005; Pasic et al., 2008). Formins are another big group of proteins that are important for filopodia formation (Yang et al., 2007; Peng et al., 2003). The actin filaments formed by formins are unbranched. The formins act through continuous barbed end nucleation and elongation (Goode and Eck, 2007). The bundling proteins such as fascin are thought to bundle the actin filaments.

1.5.2.3 Mechanisms of filopodia formation

Filopodia are often found at the leading edge of motile cells, which often protrude from lamellipodia. Filopodia often exist independent of lamellipodia in neuronal growth cones. It is possible that filopodia are generated by different mechanisms in motile cells versus growth cones.

Filopodia often protrude from lamellipodia. The physical close relation of these two structures suggests that filopodia may emerge from the lamellipodia network (Svitkina et al., 2003; Biyasheva et al., 2004; Heid et al., 2005). Electron microscopy shows that in melanoma cells filopodia are formed from the branched lamellipodial actin network and the actin bundle is continuous from the root to the tip of filopodia (Svitkina et al., 2003). A “convergent elongation model” was proposed (Svitkina et al., 2003). In this model filopodia are generated by selective coalescence and elongation of actin filaments from lamellipodia. This happens when a subset of filament barbed ends associate with the tip complex of filopodia (containing Ena/VASP actin-binding proteins and possibly formins) at the leading edge of the plasma membrane. The tip complex protects the filament from capping proteins and allows it to elongate. The adjacent filaments are bundled by the bundling protein fascin, eventually producing filopodia. This model is supported by the work done in *Listeria* (Brieher et al., 2004). But the Arp2/3 complex is absent from filopodia and it contains parallel actin bundles that grow at their tips (Svitkina and Borisy, 1999; Mallavarapu and Mitchison, 1999). In addition, lamellipodia are not essential for filopodia

formation since knockdown of WAVE proteins or subunits of the Arp2/3 complex does not affect the filopodia formation (Steffen et al., 2006). Another report shows that depletion of Arp3 by RNAi results in slightly increase in filopodia number in mouse embryonic fibroblasts (Di et al., 2005). This suggests filopodia and lamellipodia formation may be formed through different pathways. Interestingly formins can nucleate actin to generate linear actin filaments and formins are shown to localize in filopodia tips in mammalian cells and *Dictyostelium* cells (Biyasheva et al., 2004; Pellegrin and Mellor, 2005; Schirenbeck et al., 2005a). In addition, formins are shown to localize at the barbed ends of actin filaments and act as processive motors (Kovar and Pollard, 2004; Romero et al., 2004). Expression of formins is also observed to correlate with the filopodia formation with the overexpression and knockout study in *Dictyostelium* (Schirenbeck et al., 2005b). mDia2 has been shown to be implicated in filopodia formation (Pellegrin and Mellor, 2005; Peng et al., 2003; Wallar et al., 2006). Considering the fact that formins can nucleate actin *de novo* and elongate the actin filaments while protecting the tip from capping by anti-capping proteins, it is likely that these molecules can generate cellular actin structures independent of the Arp2/3 complex. These data support the idea that lamellipodia and filopodia are generated by different mechanisms, although the solid proof for the Arp2/3 independent filopodia formation is still lacking. However, the two mechanisms of filopodia formation cannot be completely separated. A recent study in melanoma cells shows that depletion of mDia2 has an inhibitive effect on both lamellipodia and filopodia. Meanwhile when a constitutively active form of mDia2 is expressed, the filopodia is formed by the gradual convergence of the mDia nucleated long lamellipodial actin filaments (Yang et al., 2007). This result suggests that different mechanisms of filopodia formation may work together which depends on the cell types and organisms.

Filopodia formation is a process coupling actin polymerization and membrane deformation. Thus another model was suggested by Mattila et al (Mattila and Lappalainen, 2008). In this model, filopodia is initiated by the convergence of the barbed ends of actin filaments (uncapped Arp2/3 nucleated filaments or formin nucleated actin filaments) through Myosin-X. The

elongation of the barbed ends of the filaments deforms the membrane. The I-BAR domain containing proteins, such as IRSp53, may also play a role in membrane deformation facilitating the filopodial membrane protrusion. Finally the actin filaments are bundled by fascin and maybe also ENA/VASP proteins to generate a stiff filopodia bundle.

1.6 Endocytosis

Endocytosis in eukaryotic cells is a process in which cells internalize the membrane components, extracellular ligands and soluble molecules into the cells. Endocytosis is implicated in various cellular functions, such as nutrient uptake, receptor recycling, synaptic vesicle recycling, cell polarity and remodeling of the plasma membrane (Maxfield and McGraw, 2004; Slepnev and de, 2000). The endocytic machinery may also be utilized by pathogens to invade into the cells. To perform these diverse roles, the endocytotic pathway is diverse and differentially regulated for different cargos.

Clathrin mediated endocytosis is the well-characterised one and there are other non-clathrin mediated endocytotic pathways, such as caveolae mediated pathway, phagocytosis, macropinocytosis, GPI-AP-enriched early endosomal compartments (GEEC) pathway and flotillin-dependent pathway (Engqvist-Goldstein and Drubin, 2003; Le Roy and Wrana, 2005; Miaczynska and Stenmark, 2008) (Figure 1.5).

1.6.1 Clathrin-mediated endocytosis

This pathway involves complex protein-protein interactions and signalings at the cell surface, which facilitate the cargo selection and provide the mechanical force for membrane deformation and vesicle scission.

1.6.1.1 Steps of clathrin mediated endocytosis

The steps of the clathrin-mediated endocytosis have been described (Brodsky et al., 2001; Higgins and McMahon, 2002; Roth and Porter, 1964). This endocytotic pathway is receptor

mediated. After ligands bind to its specific receptor, the stuff to be endocytosed including ligands, receptors and the membrane components is recruited to the endocytic site by adaptor proteins. The $\mu 2$ subunit of the adaptor protein complex AP2 binds to the cytoplasmic internalization motif of the transmembrane receptor and β subunit of AP2 complex binds to clathrin promoting polymerization. Clathrin together with the adaptor proteins and accessory proteins forms the endocytic coat of the membrane. The natural curvature of clathrin coat promotes the membrane invagination and these deformed membranes are called coated pits. Then the membrane invaginates further with the aid of protein complex including endophilin (Farsad et al., 2001), amphiphysin (Takei et al., 1999) and dynamin (Sweitzer and Hinshaw, 1998) to form a vesicle connecting to the membrane through a narrow neck. Finally, dynamin and amphiphysin form helical array at the neck of the vesicle. GTP hydrolysis induced conformational change of this apparatus provides the mechanical force for pinching off to form the endocytic vesicle (Sweitzer and Hinshaw, 1998; Stowell et al., 1999; Marks et al., 2001). The coating proteins are disassembled by synaptojanin (Cremona et al., 1999) and auxilin (Greener et al., 2001) and recycled back to the plasma membrane. The endocytic vesicles are then fused with early endosomes. From early endosomes, the internalized molecules can either be recycled back to the plasma membrane or further fused with late endosomes, and finally to lysosomes for degradation.

1.6.1.2 Clathrin-coat components

Clathrin. Clathrin exists as triskelin in solution with a molecular weight ~ 650 kDa consisting of three heavy chains and three light chains. Clathrin can self-assemble into basket-like structures by oligomerization, where the membrane is deformed into clathrin coated pits (Lafer, 2002). The intrinsic self-assembly activity of clathrin is low and the additional induction factor for clathrin assembly is required *in vivo* (Brodsky et al., 2001).

Figure 1.5 Endocytic pathways, key regulators and functions.

Color code: blue, coat proteins; red, kinases; orange, GTPases; green, adaptors; black, other proteins. The various functions of endocytosis are indicated on top. (Adapted with permission from (Miaczynska and Stenmark, 2008))

Adaptor proteins. After clathrin was identified by Pearse et al in 1976 (Pearse, 1976), many proteins have been identified as clathrin binding partners (Brett et al., 2002; Brooksbank, 2001; Kirchhausen, 1999; Kirchhausen, 2002; Traub, 2003) which can be classified into adaptor proteins and accessory proteins. Adaptor proteins bind to cargo to be internalized, PIP2 and the clathrin coat components (Kirchhausen et al., 1997), while accessory proteins play a role in the endocytic process without direct interaction with the cargo (Altschuler et al., 1998). The adaptor proteins in clathrin coat formation during endocytosis have been classified into two groups, multimeric adaptor proteins (AP2) and monomeric adaptor proteins (clathrin-associated sorting proteins, CLASP) such as epsin, AP180, Dab2 and β -arrestins (Maldonado-Baez and Wendland, 2006). Both types of adaptor proteins bind to the N-terminal domain of clathrin heavy chain through the clathrin binding motif and promote the clathrin assembly *in vitro* with similar efficiency (Maldonado-Baez and Wendland, 2006). AP-2 was the first identified adaptor complex, consisting of four subunits, two large subunits also known as adaptins (α and β 2) and two small subunits (μ 2 and σ 2). Among the four subunits, the α subunit is divergent among different APs with the other three subunits highly conserved (Kirchhausen, 1999). Two small subunits and the N-terminal domain of two large subunits form the AP-2 core, while the C-terminal domains of two large subunits form the appendages or “ears” containing the binding sites for other accessory proteins (arrestin, AP180, epsin et al.) (Gaidarov and Keen, 2005). Between the core and the “ear” is the flexible hinge region responsible for clathrin binding and assembly (Gaidarov and Keen, 2005). The AP-2 core plays a role in recruitment of AP2 to the membrane and the phosphorylation of μ 2 subunit by AAK1 kinase is required for cargo binding (Ricotta et al., 2002). AP-2 is essential for clathrin coated pit formation through participating in the clathrin coat assembly and cargo selection (Traub, 2003; Kirchhausen et al., 1997; Robinson, 2004) in the plasma membrane. AP-1 is equivalent to AP-2 with a specific localization in the clathrin-coated vesicles in trans-Golgi network (TGN), which consists of four subunits β 1, γ , μ 1 and σ 1 (Kirchhausen, 1999). Additional less-characterised AP complexes include AP-3 (β 3, δ , μ 3,

$\sigma 3$) and AP-4 ($\beta 4$, ϵ , $\mu 4$, $\sigma 4$). AP-3 is implicated in vesicle formation from endosomes and AP-4 may function at TGN for vesicle formation (Robinson and Bonifacino, 2001). Whether these two adaptors have a direct relationship with clathrin still needs further investigations.

Accessory proteins. Many accessory proteins have been identified and form a complex interacting network centered at clathrin, AP-2, Eps15. Lipid PIP₂ is also involved in organizing the complex protein network and may be responsible for the membrane localization of the proteins (Cremona et al., 1999). Eps15 may provide a link to the actin cytoskeleton (Duncan et al., 2001). Phosphorylation and dephosphorylation are important mechanisms to regulate the protein-protein and protein-lipid interactions in this complex network (Cousin and Robinson, 2001; Cremona and de, 2001).

Eps15 (EGFR pathway substrate clone 15) is identified as a component of plasma membrane clathrin-coated pits with a direct and constitutive interaction with AP-2 (Benmerah et al., 1995; Tebar et al., 1996). Eps15 also interacts with other endocytic adaptors such as epsin, AP180, Numb, Stonin 2 and ubiquitinated proteins at the plasma membrane (Maldonado-Baez and Wendland, 2006). Domain structures from sequence analysis reveal that Eps15 contains three Eps15 homology (EH) domain at the N-terminal, a coil-coiled domain implicated in the Eps15 oligomerization in the middle (Cupers et al., 1997; Tebar et al., 1996) and a poly-proline domain binding to AP-2 at the C-terminal (Benmerah et al., 1996; Iannolo et al., 1997). The N-terminal three EH domains are the binding sites for many clathrin binding proteins. The AP-2 binding site mutants of Eps15 indicate the essential role of the interaction between Eps15 and AP-2 in receptor mediated endocytosis (Benmerah et al., 1999). Other roles of Eps15 include a stimulation of the clathrin assembly activity through AP-2 and AP180 and a link of clathrin-coated pits with the actin cytoskeleton by activating the Arp2/3 stimulated actin polymerization (Lafer, 2002).

Dynamin was first identified in 1989 (Shpetner and Vallee, 1989). It is discovered later that dynamin is a superfamily consisting of three conventional dynamins (Dyn1, Dyn2 and Dyn3),

dynamamin-like proteins (DLP/DRP) implicating in mitochondrial dynamics (Labrousse et al., 1999) and the anti-viral protein Mx (MxA and MxB; (Haller and Kochs, 2002)). Dynamins express differentially in different cell types. Dyn 1 is limited to neuronal cells. Dyn 2 is ubiquitously expressed and Dyn3 is possibly only expressed in the brain, lung and testis. All conventional dynamins belong to the large GTPases with a molecular weight ~96 kDa. Dynamins share similar domain structures with important functions. There are a GTPase domain, pleckstrin homology domain (PH) and a coiled-coiled domain (GTPase Effector domain, GED) at the N-terminal. In the C-terminal there is a poly-proline rich domain (PRD). The PH domain mediates the binding of dynamin with membrane through PIP2 with the aid of an upstream domain with the lipid-bilayer penetrating activity (Burger et al., 2000). GED is a self-regulating domain which serves as an internal GTPase-activating domain (Muhlberg et al., 1997). The GED domain is thought to form direct contact with the GTPase domain of the adjacent dynamin molecule in dynamin self-assembly, thus stimulating the intrinsic dynamin GTPase activity (Muhlberg et al., 1997). The PRD is the interacting site for SH3 domain containing proteins and dynamin is also recruited to the endocytic site through the PRD (Gout et al., 1993; David et al., 1996; Wigge and McMahon, 1998; Shupliakov et al., 1997). The multiple PRDs provided by self-assembled dynamin may also serve as a platform to recruit other SH3 domain containing proteins or scaffolding proteins to the endocytic sites. The PRD binds to various proteins through their SH3 domains, including endocytic adaptor such as Grb2 (Gout et al., 1993) and intersectin (Zamanian and Kelly, 2003), BAR domain containing proteins such as amphiphysin and endophilin (Ringstad et al., 1997) and actin-regulatory proteins such as cortactin (McNiven et al., 2000) and Abp1 (Kessels et al., 2001). Many SH3 domain containing proteins play roles in actin dynamics including syndapin/paccin, amphiphysin/Rvs167, intersectin/DAP160, cortactin, Abp1, and Grb2 (Qualmann et al., 2000; Slepnev and de, 2000). Further dynamin is linked to N-WASP, an important member in actin dynamics through SH3 domain containing proteins such as syndapin, intersectin and Grb2 (Qualmann et al., 1999; Hussain et al., 2001). Dynamamin is found at several

actin-rich structures within the cells such as peripheral ruffles (McNiven et al., 2000), adhesion sites (Ochoa et al., 2000) and phagocytic cups (Gold et al., 1999). Dynamin is also linked to the activation of Cdc42 through db1 domain of intersectin.

1.6.1.3 Cdc42 and clathrin-mediated endocytosis

Rho family GTPases are involved in the regulation of the cortical actin assembly and endocytosis, such as Rac and Cdc42 (Schafer et al., 2002). Several works suggest that localized actin polymerization may increase the endocytic efficiency by providing forces for vesicle scission or and inward movement of the vesicles into the cytosol (Qualmann et al., 2000; Schafer et al., 2002).

Cdc42 is linked to the clathrin-mediated endocytosis through intersectin, a protein localizes in clathrin-coated pits (Schafer et al., 2002) and functions as an scaffold to cluster endocytic molecules such as Eps15 and dynamin (O'Bryan et al., 2001). Intersectin is a binding partner of CdGAP, a Cdc42 GAP protein and inhibits CdGAP function (Jenna et al., 2002). On the contrary, intersectin-1, a brain splice variant of intersectin, functions as a nucleotide exchange factor for Cdc42 (Hussain et al., 2001; Ridley, 2001). Interestingly, the exchange activity is stimulated by binding to N-WASP, an effector downstream of Cdc42 to activate the Arp2/3 complex (Hussain et al., 2001). Activated Cdc42 can then induce actin nucleation through the N-WASP/Arp2/3 pathway (Hussain et al., 2001). The stimulatory effect of N-WASP may provide a positive feedback promoting localized actin polymerization at clathrin-coated pits where intersectin is localized (Hussain et al., 1999). In addition, overexpression of an intersectin mutant unable to activate Cdc42 leads to endocytic defects similarly to the effect caused by actin-sequestering drug latrunculin B (McGavin et al., 2001) or absence of WASP (Zhang et al., 1999).

Another link of Cdc42 with endocytosis is through ACK (activated Cdc42-associated tyrosine kinase). ACK is a Cdc42 effector (Manser et al., 1993) and binds to clathrin (Yang et al., 2001; Teo et al., 2001; Yang et al., 2001). Overexpression of ACK affects the receptor

internalization moderately and the expression level determines whether ACK has an inhibitory or stimulatory effect (Yang et al., 2001; Teo et al., 2001). The binding of ACK to clathrin competes the binding of clathrin to AP2, which suggests that ACK may inhibit the receptor internalization by preventing recruitment of clathrin to coated pits (Yang et al., 2001). A study of ARK-1, a tyrosine kinase closely related to ACK in *C-elegance*, also suggests the stimulatory effect on the EGF receptor internalization (Hopper et al., 2000). Two groups show that ACK interacts with SH3 and PX domain containing protein 1 (SH3PX1)/sorting nexin 9 (SNX9) and ACK phosphorylates SH3PX1/SNX9 (Worby et al., 2002; Lin et al., 2002). ACK forms a complex with clathrin and SNX9. Overexpression of an N-terminal truncated ACK mutant increases the binding of SNX9 with clathrin. Overexpression of the ACK mutant that cannot bind to clathrin prevents the SNX9/clathrin binding suggests that ACK may function as a scaffold to recruit SNX9 to clathrin coated pits or vesicles (Lin et al., 2002). SNX9 is shown to bind Dock/Nck in *Drosophila*, a SH3/SH2-containing adaptor protein linking membrane receptors with the actin cytoskeleton (Worby et al., 2001; Buday et al., 2002). An interaction between DSNX9 and WASP also has been shown suggesting the involvement of actin polymerization in endosome movement (Worby et al., 2001; Suetsugu et al., 2002b). Further work shows that ACK plays a role in the vesicle traffic following internalization. Co-expression of SNX9 together with wild type ACK, but not kinase inactive ACK speeds up the traffic of internalized EGF receptors to lysosomes, which is mediated by the phosphorylation of SNX9/ SH3PX1 (Lin et al., 2002).

1.6.1.4 Intracellular trafficking of the endocytosed vesicles

The internalized cargo molecules are first delivered to early endosomes. Rab5, a small GTPase, is the main regulator in this stage of the intracellular trafficking and it is proposed that it plays a role in the organization of the early endosomal membrane through interaction with its effectors (Zerial and McBride, 2001). Rab5 has the characteristic of other small GTPases, cycling between a GTP bound form and a GDP bound form catalyzed by GAP. Rab5 also cycles

between cytosol and membrane (Zerial and McBride, 2001; Chavrier and Goud, 1999), which depends on GDI. GDI serves as a vehicle for Rab5 in the aqueous cytosol. The early endosomal protein EEA1 is one of Rab5 effectors and is recruited to early endosomes not only by GTP bound Rab5, but also phosphatidyl-inositol-3-phosphate (PI(3)P). EEA1 is essential for endosome fusion (Cavalli et al., 2001). Once delivered to early endosomes, the recycling receptors such as transferrin receptors are recycled back to the plasma membrane partially through the recycling endosomes. In contrast, the downregulated receptors such as EGF are sorted into late endosomes, and later into lysosomes for degradation (Gruenberg and Maxfield, 1995). Rab4 and Rab11 are believed to regulate the recycling process and Rab4 functions at an earlier stage than Rab11 (van der et al., 1992; Sonnichsen et al., 2000). The transport from early endosomes to late endosomes is mediated by the intermediate structures named multi-vesicular bodies (MVB) or endosomal carrier vesicles (ECV) with a characteristic multivesicular appearance (Gruenberg and Maxfield, 1995). The biogenesis of MVB is dependent on the endosomal COPs and Arf1 in mammalian cells (Gu and Gruenberg, 2000; Gu and Gruenberg, 1999). However, the signaling pathway is not clear. After the formation of MVB on early endosomes, they move to late endosomes through microtubules before docking and fusion with the late endosomes (Gruenberg and Maxfield, 1995). Again the signaling pathways are poorly defined in this step.

1.6.2 Clathrin-independent pathways

1.6.2.1 Caveolae mediated endocytosis

Caveolae mediated endocytosis is utilized by viruses (e.g. Simian Virus 40-SV40, Polyoma virus) or toxins (cholera toxin or Tetanus toxin) to internalize (Pelkmans and Helenius, 2002). This endocytic pathway also plays a role in the cell maintenance such as cholesterol homeostasis, regulation of membrane components and the uptake of the serum constituents such as albumin (Pelkmans and Helenius, 2002).

Caveolae was first observed by Palade and Yamada (Yamada, 1955) using electron microscopy of thin sections. Caveolae is a static round membrane invagination supported by cortical actin filaments underneath the plasma membrane (Thomsen et al., 2002; Pelkmans et al., 2001). Lipid compositions of caveolae are similar to those of lipid rafts, rich in cholesterol and sphingolipids and are essential for the formation and stability of caveolae (Pelkmans and Helenius, 2002). The caveolae will only be internalized into the cell and form vesicles with certain stimulations. Caveolin-1 is the main component of the protein coats of caveolae (Rothberg et al., 1992) and is essential for the formation and stability of the caveolae (Fra et al., 1995). During endocytosis, caveolin is completely internalized into the cells together with the vesicles (Pelkmans and Helenius, 2002), but the direct role of caveolin in the endocytosis is not clear. Another component of caveolae is dynamin, which is transiently localized to the neck of the caveolae invagination (Pelkmans and Helenius, 2002). Dynamin is thought to perform the similar function as that in clathrin mediated endocytosis, i.e., vesicle scission (Pelkmans and Helenius, 2002). Caveolae also consists of components for vesicle docking and fusion, GPI-anchored proteins and several receptor and non-receptor tyrosin kinases, which implies the important roles of caveolae in the signal transduction.

The affinity of the cargo to lipid raft itself is not enough to initiate the internalization process. Some induction methods are required. One way is through cross-linking caveolae components. One of these examples is GPI-anchored proteins (Parton et al., 1994; Mayor et al., 1994). Another way is to induce the phosphorylation cascade by the cargo (Anderson, 1998; Minshall et al., 2000; Pelkmans and Helenius, 2002). The signal transduction from the ligand to the phosphorylation event is not clear.

After internalization, the caveolae vesicles enter an intracellular compartment different from that of clathrin-mediated endocytosis. Caveosomes rich in caveolin-1 are the sorting compartments for caveolae mediated endocytosis. From caveosomes, the cargo is delivered to endoreticulum, Golgi, or other intracellular compartments.

1.6.2.2 Macropinocytosis

Macropinocytosis is the mechanism used by some pathologic organisms or large volume of extracellular liquids and vast area of the plasma membrane to enter the cells through big macropinosomes ($> 1 \mu\text{m}$) and is coupled to the dynamic actin cytoskeleton modulation, i. e. membrane spreading and ruffles (Cavalli et al., 2001). Macropinosomes form when the membrane ruffles and vesicles move at the tips of actin tails (Merrifield et al., 1999). Macropinocytosis can be stimulated by EGF (Haigler et al., 1979) and Ras (Bar-Sagi and Feramisco, 1986). The scission of macropinosomes does not involve dynamin (Miaczynska and Stenmark, 2008). p21-activated kinase (PAK1) downstream active Rac/Cdc42 induces the formation of membrane ruffles and thus implicated in macropinocytosis (Dharmawardhane et al., 2000). Other factors involved in this pathway include phosphatidylinositol-3-kinase (PI3K) and phosphatidylinositol (PI)-phospholipase C (Amyere et al., 2000).

1.6.2.3 GEEC pathway

Glycosyl-phosphatidylinositol (GPI)-anchored proteins (GPI-APs) are present on the plasma membrane as a small cluster dependent on cholesterol. The GPI-APs cluster serves as sorting signals for GPI-APs to be internalized through a clathrin, caveolae and dynamin independent pathway. GPI-AP enriched early endosome compartment (GEECs) mediates the internalization. In this pathway, active Cdc42 is recruited and stabilized on the plasma membrane, a process dependent on cholesterol. Active Cdc42 then induces localized actin polymerization, which is essential for this endocytosis pathway (Chadda et al., 2007). This pathway is regulated by GTPase activating protein GRAF1, an essential component of the GEECs pathway (Lundmark et al., 2008).

1.6.2.4 Flotilin pathway

Flotillins are membrane proteins and a component of the membrane microdomain, lipid raft. Coassembly of flotillin1 and flotillin2 on the plasma membrane forms laterally dynamic microdomains different from caveolae and similar to lipid raft in certain features (Frick et al., 2007). These microdomains can invaginate and pinch off as vesicles when stimulated by overexpression of flotillin (Frick et al., 2007). The primary endocytic structures are morphologically different from GEECs. This pathway is dependent on the presence of both flotillins in membrane microdomains, which are either flat or invaginated (Frick et al., 2007).

1.6.3 Endocytosis and actin cytoskeleton

Endocytosis involves dynamic membrane remodeling and the actin cytoskeleton has been shown to be involved in the endocytosis pathway in multiple organisms using diverse methodology.

1.6.3.1 Mammalian cells

Endocytosis is a process that the membrane and actin cytoskeleton is tightly coupled. The first evidence that actin is involved in the endocytotic pathway is using the inhibitor of the actin polymerization. Reagents interfering with actin turnover inhibit endocytosis and the formation of coated vesicles (Fujimoto et al., 2000; Gottlieb et al., 1993; Lamaze et al., 1997; Salisbury et al., 1980) in mammalian cells, but the inhibitory effect is not complete (Fujimoto et al., 2000; Lamaze et al., 1997; Salisbury et al., 1980) or limited to the apical surface of epithelial cells (Gottlieb et al., 1993).

1.6.3.2 *S. cerevisiae*

In yeast actin is implicated in the endocytosis manifested by the genetic studies. Using actin mutants (Kubler and Riezman, 1993), mutants of actin binding proteins (Kubler and Riezman, 1993) and deletion mutants of the actin-dependent motor proteins (Geli and Riezman, 1996) of *S.*

cerevisiae, defects in endocytosis were observed. In contrast to mammalian cells, the inhibitors of actin polymerization/depolymerization can completely block endocytosis in the yeast (Ayscough, 2000; Ayscough et al., 1997).

The observation that actin colocalizes with the endocytic proteins (Engqvist-Goldstein and Drubin, 2003; Salisbury et al., 1980; Shupliakov et al., 2002; Mulholland et al., 1994) also suggests that actin may play roles in endocytosis pathways. Additionally, protein-protein interactions have been observed between the actin cytoskeleton and the endocytic machinery by biochemical studies (Engqvist-Goldstein and Drubin, 2003; Salisbury et al., 1980; Shupliakov et al., 2002; Mulholland et al., 1994; Qualmann and Kessels, 2002), which also suggests that actin may be involved in the endocytic pathway. These results give good indications of actin involvement in endocytosis, but the exact mechanism is not clear.

1.6.3.3 Knowledge from time-lapse imaging

The actin cytoskeleton functions in clathrin-coated pits, membrane invagination and vesicle scission. Treatment of cells with latrunculin A, an actin sequestration drug, has shown that the actin cytoskeleton plays roles in several steps of the mammalian endocytic pathway. This drug inhibits the movement of clathrin-coated pits (Merrifield et al., 2002; Yarar et al., 2005). In Yarar's study, latrunculin A treatment affects both invagination and scission (Yarar et al., 2005). The role of the actin cytoskeleton during the membrane invagination is thought to provide the force (Engqvist-Goldstein and Drubin, 2003; Merrifield et al., 2004; Yarar et al., 2005). Using pH sensitive probes and latrunculin-B treatment, Merrifield et al showed that actin polymerization played a role in the vesicle scission step in endocytosis (Merrifield et al., 2005).

The time-lapse imaging has revealed more details of this pathway. Using GFP-actin or fluorescence labeled clathrin by TIRFM (Merrifield et al., 2002; Yarar et al., 2005), it was observed that actin transiently co-localized with clathrin-coated pits near the end of the life time of the clathrin-coated pits and overlapped with the internalization of the clathrin-coated vesicles.

In addition, actin recruitment accompanied the internalization of most coated pits and vesicles, which suggested that the role of actin in mammalian endocytosis was not sporadic (Merrifield et al., 2002). Thus during endocytosis, actin polymerization occurs at the site of clathrin-coated sites and stops after vesicle scission (Merrifield et al., 2005).

It has been shown that the actin polymerization during endocytosis seems to be precisely regulated. This point is supported by the observation that transient actin polymerization is precisely coordinated with the recruitment of other endocytic proteins (Merrifield et al., 2002; Jonsdottir and Li, 2004; Kaksonen et al., 2003; Kaksonen et al., 2005; Merrifield et al., 2004; Merrifield et al., 2005; Newpher et al., 2005; Sirotkin et al., 2005). The internalization is accompanied by the transient recruitment of dynamin-1 (Merrifield et al., 2002). The vesicle scission is coincident with the recruitment of cortactin, an F-actin and dynamin binding protein (Merrifield et al., 2005).

1.6.3.4 The actin cytoskeleton and endosome motility

It has also been observed that dynamic actin filaments colocalize with endosomes in several cell types (Kaksonen et al., 2000; Merrifield et al., 1999; Taunton et al., 2000; Orth et al., 2002; Rozelle et al., 2000). The actin polymerizes at one side of endosome and form the “comet tail” phenomenon when the vesicles move. This phenomenon has been observed in mammalian cells (Frischknecht et al., 1999; Kaksonen et al., 2000), *Xenopus laevis* oocytes (Taunton et al., 2000) and *Xenopus* egg extract (Kanzaki et al., 2001; Ma et al., 1998; Marchand et al., 1995; Taunton et al., 2000). This phenomenon also has been observed directly using time lapse imaging (Merrifield et al., 1999). It is thought that the driving force of endosome movement is from actin polymerization.

1.6.3.5 The actin polymerization during endocytosis is possibly through the Arp2/3 mediated pathway.

The actin polymerization is likely to be initiated by N-WASP through the Arp2/3 complex. Evidences show that N-WASP is recruited to coated pits using live cell imaging (Merrifield et al., 2002) and N-WASP knockdown by RNAi decreases the uptake rate of epidermal growth receptor (EGFR) (Benesch et al., 2005; Innocenti et al., 2005). N-WASP may be recruited by proteins containing SH3 domain, such as cortactin, endophilin, syndapin and intersectin (Kessels and Qualmann, 2002; Otsuki et al., 2003). The Arp2/3 complex has also been shown to be recruited to clathrin coated pits (Merrifield et al., 2004). Thus the actin polymerization during endocytosis is possibly through the Arp2/3 complex activated by N-WASP (Welch and Mullins, 2002). Besides the clathrin-mediated endocytosis, the Arp2/3 mediated actin polymerization is also implicated in other endocytic pathway, including macropinocytosis (Merrifield et al., 1999), phagocytosis (May and Machesky, 2001) and caveolae-mediated endocytosis (Pelkmans and Helenius, 2002).

The actin cytoskeleton is closely related to the endocytic pathway and plays multiple functions. Actin filaments near the membrane may serve as the platform, where the proteins driving the membrane are recruited. Thus the actin cytoskeleton may contribute to the clathrin coated pits formation by controlling the localization of the assembly protein complexes (Yarar et al., 2005; Bennett et al., 2001; Gaidarov et al., 1999). It may also provide mechanical forces for several steps including the membrane invagination, vesicle scission and move away of the vesicles from the membrane into the cytosol (Qualmann et al., 2000; Qualmann and Kessels, 2002; Engqvist-Goldstein and Drubin, 2003; Merrifield et al., 2004). In summary, actin polymerization appears to directly function during three steps in endocytosis: formation of the endocytic vesicles from the membrane, vesicle scission and the vesicle motility after vesicle internalization (Figure 1.6). Given all these evidences, however, the exact temporal and spatial

regulation of actin polymerization during initiation, invagination and scission of the endocytic vesicles still remains elusive in mammalian cells.

1.6.3.6 Linking molecules between the actin cytoskeleton and endocytosis.

In most cases, actin filament generation is associated with a membrane event, for example endocytosis and filopodia formation; hence it is important to investigate the functions of the molecules coupling the actin cytoskeleton and the membrane. Examples of the linking molecules between the actin cytoskeleton and the membrane include N-WASP, dynamin (Song and Schmid, 2003; Ehrlich et al., 2004; Merrifield et al., 2002; Schafer, 2004), mAbp1, Hip 1 and Hip 1R (Stamnes, 2002) and BAR domain proteins.

BAR domains were first identified in metazoan proteins BIN/Amphiphysin and yeast proteins (**R**vs161/**R**vs167; reduced viability upon starvation) prior to functional attributes of membrane bending being associated with the domain. During the course of my study, the function of BAR domains has been unravelled. BAR domain proteins deform membrane into tubules and are an important player in linking the membrane and the actin cytoskeleton in endocytosis. They play roles in diverse cellular processes including organelle biogenesis, membrane trafficking, cell division, and cell migration (Frost et al., 2009). Currently there are three distinct families of BAR domains; classical-BAR, F-BAR (FCH-BAR, e.g. Fes/CIP4 homology-BAR) and I-BAR (Inverse-BAR, e.g. IRSp53). Structural studies have revealed common elements of the BAR domains which include dimerisation modules that possess curvature and positively charged surfaces (Frost et al., 2009) (Table 1.2).

Classical BAR. The BAR domain is evolutionarily conserved. Numerous proteins contain this domain, for example, yeast protein rescue viability upon starvation (**R**vs) and metazoan protein amphiphysins (Itoh and De Camilli, 2006). Two basic residues in the BAR domain interact with the negatively charged phospholipids in the membrane. Dimerized BAR domain forms a banana-shaped structure with one pair of the basic residues in the monomer on the

concave surface and the other pair at one end. BAR domains are shown to deform membrane *in vitro* and the deformation effect is concentration-dependent. BAR domains deform the membrane into small buds, tubules and vesicles at the low, medium or high concentration respectively (Peter et al., 2004). Polymerized BAR domains around the lipid tubules are considered as inducing and stabilizing factors for further tubulation. BAR domain proteins are commonly localized at the site of membrane remodeling although these proteins are different in sequence and organization. A subset of these proteins is linked to endocytosis. These include Tuba, sorting nexins, Arfaptins, Oligophrenin, Centaurin β , ICA69 and PICK1 (Dawson et al., 2006).

N-BAR and other adapted BAR domains. The N-BAR domain is distinguished from BAR domains by an amphipathic helix in front of the classical BAR domain, which enhances its ability to bind liposomes with no effect on tubulation (Peter et al., 2004). The amphipathic helices of N-BAR domain are polarized at one side and hydrophobic at the other side. The hydrophobic side is used to insert the protein into the hydrophobic part of the lipid bilayer. This replacement causes the membrane to bend towards to the amphipathic helix. The membrane curvature is stabilized by the banana-like shape of the BAR domain. Endophilin and amphiphysin family proteins are well-studied N-BAR domain proteins. Other adaptations of BAR domains for their diverse functions include the incorporation of flanking phosphoinositide binding motifs or phox (PX) or PH domains, which enable these BAR proteins to specifically associate with certain types of membranes (Frost et al., 2009). Examples of this kind of BAR proteins include oligopherins, APPLs, GRAFs, centaurin- β s and sorting nexins 1,2,5,6,9 (Frost et al., 2009).

I-BAR. In contrast to the “banana shaped” BAR/F-BAR domains, the I-BAR (the Inverse BAR domain) domain is “zeppelin shaped” and exists as a six-helix-bundle dimer. Positively charged amino acids are located at the end of the I-BAR domain (Lee et al., 2007; Mattila et al., 2007; Millard et al., 2005; Suetsugu et al., 2006) and are the binding sites for the membrane, actin and the small GTPase Rac. The I-BAR domain binds to phosphatidylinositol-rich membranes and

Figure 1.6 Requirement for F-actin dynamics in multiple steps of clathrin mediated endocytosis.

F-actin assembly/disassembly dynamics are required for (1) CCS (clathrin coated structure) assembly, (2) CCS motility, (3) CCS constriction, and possibly (4) scission to form a CCV (clathrin coated vesicle) and (5) translocation of the nascent vesicle away from the plasma membrane. CCS invagination proceeds in the absence of actin dynamics. Actin assembly may proceed from the edge of the CCS and assemble inwards, facilitating CCS constriction, scission, and translocation. (Adapted with permission from (Yarar et al., 2005))

has membrane tubulation activity (Yamagishi et al., 2004; Mattila et al., 2007; Millard et al., 2005; Suetsugu et al., 2006). Juha et al shows that clustering of PIP₂ is formed after I-BAR binding. The membrane is deformed by electrostatic interactions and the I-BAR is still dynamically associated with the inner side of the deformed membrane tubules (Saarikangas et al., 2009). I-BAR binds to lipid bilayer through a convex surface and promotes the formation of outward protrusions of membranes such as filopodia (Mattila et al., 2007).

F-BAR. The ‘*Pombe* Cdc15 homology’ (PCH) family proteins are known as adaptor proteins involved in the cytokinesis and actin dynamics (Lippincott and Li, 2000). PCH family members are expressed in diverse eukaryotic species with limited sequence homology, but they have similar domain structures, an N-terminal Fes/CIP4 homology domain (Lippincott and Li, 2000; Greer, 2002), followed by a coiled-coil (CC) region and one or more SH3 domains at the C-terminal. The F-BAR domain, also known as extended FCH domain (EFC) in PCH family protein includes the FCH and CC domains and is only weakly homologous to the BAR domain (Itoh et al., 2005; Tsujita et al., 2006). By sequence alignment, structural, biochemical and cell biological studies, it is shown that EFC is actually an extended BAR domain and F-BAR domains share similar properties to BAR domains. The F-BAR domain plays roles in oligomerization and membrane phospholipid binding (Tsujita et al., 2006). Three pairs of basic residues are conserved in the F-BAR domain, which bind lipids and mediate membrane tubulation activity (Tsujita et al., 2006). The F-BAR domain binds specifically to certain kinds of lipids in contrast to the BAR domain, which does not have such lipid preferences. The F-BAR domain has high affinity for PIP₂ (Tsujita et al., 2006; Kovar, 2006), moderate affinity for PIP₃ and phosphatidylserine (Tsujita et al., 2006) and does not bind to lysophosphatidic acid, lysophosphocholine or sphingosine-1-phosphate (Tsujita et al., 2006). The F-BAR domain tubulates the membrane *in vitro* and *in vivo* (Tsujita et al., 2006; Itoh et al., 2005). From the crystalized structure of the F-BAR domain, Shimada et al showed that it was a gently curved

helical-bundle dimer with a length $\sim 200 \text{ \AA}$ fitting into a $\sim 600 \text{ \AA}$ tubulated membrane (Shimada et al., 2007).

There are three groups of F-BAR domain proteins based on their domain structures (Greer, 2002) (Figure 1.7). The first group is the Fps/Fes and Fer subfamily of non-receptor protein tyrosine kinases, including Fps (Fujinami poultry sarcoma) and Fes (feline sarcoma), oncogenes identified from tumor-forming retroviruses and encoding non-receptor tyrosine kinases. The second group is Rho GTPase activating proteins (RhoGAPs), which regulate the functions of GTPases. The third group is membrane scaffold proteins implicated in endocytic events.

A subset of F-BAR domain proteins including Toca-1, CIP4 and FBP17 contains an F-BAR domain at the N-terminal, an HR1 domain in the middle and an SH3 domain at the C-terminal. F-BAR domain proteins bind to lipids and deform the membrane through the F-BAR domain (Tsujita et al., 2006; Itoh et al., 2005). The membrane deformation activity of the F-BAR domain is enhanced by disruption of the actin cytoskeleton (Itoh et al., 2005; Tsujita et al., 2006). These proteins can also induce actin bundling (Chitu et al., 2005). These suggest that these proteins coordinate the membrane-cytoskeleton events. This point is further manifested by the finding that F-BAR domain proteins are implicated in the classical endocytic pathways such as EGF and transferrin uptake. Overexpression of FBP-17 and CIP 4 impairs this process and RNAi knock down of Toca-1, FBP17 and CIP4 increases the uptake (Itoh et al., 2005; Tsujita et al., 2006). Toca-1, FBP-17 and CIP4 all can bind to Cdc42 through the HR1 domain as well as N-WASP through the SH3 domain. These three molecules can regulate the activation of N-WASP (Ho et al., 2004; Tian et al., 2000; Tsujita et al., 2006; Itoh et al., 2005). Disruption of the actin cytoskeleton enhances the F-BAR domain induced tubulated membrane invaginations (Itoh et al., 2005). The SH3 domain of FBP17 and CIP4 binds to dynamin, which antagonizes the tubulation activity of FBP17 and CIP4 (Itoh et al., 2005). RNAi results suggest that there is functional

Table 1.2 Classification, structures and protein examples of BAR domains. (adapted with permission from (Frost et al., 2009))

BAR Domains		Human BAR Domain Proteins
Classical BAR		Arfaptins, ICA69, PICK1, DNMBP (Tuba)
N-BAR (N-terminal amphipathic helix-BAR)		Amphiphysins/BINs, endophilins/BIFs, nadrins
BAR-PH (BAR-Pleckstrin Homology)		Oligophrenins, APPLs, GRAFs, centaurin-βs
PX-BAR (PhoX-BAR)		Sorting nexins 1,2,5,6,9
F-BAR (FCH-BAR, Fes/CIP4 Homology-BAR)		Toca-1, FBP17, CIP4 FCHo1, FCHo2, Fes/Fer kinases, syndapins, srGAPs, PSTPIP-1, PSTPIP-2
I-BAR (Inverse-BAR; IMD-BAR, or IRSp53-MIM homology domain)		IRSp53, MIM, IRTKS

Figure 1.7. Classification and domain structures of BAR proteins.

(A) Phylogenetic tree of the BAR/F-BAR domain family. (B) Subfamilies of BAR and F-BAR domains are highlighted in light blue and pink, respectively (adapted with permission from (Itoh et al., 2005)).

A

B

redundancy between Toca-1, FBP17 and CIP4 (Itoh et al., 2005). These results suggest that this group of F-BAR domain proteins plays an important role in linking membrane dynamics and the actin cytoskeleton during endocytosis. Using an *in vitro* system, Kazunari *et al* investigated the mechanism of the Toca-1/FBP17 mediated actin polymerization. They showed that the Toca-1/FBP17 recruited N-WASP-WIP to the membrane and the membrane curvature generated by Toca-1/FBP17 activated the N-WASP-WIP mediated actin polymerization (Takano et al., 2008).

Syndapins (synaptic dynamin-associated proteins), also known as PACSINs, are another group of F-BAR domain proteins that have been studied in detail (Kessels and Qualmann, 2004). Syndapins have three isoforms and they are expressed in a tissue-specific manner implicating in the regulation of the endocytic events (Modregger et al., 2000; Anggono et al., 2006; Kessels et al., 2006; Kessels and Qualmann, 2006; Braun et al., 2005; Kessels and Qualmann, 2002). Syndapins contain an F-BAR domain at the N-terminal and an SH3 domain at the C-terminal. There are no Cdc42 binding sites in syndapins. Overexpression of the SH3 domain of syndapins inhibits the receptor-mediated endocytosis, while the overexpression of the full-length protein generates microspikes and lamellipodia-like structures. This shows that these proteins participate in both endocytosis and the actin polymerization (Qualmann and Kelly, 2000). Syndapins can oligomerize through the N-terminal coiled-coil domain (Qualmann and Kelly, 2000; Kessels and Qualmann, 2006). The oligomerization is thought to increase the concentration of the SH3 domain for the recruitment of N-WASP and dynamin. Syndapins bind to synaptojanin and sos, which are important proteins in endocytosis (Kessels and Qualmann, 2004). Other functions of syndapins include regulation of the membrane trafficking events at the trans-Golgi network (Qualmann and Kelly, 2000) and endosomal recycling (Braun et al., 2005). Recently the function of syndapin in the formation of a postsynaptic membrane system in *Drosophila* has been documented (Kumar et al., 2009). The structure study by Wang et al., (Wang et al., 2009) revealed that the specific characteristics of the F-BAR domain of syndapins contributed to its

ability in generating small tubules and tubule constriction in contrast to the wide tubules generated by other F-BAR domains. Additionally, full length syndapin adopted an autoinhibited conformation and the membrane tubulation activity was inhibited.

In general, BAR and EFC domains may deform the membrane in the process of vesicle formation. Alternately they may function as a sensor for the membrane curvature and recruit WASP /N-WASP or other actin related proteins (Takano et al., 2008).

1.7 Aims

Toca-1 was identified by Ho et al in 2004 (Ho et al., 2004). Toca-1 belongs to the PCF (pombe Cdc15 homology) protein family. It contains an FCH and a coiled-coil domain which together form the F-BAR domain at the N-terminal, an HR1 domain in the middle and an SH3 domain at the C-terminal. The HR1 domain binds to Cdc42 and the SH3 domain binds to N-WASP and dynamin. Toca-1 has been shown to be an essential component of Cdc42 mediated actin polymerization and the function of Toca-1 is to either directly or indirectly activate N-WASP using *Xenopus* extract. I aim to answer the following three questions in my thesis. What is the role of Toca-1 in vivo? What Toca-1 and N-WASP do inside the mammalian cells? Toca-1 and N-WASP both have Cdc42 binding sites. What is the role of Cdc42 in regulating Toca-1 and N-WASP function? Cdc42 and its effectors are involved in the filopodia formation. The first part of my work will focus on the morphological role of Toca-1 and the Toca-1-N-WASP complex using the N1E115 neuroblastoma cell line. I will study the effect of Toca-1 and Toca-1-N-WASP complex on cellular protrusions such as filopodia. Furthermore, I will look into the cellular localization of Toca-1 and N-WASP, which is important to understand their functions. The spatial interaction of Toca-1 and N-WASP will be investigated using FRET. During the course of my study, BAR/F-BAR domain proteins emerged as important molecules coupling membrane deformation and actin dynamics and they are implicated in endocytosis. However, the underlying mechanism and signaling pathway are not well understood. Toca-1 and N-WASP both are

effectors of Cdc42. Toca-1-N-WASP complex is involved in endocytosis. Cdc42 may play a role in endocytosis through regulating Toca-1-N-WASP activity. The second part of the study will thus focus on the functions Cdc42 in regulating the Toca-1-N-WASP complex in membrane deformation and actin polymerization during endocytosis.

CHAPTER 2 Materials and methods

2.1 Materials

2.1.1 General laboratory chemicals

General laboratory chemical including ethanol, isopropanol, methanol, glacial acetic acid, bromophenoblu, TritonX-100, NP-40, Tween-20, sodium hydroxide, EDTA, Tris-base, Tris-Hcl, Sodium Chloride, Sodium dodecyl sulfate (SDS), bromophenol blue and bovine serum albumin (BSA) were from Sigma or BDH. Glycerol and agarose were from Invitrogen.

2.1.2 Materials for cloning and mutagenesis

DNA oligos were synthesized by Proligo. Restriction endonucleases, Vent DNA polymerase and T4 DNA ligase were purchased from New England Biolab. DH5 α chemical competent cells were from Invitrogen. QIAEX II gel extraction kit, plasmid miniprep, midi-prep and maxi-prep kits were from QIAGEN, Germany. Site-directed mutagenesis kit was from Stratagene. Ampicillin and Kanamycin were from Sigma. Petri dish was from Sterilin. Bigdye 3.1 for DNA sequencing was from Applied Biosystems. DNA sequencing was performed by DNA sequencing facility (BSF, Biopolis).

2.1.3 Materials for cell culture, transfection, manipulation, fixation and staining

Plastic culturewares were from Nunc. 35 mm glass bottom culture dish was from MatTek Corporation. DMEM (4500 mg/L glucose), DMEM (1000 mg/L) and 0.125% trypsin were obtained from Media preparation facility (Biopolis shared facility Singapore). F12-Kaighn's modification medium was from Invitrogen. Fetal bovine serum was from Hyclone. Lipofactamine2000 transfection reagent, mouse laminin and Penicillin/Streptomycin were from Invitrogen. Eugene6 transfection reagent was from Roche. Cytochalasin D was from Calbiochem and nocodazole was from Sigma. Paraformaldehyde, normal goat serum and DMSO were from

Sigma. Hydromount medium was from National Diagnostics. siRNAs were from Invitrogen or Dharmacon.

Anti-Rab5, Rab7 and Lamp-1 antibodies were from Santa Cruz. Anti-GM130 antibody was from BD Biosciences. Anti-CHC antibody was from Abcam. Anti-actin antibody was from Sigma and anti-GAPDH antibody was from Chemicon. LysoTracker, mitotracker and fluophore conjugated secondary antibody were from Invitrogen. Fluorophore conjugated phalloidins were from Sigma.

2.1.4 Materials for GST tagged protein expression, purification and detection

BL21 (DE3) competent cells were from Stratagene. IPTG was from Invitrogen. Complete proteinase inhibitor tablet was from Roche diagnostics. Glutathione sepharose™ 4B was from Amersham Biosciences. Glutathione and Lysozyme were from Sigma. Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad. Acrylamide/bis-acrylamide (30%, 37.5:1), Ammonium persulfate, Precision plus prestained protein marker, Coomassie brilliant blue and blotting filter paper were from Bio-Rad. TEMED and β -mercaptoethanol were from BDH. Polyscreen PVDF membrane was from Perkin Elmer. SuperSignal West Pico Substrate was from PIERCE. Hyperfilm ECL™ was from Amersham. Anti-Toca-1 antibody was from Prof Marc W. Kirschner (Harvard University, Boston, USA) and anti-actin antibody from Sigma. Horseradish peroxidase (HRP)-conjugated goat-anti-mouse/goat-anti-rabbit IgG was from Santa Cruz.

2.1.5 Materials for liposome binding assay

Total bovine brain lipids (Folch fraction 1), PS, PE, PC and PIP2 were from Sigma.

2.1.6 Bacteria and mammalian cell lines

Escherichia coli:

DH5 α *supE44 lacU169 (ϕ 80 lacZ ρ M115) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*

BL21(DE3) *F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*

Working cultures were maintained on agar plates at 4 °C and subcultured when needed. Stock for long-term storage was in liquid medium with 20% glycerol and kept at –70 °C .

Mammalian cell line: CHO-K1, HeLa, N1E115 and COS7 cells were from American Tyre Culture Collection. N-WASP wild type and knockout cell lines were obtained from Dr Klemens Rottner (HCIR, Germany). For long-term storage, cells were suspended in growth medium and 10% dimethyl sulfoxide (DMSO)) and kept in liquid nitrogen.

2.1.7 Plasmids

GFP/pXJ40 and GFP-actin/pXJ40 were obtained from Dr Dong Jingming (GLAXO, IMCB). Myc-Toca-1/pCS+ was obtained from Prof Marc W. Kirschner (Harvard University, Boston, USA). GFP-clathrin was from Prof James Keen (Gaidarov et al., 1999). Caveolin 1-mRed was from Prof Richard E. Pagano (Sharma et al., 2004)(Addgene plasmid 12681). CFP-PH (Akt), CFP-PH (PLC δ) and GFP-PH (Btk) were provided by Dr Koichi Okumura (NUS, Singapore). GFP-Eps15 Δ 95/295 and GFP-Eps15 D3 Δ 2 were from Prof Alice Dautry-Varsat (Pasteur Institute, Paris, France). GFP-Dynamin2 was from Mark A. McNiven. HA-Dynamin1 and HA-Dynamin1-K44A were from Dr Walter Hunziker (IMCB, Singapore). ABP-mCherry was from Prof Philippe Chavrier (Pasteur Institute, Paris, France). HA-N-WASP/pXJ40 was from Dr. Thomas Leung (Glaxo-IMCB, Singapore). GFP-N-WASP and GFP-N-WASP Δ WA were from were from Dr. Silvia Lommel (IZB, Germany). GFP-Cdc42-CA and GFP-Cdc42-DN were from Prof Sohail Ahmed. Cdc42 interacting domain of WASP (amino acid residues 215-295) was a kind gift from David B. Sacks (Harvard Medical School, Boston).

2.1.8 Media and solutions

Sterilized and deionized water, phosphate buffered saline (PBS) and LB medium were prepared by Media preparation facility (BSF, Biopolis). The formula for the media and solutions used in this study were listed in Appendix 1.

2.2 Methods

2.2.1 DNA manipulation methods

2.2.1.1 Isolation of plasmid DNA

Small-scale preparation of plasmid DNA (QIAGEN miniprep kit.). Typically a 1.5 ml of overnight culture bacterial cells was pelleted in a microcentrifuge (Beckman) at $12000 \times g$ for 30 secs and the supernatant was removed. The pellet was resuspended in 250 μ l of buffer P1 by vortexing. Two hundred and fifty μ l of buffer P2 was added for cell lysis followed by addition of 350 μ l of buffer P3 for neutralization. The tubes were inverted 4-6 times between steps. The mixture was centrifuged for 10 mins at $12,000 \times g$ at room temperature. The supernatant was transferred into miniprep spin column and spin at $12,000 \times g$ for 1 min. The flowthrough was discarded and the plasmid bound to the membrane was washed with 750 μ l of 70% ethanol. After a further centrifugation for 1 min, the flowthrough was discarded and membrane-bound DNA was eluted with 50 μ l of TE buffer.

Large-scale preparation of plasmid DNA (QIAFilter midi and maxi prep kit). Two ml of terrific broth was inoculated with a single colony and the culture was incubated in a 37 °C shaker (~ 200 rpm) for 4 hrs with shaking. This 2 ml culture was then used to inoculate 100 ml of LB/antibiotic medium and the culture was incubated at 37 °C for 16 hrs with vigorous shaking (~ 200 rpm). Bacterial cells were pelleted by centrifugation at $6000 \times g$ for 15 mins at 4 °C. The bacterial pellet was resuspended in 4 ml of Buffer P1. Four ml of Buffer P2 was then added, and the suspension was mixed gently but thoroughly by inverting 4-6 times, followed by incubation at room temperature for 5 mins. Four ml of chilled Buffer P3 was then added to the lysate, and the content was mixed immediately but gently by inverting 4-6 times. The lysate was transferred into the barrel of the QIAFilter Cartridge, and incubated at room temperature for 10 mins. A QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT and the column was allowed to

empty by gravity flow. The cell lysate was then introduced into a previously equilibrated QIAGEN-tip via the QIAFilter cartridges. The cleared lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed with 2×30 ml of Buffer QC, and bound DNA eluted with 15 ml of Buffer QF. Eluted DNA was precipitated by adding 10.5 ml (0.7 volumes) of isopropanol to the eluted DNA solution at room temperature. The mixture was centrifuged immediately at $15000 \times g$ for 30 mins at 4°C . The DNA pellet was washed with 5 ml of room-temperature 70% ethanol and centrifuged at $15000 \times g$ for another 10 mins. The pellet was air-dried for 10 mins and redissolved in 400 μl of TE.

2.2.1.2 Agarose gel electrophoresis of DNA

Analysis of plasmid and DNA fragment from 0.2 to 8 kb in size was carried out by electrophoresis on horizontal agarose gels. Typically, gels used contain 0.8 to 1.5% (w/v) of electrophoresis grade agarose in TAE buffer and ethidium bromide at a concentration of 0.2 $\mu\text{g/ml}$. Molten agarose was cooled to 55°C and poured into a sealed horizontal gel casting tray with a comb positioned at one end. The gel was allowed to set. After the comb was removed, the gel tray was submerged in an electrophoresis tank containing $1 \times$ TAE. The DNA sample was then mixed with 1/5 volume of $6 \times$ DNA loading dye and loaded into the wells. Electrophoresis was performed at a constant voltage of 9 V/cm. The migration of DNA was monitored by the migration of the bromophenol blue dye in the loading buffer and DNA bands were visualized under UV light. An instant photograph was taken for record using gel documentation system.

2.2.1.3 Digestion of DNA with restriction endonucleases

A typical digestion reaction was set up with the following component, $1 \times$ appropriate buffer, restriction endonuclease (volumn less than 1/10 of the total volumn), DNA and $1 \times$ BSA (required for some enzymes). The volumn was topped up to final using sterile ddH_2O . The total volumn varies between 10 ~ 50 μl depending on the amount of DNA to be digested. The mixture was

typically incubated at 37 °C for 1 hr to overnight depending on the digestion activity of the restriction endonuclease.

2.2.1.4 Purification of DNA fragments with QIAEX II DNA gel extraction kit

At the end of electrophoresis, a DNA fragment from the agarose gel was excised and weighed. Three volumes of buffer QXI were added to every 100 mg of gel slice and 10 µl of QIAEX II beads was added to the tube. QIAEX beads specifically bind DNA in the high salt concentration of QXI buffer. The mixture was vortexed vigorously and incubated at 50 °C for ten mins. The mixture was vortexed every two mins to keep the particles in suspension. The mixture was centrifuged at $14000 \times g$ for 30 secs. The supernatant was discarded. The beads were then washed once with 500 µl of Buffer QXI and twice with 500 µl of Buffer PE. The pellet was air-dried for 10-15 mins. Ten µl of sterile water was added to elute the bound DNA.

2.2.1.5 Purification of PCR mixture using QIAGEN PCR purification kit

Five volumes of buffer PB was added into PCR mixture. The sample was then transferred into QIAquick spin column and centrifuged at 13,000 rpm for 1 min. The flowthrough was discarded and the DNA bound to column was washed with 750 µl of buffer PE. The flowthrough was discarded and the column was centrifuged another 1 min. To elute DNA, 30 µl of sterile water was added to the center of the QIAquick membrane and incubate at room temperature for 1 min. The column was finally centrifuged for 1 min to obtain the purified DNA.

2.2.1.6 Ligation of DNA insert with the vector

T4 DNA ligase was used for ligation of DNA fragment into vector DNA. The volume of a typical ligation mixture was 10 µl. The amount of vector used in each ligation reaction was 10 ng. The molar ratio of the insert DNA and the vector was about 3:1. The ligation was performed at 16 °C overnight.

2.2.1.7 Transformation of competent cells by heat-shock

Competent cells were taken out from -70 °C and thawed on ice. Five µl of ligation mixture was added to 100 µl of the freshly thawed competent cells. The mixture was incubated on ice for 30 mins, transferred to a 42 °C heating block for 1 minute and chilled immediately on ice for 2 mins. Four hundred µl of pre-warmed SOC was added and the culture was incubated at 37°C with shaking (~ 200 rpm) for 1 hr. The transformation mixture was plated onto LB-agar plates containing appropriate antibiotics and incubated at 37 °C overnight.

2.2.1.8 DNA sequencing

One µg of plasmid DNA sample was mixed with 200 ng sequencing primer and 8.0 µl of BigDye Terminator Sequencing Reagent (Dye deoxy terminator cycle sequencing kit, Applied Biosystems Inc., USA) in a 20 µl volume. The mixture was subjected to 25 thermal cycles with parameters as below using PTC-100 TM Programmeable Thermal Controller, MJ Research Inc., Watertown, MA, USA. DNA was denatured at 96 °C for 30 secs. Annealing was performed at 50 °C for 15 secs and extension at 60 °C for 3 mins. The sample was sent to the DNA sequencing facility (BSF, Biopolis) for sequencing.

2.2.1.9 DNA sequence analysis

DNA sequence analysis was performed using the Editseq program of DNASTAR (DNASTAR Ltd., UK). The nucleotide sequence homologies were analyzed using the MegAlign program of DNASTAR.

2.2.1.10 Polymerase Chain Reaction (PCR) amplification

PCR. The PCR reaction was typically performed using *Pfu* DNA polymerase. A typical PCR reaction mixture consisted of 100 ng of template DNA, 200 ng of each of the primers, 2 mM MgCl₂, 200 µM dNTPs, 1× *Pfu* DNA polymerase reaction buffer and 2.5 u *Pfu* DNA polymerase in a volume of 100 µl. The reaction was carried out for 30 cycles of 45 secs at 95 °C (denaturing),

30 secs at 55 °C (annealing), and 2 mins/kb at 72 °C (extension) with one final cycle for 20 mins at 72 °C. The PCR reaction was performed using a PTC-100 TM Programmeable Thermal Controller, (MJ Research, Inc. Watertown, MA, USA). The amplified product was analyzed by agarose gel electrophoresis.

Site-directed mutagenesis using PCR. Circular plasmid DNA of about 8 kb in length was used as a template. A pair of complementary primers was designed to contain the desired mutation in the middle. The reaction was carried out for 16 cycles of thirty secs at 95 °C, thirty secs at 55 °C and sixteen mins at 68 °C. The PCR mixture was purified using PCR purification kit and eluted with sterile water followed by digestion with *Dpn* I at 37 °C for 5 hrs. Five µl of digested DNA was used to transform into *E. coli*. The mixture was plated onto LB/antibiotic plates and the plates were incubated at 37 °C overnight. Single colonies were picked and grew in 5 ml of LB/antibiotic at 37 °C overnight. The DNA was extracted with the QIAGEN miniprep kit and the mutation was confirmed by sequencing with appropriate primers.

Subcloning and site directed mutagenesis of Toca-1 and N-WASP constructs. Human Toca-1 (a gift from Prof Marc W. Kirschner, Harvard University, Boston, USA) was amplified by PCR and subcloned from pCS+ vector into pXJ40-mRFP vector between HindIII and NotI site. The same primer was used to generate GST-F-BAR, GST-F-BAR-K33QR35Q, GST-F-BAR-K51QK52Q and GST-F-BAR-R112QK113Q. The insert DNA was amplified by PCR and subcloned between BamHI and XhoI site. Myc-Toca-1-W518K, mRFP-Toca-1-W518K, mRFP-Toca-1-K33QR35Q, mRFP-Toca-1-K51QK52Q, mRFP-Toca-1-R112QK113Q, and mRFP-Toca-1MGD383-385IST GFP-NWASPH208D were generated by mutated primer pairs using site-directed mutagenesis kit (Stratagene). The mutations were confirmed by DNA sequencing. The primer sequences are listed below.

The primer sequences are listed as below.

Toca-1/pXJ40-mRFP

FP: 5'-CCCAAGCTTAGCTGGGGCACGGAGCTGTG-3'

RP: 5'-TTTTCCTTTTGC GGCCGCTCAGGAACCTTTACTGTTTTTCTC-3'

Myc-Toca-1-W518K/pCS+ and mRFP-Toca-1W518K/pXJ40

FP: 5'-GACAAAGGTGACGGAAAGACAAGAGCTCGGAG-3'

RP: 5'-CTCCGAGCTCTTGTCTTTCCGTCACCTTTGTC-3'

mRFP-Toca-1MGD383-385IST

FP: 5'-GAAGAATCCACAAATAAGCACTCCAGGGAGTTTGCAG-3'

RP: 5'-CTGCAAACCTCCCTGGAGTGCTTATTTGTGGATTCTTC-3'

mRFP-Toca-1-K33Q/R35Q/pXJ40

FP: 5'-GATATGCCAAATTTGTTCAAGAGCAGATAGAAATTGAACAG-3'

RP: 5'-CTGTTCAATTTCTATCTGCTCTTGAACAAATTTGGCATATC-3'

mRFP-Toca-1-K51Q/K52Q/pXJ40

FP: 5'-GAGAAATCTGGTTCAGCAGTACTGCCCCAAACG-3'

RP: 5'-CGTTTGGGGCAGTACTGCTGAACCAGATTTCTC-3'

mRFP-Toca-1-R112Q/K113Q/pXJ40

FP: 5'-GATCTGAAAACCTGAACAACAAATGCATCTGCAAG-3'

RP: 5'-CTTGCAGATGCATTTGTTGTTTCAGTTTTCAGATC-3'

GFP-N-WASPH208D

FP: 5'-CACCAAGTAATTTCCAGGACATTGGACATGTTGG-3'

RP: 5'-CCAACATGTCCAATGTCCTGGAAATTACTTGGTG-3'

GST-F-BAR

GST-F-BAR-K33QR35Q

GST-F-BAR-K51QK52Q

GST-F-BAR-R112QK113Q

FP: 5'-CGCGGATCCAGCTGGGGCACGGAGCTGTG-3'

RP: 5'-CCGCTCGAGCTAGGTTCTATATATATGTTGACTGTAATC-3'

2.2.2 Methods for cell culture

2.2.2.1 Mammalian cell culture

CHO cells were grown in F12-Kaighn's modification medium containing 10% fetal bovine serum at 37 °C and maintained in a humidified atmosphere containing 5% CO₂ and 95% air. The medium was changed every 2-3 days and the cells were subcultured when confluence was

reached. To subculture, the medium was removed from the flasks and the cell layer was rinsed with $1 \times$ PBS. Sterile 0.125% trypsin was added ($1 \text{ ml}/\text{cm}^2$) and the flask was incubated at 37°C for 3 mins. Complete growth medium was added to stop the trypsinization and the cells were resuspended by repeated pipetting. The cells were seeded at $1.5 \times 10^5/\text{well}$ in 6-well plates and incubated at 37°C , 5% CO_2 incubator overnight. Seventy percent confluence was reached the next day and the cells were then ready for transfection. CHO-K1 cells were transfected using Eugene 6 transfection reagent.

Hela cells and COS7 cells were maintained in DMEM (4500 mg/L) medium supplemented with 10% FBS. The other manipulations for cell culture are similar to CHO-K1 cells. Hela and COS7 cells were transfected using Lipofectamine 2000.

N1E115 cells were maintained in DMEM (4500 mg/L) medium supplemented with 10% FBS. To subculture, the cells were dislodged from the culture dish by pipetting up and down. The other manipulations for cell culture are similar to CHO-K1 cells. N1E115 cells were transfected using Eugene6 transfection reagent. Specifically, the coverslips for seeding N1E115 cells were coated with $10 \mu\text{g}/\text{ml}$ of laminin and N1E115 cells were serum-starved for 1 hr before transfection.

N-WASP wild type cells were cultured in DMEM (1000 mg/L) medium supplemented with 10% FBS at 32°C and maintained in a humidified atmosphere containing 5% CO_2 and 95% air. The other manipulations for cell culture are similar to CHO-K1 cells.

2.2.2.2 Transfection

Transfection of mammalian cells using LIPOFECTAMINETM 2000 Reagent. Cells were seeded into a 6-well plate at 1.5×10^5 per well. The cells were grown at 37°C , 5% CO_2 incubator overnight to 70% confluence. One hundred μl of serum free DMEM was mixed with 6 μl of LIPOFECTAMINETM Reagent. Another 100 μl of serum free DMEM was mixed with 1 μg of plasmid DNA (0.5 μg of each plasmid DNA was used in co-transfection). The

LIPOFECTAMINETM Reagent solution was combined with the plasmid solution and mixed. The mixture was incubated at room temperature for 45 mins, during which it was mixed by tapping the tube every 10 mins. Eight hundred μ l of serum-free DMEM was then added to the transfection mixture. The mixture was gently mixed and transferred to each of the wells. The cells were incubated at 37 °C for 5 hrs. The transfection mixture was then removed and 2 ml of complete culture medium containing 10% fetal calf serum was added. The cells were then incubated overnight.

Transfection of mammalian cells using Fugene 6 transfection reagent. Cells were seeded into a 6-well plate at 1.5×10^5 per well. The cells were grown at 37 °C, 5% CO₂ incubator overnight to 70% confluence. One hundred μ l of serum free F12-Kaighn's modification medium was mixed with 6 μ l of Fugene 6 Reagent. The mixture was incubated at room temperature for 5 mins and 1 μ g of DNA was added into the same tube. The mixture was further incubated at room temperature for 30 mins and 1 ml of growth medium was added. The growth medium of the cells was replaced with the transfection mixture. The cells were incubated for 24-36 hrs before the analysis.

Transfection of siRNA into mammalian cells. (1) Toca-1 knockdown. Toca-1 short interference (si)RNA sequences were synthesized by Invitrogen according to the published work (Itoh et al., 2005). The Toca-1 RNAi sequence was GCGAGAAGUUGUAGCAGAAGAAAUG and the control RNAi sequence was CCAUCAGCAAGAAGUAAGUACAUA. HeLa cells were transfected with the RNAi twice (day 0 and day 2) as described in the manufactures manual. The cells were lysed 48 hrs after second transfection. Immunoblot was performed to determine the protein level in the cell lysate. Anti-Toca-1 antibody was a kind gift from Prof Marc W. Kirschner (Harvard, USA). To determine the effect of Toca-1 knockdown on the N-WASP phenotype, the cells were transfected with HA-N-WASP and GFP-actin. The filopodia number per cell was counted 24 hrs after transfection. (2) CHC knockdown. Two independent RNAi's

were used to investigate the effects of CHC KD. The target sequences were; GGAAAUGGAUCUCUUUGAA (CHC oligo I) and UAAUCCAAUUCGAAGACCA (CHC oligo II) (Dharmacon, Lafayette, CO, USA). Sequences were submitted to BLAST searches against the mouse genome sequence to ensure that only the desired mRNA was targeted. Stealth RNAi negative control duplexes (CA, Invitrogen) were used as non-silencing control. Based on individual and pooled KD efficiency, pooled CHC oligos (oligo I + II) were used for subsequent experiments. N1E115 cells were seeded at a density of 150,000 per well in 6-well plates. 1 hr after seeding, N1E115 were transfected with 10 nM CHC or negative control duplexes using HiPerfect (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. At 24 hrs, cells were transfected with 0.5 μ g myc-Toca-1 using Lipofectamine 2000 (Invitrogen). N1E115 cells were then processed at 48 hrs for Western blotting or immunofluorescence. CHC was visualized by rabbit anti-CHC (Abcam) at a dilution of 1:100 during immunofluorescence. CHC was detected in whole cell lysates by mouse anti-CHC (Clone TD.1, Sigma) at a dilution factor of 1:1000 during Western blotting. Equal loading in SDS-PAGE was ensured by protein quantitation using Bradford assay (Biorad). Mouse anti-GAPDH (Chemicon) was used to normalize for protein loading in each lane.

2.2.2.3 Microinjection of mammalian cells

Cells were seeded at a density of $\sim 10^5$ per glass bottom dish and grown overnight. Appropriate plasmid DNA in sterile water at a concentration of 50 ng/ μ l was cleared from aggregates through centrifugation at 16,000 x g at 4 °C for 30 mins. 6 μ l of the DNA solutions was loaded into a microinjection needle and cells were injected at a constant pressure of 20 psi for 100 msec. The microinjection system is a custom setup on an Olympus microscope (IMT-10). About 100 ~ 150 cells per dish were injected and left in incubator for 1 ~ 6 hrs before further analysis.

2.2.2.4 Immunofluorescence.

24 hrs after transfection, the cells were fixed in 4% paraformaldehyde for 15 mins followed by three washes with PBS for five mins each. Then the cells were permeabilized in 0.2% TritonX-100 for 5 mins. After three washes with PBS, the cells were blocked in 5% normal goat serum for 30 mins washed and incubated in PBS diluted primary antibody (anti-Myc (1:300), anti-Rab5 (1:50), anti-Rab7 (1:50), anti-Lamp1 (1:50) all from Santa Cruz) at 4 °C overnight. Following the washing steps, the cells were incubated with Alexa488 or Alexa647 conjugated secondary antibody (Molecular Probes, Invitrogen). Finally the cells were washed and mounted onto the glass slides using Hydromount (National Diagnostics, USA).

2.2.2.5 Blockers of endocytosis.

EGF phosphorylation substrate **15** (Eps15) is a key component for assembling functional clathrin-coated pits (Benmerah et al., 1999). Plasmid constructs of Eps15 (GFP-Δ95/295 dominant negative and GFP-D3Δ2) were kindly provided by Alice Dantry (Pasteur Institute, Paris, France). The GTPase Dynamin is required for both clathrin- and caveolae-dependent endocytosis (Sever et al., 2000). Plasmid constructs of dynamin, HA-dynamin 1 K44A dominant negative and HA-dynamin 1 WT, were used as endocytosis blocker and control, respectively. Alexa Fluor 488-/647-transferrin (TF; Molecular Probes, Invitrogen) were used as markers for clathrin-mediated endocytosis.

2.2.2.6 Transferrin labeling.

24 hrs after transfection, cells grown on coverslips to 70-80% confluency were rinsed twice with Dulbecco's PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (D-PBS) (Invitrogen) and serum-starved for 2 hrs in serum-free DMEM. Cells were then incubated with 25 µg/ml Alexa-transferrin (Molecular Probes) in serum-free DMEM for 10 mins at 37 °C and 5% CO₂. Subsequently, coverslips were placed on ice and rinsed twice with ice-cold D-PBS.

2.2.2.7 Cholera toxin B uptake assay

Cells grown on coverslips to 70-80% confluency were rinsed twice with Dulbecco's PBS containing 1 mM CaCl_2 and 0.5 mM MgCl_2 (D-PBS) (Invitrogen) and serum-starved for 1 h in serum-free medium. Cells were incubated with 5 $\mu\text{g/ml}$ Alexa 488-cholera toxin B (Molecular Probes) in serum-free DMEM for 30 mins at 37 °C and 5% CO_2 . Subsequently, coverslips were placed on ice and rinsed twice with ice-cold D-PBS and mounted onto glass slide using hydromount and examined with confocal microscopy.

2.2.2.8 Dextran uptake assay

Cells were serum starved for 1 hr and dextran was added into the culture medium at a final concentration of 1 mg/ml. The cells were then incubated in 37 °C for 30 min. Subsequently the cells were washed twice with ice-cold D-PBS and mounted using hydromount.

2.2.2.9 Determination of endocytosis inhibition using labeled transferrin/cholera toxin B/dextran.

The sample preparation was described as in 2.2.2.7 and 2.2.2.8 and the images were taken on Olympus confocal microscopy. Quantification of fluorescence intensities was performed using Metamorph software (Universal Imaging, PA, USA), (Balklava et al., 2007). The background fluorescence was subtracted. Fluorescence intensities were measured by thresholding and outlining whole individual cells, followed by determination of integrated fluorescence intensities, which were then normalized to cell area. Average values were expressed as a ratio relative to control cells without endocytosis blockers.

2.2.2.10 Filopodia assay

Toca-1 and N-WASP-induced filopodia were scored on neurites of live N1E115 cells, as previously described (Govind et al., 2001; Lim et al., 2008). N1E115 cells were transfected with myc-Toca1, GFP-Eps15 or HA-dynamin 1, and corresponding mRFP- or GFP-actin. Twenty-four

to thirty-six hrs after transfection, time-lapse microscopy was carried out on a heated stage at 37 °C and 5% CO₂. Cells were imaged under phase-contrast and fluorescence with Axiovert 200 (Zeiss) with frame captures every 10 secs for 5 mins, driven by Metamorph software.

2.2.2.11 Scoring of filopodia and neurites.

Filopodia were scored as protrusions that were dynamic with a lifetime in the range of min and length (as defined in (Lim et al., 2008)). Filopodia lifetime was the time interval measured between appearance and disappearance of individual filopodia. Filopodia length was measured from the base of the structure to the tip (under DIC) and scored when the filopodia reached its maximum length. Neurites (or cell extensions) were scored as processes that protruded from the cell body and were at least one cell body length. Each experiment was repeated at least three times and the data presented is one of the representative experiments.

2.2.2.12 Intensity profiling

Cells were transfected with the relevant constructs and stained with anti-Rab5 antibody 24 hrs post transfection. The cells were then fixed and examined by confocal microscopy. A line was drawn through the region of interest and the intensity profile was measured using FV10-ASW software of the Olympus FV-1000 confocal microscope.

2.2.2.13 Colocalisation

Colocalization in multi-channel confocal images refers to the coexistence of two or more fluorescence signals at the same pixel location. Colocalization is often presented by three images in the sequence of green, red and merged images. In the merged images, yellow color stands for the places of colocalization; the overlapping of the green and red signals. The intensity distribution of the green and red channels can be presented on a scatter plot. The yellow colocalized signals are located along the diagonal. Signals close to the origin stand for the

background fluorescence in the two channels. The Pearson correlation coefficient can be calculated by selecting image ROI or scatter plot ROI and in both cases the area with predominantly colocalization was selected. The method of scatter plot ROI was used when the global colocalisation in the whole cell was measured. The method of image ROI was used when the colocalisation at specific region was measured. The Pearson correlation coefficient was used as a quantitative way to describe the extent of colocalization and calculated by Olympus Fluoview 1000 software. Where Pearson colocalization coefficient (CC) of; <0.25 indicates no colocalization (-), 0.25-0.5 indicates potential colocalization (*), 0.5-0.7 indicates some colocalization (**) and >0.7, indicates high colocalization (***)

2.2.2.14 Characterization of Rab5 positive vesicles.

Distribution of vesicles was measured by placing squares with the size $1.5 \times 1.5 \mu\text{M}$ (9×9 pixel) from the cell periphery (region 1) to the perinuclear region (region 5) within individual cells and the average fluorescence intensity of each square was measured by Metamorph. (ii) Vesicle size was analyzed by randomly picking 20 vesicles/cell measuring their diameter using Metamorph software. (iii) Vesicle number was analysed by selecting fixed areas within each cell and counting the number of vesicles present. Vesicle numbers were then normalized to the non-transfected cell. The experiments were repeated three times, with $n = 20$, as the mean $\pm$ S. D. and a representative experiment is presented.

2.2.2.15 Quantification of tubulation, vesicle number and motility.

(a) **Tubule and vesicle index.** Cells were divided into eight sectors and each sector is scored for the presence of vesicles or tubules. Each sector contributes a 12.5% morphological activity. The eight sector values for each cell were then added to give % vesicle/tubule index per cell. (b) **Vesicle number.** Vesicle number was analyzed by selecting fixed areas within each cell and counting the number of vesicles present. Vesicle numbers were then normalized to the non-

transfected cell. The experiments were repeated 2-3 times, with $n = 20$, as the mean $\pm$ S. D. and a representative experiment is presented. (c) **Vesicle motility.** Vesicle motility was tracked using Metamorph software.

2.2.2.16 Statistical analysis

In general, readings were obtained from at least 3 independent experiments, and expressed as the mean $\pm$ standard deviation (S.D.). Experimental data were analyzed by Student's t-test.

2.2.3 Methods for protein work

2.2.3.1 Sample preparation for Western blot

Cells were washed with ice-cold PBS and lysed in the wells by adding 200 μ l of ice-cold Buffer A. The plate was incubated at 4 °C for 15 mins. The plate was then tilted at an angle and the supernatant was collected. The cell debris was cleared by centrifugation at $12000 \times g$ for 30 mins at 4 °C. The supernatant was transferred to another tube. SDS loading buffer was added to the remaining pellet and the mixture was subjected to 1 cycle of boiling and chilling on ice for 3 mins each to denature the chromosomal DNA. SDS loading buffer was also added to the supernatant and both fractions were boiled for 3 mins. The samples were cleared by centrifuging at $12000 \times g$ for 15-30 mins at room temperature. The cleared solution was separated in SDS-PAGE.

2.2.3.2 Western blot

Component of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and preparation. In 30% polyacrylamide/bis-acrylamide, the ratio of these two components is 29:1. A standard discontinuous SDS-PAGE gel is made up of two layers: a stacking gel on top and a resolving gel at the bottom. A stacking gel normally contains 5% acrylamide-bis, while the percentage of resolving gel may vary depending on the molecular weight of the proteins in

question. In this study, 7.5% and 10% resolving gels were used. The components of the SDS-PAGE buffer and gels are detailed in Appendix 1.

To cast a SDS-PAGE gel, the Bio-Rad Protean-III gel apparatus was set up. The resolving gel was poured first and a layer of butanol was added on top. After the resolving gel was set, butanol was removed and the set gel rinsed with water. The stacking gel was then poured and a 1.5 mm thickness comb was inserted. After the gel was set, the comb was carefully removed and the wells were gently washed with water and submerged in the running buffer (See Appendix 1). The samples were loaded and the gel was usually run in a constant voltage of 100 volt until the dye front ran out of the edge of the gel.

2.2.3.3 Semi-Dry transfer of proteins

Proteins separated by denaturing SDS-acrylamide gels were transferred onto PVDF membrane for Western blotting. The SDS-gels were equilibrated in transfer buffer. Ultra thick filter paper and one piece of PVDF membrane were cut to a similar required size. The PVDF membrane was soaked in methanol for 30 secs and the filter paper was saturated with transfer buffer. The sandwich was set up in the following sequence from bottom to top, filter paper, PVDF membrane, SDS-acrylamide gel, filter paper. The bubbles between each layer were carefully removed. Proteins were transferred on Bio-Rad trans-blot SD semi-dry system for 1 hr at a constant voltage of 15 V at room temperature.

2.2.3.4 Western blot

Proteins separated on SDS-PAGE were transferred onto PVDF membranes using a transfer apparatus from Bio-Rad. The filter was blocked in 5% skim milk for 1 hr and rinsed with TBST. Primary antibodies of appropriate concentrations were added. The filter was incubated for 1 hour with gentle rocking. The filter was washed three times with $1 \times$ TBST (5 mins each). Horseradish peroxidase-conjugated secondary antibody (1:50000) was then added and the filter was further

incubated for 1 hour with gentle rocking. At the end of this incubation, three washes with $1 \times$ TBST were again performed (5 mins each). The filter was incubated in enhanced chemiluminescence (ECL) substrate for 5 mins. The membrane was exposed to X-ray film in a dark room for varying lengths of time (depending on the intensity of the signal) and the film was developed using a Kodak X-Omatiz developer.

2.2.3.5 Coomassie Blue staining of the SDS-PAGE gel

After electrophoresis, the gel was peeled off from the plate and soaked in staining solution (see Appendix 1) at room temperature with gentle agitation for thirty mins. The staining solution was removed and the destaining solution (see Appendix 1) was added. The destaining buffer was changed when necessary until all background staining had been removed and the protein bands were clearly visible.

2.2.3.6 GST tagged protein expression and purification

Growing and harvesting cells. The plasmid DNA was transfected into BL21 (DE3) cells and grew overnight on LB/Ampicillin agar plate. One single colony was inoculated into 20 ml of LB/Ampicillin medium and grow at 37 °C overnight with shaking at 250 rpm. Next day grow a 1/10 dilution of cells (eg add 20 ml to 200 ml) in a 1 liter baffled flask with LB/ampicillin and grow at 37 °C shaker until OD (600 nm) reaches 0.6 by spectrophotometer.

Induce the fusion protein by addition of 1 mM IPTG. Cells were mildly shaken at room temperature for 4-6 hrs. The cells were harvested in Sorvall centrifuge 9000 rpm/20 mins. After the supernatant was discarded, the cell pellet was washed once in ice-cold PBS and stored -70 degree until needed.

Cell lysis and purification. The frozen pellet (from 200 ml) were suspended in 15 ml of lysis buffer (Appendix 1) and left on ice until the suspension becomes thick (due to lysozyme). The lysate was sonicated on ice for 1 min at power 25%. The extracts were transferred to 30 ml

centrifuge tubes and centrifuged in a prechilled JA17 rotor for 30 mins at maximum speed. After the supernatant was filtered through 0.45 μ m filter, glutathione sepharose 4B (ratio: 30 μ l sepharose for ~1.2 ml lysate) was added into the supernatant. The mixture was incubated at 4 °C for 2 hrs with gentle rotation. The sepharose beads were pelleted by centrifugation at 2000 $\times$ g for 30 secs. Following three washes with GST purification buffer, the sepharose beads were incubated with elution buffer at room temperature for 15 mins.

Determining protein concentration of samples using biorad kit. 10 μ l of each sample was added into 800 μ l of PBS. Then 200 μ l of Biorad dye was added and the mixture vortexed. The mixture was incubated in room temperature for 5 mins and OD was measured at 595 nm relative to blank (PBS+dye). The concentration of the protein was obtained from the BSA standard curve at the measured OD.

2.2.3.7 Liposome binding assay

The F-BAR domain (amino acid residues 2-293) of Toca-1 and three F-BAR domain mutants (K33QR35Q/K51QK52Q/R112QR113Q) were amplified by PCR and cloned into pGEX-4T-1 vector between BamH1 and Xho I site. The mutant clones were confirmed by DNA sequencing. The GST tagged protein was expressed in BL21 and purified using standard protocol. The protein concentration was determined by Bradford protein assay. Liposome binding assay was performed as previously described (Lee et al., 2002; Itoh et al., 2005; Tsujita et al., 2006). Lipids were from Sigma. The liposomes were either made from total brain lipids (Folch fraction I) or PE (65%), PC (20%), PS (5%) and PIP2 (10%). The lipid mixtures were dried and resuspended in liposome forming buffer (Appendix 1) to a final concentration 2-5 mg/ml followed by incubation at 37 °C for 2 hrs. Before lipid binding assay, GST fusion proteins were centrifuged at 70,000 rpm for 30 mins at 4 °C. The protein concentration in the supernatant was determined by Bradford assay (Biorad). For each assay, 5 μ g of proteins were incubated with 100 μ g of liposome in lipid binding buffer (Appendix 1) at room temperature for 15 mins. The

mixture is then subject to centrifugation at $16,000 \times g$ for 15 mins at 25°C. The pellets were washed with the lipid binding buffer once. SDS-PAGE was performed for both supernatants and pellets. In the absence of liposomes, the amount of GST or GST fusion proteins in the pellet was negligible. The gel was stained by Coomassie blue and the protein bands were quantified using Image J software (National Institutes of Health).

2.2.4 Microscopy methods

2.2.4.1 Confocal Microscopy and timelapse acquisition

The immunofluorescence was examined on an Olympus FV confocal microscope (Olympus, Japan) using a 63 $\times$ oil immersion objective with a 1.4 numerical aperture and a pinhole at airy 1. GFP tagged proteins were excited by a 488 nm laser line and the emission was selected using a band-pass filter between 500-550 nm. mRFP tagged proteins were excited by a 561 nm laser line and the emission was selected using a band-pass filter between 580-680 nm. The images were taken at a speed 2 μ s/pixel sequentially at a size 512 $\times$ 512. The time lapse images were taken at 10 sec interval in a humidified chamber at 37 degree with 5% CO₂.

2.2.4.2 FRET Measurement.

FRET is the radiationless energy transfer from the donor fluorophore to the acceptor fluorophore. FRET is distance-dependent and the distance between donor fluorophore and the acceptor fluorophore must be in 1~10 nm range since FRET efficiency correlates inversely with the sixth power of the distance between donor and acceptor. The emission spectrum of the donor fluorophore should overlap with the excitation spectrum of acceptor fluorophore (>30%). In addition, donor and acceptor fluorophore dipoles must in favorable orientation for FRET to occur. In case of positive FRET, the fluorescence intensity of donor decreases and the fluorescence intensity of acceptor increases. FRET is advantageous to biochemical methods to probe protein protein interaction. Firstly, protein protein interaction is investigated inside their natural

environment-fixed or live cells. Secondly, FRET images show where the protein interaction occurs in nanometer resolution.

FRET was measured by acceptor photobleaching method (Govind et al., 2001; Lim et al., 2008; Herrick-Davis et al., 2006) by making appropriate settings in a Zeiss LSM 510 confocal microscope with the objective of C-Apochromat 63 x 1.2 W objective. The fusion proteins of GFP/mRFP were excited using 488 and 561nm laser line as excitation source, by selecting 405/488/561 dichoric mirror and 490,565 secondary dichoric mirrors for GFP and mRFP emission respectively. The emission was monitored by selecting GFP (BP 505-550) and Red (Longpass 575) emission filters to record the fluorescence intensity. ROI was selected and photo bleached using 70% of 561nm laser power by selecting 50 iterations. Bleaching was performed following pre and post scan images. The increase in GFP fluorescence intensity followed by mRFP bleaching was measured as FRET. FRET efficiency was calculated using the change in background subtracted fluorescence intensity as $100 \times [(post-bleach\ intensity) - (pre-bleach\ intensity) / (post-bleach\ intensity)]$. Based on the positive control (tandem GFP-mRFP) and negative control (cytosolic GFP and mRFP), positive FRET is defined as $> 3\%$.

In order to verify the increase in GFP intensity due to any possible artifact I obtained the Pearson product moment correlation coefficient r , a dimensionless index that ranges from -1.0 to 1.0 inclusive and reflects the extend of a linear relationship between the two fluorescence intensity data of GFP and mRFP while bleaching. In our case I expect -1.0 as the perfect fitting of the linear relation. However I selected the range of -0.7 to -1.0 as the best range of index.

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

Where x and y are the sample means average (array1, GFP intensity) and average (array2, mRFP intensity). For complete details of AP-FRET method see (Lim et al., 2008).

2.2.4.3 Fluorescence Lifetime Imaging Microscopy (FLIM).

FLIM can be used to detect protein-protein interaction. The two proteins are fused with donor and acceptor fluorescence molecules. The interaction of the two proteins will result in FRET between the donor and acceptor molecules. The lifetime of the donor molecule will be decreased, which is detected by FLIM.

FLIM experiments were performed with the LIFA system (Lambert Instruments, The Netherlands) on an inverted wide-field fluorescence microscope (Olympus IX71, Center Valley, PA) with 60×1.35 oil immersion objective. Fluorescence lifetime was measured using software provided by Lambert Instruments. GFP was excited by a sinusoidally modulated 4 mW 470 nm light-emitting diode at 40 MHz. The GFP filter set was used for excitation and emission signals. The emission was collected by a modulated image intensifier with CCD camera (Fig 2.1). The image intensifier was used to extract the phase-shift and modulation depth of the fluorescence light. The sensitivity of the intensifier was modulated with the same frequency as the excitation but the phase shift is adjustable. The level of detector signal was determined by the phase-shift of the fluorescence signal relative to the the modulated sensitivity of the detector (Fig. 2.2). Fluorescein isothiocyanate was used as a standard lifetime reference of 4 ns. 12 images were captured each taken at a different intensifier phase-shift (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 degrees) to generate the whole life time image. The data points for each pixel were fitted with a sinus function and used for the calculation of lifetime (for further details see Ref. (Clayton et al., 2005), Fig 2.2). The lifetimes from 50 regions of interest (ROIs) were taken from different cells are averaged to give the average lifetime and standard deviation. The experiments were repeated three times and data from a single representative experiment were shown. The lifetime (nanoseconds) was shown in pseudocolors.

Figure 2.1 The excitation intensity is modulated in the frequency domain FLIM. The fluorescence intensity shows a phase-shift and a smaller relative modulation, but the frequency of the modulation is the same as the excitation. The dashed and dotted lines describe the simulated emission of the dyes with a lifetime of 1 ns and 4 ns respectively. The lifetime is calculated by the phase-shift and the relative modulation.(from (L.K. van Geest, et al., 2003) with permission).

Figure 2.2 Life time measurement by frequency domain method.

(a) The modulated fluorescence signal (solid line) is detected with the intensifier with modulated intensity (dashed). The resulting detector signal (dotted) is demodulated and averaged in time by the intensifier to obtain the pixel intensity (single headed arrow). (b-d): The modulated detector sensitivity is phase shifted relative to the phase of the excitation modulation to obtain a series of measurements of the pixel intensity. (e) The series of measurements is plotted as a function of the measure phase (ϕ) and a sine is fitted through the data points. The fluorescence decay constants can be calculated from the phase-shift (ϕ) and the modulation depth (m) of this sine. (from (L.K. van Geest, et al., 2003) with permission).

2.2.4.4 Fluorescence Recovery After Photobleaching (FRAP)

FRAP was performed on a Zeiss inverted laser scanning confocal microscope (LSM510) with C-Apochromat 63x1.2 W objective and a pinhole size 1 airy unit. The temperature was maintained at 37°C in a humidified atmosphere with 5% CO₂. GFP tagged proteins were bleached in a defined rectangular region using a 488 nm laser line at 100% power and 25 iterations. The fluorescence recovery was recorded by a 488 nm laser line at 5% laser power every 1 second. In case of mRFP FRAP, a 561 nm laser line was used to bleaching at 100% laser power and 25 iterations. The imaging was performed using a 561 nm laser at a 10% laser power. The fluorescence intensity of the background, the whole cell and the bleached region were recorded before bleaching, just after bleaching and during the recovery. In the analysis of the FRAP data, the background fluorescence intensity was subtracted and the fluorescence intensity was normalized against fluorescence intensity of whole cell in order to correct the effect of the focal plane drifting and the photobleaching in the imaging process. Then each data point was normalized to the fluorescence intensity before bleaching to get the relative fluorescence intensity. The relative intensity (RI) was calculated using the following equation: $RI = [I(t) - ROI(t)] / [I(0) - ROI(0)]$, $I(t)$, whole cell intensity at a certain time point; $I(0)$, whole cell intensity before bleaching; $ROI(t)$, intensity of bleached region at a certain time point; $ROI(0)$, intensity of bleached region before bleaching. T_{1/2} was calculated as the time required recovering to 50% of the fluorescence before bleaching.

2.2.4.5 Total Internal Reflection Fluorescence Microscopy (TIRFM)

To examine the vesicle dynamics at the cell surface, TIRF-FM was performed on an inverted Olympus FV TIRF microscope in a humidified atmosphere with 5% CO₂. Plan APO 63 × /NA 1.45 oil-immersion objective was used and 488nm/561nm laser line were used to excite GFP and mRFP sequentially. The penetration depth was set at around 100 nm. Light emitted by the fluorophores was detected by a charge-coupled device camera. Metamorph software controls the

multidimensional time-lapse acquisition. The exposure time was 200 ms and total 360 frames were taken with 5 secs interval. The stack files were processed using Metamorph to generate movie files.

2.2.4.6 Electron microscopy

For electron microscopy and immunogold staining, harvested cells were fixed in 4% paraformaldehyde in PIPES and dehydrated through a graded series of ethanol to 100% ethanol. They were then infiltrated with LR White resin and embedded in fresh LR White in gelatin capsules at 50°C for 48 hrs. Thin sections were cut and mounted on Formvar-carbon-coated nickel grids. All incubation and rinses were performed by floating the grids on small drops of reagents. The grids were incubated in PBS containing 10% fetal calf serum (FCS) for 30 mins, rinsed four times for 10 mins with PBS, and subsequently incubated for overnight at 4°C in a drop of a 1:10 dilution of primary antibody, affinity purified rabbit polyclonal anti-Rab5 (Santa Cruz). Following several rinses on drops of PBS, for a total of 30 mins, the grids were then incubated with a 1:15 dilution of 12 nm colloidal gold conjugated goat anti-rabbit (Jackson ImmunoResearch) for 1-2 hrs at room temperature. Grids were rinsed in PBS for 30 mins followed by a wash in distilled water for 30-40 secs. Finally, the grids were stained for 5 mins on drops of saturated uranyl acetate/50% methanol, rinsed in distilled water and allowed to dry in air. Grids were then examined using a transmission electron microscope (FEI Tecnai F20).

CHAPTER 3 The Toca-1-N-WASP complex links filopodia formation to endocytosis

3.1 Toca-1 and N-WASP induce filopodia and neurite formation.

Mouse neuroblastoma N1E115 cells have been used as a model neuronal cells to examine the morphological role of Ras, PI-3 kinase, Rho GTPases and their effectors (Kozma et al., 1995; Kozma et al., 1996; Kozma et al., 1997). In particular, it has been shown that Cdc42 and one of its effectors, IRSp53, induces filopodia and neurites in these cells (Govind et al., 2001). Since Toca-1 is also a Cdc42 effector, N1E115 cells were chosen as the model system to understand Toca-1 function. Toca-1 was expressed with GFP-actin in N1E115 cells to monitor the phenotype. Toca-1 induced filopodia and neurite formation in N1E115 cells (Fig. 3.1). [For time lapse see movie 3.1]. To show the specificity of the Toca-1 phenotype a mutant Toca-1W518K (with a defective SH3 domain) was used. Toca-1 W518K failed to induce filopodia but was still competent to induce neurites (Fig. 1A). Toca-1 induced multiple neurites that were highly branched. In addition, the neurites were longer than that observed after serum starvation of N1E115 cells (Govind et al., 2001). N-WASP expression in N1E115 cells had a similar phenotype to Toca-1; N-WASP induced filopodia and neurites (Fig. 3.1C) [For time lapse see movie 3.2].

Using sequential fluorescence/DIC time-lapse microscopy with GFP-actin transfected cells, I was able to show that Toca-1 induced *bona fide* filopodia. Mammalian filopodia have been defined as cell protrusions that contain actin and have a lifetime of 142-187 sec and a length of 7-15 μ M (Lim et al., 2008). The Toca-1 induced filopodia had the following characteristics; an average lifetime of 127.5 secs and an average length of 6.6 μ M (Table 3.1). These filopodial characteristics were similar to endogenous structures as well as those induced by Cdc42/RacN17, IRSp53 and N-WASP (Table 3.1).

FIGURE 3.1. Toca-1 and N-WASP induce filopodia and neurites in N1E115 cells.

(A) N1E115 cells were transfected with GFP-actin, GFP-actin/Myc-Toca-1, or GFP-actin/Myc-Toca-1W518K and GFP-actin/HA-N-WASP. GFP-fluorescence was monitored by confocal microscopy. The panels below the arrows show enlarged neurites to allow monitoring of complexity and filopodia formation. Bar = 50 μ M.

(B) Bar charts show statistical analysis (for at least three independent experiments, $n = 15$ cells). Cells were scored for neurites and filopodia as described in the Materials and Methods section. Data are presented as mean $\pm$ S.D.

(C) To measure filopodia characteristics, DIC time-lapse microscopy was used. Individual filopodia were followed from appearance to disappearance and the time required defined as the lifetime. Length of filopodia is the maximum length as measured from the base to the tip under DIC. All these experiments include sequential GFP-actin imaging to ensure that the structures contain actin. To assess the characteristics of the Toca-1 induced filopodia, DIC time-lapse experiments of transfected cells were carried out. The DIC image shows a typical neurite with associated Toca-1 induced filopodia over a time course as indicated in individual frames. Arrowheads (empty, white and black) follow three independent filopodia over the time course. Bar = 4 μ M.

Table 3.1. Characteristics of endogenous and induced filopodia in N1E115 cells.

The table shows a comparison of the lifetime and length of endogenous filopodia with those induced by cDNA transfections Cdc42/RacN17, IRSp53, N-WASP (Ref. (Lim et al., 2008)) and Toca-1. Details of the measurements are described in the Materials and Methods section.

Treatment	Filopodia length (μM)	Filopodia lifetime (sec)	Reference
None (endogenous)	15 +/- 7.68	142 +/- 101	(Lim et al., 2008)
Cdc42V12/RacN17	8.37 +/- 1.59	157 +/- 30	(Lim et al., 2008)
IRSp53	6.83 +/- 1.88	187 +/- 38	(Lim et al., 2008)
N-WASP	7.35 +/- 0.97	154 +/- 20	(Lim et al., 2008)
Toca-1	6.6 +/- 1.5	127.5 +/- 41.4	This study

3.2 Domains of Toca-1 required for filopodia and neurite formation.

Toca-1 F-BAR domain mutations (K33Q/R35Q, R51Q/K52K and R112Q/K113Q) and a Cdc42 binding site mutant (MGD383-385IST) were generated. The F-BAR domain mutants were based on CIP4 mutants which fail to induce tubulation in cells and have reduced lipid-binding ability (Tsujita et al., 2006). The ability of these Toca-1 mutants to bind brain lipids and phosphatidylinositol-4-5-bis phosphate (PI(4,5)P₂) was also substantially reduced compared to the wild-type (Fig. 3.2D). Toca-1 mutants were expressed in N1E115 cells independently and phenotypes were scored. Filopodia formation by Toca-1 required its F-BAR domain, Cdc42 binding site and the SH3 domain (Fig. 3.2). However, only the Cdc42 binding site was essential for neurite formation.

3.3 Toca-1 requires N-WASP for filopodia formation.

The inability of the Toca-1W518K mutant to induce filopodia suggested that interaction with N-WASP was important for this phenotype. To determine the role of N-WASP in Toca-1 phenotypes, Toca-1 was expressed in N-WASP WT and KO cells and the phenotype obtained were compared with wild-type and control cells. N-WASP knock out fibroblasts were derived from N-WASP knockout mouse embryo by immortalization and subcloning. N-WASP wild type cells were fibroblast cells from the control mouse embryo (Lommel et al., 2001). Toca-1 failed to induce filopodia (or cell extensions) in N-WASP KO cells (Fig. 3.3A). Toca-1W518K failed to induce filopodia in either wild-type cells or N-WASP KO cells (Fig. 3.3B).

Next reconstitution experiments were carried out by including low amounts of N-WASP cDNA with Toca-1 and subsequently filopodia formation was measured. Filopodia formation could be reconstituted in N-WASP KO cells by co-injection of Toca-1/N-WASP (Fig. 3.3C).

FIGURE 3.2. Effect of mutations in Toca-1 on filopodia formation.

(A) A schematic of the domain structure of Toca-1 is shown with positions of the mutations used. Three distinct domain mutations were generated; F-BAR domain, HR1 domain (Cdc42 binding site) and SH3 domain (N-WASP interacting site).

(B) Mutant Toca-1 genes were transfected into N1E115 cells as mRFP fusions with GFP-actin. The main panel shows three images; mRFP, GFP and the merge with the different version of Toca-1 transfected. Three F-BAR domain mutants, K33Q/R35Q, K51Q/K52Q, and R112Q/K113 were used. The Cdc42 binding mutant was MGD383-385IST. The SH3 domain mutant was W518K.

(C) Bar charts show statistical analysis of the experiments. Three independent experiments were performed (with $n = 15-20$). Transfected cells were scored for neurite formation and filopodia formation. The data are presented as mean $\pm$ S.D. Bar = 10 μ M.

(D) Toca-1 F-BAR domain and mutants were purified as GST-fusion proteins and lipid sedimentation assays carried out as described in the Materials and Methods section. S – supernatant, P – pellet. The data are presented as mean $\pm$ S. D. ($n = 6$) from 2 experiments.

FIGURE 3.3. Toca-1 does not generate filopodia in N-WASP KO cells.

(A) Toca-1 was transfected into N-WASP WT (flox) and N-WASP KO cells (1h51) and filopodia number per cell was scored as described in the Materials and methods section. Flox and 1h51 N-WASP KO cells were from Ref. (Lommel et al., 2001), Lommel et. al. Panels a and b WT cells, panel c, N-WASP KO cells. Three independent experiments were performed (n = 20) and one representative example is shown. Data shown are mean $\pm$ S. D. Bar = 10 μ M. (B) Toca-1 W518K was transfected into WT and N-WASP KO cells and morphology monitored. (C) N-WASP KO cells were transfected with Toca-1 and N-WASP to allow reconstitution of the cells. The right panel shows a DIC time-lapse sequence (in secs; 0, 50, 100, 150, 200, 250, and 300) of the three combinations. Toca-1 in N-WASP WT cells, Toca-1 in N-WASP KO cells and Toca-1 with N-WASP and N-WASP KO cells. Tracings show structures generated; red – filopodia and blue – lamellipodia. In parts A, B and C the bar charts show a statistical analysis of the experiments. Three independent experiments were performed (with n = 15-20). Data shown are mean $\pm$ S. D. Bar = 10 μ M. (Liz Lim helped me with the N-WASP KO cell culture, transfection and imaging).

A

B

C

C

3.4 N-WASP requires Toca-1 for filopodia formation.

Having established that Toca-1 does not induce filopodia in the absence of N-WASP, I now asked the reverse question. Does N-WASP require Toca-1 for filopodia formation? When N-WASP and Toca-1 were co-transfected, there was a synergistic induction of filopodia formation with the average cell possessing approximately 44 filopodia/cell (Fig. 3.4A). In contrast, when N-WASP was cotransfected with dominant negative versions of Toca-1 (mutants of the F-BAR domain, [K33Q/R35Q], [K51Q/K52Q], or [R112Q/K113Q]) filopodia formation was dramatically reduced (Fig. 3.4A). The effect of Toca-1 KD on N-WASP induced filopodia formation was also examined. In support of the data obtained with dominant negative Toca-1 mutants, KD of Toca-1 to negligible levels eliminated N-WASP induced filopodia formation (Fig. 3.4B). Interestingly, the absence of Toca-1 N-WASP expression did not lead to a breakdown of stress fibres (Fig. 3.4B). Thus Toca-1 is essential for N-WASP induced filopodia formation.

3.5 Localization of the Toca-1 and N-WASP.

Since N-WASP is associated with endocytosis, I decided to investigate the localization of Toca-1/N-WASP with markers associated with membrane trafficking. Of the markers (mitotracker, GM130 [Golgi], and Rab5) for vesicular structures tested, Rab5 was identified as the only marker that co-localized with Toca-1. Next cells were transfected with N-WASP, Toca-1 or Toca-1W518K alone or N-WASP/Toca-1 and followed by localization of the proteins with anti-Rab5 antibodies. N-WASP and Toca-1W518K did not co-localize with Rab5 to any significant degree (Fig. 3.5A and B). In contrast, Toca-1 partially colocalized with Rab5. After Toca-1 and N-WASP were coexpressed, these proteins were strongly colocalized in vesicular structures (Fig. 3.5B). To determine whether the membranes where Toca-1/N-WASP was localized were positive for Rab5, I carried out a co-localization analysis on the images (Fig. 3.5B, part b and c). Individual vesicles for Toca-1/Rab5 co-localization were examined. A representative group of twelve vesicles, with differing Toca-1 intensities, each

FIGURE 3.4. N-WASP induced filopodia formation requires and synergizes with Toca-1.

(A) N1E115 cells were transfected with N-WASP alone, N-WASP with Toca-1 mutants, and N-WASP with Toca-1 (a). All transfections were carried out with GFP-actin. Panels show GFP and DIC images with a merge. The bar chart shows a statistical analysis of the experiments (b). Neurites and filopodia formation were scored as described in the Materials and Methods. Toca-1 F-BAR domain and Cdc42 binding mutants were as described in figure 2. Three independent experiments were performed (with $n = 10-15$). Data shown are mean $\pm$ S. D. Bar = 10 μ M.

(B) Toca-1 was KD using RNAi and the effect of N-WASP on filopodia formation examined. (a) Toca-1 protein levels with control RNAi and with Toca-1 RNAi. (b) Left panel, N-WASP transfection with control RNAi. Right panel, N-WASP transfection with Toca-1 RNAi. Lower panel shows tracing of filopodia in red. (c) Statistical analysis of experiments similar to those presented in (b) of single cells. Data shown are mean $\pm$ S. D ($n = 20$).

A

b

B

stained positive for Rab5 (Fig. 3.5B, part b). Figure 3.5B, part c compares intensity profiles of selected Rab5 membranes in the presence of Toca-1/N-WASP or Toca-1W518K. The intensity traces show that Toca-1 but not Toca-1W518K co-localizes with Rab5 (Fig. 3.5B, part c) with correlation coefficients of 0.72 ± 0.09 (n=12) and 0.04 ± 0.03 (n=10), respectively.

Next, cells were transfected with Toca-1 and GFP-actin to determine where Toca-1 localized in relation to the filopodia and neurites that were being induced. Actin and Toca-1 co-localized in vesicular/punctate structures along the length of neurites and at the base of filopodia but not within filopodia. (Fig. 3.6B a-c). The correlation coefficient obtained for Toca-1 actin co-localization was 0.90 ± 0.02 (n=10). In contrast, N-WASP was found in filopodia and co-localized with actin (Fig. 3.6A). When N-WASP and Toca-1 were expressed together there was a synergistic effect on filopodia and neurite formation (Fig. 3.4). Under these conditions Toca-1 was found in filopodia (Fig. 3.6A, part b).

3.6 N-WASP and Toca-1-N-WASP expression affects the distribution, size and number of Rab5 membranes.

Having established that Toca-1/N-WASP co-localize with Rab5, I next tried to determine whether expression of N-WASP, Toca-1 and Toca-1W518K had any effect on the size and distribution of Rab5 membranes. In control cells Rab5 membranes were primarily found around the perinuclear area. Expression of N-WASP or N-WASP/Toca-1 changed the distribution of Rab5 membranes such that they were found evenly between cell periphery and nucleus (Fig. 3.7A). Interestingly, Toca-1W518K expression with N-WASP blocked the change in vesicle distribution suggesting that N-WASP interaction with Toca-1 was important. Next I compared the size of fourteen randomly picked Rab5 membranes from control (non-transfected) cells with Toca-1-N-WASP transfected cells (Fig. 3.7B). The size of the Rab5 membranes in Toca-1/N-WASP transfected (shown in the grid) was $0.59 \pm 0.07 \mu\text{M}$ (n=14) while those from control cells

FIGURE 3.5. Localization of N-WASP and Toca-1 with Rab5.

(A) N1E115 cells were transfected with N-WASP, Toca-1 or Toca-1W518K and stained for Rab5 with anti-Rab5 antibody. Images follow the sequence, N-WASP or Toca-1 (green), Rab5 (red) and merge (a). Bar = 10 μ M. Magnification of the images presented in (A) to show potential co-localization and vesicle morphology (b).

(B) Cells were transfected with Toca-1/N-WASP and then stained for Rab5. (a) Shows a series of three images of a typical cell (in the merge panel; red-Toca-1, green-Rab5). Bar = 10 μ M. The lower set of image panels are enlarged (zoom) areas of the box in the merge panel. Bar = 2.5 μ M. (b) Twelve randomly picked vesicles were placed in a grid pattern to allow direct side-by-side comparison and co-localization analysis. (c) A group of Rab5 membranes from either Toca-1/N-WASP or Toca-1W518K transfection were analyzed for co-localization by intensity tracing through the vesicle. First two panels in (c) following intensity tracing are the actual vesicles that were examined. The schematic of the vesicles (third panel) shows the intensity line. Intensity analysis was carried out as described in Materials and methods.

A

a

b

B

FIGURE 3.6. Localization of N-WASP and Toca-1 with actin.

N1E115 cells were transfected with Toca-1 with GFP-actin, Toca-1 with N-WASP or N-WASP with mRFP-actin and filopodia and vesicles followed. Images are in a series of three; GFP, mRFP and overlay. (A) (a) Localization of Toca-1/actin, (b) Toca-1/N-WASP, and (c) N-WASP/actin in filopodia. (B) GFP-actin/mRFP-Toca-1 expression in N1E115 cells. Lower panels of (a) are duplicates that have tracings showing the relationship between filopodia and Toca-1 localization. Toca-1 is found at the base of filopodia. Localization of Toca-1 with actin, at (a) the leading edge and (b) in neurites (the insert is an enlargement of the box area), (c) Intensity profile of two of the vesicles from (b) showing that Toca-1 and actin co-localize. Bar = 10 μ M.

A

B

was $0.28 \pm 0.05 \mu\text{M}$ ($n=14$). Thus there was a two-fold increase in vesicle size. The size of the vesicles was also affected by N-WASP expression and Toca-1W518K blocked the N-WASP induced change (Fig. 3.7B). The size change of the vesicles is also confirmed by electron microscopy (Fig. 3.7D). The vesicles where Toca-1/N-WASP was localized were motile with rates of movement similar to those reported for endocytic vesicles (see Chapter 4). Lastly, the total number of vesicles was dramatically increased by expression of N-WASP/Toca-1. N-WASP alone also increased vesicle number. In other cell types including; CHO, HeLa, and COS7, Toca-1/N-WASP coexpression also induces vesicle formation with strong colocalization of these proteins in vesicular structures (see Chapter 4). Taken together with the activities associated with N-WASP (Arp2/3 activation) and Toca-1 (F-BAR mediated membrane deformation) these data on Rab5 membranes suggest that the Toca-1-N-WASP complex plays a role in their formation and motility.

3.7 Toca-1 interacts with N-WASP in filopodia and Rab5 positive membranes.

Previous work has shown that Toca-1 and N-WASP form a complex in cell lysates but the spatial distribution of the complex in cells has not been investigated. To determine where in cells Toca-1 and N-WASP interact with, acceptor photobleaching-FRET (AP-FRET) and FLIM were used. Expression of GFP-N-WASP and mRFP-Toca-1 induced a clear phenotype of filopodia and apparent vesicle formation, with the vesicles representing the major site of Toca-N-WASP interaction as seen by acceptor-photobleaching FRET (AP-FRET; Fig. 3.8A). As controls I used unfused cytosolic GFP and mRFP (negative) and a fused mRFP-GFP tandem protein (positive). The correlation coefficient (CC) is a measure of whether there is a relationship between rates of increase of donor signal on bleaching the acceptor. If FRET is occurring I predict the CC value to be between -0.7 and -1.0. For full details of AP-FRET and controls see Materials and Methods section.

To confirm further the protein-protein interaction as seen by AP-FRET a donor lifetime (GFP) values was used to follow FRET between N-WASP and Toca-1. GFP lifetimes were measured using the phase modulation method (see Materials and methods for details). GFP lifetimes in mammalian cells are around 2.37 ns. GFP-N-WASP in the presence of mRFP-Toca-1 reduced the lifetime to 1.80 ns and in the presence of the mutant W518K, the lifetime was 2.30 ns (Fig. 3.8B). Spatial resolution of this frequency domain FLIM measurement was limited and did not allow me to distinguish filopodia and vesicles (Fig. 3.8B).

3.8 Inhibitors of endocytosis inhibit Toca-1 induced filopodia formation.

Toca-1 induces filopodia formation and localises to endocytic vesicles (and associated actin) and interacts with N-WASP in the filopodia and vesicles. The increased formation of vesicles observed with coexpression of Toca-1 with N-WASP suggested that Toca-1 uses N-WASP to drive actin polymerization and pinching off of vesicles. Taken together, this data suggests that filopodia formation and endocytosis may be linked through the Toca-1-N-WASP complex. To investigate this possibility I examined the effect of two inhibitors of endocytosis on Toca-1 induced filopodia formation. Cells were transfected with Toca-1 to induce filopodia and then with either dynamin (wild-type and a DN mutant K44A) or Eps15 (D3Δ2 and DN mutant Δ95/295) under conditions where endocytosis was significantly inhibited (as monitored by transferrin uptake; Fig. 3.9A ,B and C). Transferrin uptake at 10 min is in the linear range of the uptake curve and stands for the initial uptake rate over 10 min. Toca-1 was coexpressed with the dynamin or Eps15 proteins and filopodia number scored. Both DN dynamin and DN Eps15 protein significantly inhibited filopodia formation (Fig. 3.9B and C). To examine whether clathrin mediated endocytosis was involved with Toca-1 induced filopodia formation, I used RNAi-mediated KD of clathrin heavy chain (CHC). Using RNAi (Dharmacon) I was able to reduce CHC by over 85% (Fig.3.9D). Under these conditions there was a significant reduction in Toca-1 mediated filopodia formation. However, Toca-1 mediated neurite formation was not

affected (Fig.3.9D). Thus three different inhibitors of endocytosis blocked Toca-1 induced filopodia formation.

FIGURE 3.7. Effect of N-WASP and Toca-1 on the distribution, size and number of Rab5 membranes.

(A) N1E115 cells were transfected with constructs, N-WASP, Toca-1/N-WASP, Toca-1, Toca-1W518K, and Toca-1W518K/N-WASP, and then cells stained with Rab5 antibody. Intensity tracing below images follows the region of interest open white arrow, from perinuclear area to cell membrane, of the individual cell. Distribution of Rab5 membranes was determined by creating five zones (perinuclear to cell membrane) within the cell and counting the fluorescence intensity within each zone (details can be found in the Materials and Methods section). The bar charts show a statistical analysis of the intensity distributions in the five zones with. A schematic of a cell showing the five zones used in the statistical analysis is shown at the bottom of the figure. Bar = 10 μ M. Please note that the Bar in Toca-1/N-WASP transfection panel is smaller than the other panels. (B) Fourteen randomly picked vesicular structures from either control or Toca-1/N-WASP transfected cells were placed in a grid to allow side-by side comparison. Vesicle size was determined by enlarging images so that the intensity boundary could be seen and then using Metamorph software to measure diameters. A schematic of the vesicle size is shown below the actual images to reflect the observed boundary. The bar chart shows statistical analysis of the vesicle size with different transfections. (C) The bar chart shows a statistical analysis of vesicle number with different transfections. At least three independent experiments were carried out (with $n = 20$). Data shown are mean $\pm$ S. D. Bar = 2 μ M. (D) N1E 115 cells were transfected with Toca-1 and N-WASP. The cells were then processed and stained with anti-Rab5 antibody followed by staining with colloid gold conjugated secondary antibody. Finally the specimen was examined using transmission electron microscopy. The experimental details can be found in the Materials and methods section. Upper panel shows cells under lower magnification (N for nucleus) and lower panel shows the zoomed images of the vesicles. The vesicles are positive for Rab5 as shown by gold particles. Black arrows show the gold particles. The bar chart shows the statistics of the size of the Rab5 vesicles in control and Toca-1-N-WASP transfected cells (with $n = 10$). Data shown are mean $\pm$ S. D. Two independent experiments were carried out. (Mike Yu helped me with the EM sample processing and imaging).

A

B

C

FIGURE 3.8. Toca-1 and N-WASP interact directly in filopodia and vesicles.

(A) N1E115 cells were transfected with mRFP-Toca-1 with GFP-N-WASP, or mRFP-Toca-1W518K with GFP-N-WASP, mRFP-Toca-1 with GFP-actin, and mRFP-Toca-1W518K with GFP-actin. FRET was measured by acceptor photobleaching as described in the Materials and Methods section. Images show a series of three panels; mRFP, GFP and merge. Boxes within the image show areas where bleaching was carried out. Panels in the extreme right show signal intensity from GFP (green line) and mRFP (red line) channels, pre and post bleach, as a function of time. Table shows statistical analysis of the data generated in A. FRET efficiency and the Pearson correlation coefficient were calculated as described in the Materials and methods section. Three independent experiments were performed and one representative example is shown. Data are presented as a mean $\pm$ S. D (n = 10). Bar = 10 μ M.

(B) Cells were generated as described above and GFP lifetime measurements made using the frequency domain method (see Material and methods and Ref. (Clayton et al., 2005)). GFP lifetimes were determined using Lambert Instruments (Netherlands) software. Images were acquired sequentially over a period of 4-5 secs through 12 phase settings. Data are presented as a mean $\pm$ S. D (n = 10).

A

Controls	FRET Efficiency ± S.D. (%)	Correlation Coefficient ± S.D.
mRFP-GFP (Tandem)	15.25 ± 2.43	-0.99 ± 0.003
GFP and mRFP	0.67 ± 2.87	0.30 ± 0.17
FRET pairs		
mRFP-Toca-1 and GFP-N-WASP (vesicle)	10.17 ± 1.25	-0.99 ± 0.005
mRFP-Toca-1 and GFP-N-WASP (filopodia)	11.3 ± 3.82	-0.88 ± 0.11
mRFP-Toca-1W518K and GFP-N-WASP	0.50 ± 2.07	0.63 ± 0.25
mRFP-Toca-1 and GFP-actin	2.14 ± 1.95	0.57 ± 0.38
mRFP-Toca-1W518K and GFP-actin	-0.42 ± 2.37	0.61 ± 0.29

B

FLIM pairs	AVE (nano secs)	± S.D.
GFP-N-WASP	2.37	0.064
mRFP-Toca-1 and GFP-N-WASP	1.80	0.047
mRFP-Toca-1W518K and GFP-N-WASP	2.30	0.053

FIGURE 3.9. Effect of inhibitors of endocytosis on filopodia formation.

(A) N1E115 cells were transfected with Toca-1, GFP or mRFP-actin, and either Eps15 (inactive variant or dominant negative) or dynamin (wild-type or dominant negative). Cells were then imaged in the GFP and DIC channels. Imaging was carried out using Olympus FV1000 confocal microscope.

(B) N1E115 cells were transfected with either WT/DN dynamin or Eps15. 24 hrs after transfection, the transferrin uptake assay was performed as described in Material and methods section. After that the cells were stained, fixed and examined by confocal microscopy. Left panels are images of transferrin and the right panels are the corresponding images of dynamin or Eps15. Arrows indicate the cells expressing dynamin or Eps15. Bar = 20 μ M.

(C) Endocytosis and filopodia formation were measured as described in the Materials and methods section. N1E115 cells were transfected with dynamin or Eps15 and the transferrin uptake assay was performed. The arrows indicate the cells transfected with dynamin or Eps15. Bar = 20 μ M. Bar charts (a) and (b), show transferrin (TF) uptake. Bar charts (c) and (d) show filopodia number per cell. Three independent experiments were carried out (with $n = 10-15$) and one representative example is presented.

(D) Protein expression of CHC was analyzed by Western blotting of cell extracts obtained with or without RNAi (a) as described in the Materials and methods section. (b) Toca-1 mediated filopodia formation and neurite outgrowth were measured in cells transfected with Toca-1, GFP-actin and +/- RNAi, panel 1. Panel 2 shows an enlargement of the neurite of the cell. Panel 3 shows a tracing with filopodia indicated in red. Statistical analysis of the experiment is presented as a bar chart in (c). Three independent experiments were carried out (with $n = 10-15$) and one representative example is presented.

Data are presented as mean $\pm$ S. D. Experimental data were analyzed by Student's t-test. Difference was significant when $p < 0.05$. (* stands for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$). Bar = 10 μ M. (Amy Chou helped me with the experiment of endocytosis inhibitors).

A

B

C a

b

c

d

D

Movie 3.1 Toca-1 induces neurite outgrowth and filopodia formation in N1E115 cells. The movie was taken in 10 min with 60 frames and 10 sec interval.

Movie 3.2 N-WASP induces neurite outgrowth and filopodia formation in N1E115 cells. The movie was taken in 10 min with 60 frames and 10 sec interval.

CHAPTER 4 The Toca-1-N-WASP complex induces membrane tubulation and vesicle formation.

4.1 Toca-1-N-WASP interaction induces the formation of dynamic membrane tubules and vesicles.

FBP17 and CIP4 and their F-BAR domains alone tubulate membranes *in vivo* (Kamioka et al., 2004; Lim et al., 2008; Itoh and De Camilli, 2006; Shimada et al., 2007); tubulation reflects membrane deformation activity, the first step in the formation of endocytic vesicles. In contrast, the FCH, F-BAR domain, or full-length Toca-1 protein did not induce membrane tubulation (Fig.4.1 A-C) and expression of the FCH or F-BAR domain does not affect F-actin cytoskeleton (Fig.4.1 D). This suggests that Toca-1 may adopt an autoinhibited conformation in absence of the activators/interactors. Interestingly, coexpression of Toca-1 with N-WASP was sufficient to induce the formation of membrane tubules and vesicles in CHO cells (Fig.4.2A), HeLa (Fig.4.2B), COS7 (Fig. 4.2D) and N1E115 cells (Fig 4.2C). Using time-lapse TIRF microscopy I examined the dynamics of the processes of membrane tubulation and vesicle formation. In cells that contained few tubules, the vesicles were seen to align and form tubules. This sequence was reversible as tubules could be seen to disintegrate and give vesicles (Fig. 4.2E, a). Thus tubule formation is a result of dynamic membrane deformation (remodeling) in the absence of vesicle scission. Using GFP-actin mRFP-Toca-1/HA-N-WASP, I was able to observe vesicle formation from tubules (Fig. 4.2E, b). Vesicle formation from the tubule was possibly driven by actin polymerization. The vesicles themselves were motile with a speed of approx. 60 nm/sec (Fig. 4.2E, c). Both vesicle formation and motility were associated with an actin comet emerging behind the direction of movement (Fig. 4.2E, c) reminiscent of viral/bacterial movement in mammalian cells (e.g. refs (Rozelle et al., 2000; Burton et al., 2005)).

Figure 4.1. Phenotypes of N-WASP, Toca-1 and domains.

Cells were transfected with cDNA encoding; (A). (a) GFP alone (panel a), GFP-FCH domain (panels b,b'), GFP-F-BAR domain (panels c,c'). (B). mRFP-Toca-1 or GFP-N-WASP in HeLa cells. (C) mRFP-Toca-1 or GFP-N-WASP in CHO cells. (D) (a) GFP-FCH domain. (b) GFP-F-BAR domain. Cells in panel D were stained with TRITC conjugated phalloidin after protein expression. The cells were examined by confocal microscopy following washing and fixation. Toca-1 and N-WASP expressed individually induce filopodia formation as described in (Bu et. al., 2009) but not tubules or vesicles.

b

Figure 4.2. Characteristics of Toca-1/N-WASP induced membrane tubulation, vesicle scission and motility.

CHO, Hela, N1E115 or COS7 cells were transfected with mRFP-Toca-1 and GFP-N-WASP (A, B, C, D and E; part a) or with mRFP-Toca-1/HA-N-WASP and GFP-actin (E, parts b and c) as described in the Material and methods section. (A) Confocal image of cells expressing mRFP-Toca-1/GFP-N-WASP. mRFP-Toca-1 (a and d), GFP-N-WASP (b and e) and the merge (c and f), green – N-WASP and red – Toca-1. Upper panels show vesicles (a-c) and lower panels (d-f) tubules. (B) Hela cells expressing Toca-1 and N-WASP. Left panel-Toca-1; middle panel-N-WASP; right panel-merged image. Panels a-c show vesicles and d-f show tubules. Inserts in d-f show the zoomed tubules. (C) N1E115 cells expressing Toca-1 and N-WASP. The sequence of the images from left to right is Toca-1, N-WASP and merged image. Panels a-c show tubules and panels d-f show vesicles. Inserts in a-c show the zoomed tubules. (D) COS7 cells expressing Toca-1 and N-WASP. Sequence of images from left to right is Toca-1, N-WASP and merged image. (E). TIRF live cell microscopy was used to follow the dynamics of tubule and membrane vesicle formation at the membrane (a). A time lapse sequence of Toca-1 is shown with numbers in sec. The lower panels show a schematic of the time-lapse sequence illustrating how vesicles align to form tubules which then disassemble to give vesicles. (b) The process of vesicle scission from tubules is shown with mRFP-Toca-1 and GFP-actin as the two labels being followed. N-WASP is present but silent in HA tagged form. (c) (i) mRFP-Toca/HA-N-WASP and GFP-actin were used to follow actin polymerization during vesicle motility. One vesicle was selected to analyze. Background signals were removed to allow clear visualization of the relationship between mRFP-Toca-1 and GFP-actin. (ii) CHO cells were transfected with GFP-N-WASP/myc-Toca-1 and ABP-mCherry and the timelapse acquisition was carried out at 2.3 secs interval 24hrs post transfection. Bar = 10 μ m. Movies 4.1-4.3 illustrate the dynamics of the Toca-1-N-WASP phenotype (see CD).

E

a TIRFM

mRFP-Toca-1 and GFP-N-WASP

b

mRFP-Toca-1 and HA-N-WASP

c

mRFP-Toca-1, HA-N-WASP and GFP-actin

GFP-N-WASP, Myc-Toca-1 and ABP-mCherry (2.3 sec interval)

ii

4.2 Characteristics of Toca-1-N-WASP induced membrane tubules and vesicles.

The tubules and vesicles induced by Toca-1-N-WASP were followed with marker antibodies; Rab5, Rab7 and Lamp1-Cy5 (Fig 4.3 A and F). Toca-1 and N-WASP colocalised with Rab5 and to some degree Rab7 but not Lamp1-Cy5. Clathrin colocalised with Toca-1 in the membrane tubules and vesicles but caveolin did not (Fig 4.3 C, D and F). From this marker analysis I conclude that the vesicles and tubules induced by Toca-1-N-WASP expression are probably endocytic structures. The Toca-1-N-WASP induced membrane tubules and vesicles colocalised with GFP-actin and the polymerized actin as seen with the phalloidin staining (Fig 4.3 B and F). To examine the relationship between actin and Toca-1, I used GFP-actin expression together with mRFP-Toca-1/HA-N-WASP. GFP-actin was found to colocalise with vesicles and tubules (Fig 4.3 B). Lastly, the PH domain probes colocalised in the membrane tubules and vesicles suggesting the involvement of phosphoinositides and PIP₂ in particular (Fig 4.3 E and F).

I next examined which membrane uptake pathway was affected by coexpression of Toca-1-N-WASP. Using transferrin, cholera toxin B and dextran uptake I found that only the transferrin uptake process was inhibited while others were comparable to the control. This was dependent on the interaction of Toca-1-N-WASP as a mutant Toca-1W518K coexpressed with N-WASP did not inhibit transferrin uptake. Furthermore, expression of Toca-1 or N-WASP alone did not inhibit transferrin uptake significantly (Fig 4.4).

4.3 Toca-1 and N-WASP interact in tubules and vesicles.

To determine whether the interaction of Toca-1 with N-WASP was important for the induction of membrane tubules and vesicles, the SH3 domain defective W518K mutant of Toca-1 that does not interact with N-WASP (Ho et al., 2004) was used. Coexpression of Toca-1W518K with N-WASP did not induce membrane tubulation or the formation of vesicles (Fig. 4.5). This

Figure 4.3 Vesicles and tubules induced by Toca-1 and N-WASP colocalise with Rab5, Rab7, clathrin, actin and PH domain probes, but not caveolin.

(A) CHO cells were transfected with mRFP-Toca-1 and HA-N-WASP followed with antibody (Rab5, Rab7 or Lamp-1) staining. Vesicles or tubules induced by Toca-1 and N-WASP were analyzed for co-localization by intensity tracing through the vesicle/tubules. First two panels following intensity tracing are the actual vesicles/tubules that were examined. The schematic of the vesicles (*third panel*) shows the intensity line. Intensity analysis was carried out as described under “Materials and Methods.” Intensity tracings in other panels (B-E) are done similarly as that of panel A. (B) i. CHO cells transfected with mRFP-Toca-1, HA-N-WASP and GFP-actin were fixed and examined with confocal microscopy. left panel -Toca-1, middle panel-actin, right panel-merge. ii. CHO cells are transfected with mRFP-Toca-1 and HA-N-WASP followed by staining with FITC conjugated phalloidin. The Upper panel shows the cell with tubules and the lower panel shows the cell with vesicles. left panel-Toca-1, middle panel-phalloidin, right panel-merge. (C) CHO cells transfected with mRFP-Toca-1 and GFP-clathrin (a), or mRFP-Toca-1, HA-N-WASP and GFP-clathrin (b and c) were fixed and examined with confocal microscopy. left panel -Toca-1, middle panel-clathrin, right panel-merge (D) CHO cells transfected with Myc-Toca-1, GFP-N-WASP and RFP-caveolin were fixed and examined with confocal microscopy. left panel -N-WASP, middle panel-caveolin, right panel-merge. (E) CHO cells were transfected with mRFP-Toca-1, HA-N-WASP and CFP-PLC δ -PH (a), mRFP-Toca-1, HA-N-WASP and CFP-Akt-PH (b and c), mRFP-Toca-1, HA-N-WASP and GFP-BTK-PH (d and e). The cells were fixed and imaged with confocal microscopy 24 hrs after transfection. left panel-Toca-1, middle panel-individual PH domain probe, right panel-merge. (F) Colocalization analysis of Toca-1 and N-WASP expressing cells. Cells were transfected with Toca-1 and N-WASP and left for 24-36 hours. After fixation cells were incubated with primary antibody followed by secondary antibody for markers of membrane trafficking pathways as described in the Material and methods section. PH domains were visualized with GFP, or CFP. Olympus FV1000 confocal microscope software was used for colocalization analysis. Where Pearson colocalization coefficient (CC) of; <0.25 indicates no colocalization (-), 0.25-0.5 indicates potential colocalization (*), 0.5-0.7 indicates some colocalization (**), and >0.7, indicates high colocalization (***). The CC is an average $\pm$ S. D., with n = 8-10, from 3 experiments.

A

mRFP-Toca-1 and HA-NWASP

B

ii

D

E

Tracing

F

Vesicle		
Marker	Colocalization	Pearson CC $\pm$ S.D.
GFP-actin	**	0.55 ± 0.09
GFP-clathrin	***	0.73 ± 0.04
Rab5 staining	**	0.67 ± 0.10
Rab7 staining	**	0.51 ± 0.12
Lamp-1 staining	-	0.16 ± 0.08
RFP-caveolin	-	0.10 ± 0.08
CFP-PLC δ -PH	***	0.71 ± 0.04
CFP-Akt-PH	***	0.55 ± 0.06
GFP-Btk-PH	***	0.74 ± 0.05
Phalloidin	**	0.53 ± 0.11
Tubule		
Marker	Colocalization	Pearson CC $\pm$ S.D.
GFP-actin	***	0.72 ± 0.05
CFP-PLC δ -PH	***	0.70 ± 0.06
CFP-Akt-PH	***	0.60 ± 0.05
GFP-Btk-PH	***	0.75 ± 0.12
Phalloidin	***	0.75 ± 0.05
GFP-clathrin	***	0.75 ± 0.07

Figure 4.4 Uptake assays of endocytic markers by Toca-1 and N-WASP expressing cells.

Cells were transfected with Toca-1 and N-WASP and left to express the proteins for 24-36 hours. Markers for uptake pathways were then added and uptake monitored for 10 min for transferrin (A and B)) and 30 min for dextran and cholera toxin B (C). The cells were then washed, fixed and examined by confocal microscopy. Bar = 10 μ M. (D) Quantification of the % uptake of different markers in cells transfected with Toca-1-N-WASP compared with control cells as described in Material and methods section. The % uptake is an average $\pm$ S. D., with n = 10, from 3 experiments.

B

D

Endocytic pathway	Marker	Toca-1-N-WASP expression (% of control)
Clathrin-mediated endocytosis	Transferrin	50.2 ± 11.6
Caveolin-mediated endocytosis	Cholera toxin B	103.2 ± 8.7
Pinocytosis	Dextran	112.1 ± 19.3

result suggests that N-WASP plays an important role in the control of the membrane deforming activity of Toca-1.

To show conclusively that Toca-1-N-WASP interaction was important for the induction of membrane tubules and vesicles, I carried out FRET experiments on these cellular structures. If N-WASP and Toca-1 are binding to each other and the distance between GFP and mRFP is less than 10 nm, FRET can occur. GFP-N-WASP and mRFP-Toca-1 were coexpressed and acceptor photobleaching (AP)-FRET was carried out on fixed samples. In this AP-FRET method the mRFP fluorescence is bleached and the GFP fluorescence followed pre and post bleach. In the FRET scenario GFP intensity will increase on mRFP bleaching. Further, in the FRET scenario the rates of change in GFP/mRFP fluorescence should be inversely correlated. The intensity changes following bleaching are expressed as a cross correlation coefficient (CC). In addition to these experiments I also carried out a number of controls. These include using free GFP/mRFP proteins as well as a GFP-mRFP fused in tandem. I define a positive FRET as $\%FE > 3\%$ and a CC between -0.7 to -1.0. Full details of this methodology can be found in Lim et al (Lim et al., 2008). Figure 4.5 shows examples of FRET experiments focusing on either membrane vesicles or tubules. Toca-1 and N-WASP not only colocalise in these membrane vesicles/tubules but also interact as seen by the positive FRET. In contrast, Toca-1W518K fails to FRET with N-WASP (Fig. 4.5A-C). To confirm the AP-FRET results showing direct interaction of Toca-1 with N-WASP by an independent method I used frequency-domain FLIM. In this method the lifetime of the donor is used as an indicator of protein-protein interaction. The lifetime of GFP in cells is approx. 2.2 ns. If GFP is within 10 nm of an acceptor, such as mRFP, FRET can occur. With cytosolic GFP and mRFP FRET does not occur and coexpression does not affect GFP lifetimes (Fig. 4.5D). Using GFP-N-WASP and mRFP-Toca-1 expressing cells I measured the GFP lifetimes using the frequency domain method. With this method I was able to combine intensity measurements with lifetimes and obtain some spatial resolution of sites where Toca-1 interacted with N-WASP were found (Fig. 4.5D and E). Supporting the AP-FRET analysis, Toca-1

interacted with N-WASP in vesicles with a lifetime of 1.64 ns and tubules with a lifetime of 1.59 ns (Fig. 4.5D).

Figure 4.5. FRET and FLIM analysis of the Toca-1-N-WASP interaction.

Cells were transfected with mRFP-Toca-1 and GFP-N-WASP cDNA and left to express mRFP/GFP for 36 hours as described in the Experimental procedures section. (A) Cells were these selected for either a vesicle or tubule phenotype. ROIs focusing on these structures were chosen, as shown, and acceptor photobleaching (AP)-FRET carried out as described in the Experimental procedures section. Green/red traces to the left of the cell images indicate mRFP/GFP fluorescence pre and post-bleach. The time course of these experiments is approx. 60 sec.

(B) The Toca-1 SH3 domain mutant W518K (which is unable to bind N-WASP) was analyzed as shown for the wild-type in (A).

(C) A summary of data obtained for controls as well as FRET pairs is shown. The Pearson correlation coefficient (here on abbreviated as CC) shows the relationship between the mRFP and GFP signals during AP-FRET. GFP/mRFP FRET controls were as described in (Lim et. al., 2008; Bu et. al., 2009). For the positive FRET scenario I expect high negative cross correlation between donor and acceptor signals. We define positive FRET when the FRET efficiency (FE) > 3% and CC > -0.7.

(D) Cells are analyzed by frequency-domain FLIM as described in the Experimental procedures section. (a) Lifetimes are colour coded (1.0-3.0 ns). Cells were chosen as for AP-FRET for the presence of either tubules or vesicles and then 12 phase shifted images captured and processed to generate a lifetime image. (b) These images were then processed to demonstrate more clearly morphological structures as follows; the higher lifetime signals (the non-interacting signals) from the original FLIM image were masked in Photoshop and then (using Metamorph) the masked image was given a pseudo color and overlaid with intensity image. Lifetime images are shown with the intensity images below. The lifetimes of GFP-N-WASP within tubules and vesicles can be obtained using this analysis. (c) Summary of lifetimes obtained for controls, N-WASP alone, Toca-1/N-WASP and Toca-1W518K/N-WASP. Bar = 10 μ m. For statistical analysis numbers are averages $\pm$ S. D., with n = 7-15, from 2-3 experiments.

b Processed image (mRFP-Toca-1 and GFP- N-WASP)

C

FLIM pair	life time (nsec)	± SD	FRET efficiency (%) ± SD
GFP-N-WASP	2.21	0.06	-
mRFP-Toca-1 and GFP-N-WASP (vesicle)	1.64	0.19	25.79 ± 4.90
mRFP-Toca-1 and GFP-N-WASP (tubule)	1.59	0.08	28.05 ± 2.24
mRFP-Toca-1W518K and GFP-N-WASP	2.24	0.14	-1.45 ± 6.32

Movie 4.1: Toca-1/N-WASP vesicle dynamics.

CHO cells were transfected with cDNA encoding mRFP-Toca-1 and GFP-N-WASP. After 24 hrs, the time-lapse acquisition was performed on an inverted Olympus FV1000 confocal microscope at 10 sec interval for 4 min. Image acquisition in the mRFP and GFP channels was taken sequentially. Bar = 10 μ M.

Movie 4.2: mRFP-Toca-1/GFP-N-WASP induced tubule/vesicle transitions with TIRF microscopy.

CHO cells were transfected with cDNA encoding mRFP-Toca-1 and GFP-N-WASP. After 24 hours, the time-lapse acquisition was performed on an inverted Olympus FV1000 TIRF microscope at 10 sec interval for 60 min. The penetration depth was approx 100 nm from the coverslip. Image acquisition in the mRFP and GFP channels was taken sequentially. Bar = 10 μ M.

Movie 4.3: mRFP-Toca-1/HA-N-WASP/GFP-actin induced vesicle motility.

CHO cells were transfected with cDNA encoding mRFP-Toca-1, HA-N-WASP and GFP-actin. After 24 hours, the time-lapse acquisition was performed on an inverted Olympus FV1000 confocal microscope at 2.3 sec interval for ~ 2 min. Image acquisition in the mRFP and GFP channels was taken sequentially. Bar = 10 μ M.

CHAPTER 5 Effect of Toca-1 F-BAR mutants, dynamin, F-actin and microtubules on Toca-1-N-WASP induced tubules and vesicles.

5.1 F-BAR domain of Toca-1 is essential for membrane tubulation induced by Toca-1 and N-WASP.

F-BAR domain of CIP4 and FBP17 has been shown to be crucial for the membrane deformation activity *in vitro* and *in vivo* (Itoh et al., 2005; Tsujita et al., 2006). Thus F-BAR domain mutant of Toca-1 was cotransfected with N-WASP and the phenotype monitored. Toca-1 F-BAR domain mutants failed to form tubules when expressed with N-WASP suggesting that F-BAR domain activity is important for tubule formation (Fig.5.1A). Interestingly, the F-BAR domain mutants did not affect vesicle formation. However, the vesicles were smaller and were more motile (Fig. 5.1 B and C).

5.2 Dynamin is essential for vesicle formation but not for tubulation.

Since Toca-1 has been reported to bind dynamin (Tsujita et al., 2006), I also examined the phenotype of Toca-1-dynamin coexpression (Fig. 5.2A). mRFP-Toca-1 was expressed with GFP-dynamin and both proteins were localized in membrane vesicles but tubules were not generated. Interestingly, the vesicles were not motile and were morphologically distinct from Toca-1-N-WASP vesicles and similar to the dynamin vesicles found in the absence of Toca-1 expression. Toca-1 was in complex with dynamin in these membrane vesicles as seen by FRET analysis (Fig. 5.2A, b). It was speculated that Toca-1/N-WASP mediated tubule formation arose because of limiting amounts of dynamin. To investigate this possibility further, I used wild-type (WT) and K44A-Dominant negative (DN)-dynamin. DN-dynamin prevented the formation of membrane vesicles and all transfected cells contained membrane tubules. In contrast, expression of WT-dynamin with Toca-1-N-WASP eliminated membrane tubules and cells were rich in membrane vesicles (Fig. 5.2B).

Figure 5.1. Effect of Toca-1 F-BAR mutants on Toca-1/N-WASP phenotypes.

Cells were transfected with mRFP-Toca-1 F-BAR mutants with N-WASP as outlined in the figures. (A). Phenotypes of Toca-1/N-WASP combinations. (B) Motility of wild-type vesicles and Toca-1 F-BAR domain mutant generated vesicles. (C) FRAP analysis of Toca-1F-BAR domain mutant generated vesicles (a-d). Effect of Toca-1 F-BAR domain mutants on vesicle size (e) and motility (f). Bar = 10 μ m. For statistical analysis numbers are averages $\pm$ S. D., with n = 5-15, from 2-3 experiments.

B

C

Figure 5.2. Dynamin function, Toca-1 interaction and phenotype.

(A). (a) Cells were transfected with mRFP-Toca-1 and GFP-dynamin2 as described in the Material and method section. The top two panels (i and ii) show the cell phenotypes of individual protein expression. The lower panels show the double expression (iii). Bar = 10 μ m. Numerical data are derived from AP-FRET analysis of mRFP-Toca-1/GFP-dynamin2. (B) mRFP-Toca-1.GFP-N-WASP transfections were carried as before (see Material and methods section for details) and either dynamin1 WT (upper panels) or Dynamin1-K44A (dominant negative) cDNA (lower panels) included. Three images for each combination is shown; Toca-1, N-WASP and merge (red – Toca-1; green – N-WASP). Bar = 10 μ m. For statistical analysis numbers are averages $\pm$ S. D., with n =8-10, from 2-3 experiments.

b

FRET pair	FRET efficiency $\pm$ S.D. (%)	CC $\pm$ S.D.
GFP-Dynamin2 and mRFP-Toca-1	11.7 $\pm$ 3.8	-0.99 $\pm$ 0.01

B

5.3 Role of F-actin and microtubules on Toca-1/N-WASP phenotypes and dynamics.

As mentioned above, actin and actin filaments associate with the Toca-1-N-WASP induced membrane tubules and vesicles. To determine whether destruction of either F-actin microfilaments or microtubules (as control) affected the phenotype, cytochalasin D (Cyt. D) and nocodazole were used, respectively (Fig. 5.3). Interestingly, the two drugs had opposite effects on the Toca-1-N-WASP phenotype. Cyt. D inhibited vesicle formation and the tubules were morphologically different (thicker and stabilized; see FRAP analysis below). With nocodazole, tubule formation was inhibited and vesicle formation stimulated. This latter result suggests that microtubules are involved in inhibiting vesicle formation (and therefore the membrane scission event). Neither Cyt. D or nocodazole affected the interaction of Toca-1 and N-WASP in membrane tubules and vesicles as seen by AP-FRET (Fig. 5.3 C). Cyt. D but not nocodazole inhibited vesicle motility.

To compare protein dynamics within membrane vesicles and tubules under the different experimental conditions fluorescence recovery after photobleaching (FRAP) was used. Cells were transfected with Toca-1 and N-WASP cDNA (or mutants), allowed to express for 36 h and their phenotypes were followed. Fluorescence was monitored for approx. 30 sec pre-bleach to obtain a baseline, bleaching carried out and then recovery monitored for approx. 300 sec (Fig. 5.3D). For Cyt. D and nocodazole treatment, FRAP analysis was carried 60 min after treatment. FRAP analysis was carried out in an ROI incorporating either membrane vesicles or tubules. Toca-1-N-WASP induced membrane tubules and vesicles had similar recovery rates of approx. 44 sec. F-BAR domain mutants had a more rapid recovery rate compared to wild-type of approx. 20 sec (Fig. 5.1 C). Nocodazole treatment which eliminated tubules, did not affect recovery time. However, the fluorescence increased above baseline after recovery suggesting that there was greater exchange between pools of protein compared to the control situation (Fig. 5.3D, panel e'). Interestingly, Cyt. D treatment, which eliminated membrane vesicle formation but not tubules

formation, prevented fluorescence recovery after photobleaching (Fig. 5.3D, panel e). This suggests that actin polymerization and or filaments are required for tubule dynamics. The addition of either Cyt. D or Nocodazole did not affect the interaction of Toca-1 with N-WASP as seen by AP-FRET analysis (Fig. 5.3C).

The Toca-1-N-WASP coexpression experiments so far have established conditions where I can monitor steps related to the endocytic process; membrane tubulation – membrane deformation (F-BAR domain activity), vesicle numbers/index (vesicle formation) and vesicle motility. Cyt. D acts at three places; (i) Inhibiting protein dynamics within tubules and inducing static/stabilised tubules. (ii) Inhibiting vesicle formation and (iii) Inhibiting vesicle motility (Fig. 5.3 A, B). Actin dynamics are therefore intimately linked with these processes and are crucial for vesicle formation and tubule/vesicle dynamics.

Figure 5.3. Effect of cytochalasin D and nocodazole on the Toca-1/N-WASP phenotypes.

(A). Cells were transfected with Toca-1 and N-WASP cDNA as described in the Experimental procedures sections. After 36 hours cells were chosen for either tubulation or membrane vesicle formation. Using time-lapse microscopy the change in phenotype was followed after addition of either Cytochalasin D or nocodazole for 60 min. Panels show images at zero time (a, a' and c, c') and at 60 min (b, b' and d, d') of a representative cell. (a'b'c' and d') are enlargements of areas in panels a-d, respectively, showing (a'b', nocodazole) tubule to vesicle, and (c'd', cytochalasin D) vesicle to tubule transitions. Bar = 10 μm .

(B). Cells were then scored for presence of vesicles (vesicle index), tubules (tubule index) and vesicle motility as described in the Material and methods section.

(C) Shows AP-FRET analysis of Toca-1/N-WASP in tubules or vesicles after chemical treatment; (a) cytochalasin D, (b) nocodazole. Images show typical cells used and the ROI. Green/red tracings show changes in intensity of the ROI before and after photobleaching. (c) A statistical analysis of FRET data with controls and FRET pairs. Bar = 10 μm .

(D) FRAP analysis of the protein dynamics within the tubules/vesicles, with and without Cytochalasin D or nocodazole. Images show typical cells used and the graphs below show the bleach followed by the recovery profile. Time in sec. Bar = 10 μm . For statistical analysis numbers are averages $\pm$ S. D., with n = 7-10, from 2-3 experiments.

A

B

c

Control	FRET efficiency (%) $\pm$ S.D.	CC $\pm$ S.D.
mRFP-GFP (tandem)	28.64 $\pm$ 3.69	-0.99 $\pm$ 0.002
GFP and mRFP	1.91 $\pm$ 1.49	0.62 $\pm$ 0.38
FRET pair		
Toca-1 and N-WASP(DMSO)	17.57 $\pm$ 4.64	-0.99 $\pm$ 0.003
Toca-1 and N-WASP (cytochalasin D)	17.51 $\pm$ 1.47	-0.99 $\pm$ 0.005
Toca-1 and N-WASP(nocodazole)	15 $\pm$ 4.00	-0.99 $\pm$ 0.003

CHAPTER 6 Cdc42 regulates Toca-1-N-WASP complex formation.

6.1 Cdc42 forms complex with Toca-1-N-WASP on tubules and vesicles.

Both Toca-1 and N-WASP interact with Cdc42. I next investigated whether Cdc42 was required for Toca-1-N-WASP complex formation. First I determined whether Cdc42 did indeed interact directly with Toca-1 and/or N-WASP within the Toca-1-N-WASP complex in tubules and membrane vesicles using AP-FRET. These experiments involved using low level expression of GFP-Cdc42G12V in combination with either mRFP-Toca-1/HA-N-WASP or mRFP-N-WASP/myc-Toca-1. Cdc42G12V is a constitutively active mutant of Cdc42 and predominantly GTP bound, which is defective in interaction with Rho GDP-dissociation inhibitor. Cdc42G12V interacted with Toca-1 in tubules but not in vesicles (Fig. 6.1A). Cdc42 interacted with N-WASP in both tubules and membrane vesicles. Thus the Cdc42-N-WASP-Toca-1 complex does exist on both tubules and vesicles. However, there are differences in the Cdc42-Toca-1-N-WASP complexes on tubules and on vesicles, during membrane deformation and vesicle motility.

To determine whether Cdc42 interaction was necessary for the formation of the Toca-1-N-WASP complex, I used Cdc42 binding defective mutants, Toca-1MGD383-385IST and N-WASPH208D. Interestingly, N-WASPH208D was not competent to make a complex with Toca-1 suggesting that Cdc42 binding to N-WASP was essential for the formation of a trimer complex (Fig. 6.1B). For comparison, I also examined whether the N-WASP Δ WA mutant formed a complex with Toca-1 and found that it did. However, the Toca-1-N-WASP Δ WA complex had a null phenotype (Fig. 6.1C). The Toca-1 Cdc42 binding dead mutant MGD383-385IST was still able to complex with N-WASP and did not alter the phenotype of the complex dramatically (Fig 6.1 B and C).

6.2 Cdc42 interaction with Toca-1 and N-WASP regulates membrane tubulation, vesicle formation and motility.

When the Cdc42 binding dead Toca-1MGD383-385IST mutant was expressed with N-WASP, the phenotype was similar to the Toca-1-N-WASP phenotype, both tubules and vesicles were formed, and the vesicles were motile (Fig. 6.2B). To investigate the Toca-1MGD383-385IST phenotype in more detail, I measured the length of tubules. Figure 6.2 A shows that tubule length is reduced by the mutation of the Cdc42 binding site of Toca-1 by around 50%. In addition, vesicle size was reduced by the Toca-1 Cdc42 binding dead MGD383-385IST mutant. This result suggests that Cdc42 binding directly to Toca-1 regulates its F-BAR domain membrane deforming activity. In contrast, when N-WASPH208D mutant was used in combination with Toca-1 or Toca-1MGD383-385IST, the tubule/vesicle phenotype was absent (Fig. 6.1B and C). Thus Cdc42 interaction with N-WASP is essential for complex formation and for tubule/vesicle formation.

Since it was not possible to use N-WASPH208D to examine the role of Cdc42-N-WASP interaction in membrane tubulation, vesicle formation or motility, I turned to other approaches. This included the use of proteins that bind to active Cdc42 and block function (GBD and CRIB), Cdc42G12V and Cdc42T17N, dominant active and negative versions of Cdc42, respectively, and the exchange of N-WASPH208D into complexes of Toca-1-N-WASP. Interestingly, both CRIB and GBD reduced significantly, tubulation, vesicle formation and motility (Fig. 6.2B). Most dramatically affected by CRIB and GBD was vesicle motility. CRIB also affected the distribution of vesicles as examined by confocal sectioning of cells by making a Z-stack (Fig. 6.3). In control cells, vesicles were found throughout the cytoplasm. In CRIB cells the number of vesicles were substantially reduced and only found in the first 10 sections (3 μ m from coverslip). Together we interpret these results as suggesting that vesicle formation requires Cdc42.

Next I used Cdc42G12V/T17N to explore the role of Cdc42-N-WASP interaction in the tubule/vesicle phenotype. Cells expressing Toca-1/N-WASP with Cdc42TN17 had a null phenotype while most cells with Cdc42G12V possessed tubules but no vesicles. The tubules found in Cdc42G12V expressing cells were non-dynamic as seen in the presence of Cyt. D (Fig. 6.2C, a and b). To examine the role of Cdc42 interaction with N-WASP further, I decided to try and exchange mutant GFP-N-WASPH208D into cells that had the Toca-1/N-WASP phenotype (Fig. 6.2C, c). I was able to follow the exchange by looking for GFP in the tubules/vesicles. GFP-N-WASPH208D inhibited tubule formation and vesicle motility (Fig. 6.2C, c). Taken together, these data suggest Cdc42 interaction with N-WASP is a key event in the formation of the Cdc42-N-WASP-Toca-1 trimer complex which subsequently plays a role in three distinct steps related to endocytosis; tubulation, vesicle formation and motility.

Figure 6.1. Cdc42, Toca-1 and N-WASP interactions and phenotypes.

(A) Cells were transfected Toca-1, N-WASP and Cdc42G12V and allowed to express for 36 hours as described in the Material and methods section. To perform AP-FRET Cdc42G12V was GFP labeled and the other cDNA labeled with mRFP. The third cDNA encoded (myc or HA tagged) non-visible protein. Cells with either tubules or vesicles were then chosen and AP-FRET performed on the ROI. (a) Cdc42-Toca-1 interaction (panels 1-3 tubules, panels 4-6 vesicles). (b) Cdc42-N-WASP interaction (panels 1-3 tubules, panels 4-6 vesicles). (c) A statistical analysis of the AP-FRET data. Traces on the right of the images represent intensity values of GFP and mRFP during pre and post bleach.

(B) AP-FRET analysis of interactions between Toca-1 and N-WASP mutants. Cells were transfected with cDNAs encoding GFP and mRFP fusions and allowed to express for 36 hours as described in the Material and methods section. (a) Top two panels show single transfections, either Toca-1MGD383-385IST mutant alone or N-WASPH208D mutant alone. The subsequent 9 panels show, in groups of three; Toca-1MGD383-385IST/N-WASP (panels 1-3), Toca-1/N-WASPH208D (panels 4-6) and Toca-1MGD383-385IST/N-WASPH208D (panels 7-9). Left panels show Toca-1 and mutants, middle panels show N-WASP and mutants and right panels show merged images. (b) A statistical analysis of the AP-FRET experiments with associated phenotypes. Bar = 10 μ m

(C) Effect of Toca-1/N-WASP Δ WA combinations on F-actin as stained by phalloidin. (a) Cells were transfected with cDNAs encoding mRFP-Toca-1/GFP-N-WASP Δ WA as described in the Material and methods section. F-actin was visualized with phalloidin. First series of three images shows signals from Toca-1, N-WASP Δ WA and then the merge (1-3). Second series of three shows N-WASP Δ WA, F-actin and then the merge (4-6). Bar = 10 μ m. For statistical analysis numbers are averages $\pm$ S. D., with n = 7-10, from 2-3 experiments.

b

FRET pair	FRET efficiency (%) $\pm$ SD	CC $\pm$ SD	phenotype
mRFP-Toca-1MGD383-385IST and GFP-N-WASP	17.50 $\pm$ 8.14	-0.99 $\pm$ 0.006	vesicles and short tubules
mRFP-Toca-1 and GFP-N-WASPH208D	3.87 $\pm$ 2.34	-0.53 $\pm$ 0.49	nil
mRFP-Toca-1MGD383-385IST and GFP-N-WASPH208D	1.61 $\pm$ 3.28	-0.34 $\pm$ 0.6	nil
mRFP-Toca-1 and GFP-N-WASP Δ WA	16.56 $\pm$ 2.97	-0.99 $\pm$ 0.01	nil

C

Figure 6.2. Effect of Toca-1MGD383-385IST and Cdc42 inhibition/activation on Toca-1/N-WASP phenotypes.

(A). Cells were transfected with cDNA encoding the Cdc42 binding mutant of Toca-1(MGD383-385IST) with N-WASP as described in the Material and methods section. Cell phenotypes of Toca-1MGD383-385IST with N-WASP were analyzed for (a) tubulation, (b) vesicle number and (c) vesicle motility.

(B). Cells were transfected with cDNA encoding Toca-1/N-WASP, CRIB or GBD domains and effects on (a) tubulation, (b) vesicle number or (c) vesicle motility measured. (d) Images of cells transfected with mRFP-Toca-1, HA-N-WASP and GFP-CRIB are shown in the sequence of Toca-1, CRIB and merge. Bar = 10 μ m. (e) Images of cells transfected with mRFP-Toca-1, HA-N-WASP and GFP-GBD are shown in the sequence of Toca-1, GBD and merge. Bar = 10 μ m (f) Timelapse images of cells expressing Toca-1, N-WASP and CRIB/GBD taken at 5 secs interval. The bar chart shows the statistics of the vesicle motility. The numbers are averages $\pm$ S.D., with n=8-10, from 3 experiments. The statistical significance was analyzed by student t-test. Difference was significant when $p < 0.05$. (* stands for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$).

(C). Cells were transfected with Toca-1/N-WASP/Cdc42 combinations and allowed to express for 36 hours as described in the Material and methods section. (a) Effect of Cdc42G12V or Cdc42T17N on the Toca-1/N-WASP phenotype. (b) FRAP analysis of the protein dynamics of Cdc42G12V/Toca-1/N-WASP transfected cells. (c) Effect of N-WASPH208D exchange into Toca-1/N-WASP induced structures. The left panel shows Toca-1 image; the middle panel shows N-WASPH208D image; the right panel shows the merged images. Time lapse images of N-WASPH208D exchanged cells taken at 5 secs interval. Bar charts show statistical analysis of effects N-WASPH208D exchange into the Toca-1/N-WASP complex on tubulation and vesicle motility. The methods for measuring tubule index and vesicle motility are described in the Material and methods section. Bar = 10 μ m. For statistical analysis numbers are averages $\pm$ S. D., with n = 6-10, from 2-3 experiments.

Figure 6.3. Effect of CRIB domain on Toca-1/N-WASP phenotype.

Cells were transfected with mRFP-Toca-1 and GFP-N-WASP cDNA with and without CRIB cDNA and left to express mRFP/GFP for 36 hours as described in the Experimental procedures section. Cells were then selected based on Toca-1 mRFP fluorescence intensity and scored for (A) tubule length and (B) vesicle number per cell. In addition, (C) for each cell a Z-stack of approx 28 sections (0.3 μ M per section) was acquired and vesicle number per section measured. In (A) and (B) each point represents an individual cell. In (C) the vesicle number is an average $\pm$ S. D., with n = 4 from 2-3 experiments.

CHAPTER 7 Discussion

7.1 The Toca-1-N-WASP complex links filopodia formation to endocytosis.

Work in the mid-1990's linked signaling *via* Cdc42 to filopodia formation (Kozma et al., 1995; Nobes and Hall, 1995). A large number of Cdc42 effectors and their interacting partners have been isolated over the intervening years (Bishop and Hall, 2000; Cotteret and Chernoff, 2002). The exact role of Cdc42 interacting protein kinases (such as MRCK, PAK and ACK) in filopodia formation is unclear. Certainly there are possible roles for these kinases in modulating actin dynamics and focal complex turnover (Zhao and Manser, 2005). Cdc42 controls filopodia formation primarily by regulating the formation of protein complexes at the plasma membrane through the recruitment of proteins. The Cdc42 effector IRSp53 seems to play a dominant role in protein complex formation connected to filopodia formation (Govind et al., 2001; Krugmann et al., 2001; Disanza et al., 2006; Mattila et al., 2007). Following Cdc42 recruitment of IRSp53 to the plasma membrane, IRSp53 recruits Mena, Eps8 and N-WASP. It has been shown recently that IRSp53 generates filopodia by coupling membrane protrusion (through its I-BAR domain) with actin dynamics through interaction with N-WASP, Mena and Eps8 (Lim et al., 2008). The Rho family GTPases have also been linked to membrane trafficking events (Qualmann and Mellor, 2003). Specifically, Cdc42 is connected to the endocytic trafficking in polarised mammalian cells, *C. elegans* and yeast cells (Kroschewski et al., 1999; Adamo et al., 2001; Fares and Greenwald, 2001). The mechanism(s) by which Cdc42 regulates endocytosis in mammalian cells is unclear.

N-WASP has been linked to filopodia formation and endocytosis. Initial work with N-WASP found that it could drive filopodia formation and dominant negatives could block Cdc42 dependent filopodia formation (Miki et al., 1998a). N-WASP plays an important role in filopodia formation downstream of Cdc42 and IRSp53 (Lim et al., 2008). N-WASP has been found associated with endocytic vesicles in *Xenopus* oocytes (Taunton et al., 2000). Furthermore,

Benesch et. al., (Benesch et al., 2002), demonstrated that N-WASP is essential for PIP5 kinase induced vesicle motility. Toca-1 has also been linked with endocytosis (Tsujita et al., 2006). Toca-1 and N-WASP are both Cdc42 interacting proteins and therefore the Toca-1-N-WASP complex could play roles in filopodia formation and/or endocytosis.

7.1.1 Toca-1/N-WASP induces filopodia formation.

Toca-1 (Transducer of Cdc42 mediated actin polymerization) was identified by Ho et al using *Xenopus* extract as an essential component of the Cdc42 mediated actin polymerization (Ho et al., 2004). Toca-1 belongs to the PCH (Pombe Cdc15 homology) family and contains an F-BAR domain at the N-terminal, an HR1 domain in the middle and an SH3 domain at the C-terminal. Toca-1 can either directly or indirectly activate N-WASP downstream of Cdc42 thus stimulating actin polymerization. Little is known about its function *in vivo*. Here N1E115, a neuroblastoma cell line was used as a model to study the *in vivo* function of Toca-1. N1E 115 has been used to study the morphological roles of many proteins including Rho GTPases and effectors (Kozma et al., 1995; Kozma et al., 1996; Kozma et al., 1997; Sarner et al., 2000). Filopodia are dynamic actin-based structures that protrude from the plasma membrane and are thought to play diverse roles in a number of cellular and developmental processes such as synaptogenesis, dorsal closure in embryos and viral uptake (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008; Sherer et al., 2007). Toca-1 induces filopodia formation and neurite outgrowth. The Toca-1 induced filopodia had the following characteristics; an average lifetime of 127.5 secs and average length of 6.6 μ M comparable to the endogenous filopodia and those induced by IRSp53, N-WASP or Cdc42 (Lim et al., 2008). Toca-1 interacts with Cdc42 through the HR1 domain and N-WASP through the SH3 domain. N-WASP is a well-known Cdc42 binding protein and there are multiple studies showing that N-WASP contributes to the filopodia formation (Miki et al., 1998a; Miki and Takenawa, 1998; Nakagawa et al., 2001). Whether N-WASP contributes to filopodia formation through stimulating the Arp2/3 mediated actin

polymerization is controversial (Lommel et al., 2001; Snapper et al., 2001). Instead, N-WASP has been shown to be implicated in filopodia formation independent of its role in stimulating the Arp2/3 complex (Lim et al., 2008). IRSp53, which contains an I-BAR domain at the N-terminal, was shown to play an essential role in filopodia formation. IRSp53 directly binds to N-WASP through the SH3 domain. N-WASP may open up the self-inhibitory conformation of IRSp53 through direct binding, which further recruits other proteins that functions in actin dynamics such as N-WASP, MENA, WAVE and Eps8.

Further analysis of the various domain mutants shows that the F-BAR domain, HR1 domain and SH3 domain are required for its filopodia induction activity. The F-BAR domain dimerizes and has been shown to deform membrane *in vitro* and *in vivo* (Itoh et al., 2005; Tsujita et al., 2006). The F-BAR domain deforms membrane inwardly and it is unlikely that its membrane deformation activity plays a direct role in filopodia formation, in which membrane protrude outwardly. It was observed that Toca-1 and N-WASP colocalized in Rab5 positive vesicles. The F-BAR domain may play a role in the Toca-1/N-WASP vesicular localization/endocytosis and indirectly contribute to filopodia formation through intracellular vesicle trafficking. The HR1 domain interacts with Cdc42. The failure of the HR1 domain mutant of Toca-1 to induce filopodia formation reveals the essential role of Toca-1 as a Cdc42 effector in filopodia formation. The SH3 domain interacts with poly-proline regions found in many proteins such as N-WASP and dynamin, which in turn will recruit many other SH3 domain containing proteins that function in actin polymerization/dynamics and endocytosis.

Toca-1 also induces neurite outgrowth. In contrast to filopodia induction activity, the interaction between Toca-1 and Cdc42 was essential for neurite outgrowth, while the F-BAR and SH3 domains were dispensable. However, the mechanism of neuritogenesis is not clear. A recent study showed that filopodia were essential for the neurite formation (Dent et al., 2007). However, in this study there was no dynamic analysis to support whether the protrusive structures observed were true filopodia or not. Thus the relationship between neurite outgrowth and filopodia

formation is still not clear. In this study, it appears that filopodia formation and neuritogenesis may be two distinct processes since the requirement of domain structures for filopodia formation and neurite outgrowth is different. A study by Tetsuhiro Kakimoto et al (Kakimoto et al., 2006) showed that overexpression of Toca-1 suppressed neurite elongation in PC12 cells, which contradicts to my result. The inconsistent result obtained for Toca-1 in neurite outgrowth may be due to the different model system used. N1E115 cells have been used as a model system to study the morphological roles of Rho GTPases and effectors. While the functions of Cdc42 in N1E115 cells are well understood, the functions of Rho GTPases are not clear in PC12 cells. As a Cdc42 effector, the intracellular function of Toca-1 is more appropriate to be addressed in N1E115 neuroblastoma cells. Kakimoto et al uses PC12 cells which already have long neurites in normal growing conditions while the neuroblastoma cells I used are round or flat cells without neurites under normal growth conditions, which gives a clean background for the study. Cdc42 is important for neurite outgrowth (Hall, et al., 2001; Ishii, et al., 2001). The Cdc42 binding mutant of Toca-1 failed to induce neurite outgrowth in contrast to other Toca-1 mutants. This data suggests that the signaling pathway from Cdc42 to Toca-1 is important for neurite outgrowth possibly through regulating actin polymerization.

7.1.2 Toca-1 requires N-WASP for filopodia formation

It is established that Toca-1 and N-WASP interact with each other in *in vitro* pulldown assay. To address whether N-WASP contributes to the filopodia induction activity of Toca-1, N-WASP knockout cells were used. Toca-1 failed to induce filopodia in N-WASP knockout cells and Toca-1 can induce filopodia formation after reconstitution with low amount of N-WASP in N-WASP knockout cells. This data strongly suggests that N-WASP is essential for Toca-1 induced filopodia formation. N-WASP is shown to be involved in filopodia formation in several studies (Miki and Takenawa, 1998; Miki et al., 1998a; Nakagawa et al., 2001). N-WASP can also induce filopodia formation in N1E115 cells. When Toca-1 and N-WASP are coexpressed, there is

a synergistic effect on filopodia formation. Having established that N-WASP is essential for Toca-1 induced filopodia formation, the reverse question is whether Toca-1 is essential for N-WASP induced filopodia formation. After knockdown of Toca-1 to a negligible level by RNAi, N-WASP induced filopodia is drastically reduced. Overexpression of three F-BAR domain mutants also inhibits filopodia formation induced by overexpression of N-WASP. The conserved basic residues in the F-BAR domain are responsible for oligomerization and membrane binding. Oligomerization through the F-BAR domain is shown to be essential for induction of microspikes by syndapin, another F-BAR domain containing protein (Kessels and Qualmann, 2006). The three F-BAR domain mutants may play dominant negative roles through inhibition of oligomerization and membrane binding of Toca-1, which may be essential for N-WASP to induce filopodia formation. In combination, these data suggest that Toca-1 and N-WASP require each other to induce filopodia formation.

7.1.3 Toca-1 directly interacts with N-WASP in vesicles and filopodia

To understand the role of Toca-1 in filopodia formation, the first question is about the cellular localization of Toca-1. Toca-1 was shown to partially colocalize with the early endosome marker, Rab5. When Toca-1 was coexpressed with N-WASP, Toca-1 and N-WASP were strongly colocalized in the vesicles, which were positive for Rab5. Rab5, a small GTPase, is an important regulator of intracellular membrane trafficking in the endocytosis pathway and it is proposed that Rab5 plays a role in the organization of the early endosomal membrane through interaction with its effectors (Zerial and McBride, 2001). Coexpression of Toca-1 and N-WASP affects the size and distribution of Rab5 positive vesicles. These data suggest that Toca-1-N-WASP complex is implicated in the endocytosis/ membrane trafficking.

Toca-1 is a member of the F-BAR domain protein family and contains an F-BAR domain at the N-terminal. Toca-1 has also been linked with endocytosis (Tsujita et al., 2006). Tsujita et. al., investigated the biology of the F-BAR domains in CIP4, FBP17 and showed that they could

tubulate membranes. The SH3 domains of CIP4, FBP17 and Toca-1 recruited N-WASP and dynamin facilitating the coupling of membrane reorganisation to actin polymerization. Furthermore, they showed that KD of Toca-1, CIP4, and FBP17 inhibited endocytosis significantly, while the effects of individual KD of these proteins were less effective. These data suggest that there is significant redundancy and this makes it difficult to investigate the individual roles of Toca-1, CIP4 and FBP17 in endocytosis.

N-WASP was also found to play a role in multiple steps during endocytosis. The actin polymerization through the N-WASP-Arp2/3 pathway was shown to play multiple roles during clathrin mediated endocytosis (Qualmann and Kelly, 2000; Qualmann and Kessels, 2002; Engqvist-Goldstein and Drubin, 2003; Merrifield et al., 2004). N-WASP was shown to be recruited to coated pits using live cell imaging and N-WASP knockdown by RNAi decreased the uptake rate of epidermal growth receptor (EGFR) (Merrifield et al., 2002; Benesch et al., 2005; Innocenti et al., 2005).

The data suggests that Toca-1-N-WASP works together as a protein complex in filopodia formation and the localization of Toca-1-N-WASP in Rab5 positive vesicles indicates its role in membrane trafficking/endocytosis. Toca-1 directly binds to N-WASP and regulates its function *in vitro* (Ho et al., 2004). Here I show for the first time by FRET and FLIM that Toca-1 and N-WASP interact with each other *in vivo*. FRET will occur under certain conditions. The two molecules must be within 10 nm distance and the dipoles are in correct orientation. Furthermore the emission wavelength of donor should overlap with the excitation wavelength of acceptor. Here GFP and mRFP were used in acceptor photobleaching FRET. The use of FRET in examining protein interaction is more advantageous over biochemical methods in that FRET provides spatial information by user-defined selectively bleaching of specific region of interest (ROI). Toca-1 and N-WASP were shown to interact specifically in filopodia and vesicles by FRET.

7.1.4 Mechanism of the Toca-1-N-WASP complex induced filopodia formation

Toca-1 partially colocalized with Rab5 and was only expressed at the filopodia base and along neurites with a good colocalisation with actin in vesicular structures when it was not coexpressed with N-WASP. On the contrary, N-WASP was localized in the filopodia when it was not coexpressed with Toca-1. Interestingly, Toca-1 was found to be localized in the filopodia when it is coexpressed with N-WASP. Coexpression of Toca-1 and N-WASP synergistically induced filopodia formation. These data suggest that Toca-1 may be recruited to the filopodia by N-WASP and the Toca-1-N-WASP thus stimulates the filopodia formation.

So what is the potential mechanism by which Toca-1-N-WASP complex regulates filopodia formation? The phenotype of Toca-1 is reminiscent of work reported a few years ago on Syndapins/PACSIN by Qualmann and Kelly (Qualmann and Kelly, 2000). Syndapin has a domain structure similar to Toca-1; F-BAR and SH3 domain (but no Cdc42 interaction site). Like Toca-1, Syndapin interacts with N-WASP and is involved in endocytosis. Syndapin was also found to induce actin microspike formation, structures that resemble filopodia. With Toca-1 I show conclusively that it generates filopodia by carrying out time-lapse experiments using GFP-actin transfected cells. The lifetime and length of Toca-1 generated filopodia is similar to that of endogenous filopodia and those generated by Cdc42/RacN17, IRSp53 and N-WASP (Lim et al., 2008). More recent work with Syndapin shows that it dimerises (and oligomerises) and these events are required for its function (Shimada et al., 2007). Similarly it is possible that CIP4 and FBP17 (and by analogy Toca-1) deform membranes by forming dimers and oligomers on the membrane surface. The potential consequence of Toca-1 and/or Syndapin oligomers on the membrane is the generation of a localized concentration or “hotspots” of specific SH3 domains. These SH3 domains could recruit polyproline containing proteins such as N-WASP, Mena and Eps8; proteins that are directly implicated in filopodia formation. The hypothesis is stronger for Toca-1, CIP4 and FBP17, than for Syndapins, as these former proteins possess Cdc42-binding

sites and would thus also recruit Cdc42, creating “hotspots”, of SH3 domains and Cdc42 binding sites on the membrane. Subsequently, Cdc42 may recruit proteins such as IRSp53 that directly activate filopodia formation.

One could also visualize the reverse scenario with Cdc42-IRSp53 activation inducing filopodia formation, N-WASP and then Toca-1 recruitment to the membrane, and a subsequent stimulation of endocytosis. In this model, N-WASP plays a prominent role in the coupling of filopodia formation to endocytosis.

7.1.5 Is there a linkage between filopodia formation/actin cytoskeleton and endocytosis?

Toca-1 and N-WASP synergistically induce filopodia formation. Toca-1 localized with Rab5 positive vesicles and associated with actin. Expression of Toca-1 together with N-WASP induced the formation of Rab5 positive vesicles. N-WASP and N-WASP/Toca-1 affected the distribution, size and number of Rab5 vesicles but Toca-1 alone did not. The lack of effect of Toca-1 alone may be due to the redundancy in the system (with CIP4 and FBP17, as mentioned above). This result also suggests that the control of Rab5 vesicle trafficking may lie in the regulation of and by N-WASP. Using FRET it is established that there are two major cellular sites of Toca-1 interaction with N-WASP; filopodia and Rab5 vesicles. Taken together, these data suggest that the Toca-1-N-WASP complex can regulate the formation of both filopodia and endocytic vesicles.

The induction of filopodia formation by Toca-1 was inhibited by blockers of endocytosis. Dominant negative Eps15 and dynamin were used as blockers of endocytosis. Eps15 (EGF phosphorylation substrate 15) is a key component for assembling functional clathrin-coated pits (Benmerah et al., 1999; Sever et al., 2000). Eps15 is ubiquitously and constitutively associated with AP-2. The N-terminal of Eps15 contains three EH (Eps homology) domains which bind regulatory proteins containing NPF amino acids motif, including epsin, AP180, synaptojanin. The central domain allows for oligomerization of Eps15 into homodimers. The C-terminal domain

contains AP-2 binding site, which together with EH domains, is required for targeting of Eps15 to clathrin-coated pits. Dominant negative mutant Eps15- Δ 95/295 lacks second and third EH domains, whereas the inactive variant Eps15-D3 Δ 2 is a C-terminal domain construct lacking all AP-2 binding sites. Expression of dominant negative mutant Eps15- Δ 95/295, but not inactive variant Eps15-D3 Δ 2, inhibits clathrin-mediated endocytosis of transferrin in many cell types (Benmerah et al., 1999). Receptor endocytosis is regulated by dynamin, a GTPase that is targeted to clathrin-coated pits where it oligomerizes around the neck of budding vesicles (reviewed in (Schmid et al., 1998)). Both clathrin- and caveolae-dependent endocytosis require the activity of GTPase Dynamin (Henley et al., 1998; Sever et al., 2000). Dynamin with a point mutation in the nucleotide-binding site (K44A) interferes with the function of endogenous Dynamin by blocking vesicle internalization before membrane scission occurs (Damke et al., 1994). Both dyn1(K44A) and dyn2(K44A) were potent inhibitors of receptor-mediated endocytosis; however neither mutant directly affected other membrane trafficking events, including transport mediated by four distinct classes of vesicles budding from the TGN (Altschuler et al., 1998). Both dominant negative Eps15 and dynamin inhibit filopodia formation. This suggests that there is a link between clathrin mediated endocytosis pathway and filopodia formation. To further investigate this point, I knocked down clathrin heavy chain using RNA interference. After knockdown of the clathrin heavy chain, filopodia formation was also reduced. This supports the idea that filopodia formation is specifically connected to clathrin mediated endocytosis pathway.

The induction of filopodia formation by Toca-1 was inhibited by blockers of endocytosis. Is there a potential physiological function of linking filopodia formation to endocytosis? Lidke et al. (Lidke et al., 2005) have proposed that filopodia act as sensing organelles probing for receptor ligands facilitating activation of cell signaling pathway events through receptor endocytosis. Examples of processes that link filopodia with endocytosis include; (i) Migrating border cells of *Drosophila* where polarised receptor cycling act as guidance cues (Jekely et al., 2005; Yoneyama et al., 2004). (ii) Growth cone collapse driven by Sema3A where endocytosis is enhanced and this

is linked with changes in F-actin (Luo et al., 1993). and (iii) Growth cone guidance where cycling of adhesion molecules such as (CAM) L1 facilitates cell movement (Kamiguchi and Lemmon, 2000) (iv) Unc-51-like kinase 1/2 is required for NGF endocytosis and it inhibits excessive filopodia protrusion and axon branching in NGF induced axon outgrowth (Zhou et al., 2007).

In *Drosophila* Rab5 KO leads to defects in endocytosis and in cell polarity (Lu and Bilder, 2005). Furthermore, a recent genome wide RNAi screen of proteins involved in membrane trafficking (including endocytosis) identified proteins linked with cell polarity (Balklava et al., 2007). Prominent among the proteins implicated in endocytosis and cell polarity were Cdc42-PAR3/6-PKC complex. In yeast Marco et. al., (Marco et al., 2007) have recently modeled cell polarity using Cdc42Q61L expression and found that endocytosis is likely to play a crucial role. Thus there is evidence for a link between cell polarity and endocytosis.

In response to environmental guidance cues, such as in cell migration and growth cone guidance, cells or growth cones protrude filopodia/lamellipodia at the leading edge, which is one of the characteristics of cell polarization. The asymmetric distribution of other molecules between leading edge and the rear is also required for cell motility or growth cone advancement, for example, the cell adhesion molecules. In the leading edge, cells or growth cones need to establish solid adhesion with the extracellular matrix; while at the rear of the cell, the cell-matrix adhesion needs to be dislodged. What is the mechanism to establish the asymmetry/polarity? Endocytosis and the membrane recycling/trafficking uptake and transport molecules in certain area of the cells and play important roles in creating asymmetric distribution of proteins. For example, in the L1-dependent growth cone advancement, L1 are endocytosed in the central domain and recycled to the leading edge, which is essential for the growth cone motility (Kamiguchi and Lemmon, 2000). This suggests that coordinated endocytosis and actin cytoskeleton is important in growth cone motility. Filopodia also serve as sensing organelles in probing the environment, for example in response to the growth cone repulsive molecules Sema3A. How is the environmental cues translated into the cellular responses? It was found in Sema3A induced growth cone collapse the

endocytosis of Sema3A receptor was enhanced together with the corresponding changes in F-actin (Luo et al., 1993). Another example is the EGF signaling. Filopodia can sense the environmental EGF and transport them through retrograde flow (Lidke et al., 2005). The further signal transduction of EGF is through the EGF endocytosis at the base of the filopodia. These suggest that endocytosis may be one step of the signal transduction process after the initial response of filopodia to the environmental cues. The oligomerization of the BAR domain proteins, which play important roles in endocytosis could provide a mechanism for the link between filopodia formation and endocytosis. Oligomerization is essential for BAR domain proteins function and the oligomerization of BAR domain proteins will provide a localized high concentration of other protein domains, such as SH3 domain and Cdc42 binding domain. These hotspot domains will further recruit their binding partners, such as Cdc42, IRSp53 and N-WASP. Thus filopodia formation and endocytosis may be orchestrated.

I show here for the first time that the Toca-1-N-WASP complex localizes to filopodia and Rab5 vesicles and that this complex induces the formation of these important cellular structures.

7.2 Cdc42 interaction with N-WASP and Toca-1 regulates the membrane tubulation, vesicle formation and vesicle motility.

7.2.1 Toca-1-N-WASP induces membrane tubules and vesicles formation.

The role of BAR domains in membrane deformation has opened-up the investigation of how membranes are remodeled during cellular processes. Recent work on FBP17, CIP4 and Toca-1 suggest a role for these proteins in endocytosis (Itoh et al., 2005; Frost et al., 2009; Shimada et al., 2007). The presence of a SH3 domain in FBP17, CIP4 and Toca-1 that binds N-WASP provides a link to the actin machinery through the Arp2/3 complex. A third domain (HR1) is the Cdc42 binding site. Either full length CIP4/FBP17 or the F-BAR domain of CIP4/FBP17 can deform membrane into tubules *in vitro* and *in vivo* (Itoh et al., 2005; Tsujita et al., 2006). Unlike

FBP17 and CIP4, Toca-1 alone did not induce membrane tubulation. I found that expression of Toca-1 together with N-WASP was required to induce membrane tubules and vesicles. The Toca-1-N-WASP phenotype allowed me to investigate distinct steps in membrane trafficking connected with endocytosis. This approach is necessary as the endogenous steps of membrane tubulation, vesicle formation and motility are very difficult to follow. In the present study, I have investigated the role of Cdc42 interaction with Toca-1 and N-WASP in the mammalian cells and demonstrated how the activity of F-BAR domain is regulated.

7.2.2 Actin polymerization and Toca-1-N-WASP induced tubules and vesicles.

Time lapse and FRAP analysis showed that both vesicles and tubules were motile. What is the driving force for the motility? Toca-1 and N-WASP both bind to Cdc42 and N-WASP is well known for its function in stimulating the Arp2/3 mediated actin polymerization. Actin polymerization has been shown to be involved in multiple endocytosis pathways (Miaczynska and Stenmark, 2008). It has also been shown that dynamic actin filaments colocalize with endosomes in several cell types (Kaksonen et al., 2000; Merrifield et al., 1999; Taunton et al., 2000; Orth et al., 2002; Rozelle et al., 2000). Actin polymerizes at one side of endosomes and forms the “comet tail” phenomenon when the vesicles move. Toca-1 has been shown to be essential for PMA induced actin comet formation/vesicle mobility in *Xenopus* extract (Ho et al., 2004). GFP-actin was used to follow the actin cytoskeleton. In my study, after coexpression of Toca-1, N-WASP and GFP-actin in the mammalian cells, actin also formed “comet tail” following moving vesicles labeled either with Toca-1 or N-WASP. This result suggests that actin polymerization is responsible for the motility of the vesicles induced by Toca-1 and N-WASP. The function of actin polymerization in vesicle motility is further confirmed by treatment the cells with cytochalasin D, an actin depolymerization drug. After cytochalasin D was added into the cells overexpressed with Toca-1 and N-WASP, the vesicles lost their motility almost immediately. Furthermore the vesicles were aligned into static tubules which colocalised with F-actin. The

FRAP analysis revealed that the tubules were also dynamic. Disassembly of the actin cytoskeleton by cytochalasin D resulted in static tubules. Taken together, these results reveal that the actin cytoskeleton is essential for the dynamic membrane remodeling induced by Toca-1 and N-WASP.

7.2.3 What is responsible for the polarized actin polymerization?

During movement of vesicles, actin polymerizes only at one side of the vesicle and thus forms the “actin comet” to provide the mechanical forces required for the movement of the vesicles. What drives this polarity? N-WASP downstream of Cdc42 activates Arp2/3 to stimulate actin polymerization. Another important regulator for Cdc42-N-WASP mediated actin polymerization is PIP2, which activates N-WASP synergistically with active Cdc42. Thus localized PIP2 may activate actin polymerization in specific localization of plasma membrane. Cdc42, N-WASP and PIP2 may be the possible candidates. As N-WASP is colocalized well with Toca-1 on the whole vesicles, it is unlikely that the polarity is determined by N-WASP. However the resolution of the microscope prevented me from getting a clear-cut localization result of the above candidate proteins on the vesicles. Maybe the use of the high resolution microscopy in the future will resolve the issue.

7.2.4 Microtubules and Toca-1-N-WASP induced vesicle/tubule phenotype

Microtubules are involved in the intracellular transport either through microtubule dynamics or the motor proteins on the microtubules. To investigate whether microtubules participated in the Toca-1-N-WASP induced vesicle and tubule phenotype, nocodazole, a microtubule disrupting drug was used to treat the cells. From the fixed samples, cells with vesicular phenotype were still there, while the cells with tubules were absent. The FRAP analysis of the vesicles after nocodazole treatment showed that the vesicles were similarly motile compared to the control cells. This result indicates that microtubules are involved due to the absence of cells with tubule

phenotype. In the timelapse experiment, tubules of Toca-1-N-WASP expressing cells disappeared with time and more and more vesicles appeared. Thus microtubules prevent the vesicles formation from tubules. Toca-1 is a member of PCH family protein. The FCH domain at the N-terminal has been shown to be associated with microtubules and unpolymerized soluble tubulin (Tian et al., 2000). The FCH domain plays a role in microtubule nucleation and bundling (Takahashi et al., 2003). It is likely that Toca-1 brings microtubules into this picture. The mechanism of microtubules in Toca-1-N-WASP induced tubules/vesicles needs to be further investigated.

7.2.5 Effect of dynamin on Toca-1-N-WASP induced tubules and vesicles.

Dynamins self-assemble into polymers, which constrict upon GTP hydrolysis. The interaction of the PH domain with membrane lipids such as PIP2 or the binding of PRD domain to the SH3 domain containing proteins can result in the hydrolysis-constriction cycle (Lin et al., 1997). Several functions are postulated for this constriction process. Firstly, together with BAR-domain containing protein and actin cytoskeleton, dynamin can deform membrane (Itoh et al., 2005). Secondly, this constriction is essential for the vesicle scission during endocytosis (Marks et al., 2001). Study by Itoh et al demonstrated that dynamin/F-BAR domain protein/actin played a role in membrane tubulation and vesiculation (Itoh et al., 2005). F-BAR domain proteins deform and tubulate membrane. F-BAR domain proteins bind to dynamin. Overexpression of dynamin together with the F-BAR domain inhibited the strong tubulation activity of F-BAR domain proteins. Instead of tubulation, the vesiculation was observed in this scenario, which suggested that probably the vesicle scission activity of dynamin resulted in the vesiculation of the tubules. This vesiculation activity can be reduced by overexpression of the K44A mutant of Dyn2 or treatment with latrunculin, an actin antagonist. This result suggests that the vesicle scission activity of dynamin is dependent on the intact actin cytoskeleton. In my study, expression of wild type dynamin promoted vesicle formation which maybe due to the elevated membrane scission

activity. Consistent with the previous study (Ito, et al., 2005), expression of K44A dynamin inhibited the vesiculation and only membrane tubules were observed. Interestingly, when Toca-1 and dynamin were coexpressed, both proteins were colocalized in vesicles similar to those when dynamin was expressed alone. Toca-1 was probably recruited to the endocytic sites by dynamin. These vesicles are not motile probably because there was not enough N-WASP and the resulting actin polymerization. This result supports the critical role of N-WASP mediated actin polymerization in the generation and motility of the endocytic vesicles.

7.2.6 Functions of Cdc42 in regulating Toca-1-N-WASP induced vesicle/tubule phenotype.

Toca-1 and N-WASP both bind to Cdc42. Cdc42 acts upstream of Toca-1 and N-WASP. Furthermore, Toca-1 and N-WASP interact with each other. Thus the protein complex Cdc42-Toca-1-N-WASP may regulate the formation of vesicles and tubules. However, Cdc42, Toca-1 and N-WASP may not always work at the same time. What is the role of each protein interacting pair in the generation of phenotypes?

When Toca-1W518K, a mutant unable to bind to N-WASP, was coexpressed with N-WASP, the cells totally lost vesicles and tubules. Thus the interaction between Toca-1 and N-WASP is crucial.

What is the role of Cdc42-Toca-1 interaction in vesicle/tubule induction? I tried several approaches to answer this question. First the constitutive active or dominant negative Cdc42 was used. The use of constitutive active Cdc42 with Toca-1 and N-WASP dominantly generated tubules, in which there was a good colocalisation between Toca-1 and Cdc42. When dominant negative Cdc42 was coexpressed with Toca-1, both tubules and vesicles were inhibited. This result suggests that Cdc42 is an essential upstream regulator of the phenotype. The constitutive Cdc42 data suggests that the cycling between a GTP and GDP bound state may be important for the transition between vesicles and tubules. The second approach is to generate a Toca-1 mutant MGD383-385IST, which is unable to bind with Cdc42. When Toca-1 mutant MGD383-385IST

was coexpressed with N-WASP, the tubules were much shorter than those of the control cells. Thus Cdc42-Toca-1 interaction may regulate the membrane deformation process manifested by the extent of tubulation. In this case, the vesicles were not affected. Presumably, the domain structure in Toca-1 responsible for the membrane deformation is the F-BAR domain. However F-BAR domain or the full length Toca-1 was not enough to induce membrane tubulation and the interaction with N-WASP was required. When various conserved residues were mutated in the F-BAR domain in full length Toca-1, the phenotypes generated by these F-BAR domain mutants and N-WASP were only vesicles, but no tubules. If vesicles are formed from tubules, there should be no vesicles formed without tubules. This result may be explained by the redundant role between Toca-1/CIP4/GBP17 in membrane tubulation. CRIB and GBD domains are Cdc42 binding motifs. The CRIB domain also binds to Rac besides Cdc42 and GBD domain is specific for Cdc42 binding. Expression of either domain will inhibit functions of endogenous Cdc42. The coexpression of CRIB or GBD together with Toca-1 and N-WASP ends up with shorter tubules, which is consistent with the result using Toca-1MGD383-385IST mutant suggesting a role of Cdc42-Toca-1 interaction in membrane deformation. Another observation is that the number of vesicles is less than that of the control. This indicates that the formation of vesicles from tubules is affected by perturbation of the Cdc42 level and Cdc42 regulates the vesicle formation. Taken together, these results reveal that the full length Toca-1 and the interaction between Toca-1 and N-WASP are crucial for tubule formation. Cdc42 acts upstream of Toca-1 to regulate the membrane deformation. Dynamin regulates the vesicle formation from tubules. The data also suggests a role of Cdc42 in the vesicle formation process.

To investigate the role of Cdc42-N-WASP interaction in vesicle and tubule formation, the N-WASPH208D mutant was used. N-WASPH208D is defective in Cdc42 binding. Interestingly, both phenotypes were absent in this condition. This data suggests that the Cdc42-N-WASP interaction is crucial for the generation of both vesicles and tubules. Due to the total loss of phenotype, it is difficult to dissect the exact role of Cdc42-NWASP interaction in different steps

of the endocytosis. To address this question, it is necessary to generate the phenotype first and then perturb the system using the mutant. In this scenario, I generated the vesicle/tubule phenotype using low amount of wild type Toca-1 and N-WASP and later exchanged N-WASP with N-WASPH208D. N-WASPH208D was successfully exchanged onto the vesicles. However, the vesicles motility was lower than that of control cells. Thus it supports that the actin polymerization stimulated by the Cdc42-N-WASP interaction regulates the vesicle motility. N-WASP activates the Arp2/3 mediated actin polymerization. This result is consistent with the actin comet tail and cytochalasin D experiments, which suggest a role of actin polymerization in the vesicle mobility. Thus Cdc42 upstream of N-WASP regulates the actin polymerization process. Regarding the role of Cdc42-N-WASP interaction in the tubule formation, there is a significant reduction of membrane tubulation after N-WASPH208D exchange. The good colocalisation of F-actin with the tubules suggests a role of actin polymerization in tubulation. The FRET analysis of Cdc42-Toca-1-N-WASP complex showed that Cdc42 interacted with both Toca-1 and N-WASP on tubules while Cdc42 only interacted with N-WASP, but not Toca-1 on vesicles. This result also supports a role of Cdc42-N-WASP interaction for tubule formation. So actin polymerization is involved in the tubule formation. It may provide the force for the membrane deformation as suggested by other studies.

7.2.7 Which endocytosis pathway does the Cdc42-Toca-1-N-WASP complex regulate?

Toca-1 and N-WASP coexpression gives a dynamic and interconvertible vesicle/tubule phenotype. The motility rate and the size of the vesicles are within the range of the endocytic vesicles. Dynamin is well-known for its vesicle scission role during endocytosis pathway. In my system, wild-type dynamin coexpression with Toca-1 and N-WASP gave much more vesicles. In agreement with the function of dynamin in vesicle scission, the co-expression of dominant-negative form of dynamin with Toca-1 and N-WASP resulted in tubules possibly due to blockage

of the scission process by dominant negative dynamin. Results using various PH probes indicated that the vesicles and tubules were PIP2 enriched membrane structures.

There are multiple endocytosis pathways (clathrin dependent and independent pathways) to perform the diverse functions such as nutrient uptake, receptor recycling, synaptic vesicle recycling, cell polarity and remodeling of the plasma membrane. To test which endocytosis pathway was involved, transferrin, cholera toxin B and dextran were used as markers for clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis pathway respectively. Coexpression of Toca-1 and N-WASP inhibited transferrin uptake while the uptake of the other markers was not affected. In combination of the localization study, the vesicles were positive for Rab5 and Rab7. Vesicles and tubules colocalised with clathrin well, but not caveolin. The colocalisation marker and uptake data presented here suggest Toca-1-N-WASP complex plays a role in clathrin mediated endocytosis.

7.2.8 Summary of effects of various factors on Toca-1-N-WASP induced tubules and vesicles.

In my study, I have tried various reagents on Toca-1-N-WASP generated tubules and vesicles. Either stimulatory or inhibitory effect was observed for each reagent on the tubules/vesicle formation or tubules/vesicles dynamics as summarized in Figure 7.1.

7.2.9 The proposed model

The *in vivo* function of Toca-1 is largely unknown after it was identified in 2004 by Ho et al (Ho et al., 2004). I show here that Toca-1 together with Cdc42 and N-WASP forms a protein complex that regulates different events connected to clathrin mediated endocytosis pathway, i.e. membrane deformation, vesicle formation and vesicle motility. The regulatory factors of each event were investigated. The actin polymerization mediated by Cdc42-N-WASP plays important roles in membrane deformation, vesicle formation and vesicle motility. Besides actin

cytoskeleton, microtubules were shown for the first time to be involved in the vesicle formation process.

From the analysis of the Toca-1-N-WASP expression phenotype, I established assays for; (1) Membrane deformation – tubule index, (2) Vesicle formation (vesicle numbers/index and distribution) and (3) Vesicle motility (time-lapse analysis of GFP/mRFP labeled proteins). Having established assays for the cellular function of Toca-1-N-WASP complexes I investigated the role of Cdc42 in the phenotypes.

Tubulation. FRET analysis revealed that Cdc42 interacted with N-WASP and Toca-1 on tubules. The Toca-1MGD383-385IST mutant is not able to bind Cdc42 and with N-WASP induced formation of tubules and vesicles. However, the tubules formed were shorter and the vesicles smaller. I interpret this data as evidence that Cdc42 interaction with Toca-1 regulates but is not essential for F-BAR domain activity i.e, membrane deformation. In contrast, the N-WASP mutants, H208D and Δ WA, expressed with Toca-1 did not give a phenotype. This result suggests that Cdc42 interaction with N-WASP, and N-WASP with Arp2/3, are essential for membrane deformation. This is supported by the result obtained by substituting N-WASP with N-WASPH208D which resulted in a reduced number of tubules. FRET data showed that the N-WASPH08D mutant did not form a complex with Toca-1 and this may be the reason for the null phenotype. In contrast, the Δ WA N-WASP mutant did form a complex with Toca-1 but still had a null phenotype. From the protein-protein interaction-phenotype correlations I hypothesize that Cdc42-interaction with N-WASP is critical to “open” the protein and expose the polyproline rich and WA domains, which then bind Toca-1 SH3 domain and the Arp2/3 complex, respectively. Binding of N-WASP polyproline rich domain by Toca-1 SH3 domain may reveal its F-BAR domain allowing it to couple membrane deformation with actin polymerization via Arp2/3 complex (see Fig. 7.2 for model).

Figure 7.1 Effect of various reagents on Toca-1-N-WASP induced tubules and vesicles.

TIRFM showed that Toca-1/N-WASP generated tubules and vesicles were in dynamic interchange. Cdc42 binding-defective mutants of Toca-1 and N-WASP together gave a null phenotype suggesting the Cdc42-Toca-1-N-WASP complex was essential. AP-FRET analysis showed that Cdc42-Toca-1-N-WASP trimer complex exists on both tubule and vesicles. The role of Cdc42 in regulating the activity of the Toca-1-N-WASP complex was investigated by using; (i) protein domains that bind active Cdc42 – CRIB and GBD, (ii) dominant negative and positive versions of Cdc42, and (iii) exchange of N-WASPH208D into wild-type complexes. The data generated from these experiments suggest that Cdc42 regulates membrane tubulation, vesicle formation and motility. Proteins in red indicate inhibition. Proteins in green indicate stimulation.

Vesicle formation. Since vesicle number was unaffected by the Toca-1MGD383-385IST mutation, Cdc42 interaction with Toca-1 is not important for vesicle formation. Since the N-WASPH208D mutant had a null phenotype I could not use this mutant to investigate the role of Cdc42 interaction with N-WASP in vesicle formation or motility. To address the role of the Cdc42–N-WASP interaction in vesicle formation and motility, I used a number of modulators of the Cdc42 pathway. Firstly, CRIB and GBD reduced vesicle number significantly and CRIB affected the distribution of vesicles within the cell. Secondly, in cells expressing high levels of Cdc42V12 or Cdc42N17 with Toca-1/N-WASP no vesicles were seen. This result suggests that cycling of active/inactive Cdc42 is important for N-WASP function in vesicle formation.

Vesicle motility. FRET analysis revealed that Cdc42 interacted with N-WASP but not Toca-1 on vesicles. The vesicles generated by Toca-1/N-WASP were motile and possessed an actin tail in the shape of a comet reminiscent of bacterial/viral motility in mammalian cells. In contrast, Toca-1-Dynamin coexpression generated non-motile vesicles. Toca-1MGD383-385IST or the F-BAR domain mutants did not affect vesicle motility significantly. The addition of either GBD or CRIB to Toca-1/N-WASP cells eliminated vesicle motility. Lastly, exchange of N-WASPH208D into vesicles inhibited their motility. Thus Cdc42 interaction with N-WASP and not Toca-1 is essential for vesicle motility. As found *in vitro*, Toca-1 interaction with N-WASP may be required to activate actin polymerization *in vivo* to drive vesicle motility. Further work is necessary to investigate the role of Toca-1 in controlling N-WASP actin polymerization activity during vesicle movement.

The Cdc42, Toca-1, N-WASP expression analysis presented here allows me to make a number of conclusions about the mechanism of membrane remodeling in cells and how it is regulated. Toca-1 is more tightly regulated than CIP4 and FBP17 and requires N-WASP to induce the tubule and vesicle phenotype. The protein-protein interaction-phenotype correlations suggest that Cdc42 interaction with N-WASP is a critical first step in the formation of the Cdc42–N-WASP–Toca-1 trimer complex (Fig. 7.1). The function of this trimer complex is to couple F-

BAR domain activity with actin polymerization to give dynamic membrane deformation and the formation of motile endocytic vesicles. Dynamin regulates the vesicle formation from tubules. Cdc42-Toca-1 interactions affect tubule length and therefore F-BAR domain activity. Thus Cdc42 activity may regulate membrane trafficking pathways such as endocytosis by controlling the formation and activity of N-WASP-Toca-1 complexes, and tubulation.

7.3 Cdc42 coordinates cell morphology, endocytosis and polarity.

Cell morphology is a fundamental characteristic of all living cells and is correlated with their functions. For example, neurons developed long axons to facilitate efficient transmission of the signals to the cell periphery and other cells. In contrast epithelial cells adopt a tight cell sheet to perform their function as a protective barrier. Cells also change their morphology in various biological processes such as cell migration and mitosis. Cellular signalling pathways determine the asymmetric distribution of certain structures to give cell distinct morphology. One of the well-known polarity complex called partitioning defective (PAR) consisting of Par3, Par6 and aPKC is a downstream effector of Cdc42. GTP-Cdc42-PAR complex controls polarity in several cell types such as neurons, T-cells and epithelial cells and different cellular context such as polarized cell migration. The PAR complex determines the cell polarity by affecting diverse cellular activities, such as axon specification in neurons, the lamellipodia formation in T-cells and tight junction formation in epithelial cells.

Active Cdc42, which is localized to the membrane, serves as an organizing platform to orchestrate the cellular activities through recruiting and activating different protein complexes on the membrane. Cycling between GTP and GDP bound form and associating with certain part of the membrane, Cdc42 determines temporally and spatially the occurrence of cellular activities on the cell membrane. IRSp53, an adaptor protein with multiple domains including an I-BAR domain is recruited to the membrane by Cdc42 and regulates the actin dynamics and filopodia formation possibly through interaction with a group of proteins implicated in the actin dynamics

such as, N-WASP, Mena and Eps8. I-BAR domain of IRSp53 deforms membrane. IRSp53 generates filopodia through coupling membrane deformation with actin dynamics and contributes to cell polarity, for example in directed cell migration. Similar to IRSp53 architecture, Toca-1 belongs to F-BAR domain proteins and contains an SH3 domain. Toca-1 is also a Cdc42 effector. Toca-1 links to the proteins implicated in the actin dynamics through SH3 domain. N-WASP is such a binding partner of Toca-1 and other proteins may also be included. Toca-1-N-WASP complex possibly contributes to filopodia formation through regulation of the actin dynamics and links to the Cdc42-IRSp53 pathway.

The F-BAR domain of Toca-1 generates inward membrane deformations and Toca-1 is involved in the endocytosis pathway and membrane trafficking coupling membrane deformation (F-BAR domain) and actin dynamics (through SH3 domain). There are close similarities between the ability of the I-BAR domain protein IRSp53 (Mattila et al., 2007; Lim et al., 2008; Saarikangas et al., 2008) in generating filopodia and Toca-1 generating membrane vesicles. Both have Cdc42 binding sites and N-WASP binding sites. In the case of IRSp53 mediated filopodia formation its SH3 domain interacts with a number of proteins involved in actin dynamics; Mena (Krugmann et al., 2001), Eps8 (Disanza et al., 2006) as well as N-WASP (Lim et al., 2008). For Toca-1, its SH3 domain seems to be specific for N-WASP but there is no reason why other proteins involved in actin dynamics could not bind it. Cdc42 regulates F-BAR domain activity of Toca-1. Other BAR domain proteins also contain Cdc42 binding site such as IRSp53 and Tuba or RhoGAP domain such as oligophrenin I-1. Thus it is possible that regulation of membrane deformation activity of BAR domain proteins by Cdc42 or Rho GTPases may be a general mechanism in cells. The results presented here showing the coupling of BAR domain activity with actin dynamics as a means to remodel membranes may represent a general mechanism used in the formation of structures such as filopodia, podosomes and T-tubules (Frost et al., 2009).

Taken together, the link through Cdc42 of filopodia formation/cell motility (*via* IRSp53-N-WASP, Mena, Eps8), endocytosis (*via* Toca-1-N-WASP) as well as cell polarity (*via* PAR3-

PAR6) may provide a mechanism to coordinate environmental cues, cell signaling events and cellular/developmental processes (Fig. 7.3).

Figure 7.2. Model for Cdc42 regulation of Toca-1/N-WASP induced membrane tubulation, vesicle formation and motility.

Cdc42 interaction with N-WASP, but not with Toca-1, is essential for the tubule/vesicle phenotype. FRET data shows that the Cdc42 binding defective N-WASPH208D does not interact with Toca-1 and there is no phenotype associated with the Toca-1/N-WASPH208D combination. FRET data also show that N-WASP Δ WA although competent to bind Toca-1 does not induce a phenotype demonstrating the essential role of actin polymerization in membrane remodeling events. The model that emerges from these observations is that active Cdc42 interaction with N-WASP opens the protein, exposing its polyproline rich domain (PP, SH3 binding site) and the WA domain allowing N-WASP to interact with Toca-1 and the Arp2/3 complex (step 1). N-WASP binding to Toca-1 and the Arp2/3 complex couples membrane tubulation with actin polymerization (step 2). Dynamin then allows vesicles to undergo scission leading to the formation of motile vesicles (step 3).

Figure 7.3 Cdc42 is involved in coordinating filopodia formation, endocytosis and polarity.

GTP bound Cdc42 (activated) can recruit multiple protein complexes to the membrane and controls diverse cellular activities. Active Cdc42 may recruit IRSp53, which acts through protein complex including N-WASP, Mena and Eps8 to control filopodia formation. Cdc42 can activate Toca-1-N-WASP complex to play a role in membrane trafficking and endocytosis. Importantly the oligomerization of the proteins (IRSp53 or Toca-1) through I-BAR/F-BAR domains will provide “hotspots” of different protein binding domains such as Cdc42 binding domains and SH3 domains. These localized high concentrations of domains will bind to other protein complexes and induce downstream effects. For example, Par3/Par6/aPKC will bind to active Cdc42 to control cell polarity. This provides a mechanism to coordinate different cellular processes.

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Appendix 1 Media and solutions

LB medium

Bacto-tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	10 g/l

The pH was adjusted to 7.0 with 5 N NaOH. The medium was sterilized by autoclaving for 20 minutes at 15 lb/sq in. on liquid cycles.

SOC medium

Bacto-tryptone	2.0%
Yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
MgCl ₂	10 mM
MgSO ₄	10 mM

The pH was adjusted to 7.0 with 5 M NaOH prior to autoclaving. Upon cooling to 55 °C, filter-sterilized 20% glucose was added to a final concentration of 0.2%.

Miscellaneous solutions

IPTG

Isopropylthio- β -D-galactoside (IPTG) solution at 1 M was filter-sterilized through a 0.22 μ m filter and stored at -20 °C in 1 ml aliquots.

TE buffer

Tris-HCl (pH 7.6)	10 mM
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EDTA (pH 8.0) 1 mM

Ampicillin/Kanamycin stock solution

Ampicillin stock solutions were prepared by dissolving 100 mg of ampicillin/ 50 mg of kanamycin in 10 ml of sterile water (10 mg/ml) and sterilized by filtration through a 0.22 µm filter.

Solutions for plamid preps.

P1

Tris·Cl, pH 8.0 50 mM
EDTA 10 mM
RNase A 100 µg/ml

P2

NaOH 200 mM
SDS (w/v) 1%

P3

potassium acetate 3.0 M pH 5.5

Buffer QC

NaCl 1.0 M
MOPS, pH 7.0 50 mM
isopropanol 15% (v/v)

Buffer QF

NaCl	1.6 M
MOPS, pH 7.0	50 mM
isopropanol	15% (v/v)

Reagents for DNA electrophoresis

1 × TAE running buffer

Tris-acetate	0.04 M
EDTA (pH 8.0)	1 mM

Ethidium bromide

The stock solution was made at 10 mg/ml and stored in a light-tight bottle. The final working concentration was 0.2 µg/ml.

6 × DNA loading buffer

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30% (w/v)

Solutions for SDS-PAGE

PBS

NaCl	140 mM
KCl	2.7 mM
Na ₂ HPO ₄ ·2H ₂ O	10 mM
KH ₂ PO ₄	1.8 mM

The buffer was adjusted to pH 7.4.

1 × SDS loading buffer

Tris-HCl (pH 6.8)	50 mM
Mercaptoethanol	100 mM
SDS	2% (w/v)
Bromophenol blue	0.05% (w/v)
Glycerol	10% (w/v)

SDS-PAGE running buffer

Tris	25 mM
Glycine	250 mM
SDS	0.1% (w/v)

The buffer was adjusted to pH 8.3.

Solutions for Western blotting

Transfer buffer

Tris	25 mM
Glycine	192 mM
Methanol	20% (v/v)

10 × TBS

Tris-HCl (pH 7.5)	100 mM
NaCl	1.5 M

TBST buffer

TBS buffer with 0.1% Tween-20 (v/v)

Blocking buffer

5% (w/v) non-fat milk in TBST

7.5% resolving SDS-PAGE gel

Acrylamide-Bisacrylamide	7.5% (w/v)
SDS	0.1% (w/v)
Ammonium persulfate	0.4% (w/v)
TEMED	0.04% (w/v)
Tris-HCl (pH 8.8)	0.37 M

10% resolving SDS-PAGE gel

Acrylamide-Bisacrylamide	10% (w/v)
SDS	0.1% (w/v)
Ammonium persulfate	0.4% (w/v)
TEMED	0.04% (w/v)
Tris-HCl (pH 8.8)	0.37 M

5% stacking SDS-PAGE gel

Acrylamide-Bisacrylamide	5% (w/v)
SDS	0.1% (w/v)
Ammonium persulfate	0.05% (w/v)
TEMED	0.1% (w/v)
Tris-HCl (pH 7.0)	62.5 mM

Staining solution

Ethanol	50% (v/v)
Glacial acetic acid	10% (v/v)
Coomassie brilliant blue	2.5 g/l

Destaining solution

Ethanol	30% (v/v)
Glacial acetic acid	10% (v/v)

Cell lysis buffer (Buffer A)

20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (v/v) NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% (v/v) glycerol. Add 1× Protease Inhibitor cocktail just before use (from Roche Molecular Biochemicals/Boehringer, catalogue number 1836145).

Solutions for expression and purification of GST tagged proteins

GST purification buffer

The following components were added into PBS.

Tris (pH 8)	50 mM
Triton X-100	0.1%
Lysozyme	1 mg/ml
DTT	5 mM*
PMSF	1 mM*
MgCl ₂	0.5 mM*

*freshly added

Lysis buffer

GST purification buffer with the freshly added following components.

DTT 5 mM

PMSF 10 mM

Lysozyme 1%

Elution buffer:

The following components were added into GST purification buffer

Glutathione 10 mM

Glycerol 7%

Solutions for liposome binding assay

Liposome forming buffer

Sucrose 0.1M

Hepes 20 mM

KCl 100 mM

EDTA (pH 7.4) 1 mM

Liposome binding buffer

Hepes 20 mM

NaCl 200 mM

KCl 100 mM

EDTA (pH7.4) 1 mM

Appendix 2 Vectors

pXJ40-HA/GFP vectors

pXJ40-HA MCS

pXJ40-GFP was generated by replacing HA with GFP tag between EcoRI and BamHI site.

pGEX-4T-1 vector

pGEX-4T-1 (27-4580-01)