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博士（人間生物学）学位論文

Thesis Intended for the doctor degree (Human Biology)

Elucidation of physiological functions of the small G protein Arf6 and the Arf6 GAP ACAP3 in neurite outgrowth

(低分子量 G タンパク質 Arf6 と Arf6 GAP ACAP3 の神経突起伸長における生理機能の解明)

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Abbreviations

ACAP ArfGAP with coiled-coil, ankyrin repeat and pleckstrin homology domains

ADAP ArfGAP with dual PH domain-containing

ARAP ArfGAP with Rho GAP domain, ankyrin repeast and PH domain

AFGF ArfGAP domain and FG repeats-containing

ALS amyotrophic lateral sclerosis

ANK ankyrin

APP amyloid precursor protein

Arf ADP-ribosylation factor

Arl Arf-like

Arp Arf-related proteins

ASDs autism spectrum disorders

ASAP ArfGAP containing SH3, ankyrin repeat and PH domains

BAR Bin/Amphiphisin/Rvs

BCIP 5-bromo-4-chloro-3-indolyl phosphate

BDNF brain-derived neurotropic factor

BRAG brefeldin-resistant Arf GEF

CMV cytomegalovirus

DIV days in vitro

DMEM Dulbecco's modified Eagle's medium

DRG dorsal root ganglia

E	embryonic day
ECM	extracellular matrix
EFA6	exchange factor for Arf6
EGF	epidermal growth factor
EHD1	EH-domain-containing 1
ERM	ezrin/radixin/moesin
FTD	frontotemporal dementia
GAP	GTPase-activating protein
GAPDH	glyceraldehyde-3-phosphatedehydrogenase
GDF	GDI displacement factor
GDI	GDP-dissociation inhibitor
GEF	guanine-nucleotide-exchange factor
GGA3	Golgi-associated, g-adaptin ear-containing, Arf-binding protein 3
GGT	geranylgeranyl transferase
GLUT4	glucose transporter 4
CMV	cytomegalovirus
HA	haemaggulutinin
HEK	human embryonic kidney
HGF	hepatocyte growth factor
HSD	honest signify difference
KO	knockout
LZII	leucine zipper region II

MHC major histocompatibility complex

M2 M2-muscarinic acetylcholine

NBT Nitro Blue Tetrazolium

NgCAM Neuron-glia cell adhesion molecule

NGF nerve growth factor

P postnatal day

PA phosphatidic acid

PFA paraformaldehyde

PH pleckstrin homology

PIP5K Phosphatidylinositol 4-phosphate 5-kinase

PtdIns(4,5)P_2 phosphatidylinositol 4,5-bisphosphate

PSD95 postsynaptic density 95

REP Rab escort protein

RT reverse transcription

Sar Secretion-associated and Ras-related

SMAP small ArfGAP

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Chapter 1: Introduction

1-1 Small G protein ADP-ribosylation factor 6 (Arf6)

1-1-1 Small G proteins

Small GTP-binding proteins (G proteins) are monomeric G proteins, and their molecular masses are about 20-40 kDa. More than 100 small G proteins have been reported in eukaryotes from yeast to human, and they consist of structurally classified five subfamilies, Ras, Rho, Rab, Ran, and Arf families, which share 50-55% amino acid identity [1] (Figure 1A). All small G proteins contain consensus amino acid sequences essential for interaction with GDP and GTP, for GTPase activity, which hydrolyze bound GTP to GDP, and for a region required for binding to specific downstream effectors. In a physiological condition, small G proteins have two forms: GDP-bound inactive and GTP-bound active forms. Small G proteins function as molecular switches by cycling between GDP-bound inactive and GTP-bound active forms. This cycle is precisely regulated by GEFs (guanine-nucleotide exchange factors), which facilitate exchange of GDP for GTP on small G proteins, and GAPs (GTPase-activating proteins), that stimulate GTPase activity of small G proteins to hydrolyze GTP on that to GDP [2] (Figure 1B).

The physiological roles of small G proteins have been intensively studied and clarified: the Ras family members principally control gene expression, the Rho family members regulate cytoskeletal reorganization, Rab family members are involved in vesicle trafficking, Ran family members function in nucleocytoplasmic transport and microtubule organization, and Arf family members uniquely play important roles in

membrane trafficking and reorganization of actin filament (Figure 1B).

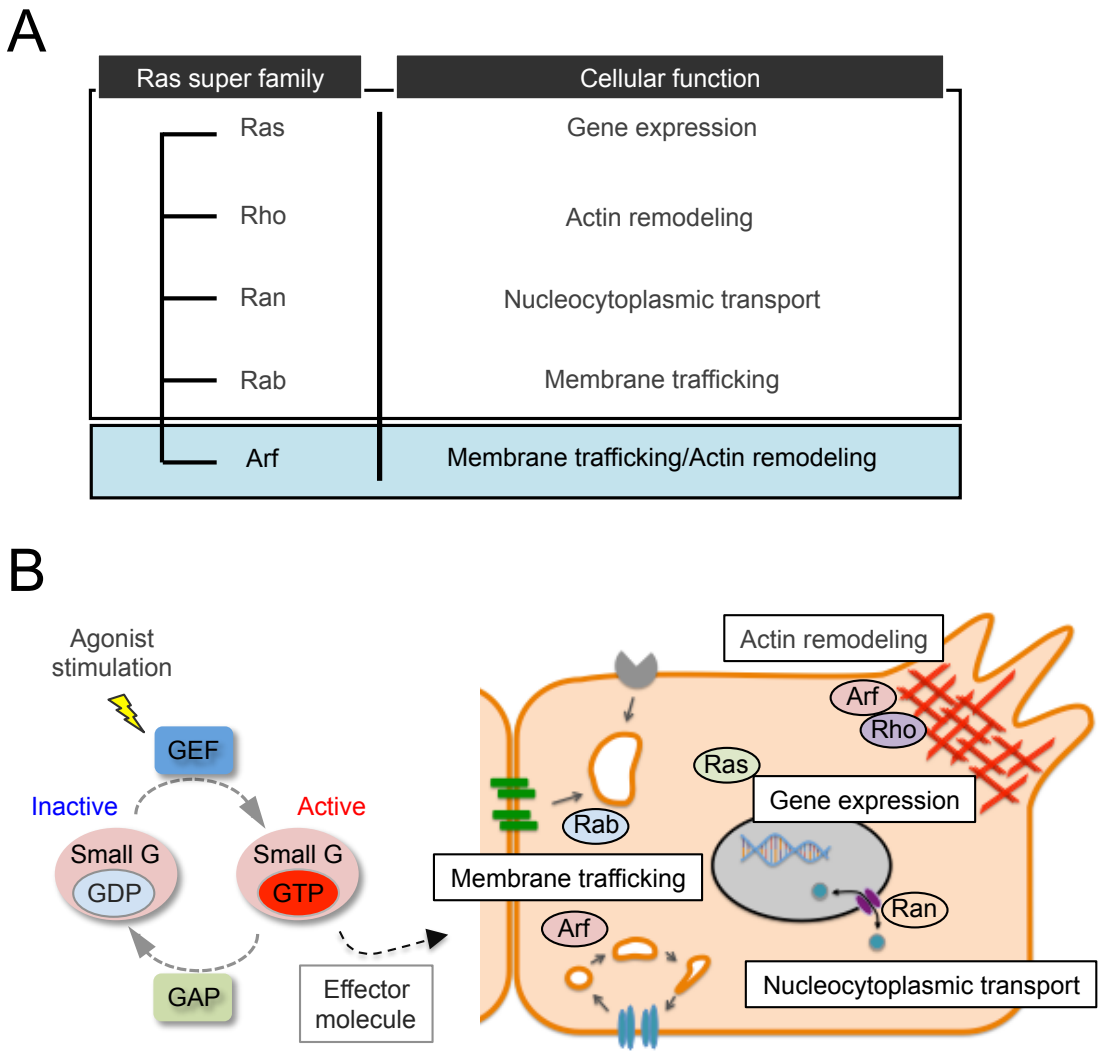


Figure 1. Ras super family small G proteins
(**A**) Ras super family of small G proteins, and their cellular functions. (**B**) Small G proteins function as molecular switches between GDP-bound inactive and GTP-bound active forms, regulated by GEFs (guanine-nucleotide exchange factors) and GAPs (GTPase-activating proteins). The small G proteins regulate variety of cell functions: the Ras family members control gene expression, the Rho family members regulate cytoskeletal reorganization, Rab family members are involved in membrane trafficking, Ran family members function in nucleocytoplasmic transport, and Arf family members play important roles in membrane trafficking and reorganization of actin filament.

1-1-2 Arf family

The Arf proteins were initially identified as a cofactor for cholera-toxin-catalyzed

ADP-ribosylation of the α-subunit of heterotrimeric G proteins, Gs [3]. Thereafter, their roles in membrane trafficking and organelle structures have been shown [4]. Arf family includes Arl (Arf-like), Arp (Arf-related proteins) and Sar (Secretion-associated and Ras-related) proteins. They broadly exist in animals, fungi, plant, and protists [5] (Figure 2A), however, their physiological roles are not well documented, yet. Of these Arf family proteins, in particular, physiological functions and biochemical properties of Arfs are intensively studied and well characterized.

The Arfs are widely expressed and their amino acid sequences are well conserved in all eukaryotes from yeast to humans with high fidelity [6]. The mammalian Arfs consist of six isoforms, Arf1-Arf6, which are further divided into three classes based on their amino acid sequence similarities [7]. The distinct cellular distributions of individual Arfs are critical for their cellular functions. Classes I and II of Arfs, including Arf1-Arf3 and Arf4 and Arf5 respectively, localize at the endoplasmic reticulum and/or Golgi to coordinately regulate structure of Golgi and secretory pathway through the recruitment of coat proteins to membrane surface of these organelles [8,9]. In contrast, sole member of the class III Arf Arf6 principally localizes to the plasma membrane and endosomal compartment [10], and regulates membrane dynamics based-cellular events including membrane trafficking [11–14] and reorganization of actin cytoskeleton [15,16] (Figure 2B).

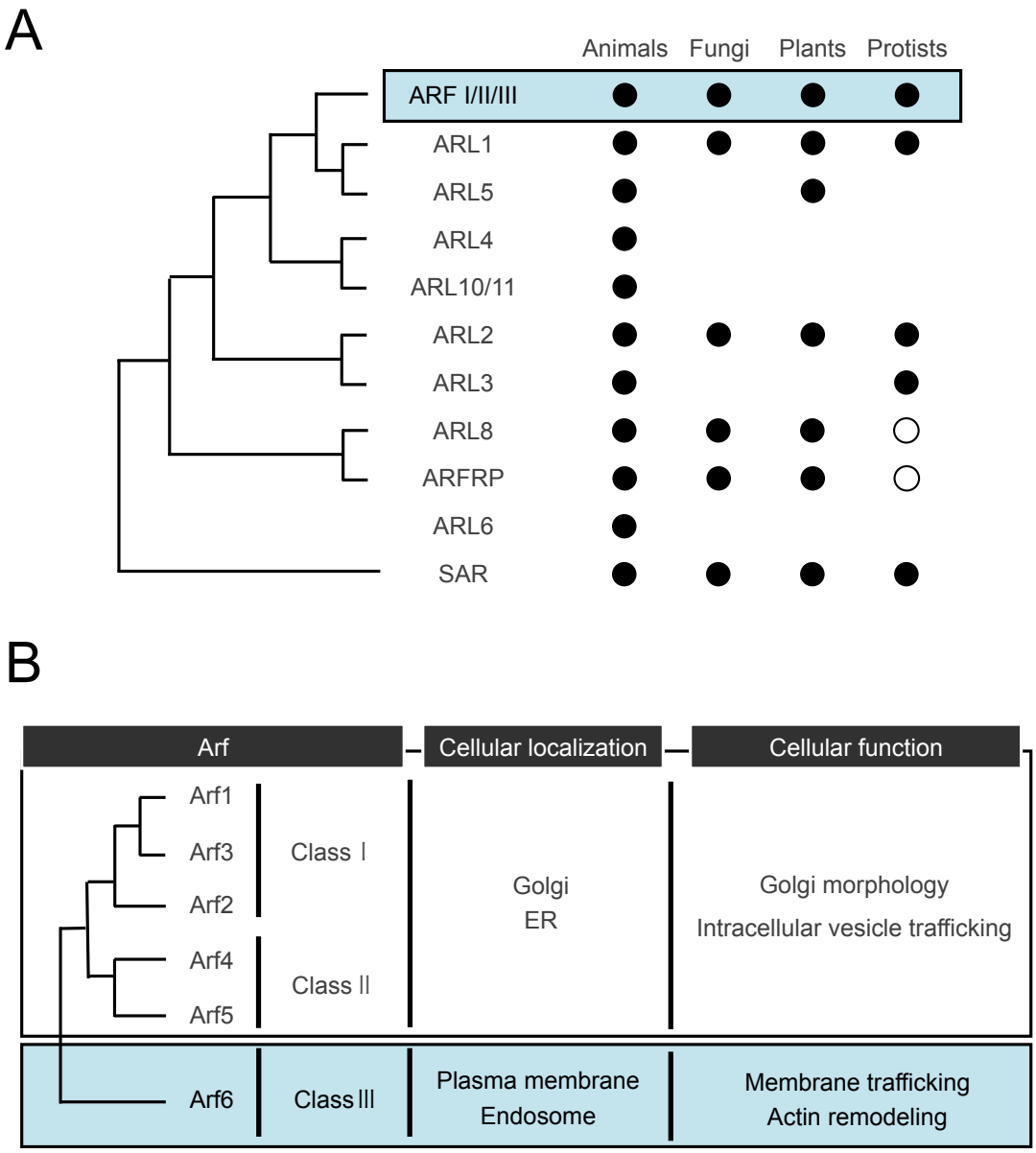


Figure 2. Arf family small G proteins
(**A**) Phylogenic relationship among Arf family homologs. The table shows the presence (•) of Arf groups in animals, fungi, plants, and protists. White circles (o) show uncertainty due to the position of *Giardia lamblia* Arf8/Arfrp in between Arl8 or Arfrp sequences. (**B**) ADP-ribosylation factors (Arfs). The mammalian Arfs consist of six isoforms, Arf1-Arf6, which are further divided into three classes based on their amino acid sequence similarities, and they play distinct roles in the cell.

1-1-3 Arf6 effectors

Like other small G proteins, the active form of Arf6 interacts with specific effectors,

and regulates their activities or subcellular locations, thereby contribute to specific cellular functions. For instance, Arf6 interacts with the lipid-metabolizing enzymes including PIP5K (phosphatidylinositol 4-phosphate 5-kinase) to generate the versatile phospholipid PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) [17], and PLD (phospholipase D), which produces the signaling lipid PA (phosphatidic acid) [18]. These lipid products play important roles in signal transduction and cellular functions. In addition, Arf6 binds to molecules involving in membrane trafficking, such as AP2, clathrin [19–21] and Nm23-H1 [22] for internalization of the proteins at the plasma membrane. Arf6 also interacts with JIP3/4 [23,24] , FIP3, Exo70p [25], and GGA3 (Golgi-associated, γ-adaptin ear-containing, Arf-binding protein 3) [26,27] to control recycling of the proteins to plasma membrane.

1-1-4 Physiological roles of Arf6

The plasma membrane proteins are taken up into inside of cells by several internalization pathways including clathrin-mediated/independent endocytosis [28] and macropinocytosis [29]. Arf6 regulates these internalization events through the interaction with effector molecules. Arf6 directly activates PIP5K to produce PtdIns(4,5)*P*2, and produced PtdIns(4,5)*P*2 plays critical roles in recruitment of AP2, AP180 and dynamin [20], which are essential components for clathrin-dependent endocytosis of β2 adrenagic receptor [30], transferrin receptor [11], and adhesion molecule E-cadherin [31] to regulate cellular signaling and cell-cell contact. Arf6 is also involved in the internalization of proteins through unique clathrin-independent pathway:

Arf6 regulates endocytosis of clathrin-independent cargos, MHC class I (major histocompatibility complex class I protein) [32], and M2 receptors (M2-muscarinic acetylcholine receptors) [33], although those molecular mechanisms remain elusive. Moreover, Arf6 is involved in recycling of the membrane proteins to the plasma membrane. Recycling of HGF (hepatocyte growth factor) receptor, c-Met [27], and neural cell-cell adhesion molecule N-cadherin [34] were regulated by Arf6 through interaction with GGA3 and FIP3 respectively, and Arf6 regulates migration of Hela cells and neurons. Arf6 also controls HGF-induced recycling of β1 integrin, which is important molecule for cell-ECM (extracellular matrix) adhesion in endothelial cells, and play key roles in endothelial cell migration and subsequent tumor angiogenesis [35]. Arf6-mediated macropinocytosis under EGF (epidermal growth factor)-stimulation [36] and macropinocytosis of APP (amyloid precursor protein) [37] have been documented, however, mechanistic insight through which Arf6 regulates these cellular events remain unclear.

Furthermore, Arf6 plays pivotal roles in the reorganization of actin cytoskeleton and membrane structures, which are essential processes for cellular functions such as migration and invasion of cancer cells, and neurite outgrowth of neurons. We have previously demonstrated that PtdIns(4,5)P_2 generated by Arf6/PIP5K induces membrane ruffling, which is a cellular process indispensible for cell migration in EGF-stimulated Hela cells [17]. Regulation of actin cytoskeleton remodeling by PtdIns(4,5)P_2 is mediated by actin-modulating proteins such as α-actinin [38], gelsolin [39] and cofilin [40], which regulate polymerization/depolymerization or

assembly/disassembly of actin filaments. In addition, PtdIns(4,5)P_2 directly binds to and regulates activity of proteins anchoring actin filaments to the plasma membrane, such as ERM (ezrin/radixin/moesin) proteins [41] and vinculin [42], to control the cell adhesion/detachment to/from extracellular matrix. Such a modulation of cell matrix adhesion is critical for regulating dynamic membrane structures such as filopodia and lamellipodia. Alternatively, it has been shown that Arf6 activates other small G protein, Rac1, which play a pivotal role in actin polymerization and reorganization [43]. Collectively, Arf6 regulates a wide range of membrane dynamics-based cellular functions. These physiological functions of Arf6 in variety of cells largely depend on precise regulation of Arf6 activity modulated by diverse Arf6 regulators, GEFs and GAPs [2,44] (Figure 3).

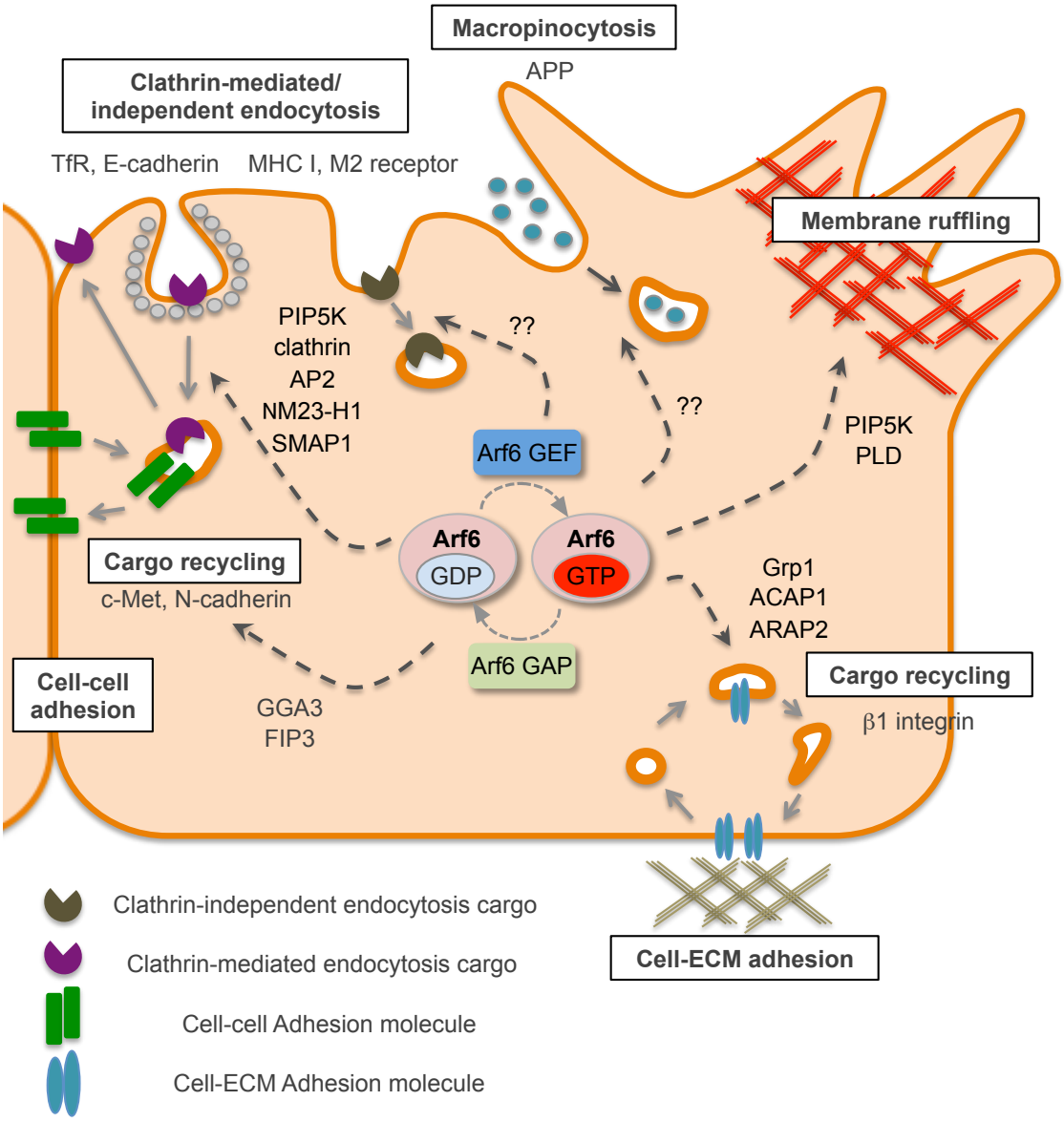


Figure 3. Physiological roles of Arf6
Arf6 functions in the internalization of proteins via clathrin-mediated/independent pathway, and macropinocytosis, through interaction with effector molecules. Arf6 also regulates recycling of cargo proteins, and membrane ruffling. Cycling between inactive and active forms of Arf6, regulated by variety of Arf6 GEFs and Arf6 GAPs has been shown to be required for appropriately regulating cellular events.

1-1-5 Arf6 guanine nucleotide exchange factors (GEFs)

To control actions of Arf6 in a wide range of cellular events, activation of Arf6 should be spatiotemporally regulated by its activators GEFs (Figure 3). Of 14 Arf GEFs, which are categorized into five GEF families, BRAG, cytohesin, EFA6, Fbx, and GBF/BIG, at least eight types of GEFs, including BRAG (brefeldin-resistant ArfGEF), EFA6 (exchange factor for Arf6) and cytohesin family GEFs have been reported to function as Arf6 GEFs [44,45]. These Arf6 GEFs are activated in distinct mechanisms upon stimulation of cells by agonist stimulations including hormones and growth factors. The Arf GEFs contain a catalytic domain, the Sec7 domain, composed of approximately 200 amino acids. A conserved glutamate residue in the Sec7 domain of Arf GEFs extrudes into the nucleotide-binding pocket of Arfs, thereby electrostatically competing with GDP. Thus, the interaction of Arf GEFs with Arfs can facilitate the dissociation of GDP from Arfs, leading to insertion of cytosolic free GTP into the nucleotide-binding pocket of Arfs for their activation. Thereafter, activated Arfs play a wide variety of cellular functions through interacting distinct effector molecules.

1-1-6 Arf6 GTPase activating proteins (GAPs)

Arf GAPs are proteins containing a characteristic module, the ArfGAP domain, which was first identified in rat ArfGAP1 as the domain responsible for stimulation of GTP hydrolysis on Arf1 [46]. ArfGAP domains are approximately 130 amino acids, and contain a characteristic C_4-type zinc finger motif and conserved arginine that is essential for its GAP activity. To date, 31 types of Arf GAPs have been identified and classified

into ten families, including ArfGAP1, ArfGAP2, ADAP (ARF GAP with dual PH domain-containing), SMAP (small ArfGAP), AFGF (ArfGAP domain and FG repeats-containing), GIT, ASAP (ArfGAP containing SH3, ankyrin repeat and PH domain), AGAP, ARAP (ArfGAP with Rho GAP domain, ankyrin repeat and PH domain), and ACAP (ArfGAP with coiled-coil, ankyrin repeat and pleckstrin homology domains) subfamilies, based on their sequence similarity [47] (Figure4). The eight types of Arf GAPs had been reported to function as Arf6 GAPs [45].

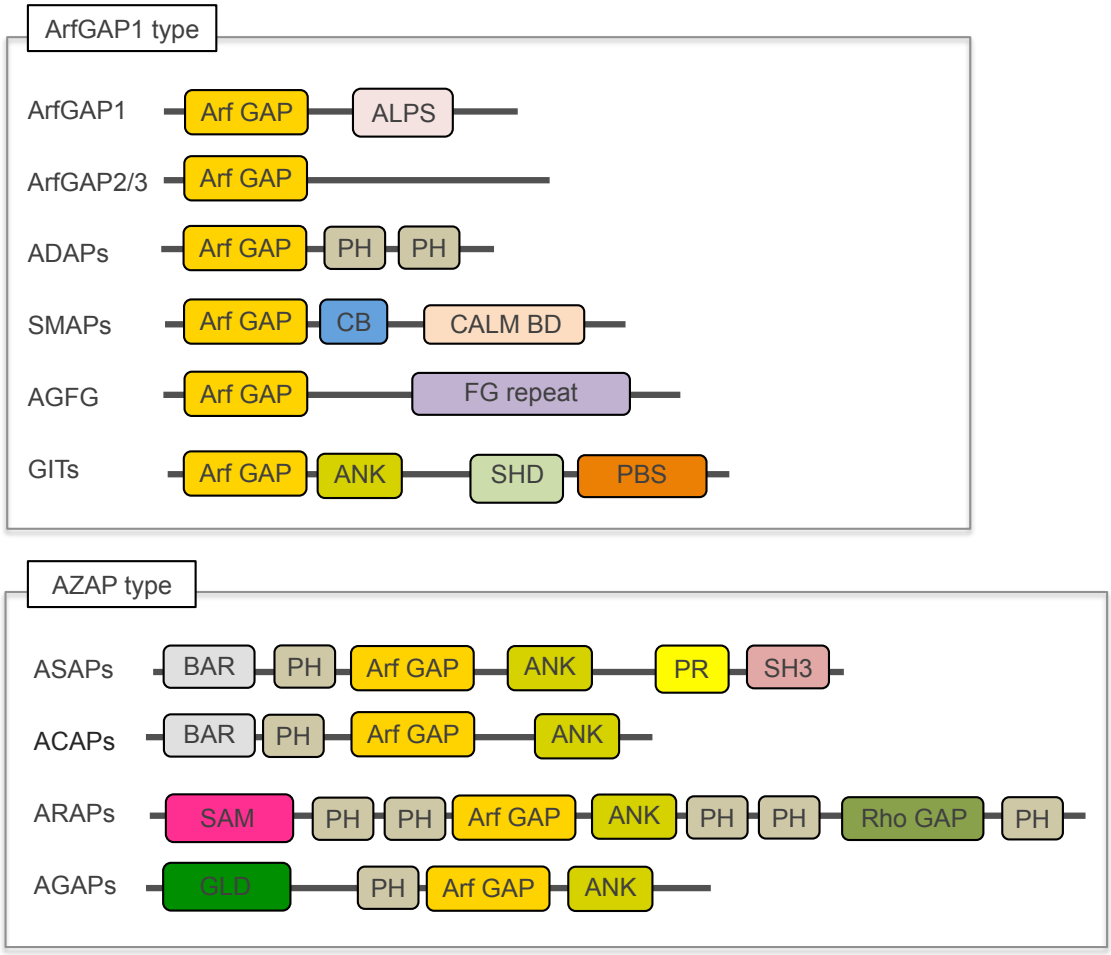


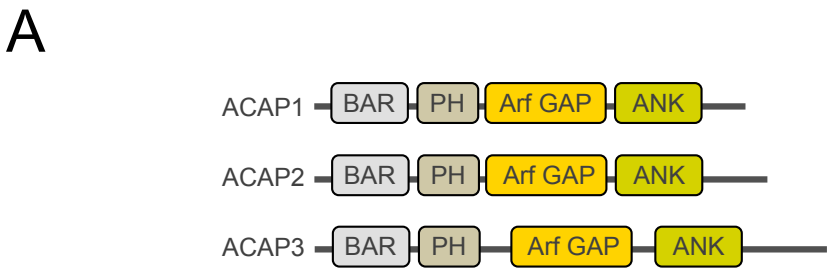
Figure 4. Domain structure of Arf GAP subfamily
To date, 31 types of Arf GAPs have been identified and classified into ten families, including ArfGAP1, ArfGAP2, ADAP, SMAP, AFGF, GIT, ASAP, AGAP, ARAP, and ACAP subfamilies, based on their sequence similarity. ALPS, ArfGAP 1 lipid-packing sensor; PH, Plekcstrin Homology; CB, Clathrin Box; CALM BD, CALM Bindin Domain; SHD, Spa Homology Domain; PBS, Paxillin Binding Sequence; BAR, Bin, Amphiphysin and Rvs167 and 161; ANK, Ankyrin repeat; PR, Proline Rich; SH3, Src Homology 3; SAM, Sterile α motif; GLD, GTP-binding protein-like domain

In a physiological setting, cycling between inactive and active forms of Arf6 seems to be required for appropriately regulating cellular events (Figure 3). This notion is strongly supported by the report showing that the Arf6 GAP SMAP1 regulates clathrin-mediated endocytosis [48]. In addition, it has been reported that ectopic expression of the Arf6 GEF EFA6 inhibits transferrin uptake/internalization [19,49],

implying that hyperactivation of Arf6 could disrupt the Arf6-regulated cell events. Thus, inactivation of Arf6 by GAPs as well as activation by GEFs could play an important role in the signal transduction to exert cell functions through Arf6.

1-1-7 ACAP family

ACAP family includes three isoforms: ACAP1-ACAP3, which contain BAR (Bin/Amphiphysin/Rvs) domain, PH (pleckstrin homology) domain, ArfGAP domain, and ANK (ankyrin) repeats (Figure 5A). ACAP1 and ACAP2 have been identified as Arf6-specific GAPs, which localize to tubular recycling endosome and regulate reorganization of actin cytoskeleton [50]. ACAP1 is involved in the recycling of β1 integrin and GLUT4 (glucose transporter 4) from the endosome to the plasma membrane [51–53]. ACAP2 is involved in FcγR-mediated phagocytosis by macrophages [54] and in neurite outgrowth of PC12 cells and hippocampal neurons [55]. In contrast, specificity of the ACAP3 GAP activity to Arf isoenzymes and its physiological functions at the cellular level remain elusive (Figure 5B).



B

	Substrate specificity of GAP activity	Cellular Localization	Cellular Functions
ACAP1	Arf6	Recycling endosome	β1 Integrin recycling Cell migration
ACAP2	Arf6		Phagocytosis Neurite outgrowth
ACAP3	?	?	?

Figure 5. ACAP family
(**A**) Domain structures of ACAPs. ACAPs contain BAR, PH, ArfGAP domain, and ANK repeats. BAR, Bin/Amphiphsin/Rvs; PH, Pleckstrin-Homology, ANK, Ankyrin repeats. (**B**) Substrate specificity of GAP activity, cellular localizations and functions of ACAPs.

1-2 Roles of Arf6 in neurite outgrowth

1-2-1 Formation of neural circuits in brain

The adult human brain weights about three ponds and consists of more than 100 billion of neurons, which have specialized two types projections, axon and dendrites (Figure 6). These neurons form complicated neural network during development of the brain, and precise interconnections among these neurons are critical for appropriate neural functions, including learning and memory, normal behaviors and motor functions. Abnormal neural circuits and formation of aberrant neural networks have been reported in several neurological and psychiatric disorders. For instance, disruption of axon

midline crossing for interconnections of the left and right sides of the brain causes mental retardation in corpus callosum agenesis [56]. Specific disconnection of the thalamo-orbitofrontal pathway has been implicated in neuropsychiatric disease such as schizophrenia [57]. In addition, a study in adult human *post-mortem* brain tissue of the patient with ASDs (autism spectrum disorders) indicates specific structural changes in prefrontal axons connectivity [58]. Thus, deciphering molecular basis underlying the formation of appropriate neural circuits in the brain is important for the development of approaches for the treatment of these disorders.

1-2-2 Neurite outgrowth

Neurite outgrowth is a pivotal process in the establishment of functional neural circuits in the brain development. Numerous *in vitro* studies using primary culture of the neurons [59], demonstrated that neurons initially extends premature several neurites from their cell bodies, and finally specify the long thin axon and several shorter dendrites [60]. After specification of the neurites, neurons develop dendritic spine structures, which contain receptors to receive neurotransmitters released from axons, and finally neurons form synaptic contacts between presynaptic axon terminals and postsynaptic dendritic spines for neural transmission [61] (Figure 6). To make these synaptic contact sites, the axon travels over long distance via axonal growth cone, which is a key structure for the navigation of the axon to target postsynaptic sites of other neurons.

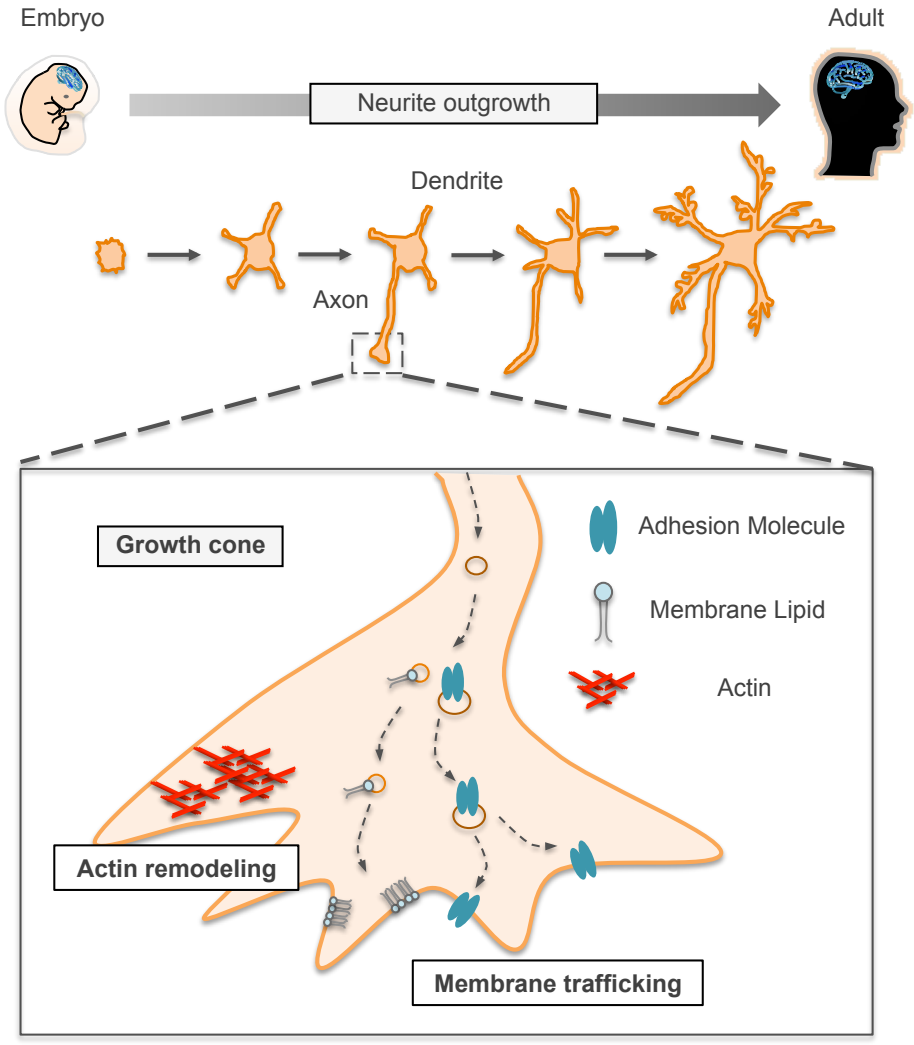


Figure 6. Growth cone dynamics in neurite outgrowth.
Neurite outgrowth is a pivotal process in the establishment of functional neural circuits in the brain. Neurons initially extends premature several neurites from their cell bodies, and finally specify the long thin axon and several shorter dendrites. After this specification of the neurites, neurons develop dendritic spine structures. The growth cone is dynamic structure at the tip of growing axon, and leads the axon to target postsynaptic sites. The dynamic behavior of the growth cone is largely depends on the reorganization of actin filaments and membrane trafficking of adhesion molecules and membrane lipid.

1-2-3 Growth cone

The growth cone, which was originally discovered by Santiago Ramon y Cajal [62], is a

structure at the tip of growing axon, and leads the axon to target postsynaptic sites (Figure 6). The growth cone repeatedly protrudes and withdraws its finger-like filopodia and lamellipodia structures, which are enriched in actin. For the navigation of the growth cone to proper places, the growth cone senses diverse surrounding extracellular molecules including tropic factors, guidance cues, and ECM components through receptors at the membrane surface of the growth cone. Continuous remodeling of the growth cone structure for extension and withdraw, finally results in reaching at target dendritic spines of other neurons during development of the neurons [63].

1-2-4 Actin remodeling and membrane trafficking in the growth cone

The dynamic behavior of the growth cone is precisely controlled by the reorganization of actin filaments [64], and membrane trafficking [65] at the growth cone.

Actin filament organization at the growth cone is required for their advance and navigation to synaptic targets. The polymerization of actin filaments, and their recycling provide the protrusive forces to push the plasma membrane of leading margin of the filopodia and lamellipodia at the growth cone. The polymerization/depolymerization of the actin filaments are precisely regulated by various actin-binding proteins including, Arp2/3 complex [66], Formin [67], ADF/cofilin [40] and gelsolin [39]. In addition, actin filaments in the peripheral region of the growth cone interact with the adhesion complexes to generate traction forces that pull the growth cone forward against adhesions to ECM, such as collagen, laminin, and fibronectin. The adhesive complexes include several actin-binding proteins, ERM proteins [41], α-/β-catenin [68,69],

vinculin [42] and α-actinin [38] (Figure 7A).

Membrane trafficking events also participate in the regulation of growth cone behaviors in multiple ways. The vesicles containing receptors for tropic factors including NGF (nerve growth factor) and BDNF (brain-derived neurotropic factor) and guidance cues such as netrin and Sema3A are involved in generation of intracellular signals that induce axon guidance and elongation. Moreover, it has been suggested that cytoskeletal molecules (e.g. Rho family small G proteins) and adhesion molecules such as β1 integrin and N-cadherin, are trafficked through exocytic and endocytic events in the growth cone [65] (Figure 7A). Artificial optogenetic approaches in primary cultured neurons demonstrated that manipulation of the recycling endosome position is sufficient to promote axon elongation, whereas facilitation of dynein-mediated retrograde transport of vesicles at the growth cone induce withdraw of growth cone [70], directly indicating the importance of membrane trafficking events for the advance of growth cone and neurite outgrowth.

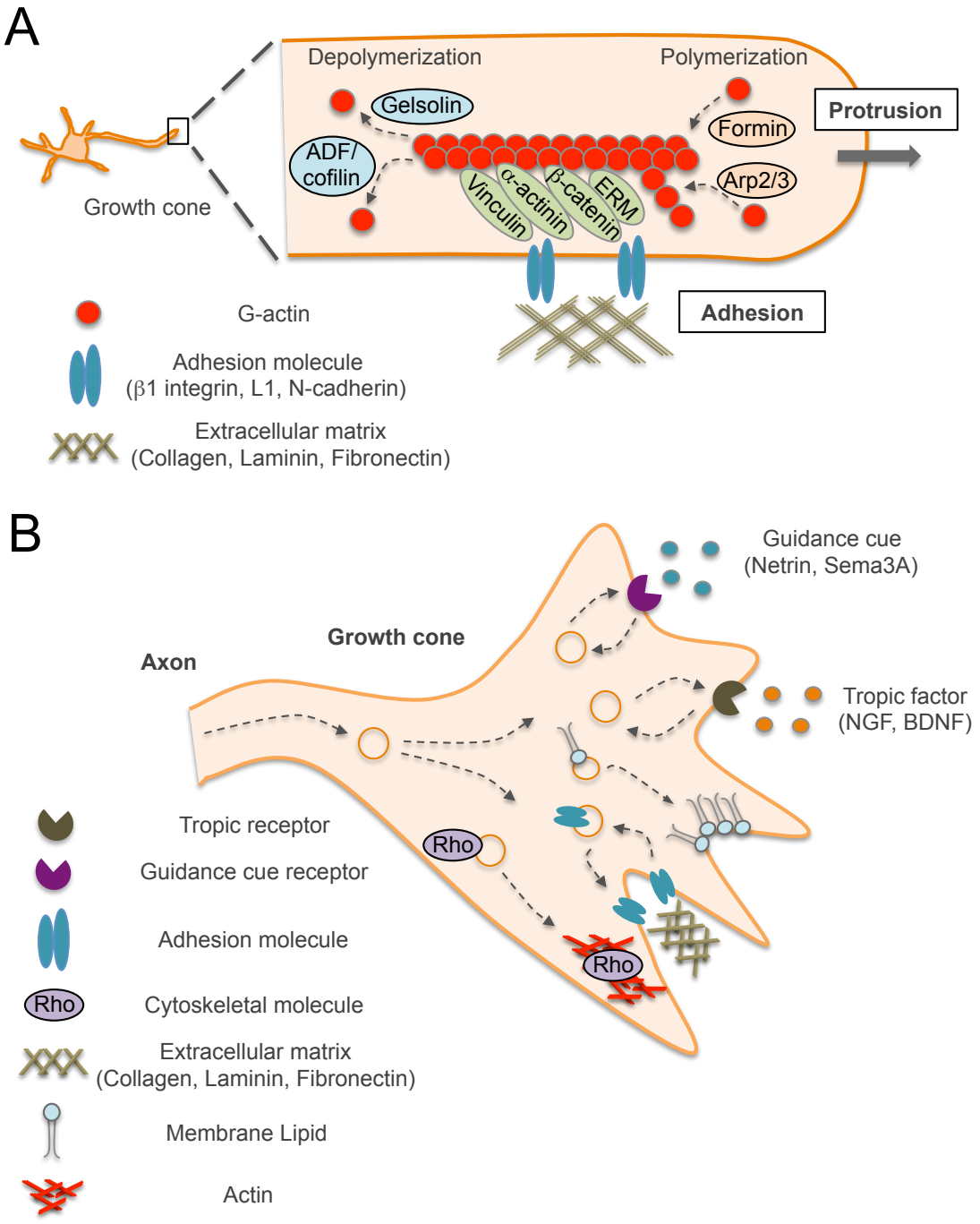


Figure 7. Actin dynamics and membrane trafficking in neurite outgrwoth
(**A**) Actin dynamics at the growth cone. The polymerization/depolymerization of the actin filaments , and adhesion at the growth cone are precisely regulated by various actin-binding proteins (**B**) Exocytic and endocytic event at the growth cone. The vesicles containing tropic receptors, guidance cue receptors, adhesion molecule, cytoskeletal molecules, and membrane lipids are trafficked through exocytic and endocytic events at the growth cone.

1-2-5 Physiological functions of Arf6 in neurite outgrowth

Arf6 is expressed in several brain regions, and in neurons [71–73]. The physiological roles of Arf6 in neurite outgrowth have been intensively studied, however, the roles of Arf6 in neurite outgrowth and its mechanistic insights remain unclear.

Ectopic expression of nucleotide-free form of Arf6 (T27N), which is used as dominant negative form of Arf6, increases length of axon in primary cultured hippocampal neurons by regulating PIP5Kα and actin binding protein Mena [74]. Moreover, the inhibitory role of Arf6 in neurite outgrowth was also reported in cortical neurons, mechanisms dependent on its effector JIP3 [75]. In DRG (dorsal root ganglia) neurons, constitutive active form of Arf6 accelerates retrograde transport of ECM adhesion molecule β1 integrin and inhibit axonal outgrowth, whereas nucleotide free forms of Arf6 facilitates anterograde transport of β1 integrin and induces axon elongation [76]. More recently, it has been reported that in motor neurons, Arf6 functions as a downstream molecule of c9orf72, which is related to motor neuron disorder ALS (amyotrophic lateral sclerosis) and FTD (frontotemporal dementia), in neurite outgrowth: deletion of c9orf72 significantly enhanced activity of Arf6, leading to increased activated LIM-kinas 1/2, and finally interrupted axonal actin dynamics and neurite outgrowth [77]. Altogether, these results support the notion that Arf6 functions as a negative regulator of neurite outgrowth of neurons.

In contrast, overexpression of FE65, an adaptor protein that binds to Arf6 and amyloid precursor protein, stimulates activation of Arf6, leading to promote neurite outgrowth in rat cortical neurons [78]. In commissural neurons, Arf6 mediates

Wnt5a-induced commissural axonal outgrowth. Arf6 interacts with Frizzled 3 for its endocytosis, and ectopic expression of Arf6-T27N and knockdown of Arf6 significantly inhibits endocytosis of Frizzled 3 at the growth cone and Wnt5a-stimulated neurite outgrowth respectively [79]. Thus, these facts suggest that Arf6 plays positive roles in neurite outgrowth. The discrepancies between negative and positive roles of Arf6 in neurite outgrowth could result from the difference in experimental conditions, in types of primary cultured neurons, and in use of ectopic expression of nucleotide free form of Arf6. Further comprehensive studies of Arf6 in neurite outgrowth are required for understanding the physiological significance of Arf6 in neurite outgrowth.

1-3 Aim of thesis project

The small G protein Arf6 plays pivotal roles in membrane dynamics-based variety of cellular and physiological functions including cancer cell progression and neurite outgrowth of neurons. The cycling of active and inactive forms of Arf6 seems to be critical for the completion of these cellular functions, indicating that Arf6 regulators, GAPs as well as GEFs play key roles in physiological functions. Although, ACAP3 belongs to the ACAP family of GAPs for the small G protein Arf, its specificity to Arf isoforms and physiological functions remain unknown. In the present study, we aimed to investigate specificity of ACAP3 to Arf isoforms, and its physiological roles, especially in neurite outgrowth of neurons, in which Arf6 seems to be significantly involved.

Chapter 2: Materials and Methods

2-1 Plasmids

cDNAs for ACAP2, ACAP3 and ArfGAP1 were amplified from total RNA prepared from immortalized mouse embryonic endothelial cells by RT (reverse transcription)-PCR, and inserted into the mammalian expression vectors pEGFP-C2 (Clontech) and pCAGGS (a gift from Dr J. Miyazaki, Osaka University, Osaka, Japan) [80] for transfection into HEK (human embryonic kidney)-293T cells and primary cultured hippocampal neurons respectively. cDNAs encoding mouse Arfs, which were gifts from Dr K. Nakayama (Kyoto University, Kyoto, Japan), were subcloned into pcDNA3 (Life Technologies) with a C-terminal HA (haemagglutinin) or Myc tag. GAP activity-deficient mutants of Arf GAPs (ACAP2 R442Q, ACAP3 R446Q and ArfGAP1 R50K), and active (Q67L), inactive (T44N), and fast cycle (T157A) mutants of Arf6 were generated by PCR-based site-directed mutagenesis. For construction of shRNA plasmids, we employed the expression vector containing dual promoters CMV (cytomegalovirus) and H1. cDNAs encoding GFP and shRNAs were inserted downstream of CMV and H1 respectively. Target sequences of shRNAs were following.

ACAP3 shRNA #1: 5'-GGTAGAAACAGATGTGGTTGA-3'

ACAP3 shRNA #2: 5'-GCACCAAGTGGTGTGGTAATG-3'

Arf6 shRNA #1: 5'-AGCTGCACCGCATTATCAA-3' [81]

Arf6 shRNA #2: 5'-CCAGGAGCTGCACCGCATTAT-3'

Control shRNA: 5'-CGAATCCTACAAAGCGCGC-3'

For the rescue experiments, shRNA-resistant *ACAP3* cDNAs were produced by mutating four nucleotides in the target region of *ACAP3* shRNA#1 without any changes in amino acid sequences. The Strawberry- and Flag-tagged Rab18 and Rab35 plasmids were kind gifts from Dr M. Fukuda (Tohoku University, Miyagi, Japan).

2-2 Mice

Generation of *Arf6*-KO (knockout) mice was described previously [82]. All experiments with mice were conducted according to the Guideline for Proper Conduct of Animal Experiments, Science Council of Japan. The protocols of the experiments were approved by the Animal Care and Use Committee, University of Tsukuba, Japan.

2-3 Tissue distribution of ACAPs

Tissue distribution of *ACAP1-ACAP3* mRNAs and ACAP3 protein were analysed by semi-quantitative RT-PCR and Western blotting respectively.

For semi-quantitative RT-PCR of *ACAP1-ACAP3* mRNAs, total RNA was extracted from tissues of C57BL/6J mice with TRIzol® reagent (Life Technologies), and reverse-transcribed with Superscript III (Life Technologies) according to the

manufacturer's protocol. PCR primers were following.

ACAP1 FW: 5'-GCAAGTCATCTGAGATGACGGTCAAGC-3'

ACAP1 RV: 5'-ACCTCTGCATTGTGGGTAAGAGCAG-3'

ACAP2 FW: 5'-AACAGCACGAGGTTGAAGAGGCTGC-3'

ACAP2 RV: 5'-CTGGAGAAATCCTTCTGCTGGATCG-3'

ACAP3 FW: 5'-TGGACAAGCTGGTCAAACTGTGCAG-3'

ACAP3 RV: 5'-TCACCAAGGACAACTCCATGTCCTC-3'

GAPDH FW: 5'-GAGGGGCCATCCACAGTCTTC-3'

GAPDH RV: 5'-CATCACCATCTTCCAGGAGCG-3'

For Western blotting of ACAP3, tissues of C57BL/6J mice were homogenized in a buffer consisting of 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1% SDS and protease inhibitor cocktail (Nacalai Tesque). Tissue lysates were sonicated on ice and centrifuged at 1000 *g* for 20 min at 4°C. Supernatants obtained were subjected to Western blotting.

2-4 Cell culture and transfection

To analyse expression levels of shRNA-resistant ACAP3 proteins and specificity of ACAP3 GAP activity to Arf isoenzymes, HEK-293T cells were employed. Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/ml glucose (Nacalai Tesque) supplemented with 10% (v/v) FBS (Life Technologies) and 1% penicillin/streptomycin (Nacalai Tesque) for 2 h, and transfected with plasmids for shRNAs and/or proteins using Lipofectamine^{TM} 2000 (Life Technologies) according to the manufacture's protocol. After incubation at 37°C for 1.5 days under 5% CO_2, cells were subjected to analyses for protein expression and ACAP3 GAP activity.

For the analysis of interaction between ACAP3 and small G protein Rabs, GFP-tagged ACAP3 was co-expressed with Flag-tagged wild type, constitutive active mutant Q67L or dominant negative mutant S22N of Rab35 in HEK-293T cells by PEI transfection method according to standard method. After incubation at 37°C for 1.5 days under 5% CO_2, cells were lysed in lysis buffer containing 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% Triton X-100 (v/v), 5 mM NaF, 2 mM Na_2VO_4, 4 mM $Na_4P_20_7$ and protease inhibitor cocktail (Nacalai Tesque). Flag-Rabs were immunoprecipitated with anti-Flag M2 resin (Sigma-Aldrich), and coimmunoprecipitated GFP-ACAP3 was detected by Western blotting probed with anti-GFP antibody.

For the experiments with hippocampal neurons, hippocampi were dissected from ICR WT (wild-type) mice and Arf6-KO mice back-crossed with C57BL/6J [82] at E (embryonic day) 17 or E18, and treated with 10 units/ml papain and 100 units/ml DNase in DMEM at 37°C for 20 min. The dissociated hippocampal neurons were plated

on polyethyleneimine-coated dishes or glass coverslips, and cultured in Neurobasal medium (Life Technologies) supplemented with B-27 (Life Technologies), 0.5 mM L-glutamine and 1% penicillin/streptomycin (Nacalai Tescque). To assess the knockdown efficiencies for ACAP3 and Arf6, hippocampal neurons were transfected with plasmids encoding shRNAs for these proteins by Nucleofector^{TM} 2b Device (AAB-1001, Lonza) with Amaxa Mouse Neuron Nucleofector Kit (VPG-1001, Lonza) at DIV (days *in vitro*) 0, and expression levels of these proteins were determined by Western blotting at DIV5. Under these conditions, transfection efficiency of the plasmid was ~60% of total cells, which was determined as the percentage of cells expressing GFP. In the experiments for the subcellular location of overexpressed ACAP3 and Arf6, primary cultured hippocampal neurons were transfected with plasmids for HA-ACAP3 and Arf6-Myc using the calcium phosphate method [83] at DIV4, and subjected to immunocytochemical analysis with anti-HA and -Myc antibodies at DIV6. The ratiometric image and the line profile for fluorescence intensity of HA-ACAP3 over that of co-expressed GFP were obtained with ImageJ software (NIH). For the assay of neurite outgrowth with hippocampal neurons prepared from ICR mice, shRNA plasmids for *ACAP3* or *Arf6* with or without plasmids encoding WT and mutant ACAP3 or Arf6 were transfected into hippocampal neurons using the calcium phosphate method at DIV0, 1, 4 and 9, and total neurite length and total neurite number were assessed at DIV3, 6, 9 and 12 respectively. In the experiment with hippocampal neurons prepared from WT and *Arf6*-KO mice, hippocampal neurons were cultured as described above, and neurite outgrowth was assessed at DIV6. In the experiment to investigate the effects

of ACAP3 knockdown on the level of the active form of Arf6, cultured hippocampal neurons were co-transfected with plasmids for *ACAP3* shRNA and for Arf6-HA using the calcium phosphate method at DIV4, and levels of the active form of Arf6-HA were assessed at DIV9.

2-5 Assay for active forms of Arfs

GTP-bound active forms of Arfs were assessed by the pull-down method with GST-conjugated GGA3 (amino acids 1-226) [26] or LZII (leucine zipper region II) of JNK (c-Jun N-terminal kinase)-interacting leucine zipper protein (amino acids 389-455) [84], followed by Western blotting.

HEK-293T cells overexpressed with Arfs-HA and GFP-Arf GAPs were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 μg/ml aprotinin and 1 μg/ml leupeptin). After centrifugation, supernatants were incubated with COSMOGEL® GST-Accept (Nacalai Tesque) pre-conjugated with GST-GGA3 at 4°C for 30 min with rotation. The beads were washed five times with the washing buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% NP-40, 10% glycerol, 1 μg/ml aprotinin and 1 μg/ml leupeptin). The active forms of Arfs bound to beads were eluted with SDS/PAGE sample buffer and detected by Western blotting with anti-HA antibody.

For assay with cultured hippocampal neurons, cells transfected with plasmids for Arf6-HA and shRNAs were lysed with the lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 0.5% sodium deoxycholate, 1% Triton X-100 and 10%

glycerol) supplemented with protease inhibitor cocktail (Nacalai Tesque), and the active form of Arf6 was pulled down with GST-LZII-conjugated beads. After the beads were washed with the buffer consisting of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM sodium deoxycholate and 10% glycerol, the active form of Arf6 was eluted with SDS/PAGE sample buffer and detected by Western blotting with anti-HA antibody.

2-6 Western blotting

Proteins of sample were separated by SDS/PAGE, and transferred on to PVDF membrane (Millipore). Membranes were blocked with Blocking-One P (Nacalai Tesque) at room temperature for 1.5 h. After washing with 0.05% Tween 20 in PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) at room temperature for 1 h, and washed with 0.05% Tween 20 in PBS. Reacted proteins were visualized with the Chemi-Lumi One enhanced detection reagents (Nacalai Tesque), and signals were detected with luminescent image analyser LAS-4000 mini (Fujifilm). Primary antibodies used were as follows: anti-HA antibody (3F10, Roche), anti-GFP antibody (598, MBL), anti-ACAP3 antibodies (17570-1-AP, Proteintech, and sc160232, Santa Cruz Biotechnology), anti-GAPDH antibody (MAB374, Millipore), anti-PSD-95 (postsynaptic density 95) antibody (MA1-045, Thermo Scientific) and anti-actin antibody (A2066, Sigma-Aldrich). Rabbit anti-Arf6 antibody was generated as described previously [73].

2-7 *In situ* hybridization

Brains dissected from P (postnatal day) 14 mice were fixed with 4% (w/v) PFA (paraformaldehyde) in PBS, and embedded in OCT compound. After preparation of cryostat sections, they were fixed with 4% (w/v) PFA in PBS at room temperature for 10 min, washed with PBS and immersed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min. They were then washed three times with PBS and blocked in the pre-hybridization solution [50% formamide, 5x SSC (1x SSC is 0.15 M NaCl/0.015 M sodium citrate), 1x Denhardt's, 250 μg/ml tRNA and 500 μg/ml herring sperm DNA] at 4°C overnight. The cRNA probes were hybridized in the hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris/HCl, pH 8.0, 5 mM EDTA, 10 mM Na_2HPO_4, 10% dextran sulfate, 1x Denhardt's, 500 μg/ml tRNA and 200 μg/ml herring sperm DNA) at 65°C overnight. The sections were washed four times with 0.2x SSC and buffer A (0.1 M Tris/HCl, pH 7.5, and 0.15 M NaCl), the sections were blocked with 10% (v/v) normal sheep serum at 4°C overnight. They were washed with buffer A supplemented with 0.1% Triton X-100 three times, and developed by incubation with NBT (Nitro Blue Tetrazolium)/BCIP (5-broom-4-chloro-3-indolyl phosphate) (1:200 NBT/BCIP stock solution, Roche) in a buffer consisting of 0.1 M Tris/HCl, pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$. Images were obtained using a Biozero BZ-8000 microscope (Keyence). PCR primers used for probe of *in situ* hybridization for *ACAP3* were following.

FW: 5'-aagctgtgcaggctagcatcgcctc-3'

RV: 5’-acttctgtgccctccagcgtcttg-3’

2-8 Immunocytochemistry

For the detection of overexpressed proteins in hippocampal neurons, cells were fixed with 4% (w/v) PFA in PBS for 20 min, permeabilized with 0.1% Triton X-100 an 0.1% Tween 20 for 10 min, blocked with 1% (w/v) BSA in PBS at room temperature for 1 h, and stained by sequential incubation with primary antibodies and Alexa Fluor® 488- or Alexa Fluor® 546-conjugated secondary antibodies (Life Technologies). Primary antibodies used were as follows: anti-HA antibody (16B12, Covance), anti-GFP antibody (598, MBL), anti-Myc antibody (562, MBL) anti-tubulin antibody (T6199, Sigma-Aldrich), and anti-RFP (PM005, MBL).

For the detection of endogenous ACAP3 and Arf6 of hippocampal neurons, cells were fixed with 4% (w/v) PFA and 4% (w/v) sucrose in PBS for 20 min, and post-fixed with trichloroacetic acid on ice for 15 min. The fixed neurons were permeabilized with 0.1% Triton X-100 for 5 min, blocked with 2% (w/v) BSA in PBS at 4°C overnight, and stained by sequential incubation with primary antibodies diluted with Can Get Signal Solution B (NKB-601, Toyobo) at 4°C overnight and with Alexa Fluor® 488- or Alexa Fluor® 546-conjugated secondary antibodies (Life Technologies). Primary antibodies used were as follows: anti-ACAP3 antibodies (17570-1-AP, Proteintech) and guinea pig anti-Arf6 antibody (a gift from Dr H. Sakagami, Kitasato University, Kanagawa, Japan) [34].

For the staining of PtdIns(4,5)P_2, cells were fixed with 0.2% glutaraldehyde (v/v)/4% (w/v) PFA/PBS for 3 h at 4°C. After washed with 50 mM NH_4Cl/PBS three times, and cells were permeabilized with 0.5% (w/v) saponin/5% (w/v) normal goat serum in pH 6.8 NaGB buffer (20 mM PIPES-NaOH, 137 mM Sodium Glutamate, 2 mM MgCl2, 1 mg/ml BSA) at 4°C for 4 h, and stained with primary antibodies diluted with 0.1% (w/v) saponin/5% (w/v) normal goat serum in NaGB buffer at 4°C overnight. After washing with NaGB buffer for 10 min twice, cells were stained with Alexa Fluor® 488- (Life Technologies) or Cy3-conjugated (Jackson Imm. Lab. Inc.) secondary antibodies and Alexa Fluor® 633 Phalloidin diluted with 0.1% (w/v) saponin /5% (w/v) normal goat serum in NaGB buffer at 4°C for 4 h, washed with NaGB buffer for 10 min twice, and post fixed with 0.2% (v/v) glutaraldehyde/4% (w/v) PFA/PBS for 10 min on ice and 5 min at r.t. Primary antibodies used were as follows: anti-PtdIns(4,5)P_2 antibodies (Z-P045, Echelon) and anti-GFP antibody (598, MBL). Fluorescence images were acquired with the confocal laser-scanning microscope FV10i (Olympus), confocal microscope Leica TCS SP5 (Leica Microsystems) or BZ-X710 (Keyence). Total neurite length and total neurite number of 33-90 neurons, fluorescence intensities for ACAP3, tubulin, PtdIns(4,5)P_2, actin, growth cone size and circularity of 39-45 neurons were analysed using ImageJ software.

2-9 Statistical analysis

Each value shows the means±S.E.M. from more than three independent experiments. Statistical significance was calculated by Student's *t* test or one-way ANOVA with Tukey's HSD (honest significant difference) or Dunnett's multiple comparison tests.

Chapter 3: Results

3-1 Abundant expression of ACAP3 in brain

To obtain the information for tissues or cells in which ACAP3 functions, tissue distribution of *ACAP3* mRNA in P56 adult mice was analysed by semi-quantitative RT-PCR in comparison with that of *ACAP1* and *ACAP2* mRNAs (Figure 8A). Consistent with the previous study [50], *ACAP1* mRNA was not detected in the cerebrum and cerebellum, but was abundantly expressed in the lung, spleen adipose tissue and testis. *ACAP2* and *ACAP3* mRNAs were ubiquitously expressed. Notably, *ACAP3* mRNA was abundant in the cerebrum, cerebellum, heart and stomach. When expression levels of the ACAP3 protein in P56 mouse tissues were analysed by Western blotting, however, extremely higher expression of ACAP3 was observed in the brain compared with other tissues (Figure 8B). Furthermore, it was found that the expression level of ACAP3 in the brain was dependent on the mouse development (Figure 8C): ACAP3 expression was detectable in the brain of E12-14 mouse embryos and increased with development, reaching a plateau by E16-E18. *In situ* hybridization analysis in the brain of P14 mice revealed evident expression of *ACAP3* mRNA in the cortex, hippocampus and cerebellum (Figure 9); in particular, the signal in the hippocampus was very strong.

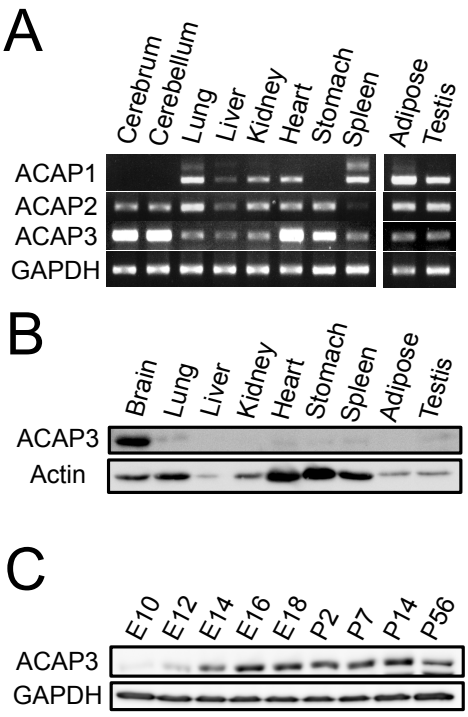


Figure 8. ACAP3 is abundantly expressed in the brain.
(**A**) mRNA levels of *ACAPs* in P56 adult mouse tissues were analyzed by semi-quantitative RT-PCR. (**B,C**) Expression levels of ACAP3 protein in P56 adult mouse tissues (**B**) and in the brain at each developing stage (**C**) were analyzed by western blotting.

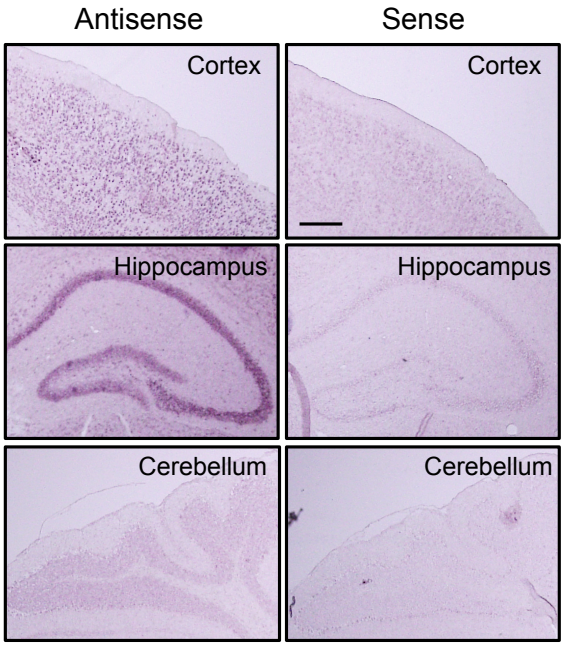


Figure 9. *In situ* hybridization for *ACAP3* in the brain.
In situ hybridization for *ACAP3* in the cortex, hippocampus and cerebellum of P14 mouse. Scale bar, 300 μm.

3-2 ACAP3 is involved in neurite outgrowth of hippocampal neurons

Since *ACAP3* mRNA was highly expressed in the hippocampus (Figure 9), it is plausible that ACAP3 functions in hippocampal neurons. In primary cultured mouse hippocampal neurons, expression level of ACAP3 was detectable at DIV6, and increased with prolonged culture, reaching a plateau by DIV12, which was almost parallel to the expression pattern of the postsynaptic marker protein PSD95 (Figure 10), suggesting that ACAP3 plays a role in the development of hippocampal neurons.

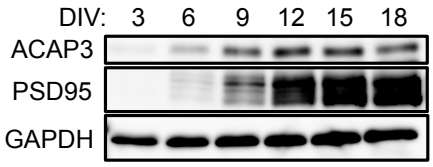


Figure 10. Expression levels of ACAP3 in hippocampal neurons.
Expression levels of the ACAP3 protein in primary cultured hippocampal neurons prepared from E17 mouse embryos were analyzed by western blotting probed with anti-ACAP3 antibody. Expression of PSD95 was also analyzed to confirm the development of neurons.

When HA-ACAP3 was expressed in hippocampal neurons, it localized in the soma and neurites, especially at the tip of the growth cone as revealed by the ratiometric image and line profile (Figure 11A,B). Consistent with this result, endogenous ACAP3 was found to locate at the tips of the growth cone in DIV6-DIV12 hippocampal neurons (Figure 11C), although it was not detectable at DIV3 due to its lower expression levels as shown in Figure 10. These results strongly suggest the involvement of ACAP3 in axonal outgrowth.

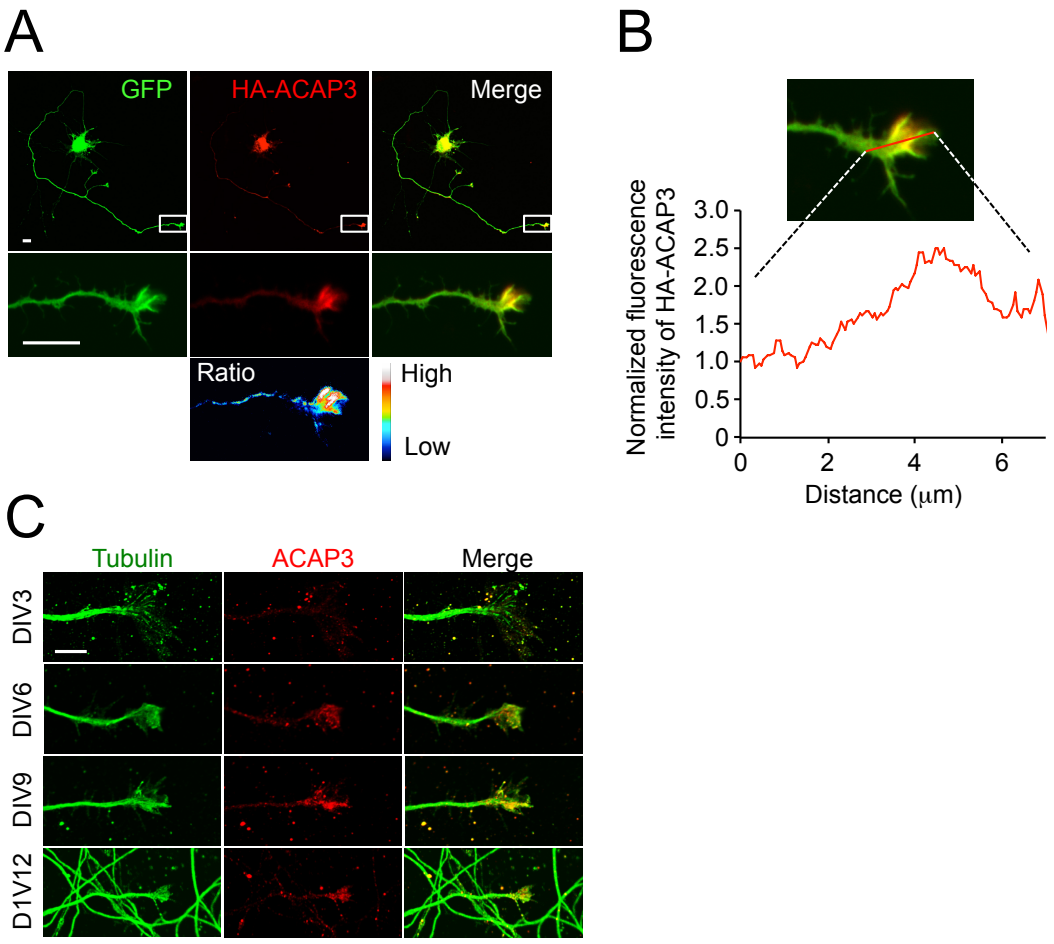


Figure 11. Subcellular localization of ACAP3 in hippocampal neurons.
(**A**) Subcellular localization of ACAP3 in hippocampal neurons. HA-ACAP3 and GFP were co-expressed in DIV4 hippocampal neurons and immunocytochemically analyzed with anti-GFP and -HA antibodies at DIV6. The signal intensity of HA-ACAP3 at the growth cone was normalized by that of co-expressed GFP, and a ratiometric image was shown in lowest panel. (**B**) The fluorescence intensity of HA-ACAP3 normalized to that of co-expressed GFP across the growth cone was plotted. (**C**) Localization of endogenous ACAP3 at the growth cone of hippocampal neurons. Endogenous ACAP3 and tubulin in primary cultured hippocampal neurons were immunocytochemically visualized at DIV3, 6, 9 and 12. Scale bar, 10 μm (**A**) and 5 μm (**C**).

To address this possibility, ACAP3 in hippocampal neurons was efficiently knocked down with shRNAs (Figure 12A). Under these conditions, total neurite length was significantly decreased, whereas total neurite number was not affected in DIV3-12 hippocampal neurons (Figure 12B-D), supporting the possibility described above that

ACAP3 is involved in neurite outgrowth throughout these developing stages, possibly axonal outgrowth, without any effects on initial formation of the neurite.

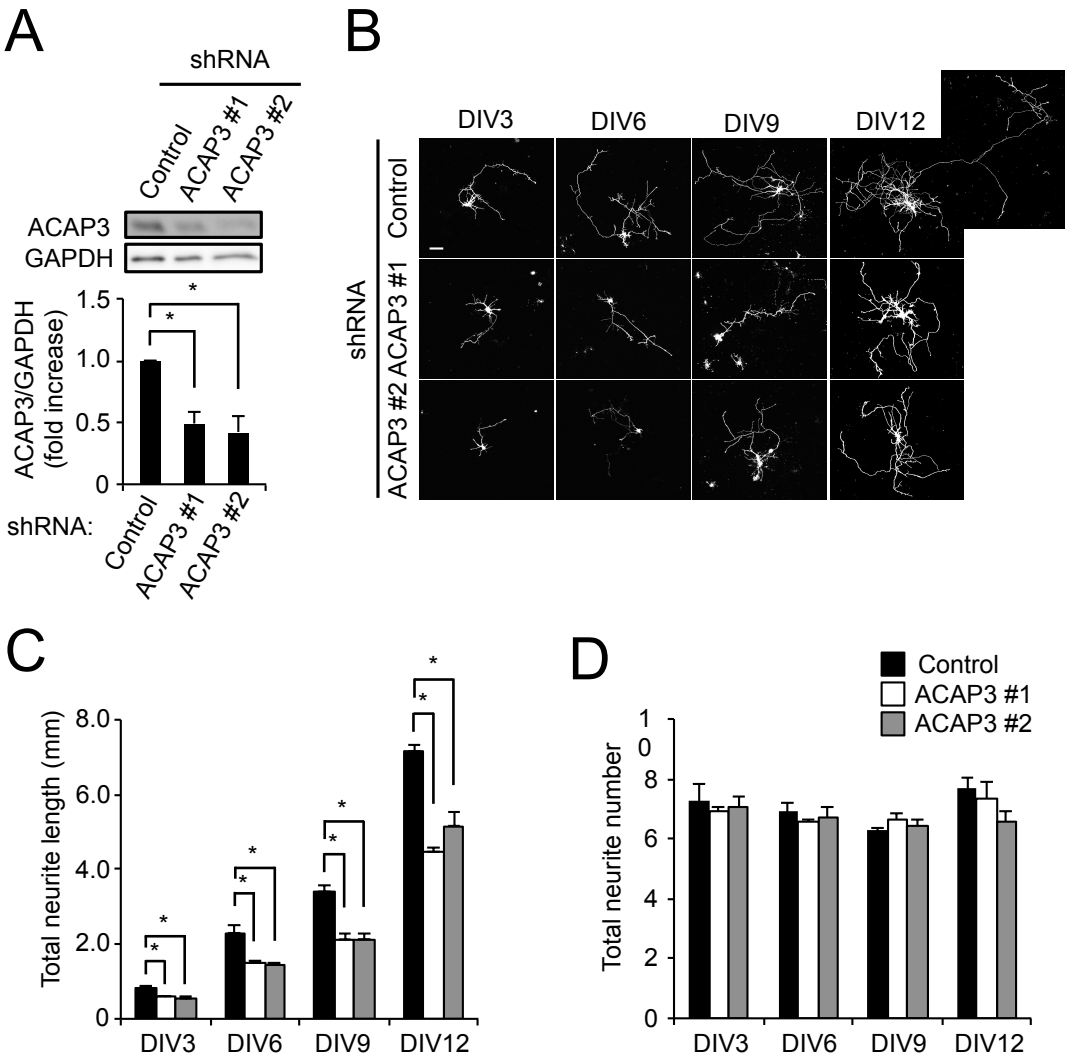


Figure 12. ACAP3 is involved in neurite outgrowth of hippocampal neurons. (**A**) Efficient knockdown of ACAP3 in hippocampal neurons. shRNAs for *ACAP3* and control were expressed in hippocampal neurons at DIV0, and expression levels of ACAP3 protein were detected by western blotting probed with anti-ACAP3 antibody at DIV5. Upper panels show representative western blots from four independent experiments and the lower panel is the quantitative data. (**B-D**) Effects of ACAP3 knockdown on neurite outgrowth. GFP and shRNAs for control and *ACAP3* were expressed in DIV0, 1, 4, and 9 cultured hippocampal neurons, and GFP were immunocytochemically stained at DIV3, 6, 9 and 12, respectively (**B**). Total neurite length (**C**) and total neurite number (**D**) were measured in 33-90 neurons. Scale bar, 100 μm (**B**). Data represent the mean ± SEM from at least three independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

3-3 ACAP3 regulates neurite outgrowth through its GAP activity specific to Arf6

To investigate whether regulation of neurite outgrowth by ACAP3 requires its GAP activity, we conducted rescue experiments of neurite outgrowth inhibited by ACAP3 knockdown with shRNA-resistant WT (WT^{RES}) and the GAP activity-deficient mutant R446Q of ACAP3 ($R446Q^{RES}$), in which the conserved arginine residue necessary for the GAP activity of ACAPs was substituted by glutamine [50]. When these molecules as well as shRNA-non-resistant WT and the R446Q mutant of ACAP3 were expressed in HEK-293T cells, expression levels of WT^{RES} and $R446Q^{RES}$ were not significantly disturbed by ACAP3 shRNA, whereas expression of the shRNA-non-resistant molecules was completely suppressed (Figure 13).

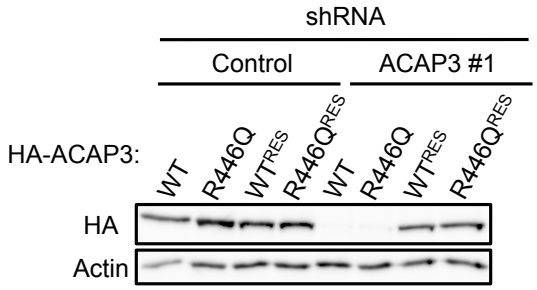


Figure 13. Expression of shRNA-non-resistant and -resistant wild type and GAP activity-deficient mutants of ACAP3.
HA-tagged shRNA-non-resistant and -resistant wild type (WT and WT^{RES}, respectively) and GAP activity-deficient mutants of ACAP3 (R446Q and $R446Q^{RES}$, respectively) were co-expressed with shRNA for control or *ACAP3* in HEK293T cells. After 1.5 days of culture, expression levels of these molecules were detected by western blotting probed with anti-HA antibody.

Also, expression of shRNA-resistant molecules was confirmed in ACAP3-knocked-down hippocampal neurons (Figure 14A-D).

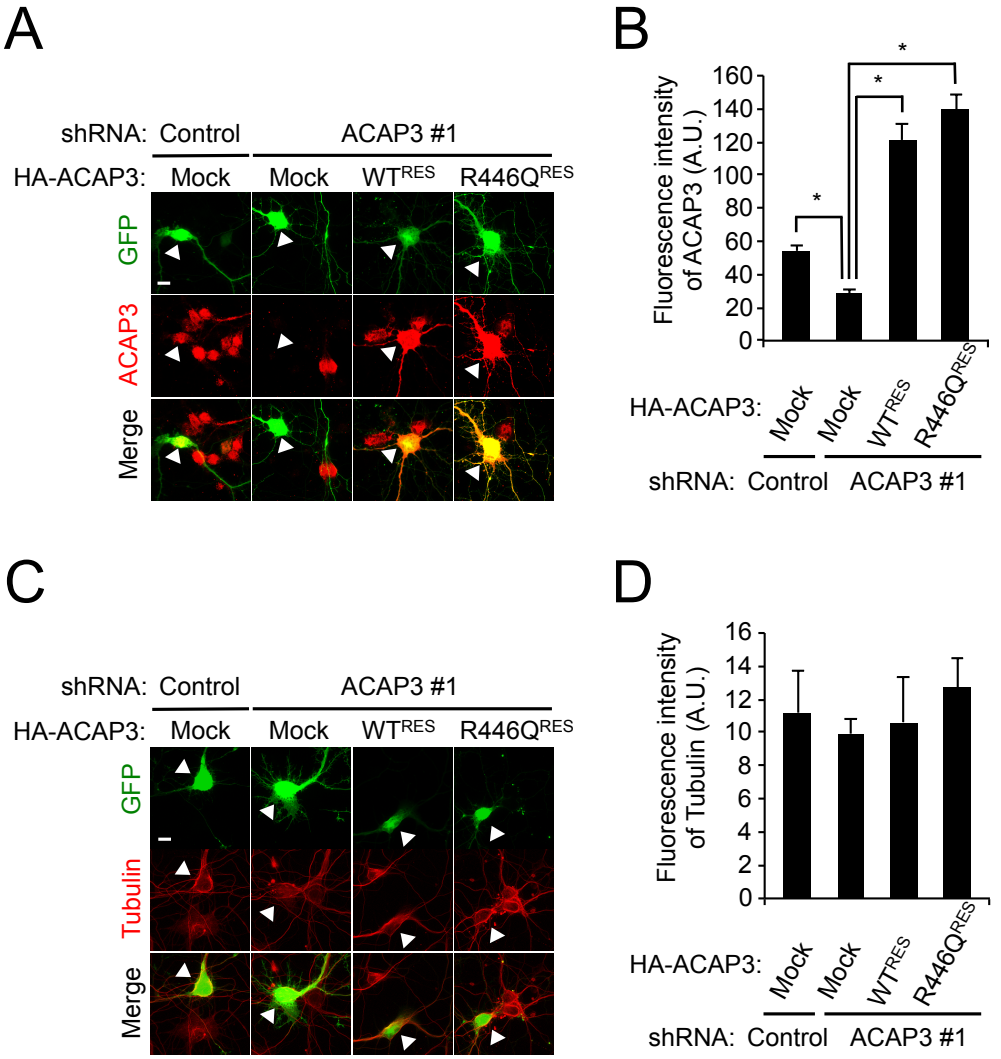


Figure 14. Expression levels of shRNA-resistant ACAP3s in ACAP3-knocked down hippocampal neurons. The expression vector encoding GFP and shRNA for control or *ACAP3* was co-transfected with the vector encoding WT^{RES} or R446Q^{RES} of HA-ACAP3 into primary cultured hippocampal neurons at DIV4. ACAP3 (**A**) or tubulin (**C**) with GFP was immunocytochemically visualized at DIV9, and fluorescence intensity of ACAP3 (**B**) or tubulin (**D**) was measured in 29-45 neurons. Allow heads indicate GFP-positive neurons. Scale Bar, 10 μm (**A**,**C**). Data show the mean ± SEM of three independent experiments. A.U., arbitrary unit. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

Under these conditions, WT^{RES}, but not R446Q^{RES}, rescued neurite outgrowth inhibited by ACAP3 knockdown (Figure 15A,B). Thus, ACAP3 regulates neurite outgrowth

through its GAP activity.

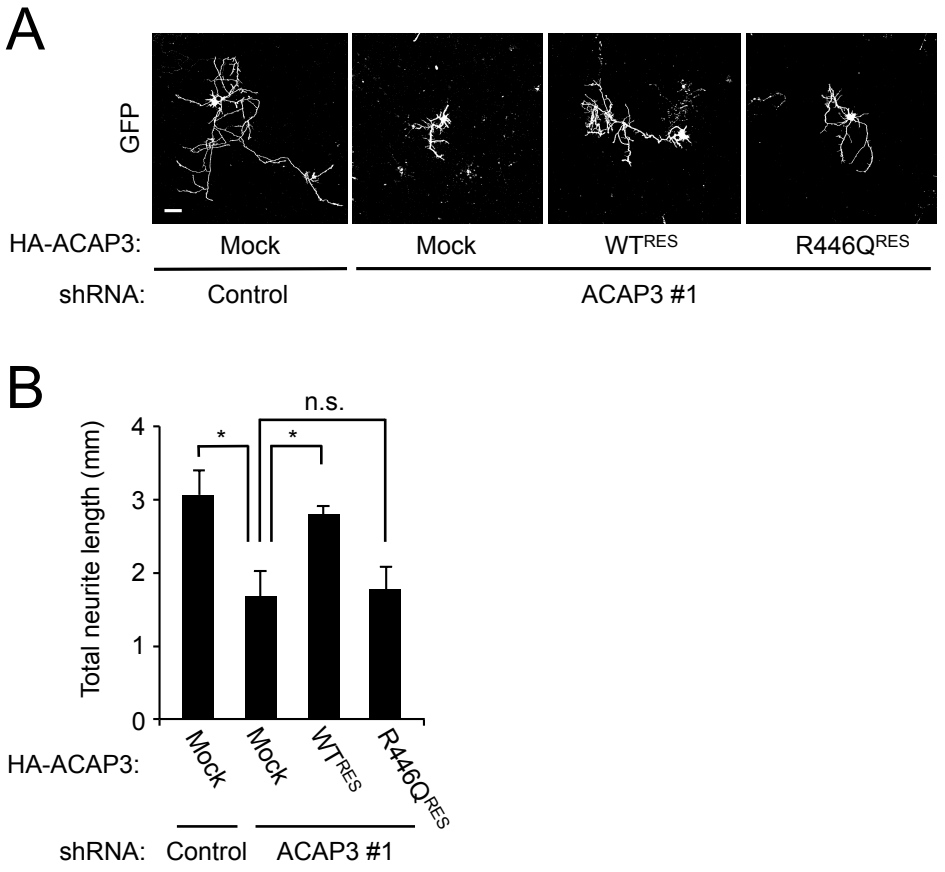


Figure 15. Regulation of neurite outgrowth by ACAP3 requires its GAP activity.
(**A**,**B**) ACAP3-WT^{RES} or -R446Q^{RES} was co-expressed with GFP and shRNA for control or *ACAP3* in DIV4 cultured hippocampal neurons. At DIV9, neurons were immunocytochemically stained for GFP (**A**), and total neurite length was measured in 20 neurons (**B**). Scale bar, 100 μm. Data represent the mean ± SEM from three independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

The result described above showing that GAP activity of ACAP3 is critical to regulate neurite outgrowth raises a possibility that regulation of neurite outgrowth by ACAP3 is mediated by an Arf isoenzyme(s). To clarify the specificity of ACAP3 GAP activity to Arf isoforms, effects of overexpressed WT and the GAP activity-deficient

mutant of ACAP3 (ACAP3 WT and ACAP3 R446Q respectively) on the levels of GTP-bound active form of ectopically expressed Arfs-HA in HEK-293T cells were analysed (Figure 16A-C). Wild type ArfGAP1 and ACAP2 specific to Arf1 and Arf6 respectively and their GAP activity-deficient mutants (ArfGAP1 R50K and ACAP2 R442Q respectively) were also overexpressed as controls. As reported [46,50], ArfGAP1 and ACAP2 had GAP activities specific to Arf1 and Arf6 respectively (Figure 16A,C). ACAP3 WT and ACAP3 R446Q did not affect the levels of the active form of Arf1 and Arf5, the representatives of classes I and II respectively (Figure 16A,B). In contrast, ACAP3 WT significantly decreased the level of the GTP-bound active form of Arf6, whereas ACAP3 R446Q did not (Figure 16C). These results demonstrate that ACAP3 functions as an Arf6-specific GAP.

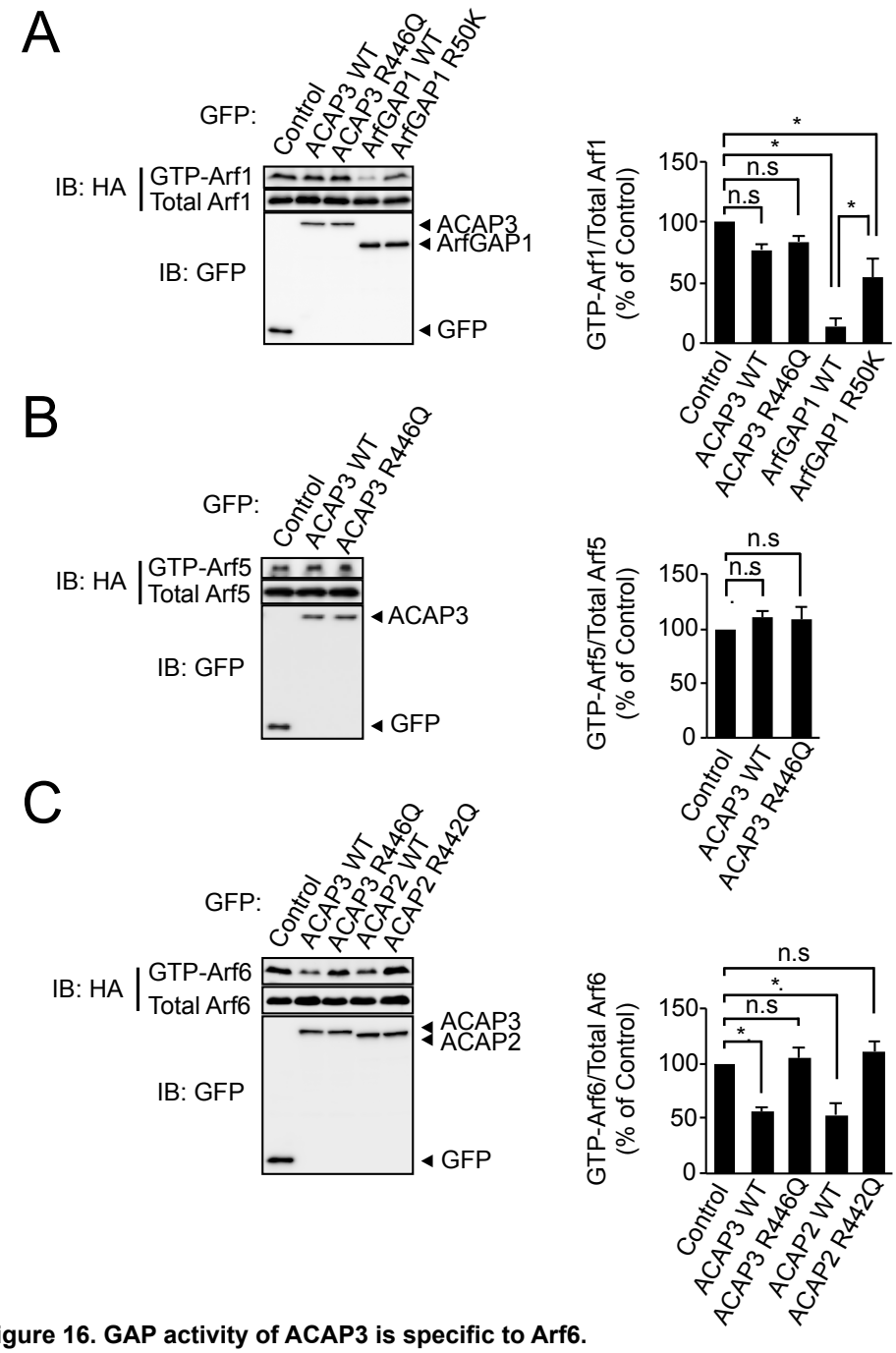


Figure 16. GAP activity of ACAP3 is specific to Arf6.
GFP-Arf GAPs and their GAP-deficient mutants were co-expressed with Arfs-HA in HEK293T cells. After 1.5 days of culture, GTP-bound active Arf1 (**A**), Arf5 (**B**) and Arf6 (**C**) were pulled-down with GST-GGA3 and detected by western blotting probed with anti-HA antibody. Expression levels of GFP-Arf GAPs and total Arfs were also detected. Left panels in each figure are the representatives from three independent experiments, and right panels quantitative data which show mean ± SEM of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's HSD for **A** and Dunnett's multiple comparison test for **B** and **C**; n.s., not significant, $^{*}P<0.05$.

An expression pattern of endogenous Arf6 during *in vitro* culture of mouse hippocampal neurons was similar to that of ACAP3: the expression levels of Arf6 increased with prolonged culture and reached a plateau at DIV9-DIV12 (Figure 17A). It has been reported that Arf6 localizes at the cell body, axons and dendrites of hippocampal neurons [72]. Consistent with this earlier paper, Arf6-Myc expressed in hippocampal neurons localized at these compartments and co-localized with co-expressed HA-ACAP3 (Figure 17B). It is noteworthy that co-localization of these two molecules was observed at the neurite tip. This was also true for endogenous Arf6 and ACAP3 (Figure 17C): co-localization of Arf6 and ACAP3 at the neurite tip was obvious at DIV6 and DIV9, although it was not clear at DIV3 and DIV12.

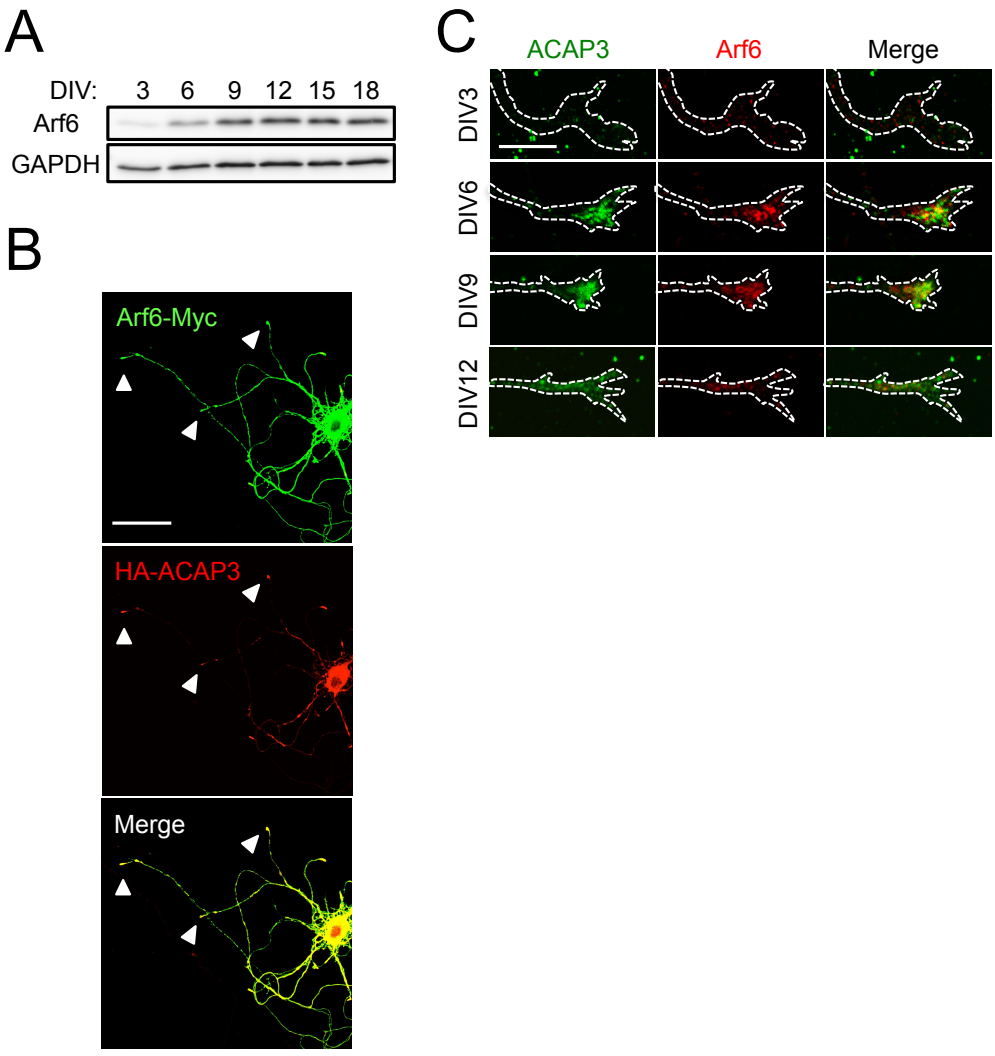


Figure 17. ACAP3 co-localizes with Arf6 in hippocampal neurons.
(**A**) Expression levels of Arf6 protein in primary cultured hippocampal neurons were analyzed by western blotting probed with anti-Arf6 antibody. (**B**) Arf6-Myc and HA-ACAP3 were co-expressed in hippocampal neurons at DIV4, and immunocytochemically stained at DIV6. Arrow heads indicate neurite tips where these two molecules co-localized. (**C**) Co-localization of ACAP3 and Arf6 at the neurite tips. Endogenous ACAP3 and Arf6 in cultured hippocampal neurons were immunocytocemically visualized at DIV3, 6, 9 and 12. Scale bar, 50 μm (**B**) and 5 μm (**C**).

Furthermore, it was found that knockdown of ACAP3 in hippocampal neurons increased the level of the active form of Arf6-HA (Figure 18), demonstrating that ACAP3 has the Arf6 GAP activity in hippocampal neurons. These results, taken

together with the results shown in Figure 15, support the notion that ACAP3 regulates neurite outgrowth by controlling Arf6 activity through its Arf6-specific GAP activity.

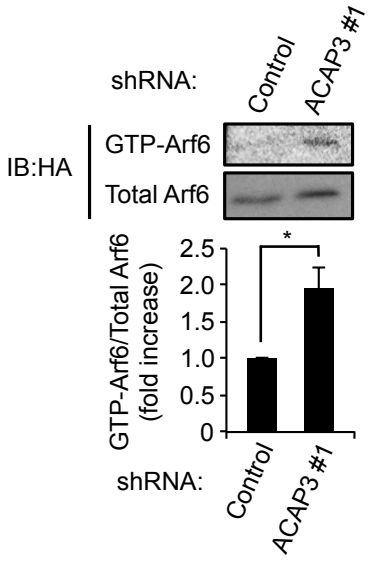


Figure 18. ACAP3 functions as Arf6 GAP in hippocampal neurons.
Arf6-HA and shRNAs for control and *ACAP3* were expressed in primary cultured hippocampal neurons at DIV4, and levels of the GTP-bound active form of Arf6-HA was detected at DIV9 (upper panels). The lower panel is the quantitative data which show the mean ± SEM of four independent experiments. Statistical significance was calculated using unpaired two-tailed Student's *t*-test; $^{*}P<0.05$.

3-4 GTP/GDP cycle of Arf6 is required for neurite outgrowth

Next, we investigated the involvement of Arf6 in neurite outgrowth and the functional relationship between ACAP3 and Arf6. When primary cultured hippocampal neurons were transfected with plasmids for *Arf6* shRNAs, the levels of Arf6 were decreased by approximately 30-40% (Figure 19A). Under these conditions, total neurite length, but not total neurite number, was inhibited by ~50% (Figure 19B-D).

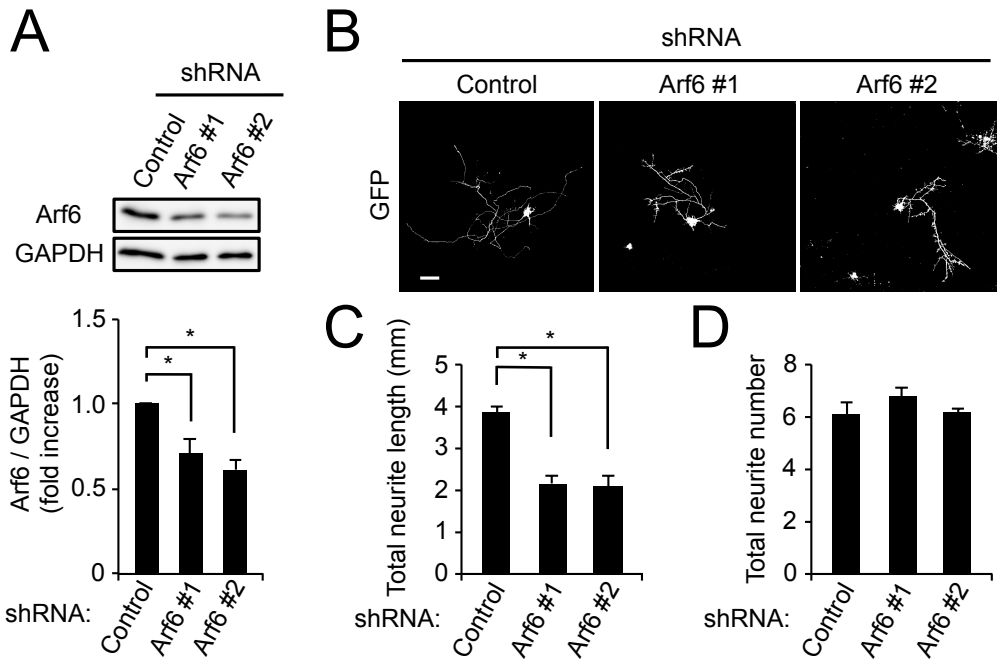


Figure 19. Involvement of Arf6 in neurite outgrowth.
(**A**) Knockdown of Arf6. shRNAs for control and *Arf6* were expressed in primary cultured hippocampal neurons with Amaxa Mouse Neuron Nucleofector Kit at DIV0, and the expression levels of Arf6 were analyzed by western blotting probed with anti-Arf6 antibody at DIV5. (**B-D**) Effect of Arf6 knockdown on neurite formation and outgrowth. The expression vector encoding GFP and shRNA for control and *Arf6* were transfected into primary cultured hippocampal neurons at DIV4. Expressed GFP was immunocytochemically visualized at DIV9 (**B**), and total neurite length (**C**) and total neurite number (**D**) were measured in 20 neurons. Scale bars, 100 μm. Data shows the mean ± SEM of at least three independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

Consistent with these results, total neurite length, but not total neurite number, of hippocampal neurons prepared from *Arf6*-KO embryos was significantly inhibited (Figure 20A-D). Thus Arf6 also positively regulates neurite outgrowth without any effects on the number of neurites.

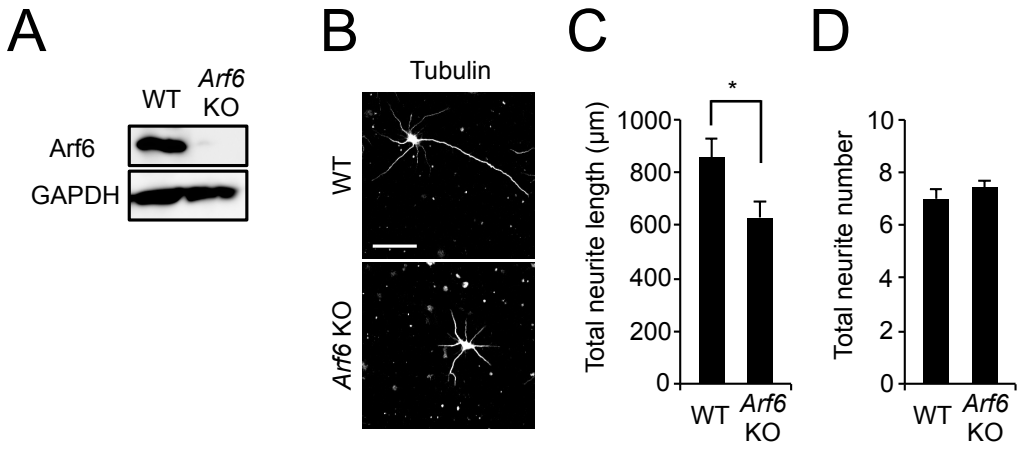


Figure 20. Neurite outgrowth in *Arf6* KO hippocampal neurons.
(**A**) Expression levels of Arf6 protein in hippocampal neurons prepared from E17-18 WT or *Arf6* KO mouse embryos were analysed by western blotting probed with anti-Arf6 antibody. (**B-D**) Effects of *Arf6* ablation on neurite formation and outgrowth. Primary cultured hippocampal neurons prepared from E17-18 WT and *Arf6* KO embryos (n = 5 and 4 for WT and *Arf6* KO, respectively) were stained for tubulin at DIV6 (**B**), and total neurite length (**C**) and neurite number (**D**) were measured in 20 neurons. Scale bars, 100 μm. Data shows the mean ± SEM of at least three independent experiments. Statistical significance was calculated using unpaired two-tailed Student's t-test; $^{*}P<0.05$.

Furthermore, it was found that neurite outgrowth suppressed by ACAP3 knockdown was rescued by overexpression of the fast cycle mutant of Arf6, T157A, which spontaneously exchange nucleotides on the mutant T157A [85], but not by WT and GTP- and GDP-locked Arf6 mutants of Arf6, Q67L and T44N [86] respectively (Figure 21A,B). These results indicate that Arf6 functions as a downstream effector of ACAP3 in the neurite outgrowth signaling and cycling between active and inactive states of Arf6 is required for neurite outgrowth.

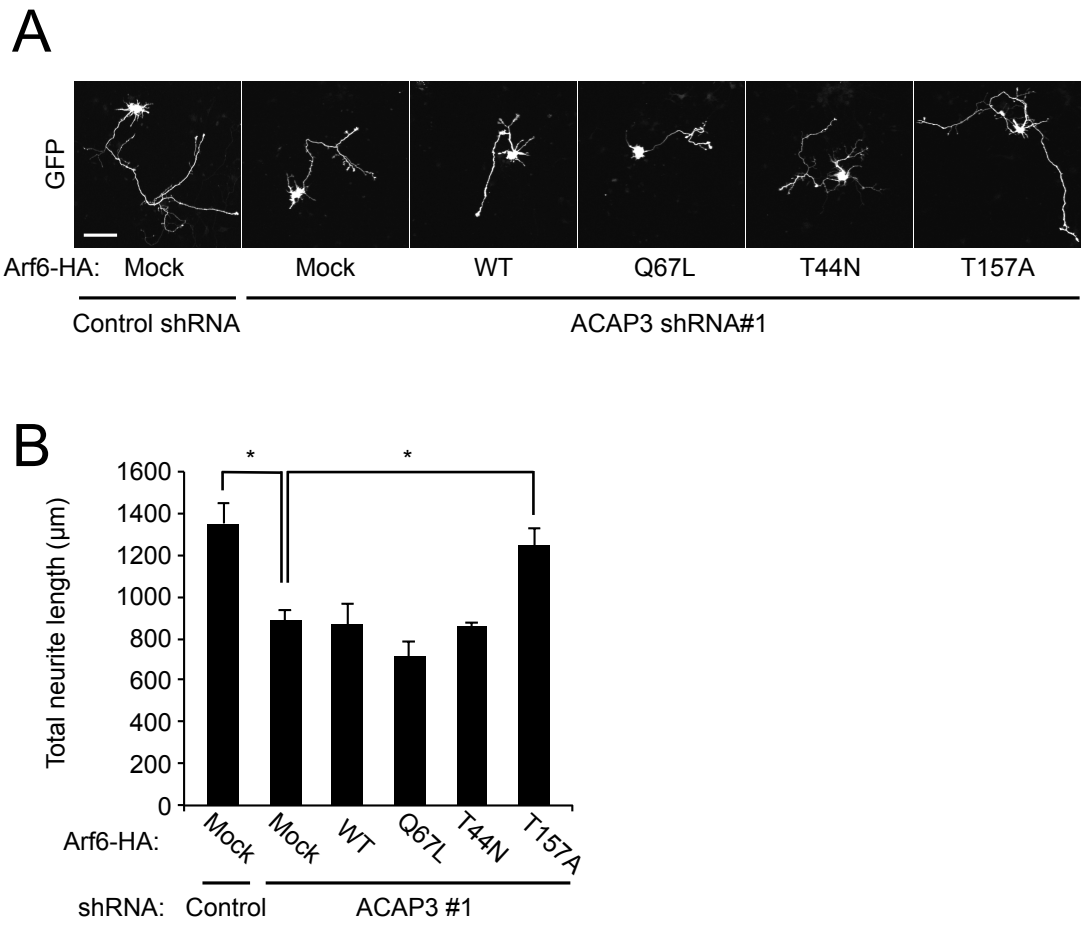


Figure 21. Effect of overexpressed Arf6 mutants on neurite outgrowth in ACAP3-knocked-down hippocampal neurons.
The expression vector encoding GFP and shRNA for control or *ACAP3* was co-transfected with the vector encoding HA-tagged Arf6-WT, -Q67L, -T44N or -T157A into primary cultured hippocampal neurons at DIV4. Expressed GFP was immunocytochemically visualized at DIV6 (**A**), and total neurite length was measured in 20 neurons (**B**). Images were the representative from four independent experiments. Scale Bar, 100 μm. Data shown in **B** is the mean ± SEM from four independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

3-5 ACAP3 controls levels of membrane phospholipid PtdIns(4,5)P_2 at the growth cone

We further investigated the levels of PtdIns(4,5)P_2 at the growth cone of hippocampal neurons in ACAP3 knocked-down cultured hippocampal neurons, since it has been reported that the PtdIns(4,5)P_2-producing enzyme PIP5K is directly activated by the

active form of Arf6 [17], and PtdIns(4,5)P_2 regulates actin filament reorganization and membrane trafficking which are essential for neurite outgrowth [64,65]. Deletion of ACAP3 significantly increased the levels of PtdIns(4,5)P_2 at the growth cone without any effect on actin remodeling (Figure 22A-E), suggesting that inactivation of Arf6 by ACAP3 downregulates PIP5K, which results in the decrease in the PtdIns(4,5)P_2 level, and PtdIns(4,5)P_2 controlled by ACAP3 is involved in neurite outgrowth by regulating membrane trafficking, but not by actin remodeling.

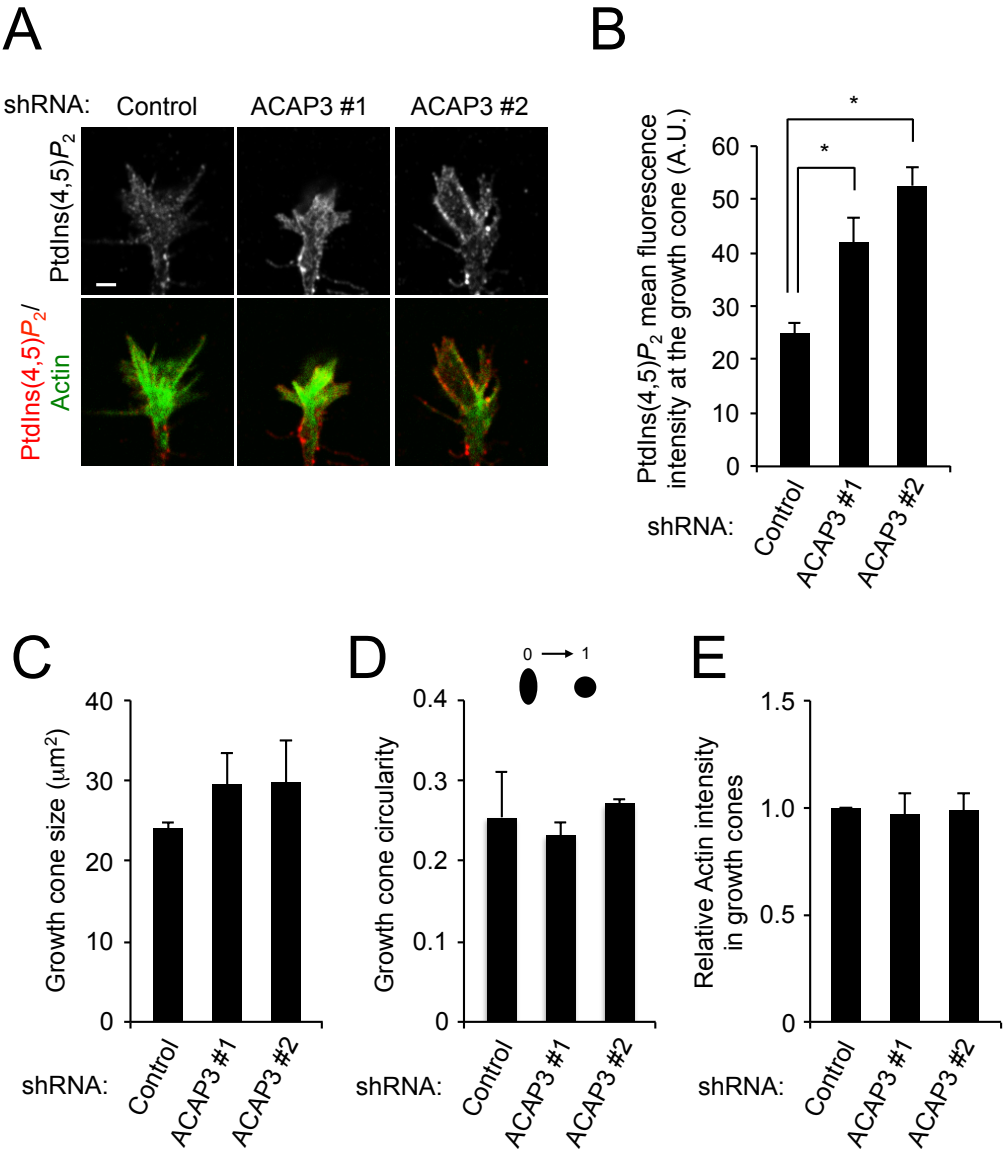


Figure 22. Deletion of ACAP3 increased the levels of PtdIns(4,5)P_2 at the growth cone of hippocampal neurons without any effect on actin remodeling

The expression vector encoding GFP and shRNA for control and *ACAP3* was transfected into primary cultured hippocampal neurons at DIV4. PtdIns(4,5)*P*2 and actin was immunocytochemically visualized at DIV6 (**A**), and fluorescence intensity of PtdIns(4,5)*P*2 (**B**) and actin (**E**), and growth cone size (**C**) and circularity (D) were measured in 39-42 neurons. Scale Bar, 2 μm (**B-E**). Data show the mean ± SEM of three independent experiments. A.U., arbitrary unit. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

3-6 ACAP3 regulates Rab35-stimulated neurite outgrowth

To further investigate whether ACAP3 regulates membrane trafficking at the growth cone, we examined the interaction between ACAP3 and other small G protein Rabs, which play key roles in membrane trafficking [87,88].

Initially synthesized Rabs are processed by multiple proteins containing REP (Rab escort protein), GGT (geranylgeranyl transferase), GDI (GDP-dissociation inhibitor) and GDF (GDI displacement factor) (Figure 23A). After processing, Rabs, as well as other small G proteins, act as molecular switches, cycling between active and inactive form, regulated by GEFs and GAPs. Through the interaction with effectors, Rabs regulate multiple aspect of membrane trafficking including vesicle formation, transport, tethering, and fusion. More than 60 members of Rabs have been reported and these Rabs are involved in distinct process of membrane trafficking in different organelles compartments of cells (Figure 23B, C).

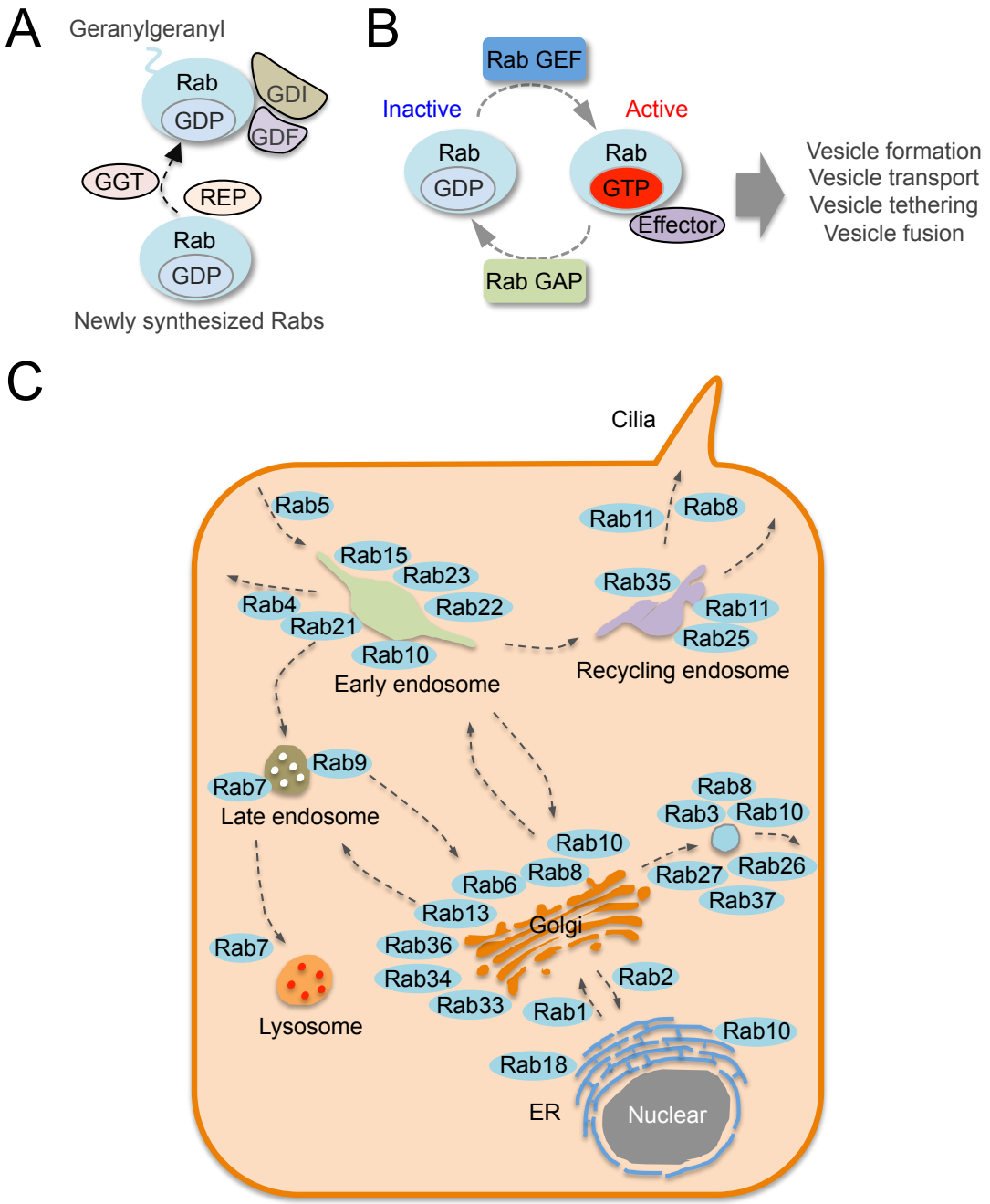


Figure 23. The Rab small G proteins
(**A**) Newly synthesized Rab proteins are recognized and escorted by REP (Rab escort protein) to GGT (geranylgeranyl transferase), which geranylgeranlates the Rabs on their carboxy terminal Cys. These geranylgeranylated-Rabs are recognized by Rab GDI (GDP dissociation inhibitor), which controls membrane cycle of the Rabs. GDF (GDI displacement factor) interacts with Rab-GDI and target it to specific membrane. (**C**) Like other small G proteins, Rabs act as molecular switches between GTP-bound active and GDP bound inactive forms, and regulate multiple steps of membrane trafficking, including vesicle formation, transport, tethering, and fusion. (**C**) Intracellular distribution of Rabs at the distinct organelles compartments.

Of note, several Rabs have been reported to be involved in neurite formation [89], and among them, we recently identified Rab18 and Rab35, as putative binding proteins of ACAP3 (unpublished data) *in vitro* GST-Rabs pulldown assay. Both proteins have been recently reported to regulate axonal growth [90,91], however, their localization in hippocampal neurons and molecular mechanisms in which these Rab proteins regulate axon growth remain elusive. In hippocampal neurons, ectopically expressed strawberry tagged-Rab18 and -Rab35 were localized at the growth cone. In particular, Rab35 was strongly co-localized with GFP-tagged ACAP3 in the growth cone (Figure 24). Although Rab18 was also co-localized with ACAP3, the co-localization between these two proteins at the growth cone was rather moderate than Rab35.

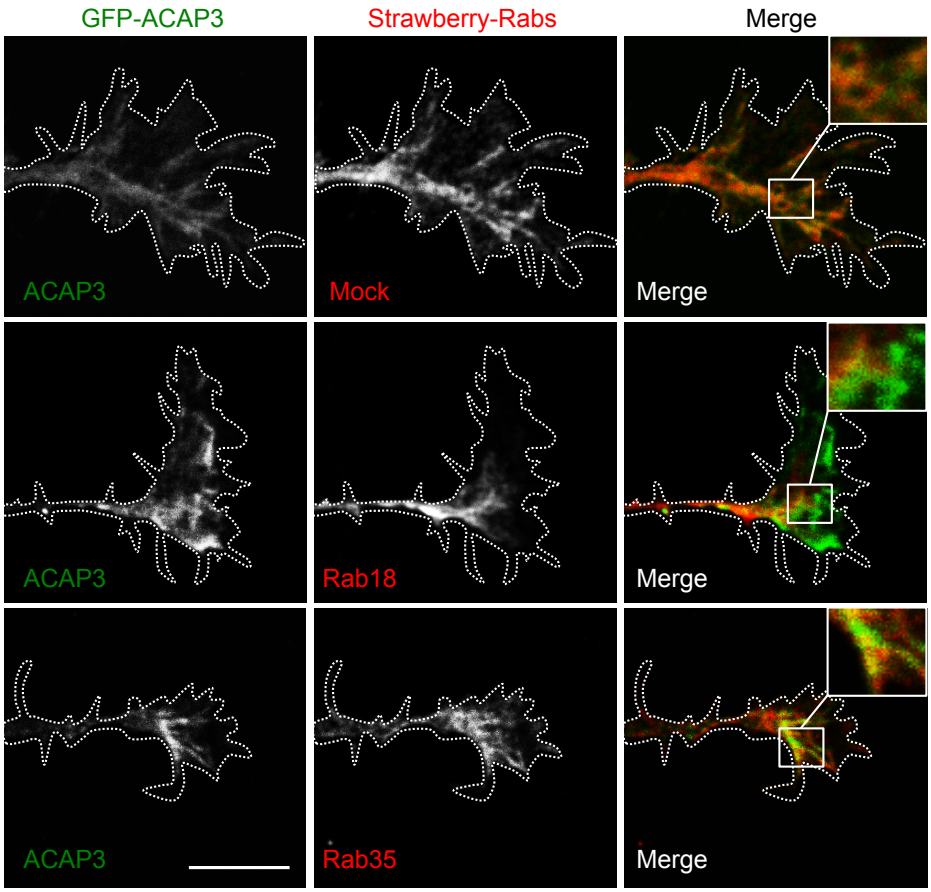


Figure 24. ACAP3 co-localizes with Rab35 at the growth cone. GFP-ACAP3 and Strawberry-Rab18 and Rab35 were co-expressed in hippocampal neurons at DIV4, and GFP and Strawberry were immunocytochemically stained at DIV6. Scale bar, 5 μm. Dotted line indicate growth cone regions.

Furthermore, we found that in HEK-293T cells, GTP-locked Rab35-Q67L mutant of Rab35 preferentially interacts with ACAP3, but not dominant negative mutant Rab35-S22N (Figure 25), suggesting that Rab35 interacts with ACAP3 in Rab35 activity-dependent manner.

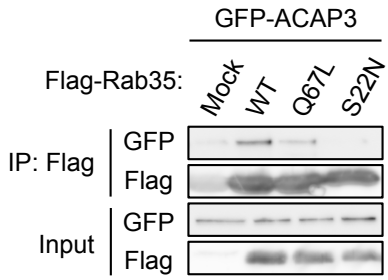


Figure 25. ACAP3 interacts with active forms of Rab35
GFP-ACAP3 was co-expressed with Flag-tagged wild type of Rab35, its constitutive active mutant Q67L and dominant negative mutant S22N in HEK293T cells. After 1.5 days of culture, Flag-Rabs were immunoprecipitated with Flag antibody beads, and coimmunoprecipitated GFP-ACAP3 was detected by western blotting probed with anti-GFP antibody.

These facts led us to speculate that ACAP3 functions as an effector molecule of Rab35 in neurite outgrowth. To address this issue, we investigated the effect of ACAP3 knockdown on Rab35-stimulated neurite outgrowth. Consistent with the report showing that Rab35 stimulates neurite outgrowth of hippocampal neuron [91], ectopic expression of Rab35 promoted neurite outgrowth of hippocampal neurons. The Rab35-stimulated neurite outgrowth was completely suppressed by knockdown of ACAP3 (Figure 26A,B). Thus, ACAP3 could function as a downstream molecule of Rab35 in neurite outgrowth, and regulate Rab35-mediated membrane trafficking at the growth cone in neurite outgrowth.

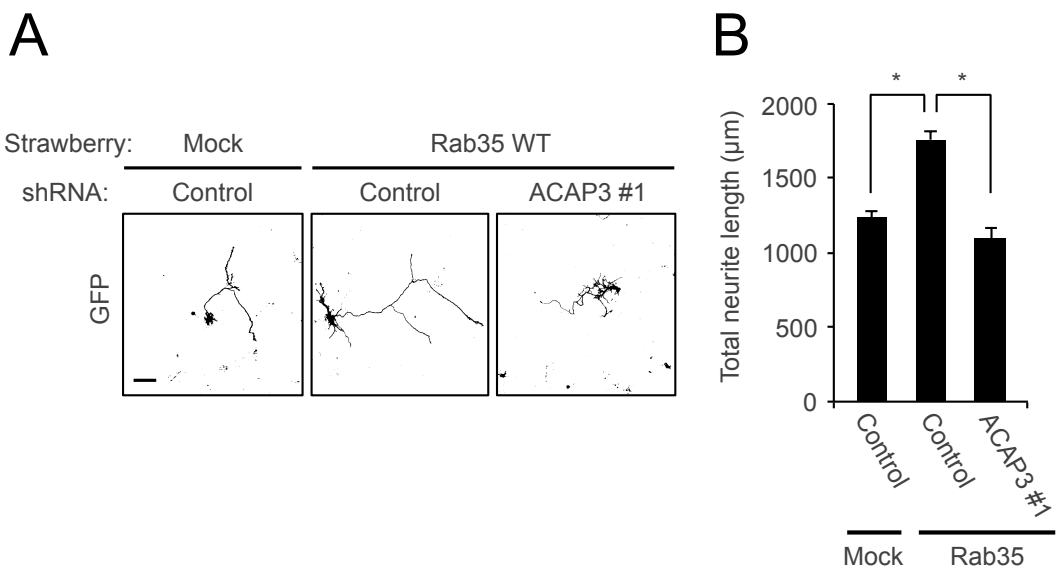


Figure 26. ACAP3 is involved in Rab35-stimulated neurite outgrowth
The expression vector encoding GFP and shRNA for control and *ACAP3* was co-transfected with the vector expressing Strawberry-tagged Rab35 wild type into primary cultured hippocampal neurons at DIV4. Expressed GFP was immunocytochemically visualized at DIV6 (**A**), and total neurite length was measured in 20 neurons (**B**). Images were the representative from three independent experiments. Scale Bar, 100 μm. Data shown in **B** is the mean ± SEM from three independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test; $^{*}P<0.05$.

Chapter 4: Discussion

In the present study, we demonstrated that the Arf GAP family member ACAP3 functions as an Arf6-specific GAP, and positively regulates neurite outgrowth of hippocampal neurons by controlling the cycle between active and inactive forms of Arf6. Furthermore, we found that ACAP3 controls levels of PtdIns(4,5)P_2 at the growth cone of hippocampal neurons, and interacts with other small G protein Rab35 at the growth cone to regulate Rab35-stimulated neurite outgrowth of hippocampal neurons (Figure 27).

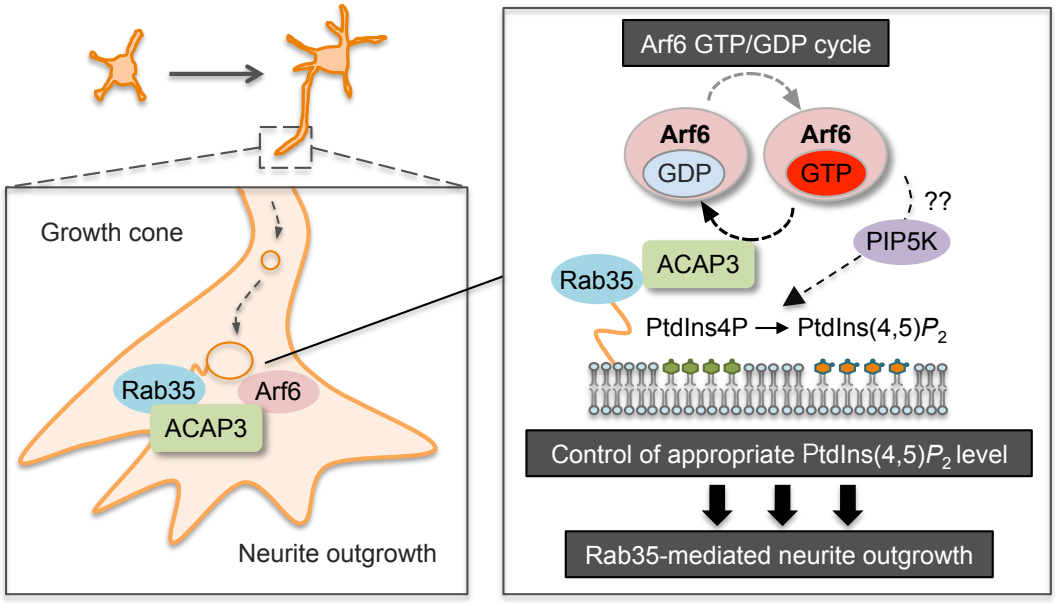


Figure 27. Proposed model of neurite outgrowth regulated by ACAP3 and Arf6
Arf GAP ACAP3 functions as an Arf6-specific GAP and positively regulates neurite outgrowth of hippocampal neurons by controlling the GTP/GDP cycle of Arf6. ACAP3 controls levels of PtdIns(4,5)P_2 at the growth cone of hippocampal neurons. ACAP3 co-localizes and interacts with other small G protein Rab35 at the growth cone to regulate Rab35-stimulated neurite outgrowth of hippocampal neurons.

4-1 GTP/GDP cycle of Arf6 regulated by ACAP3 is indispensible for neurite outgrowth

In this study, we emphasize that cycling of Arf6 between active and inactive states, regulated by ACAP3 and undefined GEF(s), is crucial for regulating neurite outgrowth. This resulted from our findings that depletion of Arf6 inhibited neurite outgrowth (Figure 19, 20), ACAP3 positively regulated neurite outgrowth through its Arf6-specific GAP activity (Figure 15) and neurite outgrowth inhibited by ACAP3 knockdown was rescued by expression of the fast cycle mutant of Arf6, but not by that of wild type, constitutively active and dominant-negative mutants of Arf6 (Figure 21). This notion is also supported by the report suggesting that hyperactivation of Arf6 interferes with the clathrin-mediated endocytosis: overexpression of the Arf6 GEF EFA6 inhibits transferrin uptake/internalization [49]. The requirement of cycling between active and inactive states of Arf6 is suggested in phagocytosis in macrophages [92] and the membrane trafficking of the membrane-associated protease MT1-MMP (membrane-type 1 matrix metalloproteinase) from the sorting endosome to invadopodia via the late endosome in the breast cancer cell line MDA-MB-231 cells, which is indispensable for extracellular matrix degradation by invadopodia of cancer cells [24,93]. Thus, cycling of GTP/GDP-bound Arf6 seems to be important to regulate a wide range of cell functions.

4-2 Downstream molecule of Arf6 in neurite outgrowth

Although downstream molecule(s) by which Arf6 cycling between the inactive and active states regulates neurite outgrowth remains to be elucidated, it is plausible that the PtdIns(4,5)P_2-producing enzyme PIP5K functions downstream of ACAP3/Arf6 in neurite outgrowth. This idea is strongly supported by the previous study and the result obtained in this study demonstrating that Arf6 directly activates PIP5K to produce the versatile phospholipid PtdIns(4,5)P_2, which is a critical regulator of reorganization of actin filament and membrane trafficking, and that knockdown of ACAP3 significantly increased the level of PtdIns(4,5)P_2 at the growth cone (Figure 22) respectively. If this is the case, PIP5K isoform regulating neurite outgrowth downstream of Arf6 might be PIP5Kγ661, since among three spliced variants of PIP5Kγ, γ635 γ661, and γ687, only PIP5Kγ661 is expressed in the hippocampal neurons in development dependent manner [84] and regulates Sema3A-induced repulsion of axonal growth cone by regulating adhesion of the neurons [94]. Although all PIP5K isoforms are expressed in brain, PIP5Kα and PIP5Kβ act as negative regulators of neurite outgrowth in PC12 cells [95], and DRG neurons [96] respectively. These facts led us to speculate that each PIP5K isoenzyme generates a distinct PtdIns(4,5)P_2 pool in spatiotemporally different manner in neurons, and ACAP3/Arf6 would regulate specific PtdIns(4,5)P_2 pool, which was produced by PIP5Kγ661, to positively regulate neurite outgrowth. Further investigation is needed to clarify this point.

4-3 Involvement of ACAP3/Arf6 in membrane trafficking at the growth cone

Deletion of ACAP3 in hippocampal neurons increased the level of PtdIns(4,5)P_2 (Figure 22A,B), which is a key component for actin remodeling and membrane trafficking. Since suppression of ACAP3 did not affect the actin remodeling in the growth cone (Figure 22C-E), and it was found that ACAP3 interacts and co-localizes with Rab35 at the growth cone of hippocampal neurons (Figure 24, 25), it is reasonable to expect that levels of PtdIns(4,5)P_2 controlled by ACAP3/Arf6 is responsible for Rab35-mediated membrane trafficking in the growth cone. During neurite extension, membrane proteins destined to the growth cone membrane and proximal axons, such as NGF receptor [Trk (tropomyosin receptor kinase) receptors] and cell adhesion molecule L1/NgCAM (neuron-glia cell adhesion molecule), should be supplied from endosomes to the plasma membrane of these areas by membrane trafficking or recycling [97,98]. Since it has been reported that Rab35 and Arf6 are involved in membrane trafficking and recycling in many types of cells, these molecules would regulate neurite outgrowth by controlling these intracellular events. Consistent with this idea, it has been suggested that in PC12 cells, Rab35 and Arf6-mediated membrane trafficking is involved in neurite outgrowth stimulated by NGF: scission of vesicles from recycling endosomes by the key player of vesicle scission EHD1 (EH-domain-containing 1) [99], whose recruitment to the endosome is regulated by Arf6 [100], is critical for the neurite outgrowth [101]. In the process of Arf6-dependent recruitment of EHD1, PtdIns4P functions as a key recruiter of EHD1 [102]. Since Arf6 is the activator of the PtdIns(4,5)P_2-producing enzyme PIP5K and an Arf6 GAP is involved in the recruitment

of EHD1 [101], it is reasonable to expect that inactivation of Arf6 by Arf6 GAP at the recycling endosomes terminates the activation of PIP5K to decrease the PtdIns(4,5)P_2 level and thereby increases PtdIns4P level. Although it has been demonstrated that the Arf6-specific GAP ACAP2, recruited by Rab35, functions in this process [101], it has not been examined whether ACAP3 is involved in this process. It is of interest to examine whether ACAP3 is also involved in the recruitment of EHD1 to the recycling endosome and regulates recycling of the cargo proteins essential for neurite outgrowth, such as Trk receptor and L1/NgCAM.

4-4 Sequential regulation of Arf6 by distinct Arf6 GEFs and GAPs in neurite outgrowth

Other ACAP family member ACAP2 is expressed in the brain upon development dependent manner as well as ACAP3, whereas ACAP1 is not expressed in the brain (Figure 8A). In addition, both ACAP2 and ACAP3 have an Arf6-specific GAP activity (Figure 16C). These results raise a possibility that ACAP2 also positively regulates neurite outgrowth by the same mechanism as ACAP3, consistent with the report that in PC12 cells and hippocampal neurons knockdown of ACAP2 suppresses neurite outgrowth [55], although we did not investigate the involvement of ACAP2 in neurite outgrowth in the present study. Should this be the case, a question arises as to how ACAP2 and ACAP3 share their functions to regulate neurite outgrowth. Recycling of endosomal proteins to the plasma membrane includes multiple steps, e.g. budding of vesicles from endosomes, transport of the vesicles to the plasma membrane and tethering/fusion of the vesicles to/with the plasma membrane. This fact led us to

speculate that different populations of Arf6 regulate distinct steps of the recycling process in neurite outgrowth due to the spatiotemporal regulation of Arf6 by distinct Arf6 GAPs in concert with Arf6 GEFs. This speculation is consistent with the reports suggesting that Arf6 is implicated in several steps for recycling of GLUT4 in differentiated 3T3-L1 adipocytes [103] and of β1 integrin in vascular endothelial cells [35]. Thus, sequential regulation of Arf6 by distinct Arf6 GAPs including ACAP2 and ACAP3 may drive the entire process of membrane recycling in neurite outgrowth. These points should be clarified by further investigation.

4-5 Interaction between Rab proteins and ACAP3 in neurite outgrowth

Although molecular mechanism through which Rab35 regulates ACAP3 and Arf6 in neurite outgrowth remain elusive, Rab35 may recruit ACAP3 to Arf6, and control GTP/GDP cycle of Arf6 for neurite outgrowth. This idea is well consistent with the evidence showing that recruitment of Arf GAP ACAP2 by Rab35 to Arf6-positive endosome is essential process for NGF-induced neurite outgrowth in PC12 cells [55,101]. In addition, the idea is further supported by the report that knockdown of Rab35 resulted in hyperactivation of Arf6 [104]. Thus, ACAP3, as well as ACAP2, could take part in the cross-talk between Arf6 and Rab35 in neurite outgrowth. As discussed above, differences in the molecular mechanisms in which ACAP2 and ACAP3 regulate Arf6 and neurite outgrowth should be further studied.

Another issue to be clarified is the interaction between ACAP3 and Rab18 in neurite outgrowth. Although co-localization between Rab18 and ACAP3 was moderate

than that between Rab35 and ACAP3 at the growth cone, Rab18 seems to be co-localized with ACAP3 at the axonal region of neurons (Figure 24). Rab18 has been shown to regulate neurite outgrowth of cortical neurons by controlling trafficking of N-cadherin [90]. Notably, loss of function mutation in the gene of *Rab18* causes Warburg micro syndrome, which is characterized by neurodevelopmental defect with axonal degeneration [105,106]. Thus, it is of interest to investigate whether interaction between ACAP3 and Rab18 has pathophysiological significance in the neurite outgrowth and Warburg micro syndrome.

Chapter 5: Conclusion

In the present study, we aimed to investigate the physiological role of Arf GAP ACAP3 and its specificity to Arf isoform.

First, we found that ACAP3 is highly expressed in mouse brain, especially in hippocampus. In primary cultured hippocampal neurons, we demonstrated that ACAP3 localizes to the tip of growth cone, and positively regulates neurite outgrowth. The regulation of neurite outgrowth by ACAP3 required its Arf GAP activity, and GAP activity of ACAP3 was specific to class III Arf, Arf6. It was also found that ACAP3 co-localizes with Arf6 at the neurite tip, and ACAP3 functions as Arf6 GAP in hippocampal neurons, as well. In addition, we revealed that Arf6 functions as a downstream effector of ACAP3 in the neurite outgrowth, and cycling between active and inactive states of Arf6 regulated by ACAP3 is essential for neurite outgrowth. Further analysis demonstrated that ACAP3 controls the levels of membrane phospholipid PtdIns(4,5)*P*2 at the growth cone, and ACAP3 interacts and co-localizes with the small G protein Rab35 at the growth cone, to regulate Rab35-stimulated neurite outgrowth.

Collectively, the present study shed light on the physiological significance of ACAP3 and Arf6 in neurite outgrowth of hippocampal neurons. This study also provides new insight that GTP/GDP cycling of Arf6 regulated by ACAP3 in concert with undefined GEF(s) is absolutely required for neurite outgrowth of the hippocampal

neurons. Furthermore, this study suggests the significance of GTP/GDP cycling of Arf6 regulated by ACAP3 for precisely modulating the levels of membrane phospholipid PtdIns(4,5)P_2, which may control Rab35-mediated membrane trafficking at the growth cone in neurons. Thus, the results obtained in this study open new avenues of research for the regulation of membrane phospholipid for membrane trafficking at the growth cone in neurite outgrowth.

Moreover, involvement of neurite outgrowth regulated by Arf6, has been implicated in several neurological diseases. Thus, I believe that the present study will also contribute to understanding of the neurological diseases with dysfunctions of neurite outgrowth.

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