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ARF6 CONTROLS LYSOSOMAL TRANSPORT OF APP AND Ap42 PRODUCTION

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ABSTRACT

Alzheimer's disease (AD) is characterized by the deposition of Beta-Amyloid (Aβ) peptide plaques in the brain. Aβ peptides are generated by the sequential cleavage of the Amyloid Precursor Protein (APP). The Aβ42 cleavage product is the most neurotoxic form. Previous studies in our lab have uncovered a novel rapid lysosomal APP trafficking pathway that bypasses the early and late endosomal compartments. We set out to characterize this transport pathway using APP constructs with an N-terminal HA-tag, allowing us to label APP at the cell surface with a fluorescently labeled antibody. SN56 cells and mouse cortical neurons were also co-transfected with fluorescently-tagged compartment marker proteins and a panel of endocytosis regulatory proteins bearing dominant negative and constitutively activating mutations. Rapid APP internalization to lysosomes is stimulated by antibody binding and is increased when Arf1 activity was inhibited, but decreased when Arf6 activity was inhibited. In addition, disruption of either Arf6 or Arf1 was able to significantly reduce Aβ42 secretion into the media. Our findings suggest that rapid APP transport to lysosomes is regulated by Arf6 and is an important, and potentially targetable, mechanism that regulates Aβ42 production, while Arf1 regulates secretion of Aβ42 into the media.

Keywords: Alzheimer's disease, APP, Aβ42, Aβ40, Arf6, Arf1, macropinocytosis, lysosomes, intracellular trafficking

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LIST OF ABBREVIATIONS

AP-1,3,4	Adaptor protein complex 1,3,4
Arf	ADP-ribosylation factor
AD	Alzheimer's disease
APS	Ammonium persulfate
APLP	Amyloid precursor-like protein
APP	Amyloid precursor protein
AICD	APP intracellular domain
ASGP	Asialoglycoprotein
ASGP-R	Asialoglycoprotein receptor
D	Aspartic acid
Aβ	Beta-amyloid
Aβ42	Beta-amyloid 42 residue peptide
Aβ40	Beta amyloid 40 residue peptide
BFP	Blue fluorescent protein
CSL	CBF1, Suppressor of Hairless and Lag-1
CLIC	Clathrin independent carriers
CURL	Compartment of uncoupling receptor and ligand
CR3	Complement receptor 3
CtBP1	C-terminal Binding Protein 1
CFP	Cyan fluorescent protein
°C	Degrees Celcius
dbcAMP	Dibutyryl cyclic AMP
DMSO	Dimethyl sulfoxide
DTT	DL-Dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
ER	Endoplasmic reticulum
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EDT	1,2-ethanedithiol
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
GPI-AP	Glycosylphosphatidylinositol-anchored proteins
GFP	Green fluorescent protein
GEEC	GPI-enriched early endosomal Compartments
GED	GTPase effector domain
GAP	GTPase-activating proteins
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GTPase	Guanosine triphosphate hydrolase
HA	Haemagglutinin
HBSS	Hank's balanced saline solution
H	Histidine

HRP	Horse radish peroxidase
kDa	kilodalton
K	Lysine
LAMP1	Lysosome associated membrane protein 1
MHC	Major histocompatability complex
µl	Microliter
µm	Micrometer
µM	Micromolar
ml	Milliliter
mM	Millimolar
Min	Minute(s)
M	Molar
mRFP	Monomeric red fluorescent protein
nm	Nanometer
NICD	Notch intracellular domain
siRNA	Short interfering ribonucleic acid
PFA	Paraformaldehyde
P/S	Penicillin/Streptomycin
PA	Phosphatidic acid
PC	Phosphatidylcholine
PI	Phosphatidylinositol
PITP	Phosphatidylinositol transfer protein
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol triphosphate
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PLD	Phospholipase D
PX	Phox sequence
PH	Plekstrin domain
PVDF	Polyvinylidene fluoride
PS1/PS2	Presenilin1/Presenilin2
PRD	Proline/Arginine-rich domain
PrP	Prion protein
PAK1	p21 activated kinase
SEM	Standard Error of the Mean
TEMED	Tetramethylethylenediamine
Tf	Transferrin
TGN	Trans-golgi network
YFP	Yellow fluorescent protein

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is characterized by the deposition of senile beta-amyloid (Aβ) plaques in the brain. It affects 10% of the population over the age of 65 and roughly 50% of the population over 85 (Herbert 1995). Currently, it is estimated that nearly 500,000 Canadians suffer from AD and that this number will drastically rise to more than 1 million by 2038 (Smetanin 2009). However, what is most concerning to our society and health care system is the increasing cost of caring for patients with AD in the future. The Alzheimer's Society of Canada projects that the current cost of care, estimated at $15 billion per year, will sharply rise to $158 billion by 2038 (Smetanin 2009).

Aβ peptides are produced from the sequential cleavage of the amyloid precursor protein (APP). The first cleavage is at a β site by β-secretase (BACE1) within the extracellular domain to release the large APP extracellular domain (Figure 1). This leaves behind the 10 kDa C99 carboxyl terminal stub. Alternatively, APP can be processed through the non-amyloidogenic pathway where it is cleaved within its Aβ domain by α-secretase, thereby preventing Aβ production by shortening the resultant Aβ peptide (Strooper 2000, Esch 1990). The C-terminal stub is then cleaved at a variable γ-cleavage site within the transmembrane domain by the presenilin component of the γ-secretase complex (Thinakaran 2008), releasing the 38-43 amino acid Aβ peptide and the APP intracellular domain (AICD). This final cleavage step is important because it

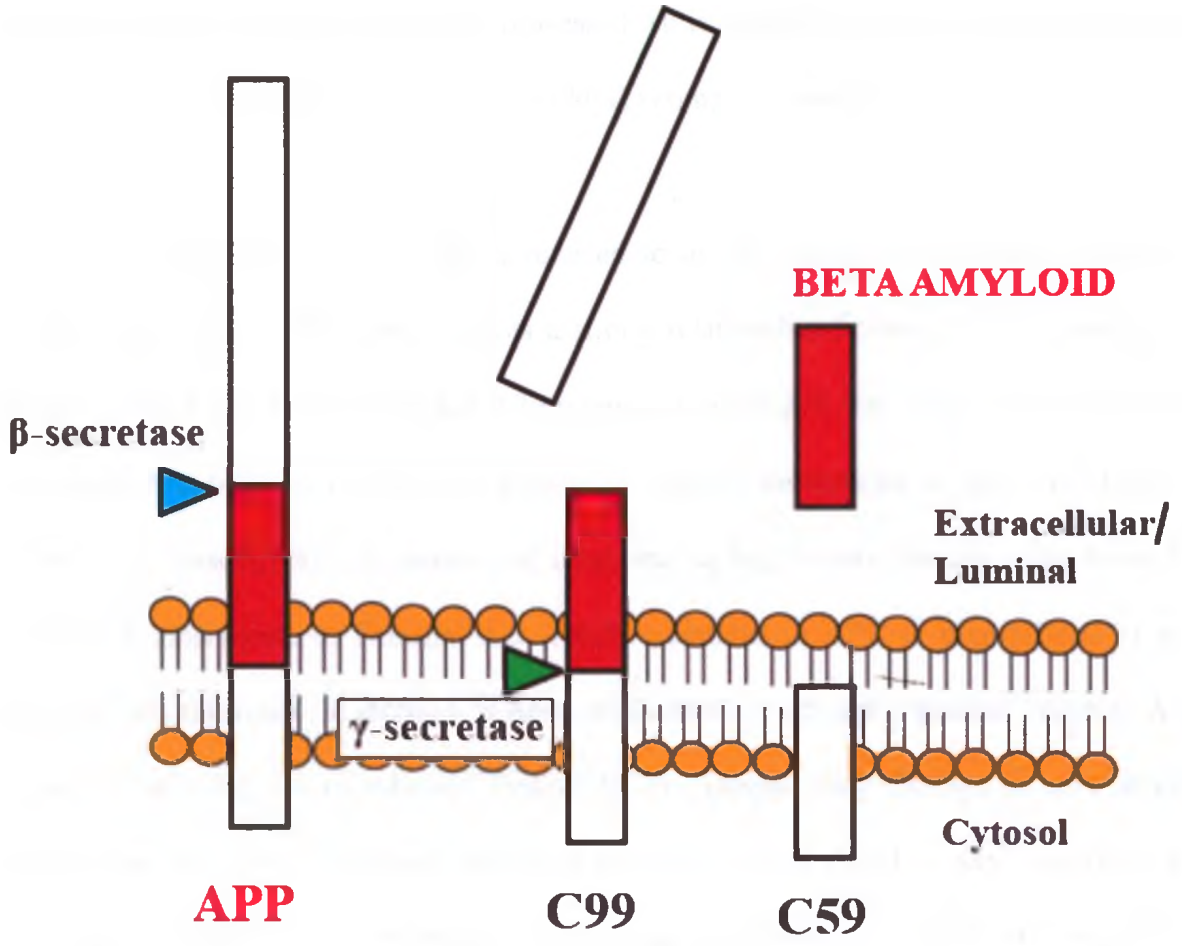


Figure 1. *APP cleavage events to yield Aβ.* Amyloid domain of APP is highlighted in red. BACE, the functional component of β-secretase, cleaves APP at the β-cleavage site (blue triangle) in the extracellular domain. The remaining C99 C-terminal is then cleaved by the Presenilin component of γ-secretase at the γ-cleavage site (green triangle) to release the Aβ peptide from the remaining C-terminal stub. Due to the variable nature of γ-cleavage, the Aβ peptide can range between 38-43 amino acids in size. The 42 amino acid form (Aβ42) is the most neurotoxic.

regulates the total amount of Aβ produced as well as the relative amount of toxic Aβ42 to other Aβ species. Nearly 90% of all processed Aβ is secreted as the 40-amino acid form (Aβ40), whereas the 42-amino acid form (Aβ42) comprises less than 10%.

The leading model of AD is referred to as the Amyloid Cascade Hypothesis (Hardy and Selkoe 2002) and suggests a strong relationship between the initiation and progression of AD to the APP and Aβ proteins. Simply put, this model states that AD pathology first begins with events that result in a significant increase in Aβ42/Aβ40 ratio. Imbalances between Aβ production and clearance leading to this rise can occur through either the inheritance of Familial AD (FAD) mutations in APP or presenilin (PS1 or PS2), which can cause an increase in Aβ42 production, or through a gradual failure in Aβ clearance mechanisms (Hardy and Selkoe 2002). Despite their linkage to devastating early onset AD, FAD mutations represent less than 1% of all AD cases. Factors that enhance the release of Aβ monomers increase the likelihood that soluble Aβ oligomers will assemble (Walsh 2007). These soluble oligomers initially have subtle effects on neuronal synapses. Eventually long term potentiation becomes impaired, causing synapse remodeling and ultimately, synapse loss. Neuronal homeostasis mechanisms then become severely altered, culminating in cell death and the onset of irreversible dementia (Hardy and Selkoe 2002).

1.2 Amyloid Precursor Protein (APP)

APP is a member of a small gene family which also includes APLP1 (amyloid precursor-like protein) and APLP2 in humans. It is a type I membrane protein with a

large extracellular domain and a relatively short cytoplasmic domain. The APP gene in humans is located on chromosome 21 (Hardy and Selkoe 2002). Multiple isoforms of APP arise through alternative splicing of its 19 exons, with different isoforms of different sizes being expressed in specific tissues. The most common isoforms are APP695, the neuronal form, and APP770 and APP751, which are both expressed ubiquitously. APP is the only member of the APP-related genes to contain an Aβ domain.

1.2.1 Physiological Functions of APP

Several physiological processes have been attributed to APP, but its actual role in mammalian physiology remains largely unknown. In a study by Saito *et al* (1989), fibroblasts that were depleted of APP showed signs of growth retardation. However, this effect was restored with the application of exogenous APP molecules. A later study identified that the active RERMS pentapeptide domain in the APP extracellular region was responsible for linking APP to fibroblast growth (Ninomiya 1993). The apparent autocrine and paracrine functions of APP's extracellular domain in regulating cellular growth were also shown to be present in neurons as well, where infusion of either the RERMS domain or exogenous APP molecules was able to improve the memory retention and synaptic density in animals (Meziane 1998, Roch 1994). Studies have also strongly suggested that APP functions to induce neuronal maturation and neurite outgrowth (Hung 1992) as well as helping to regulate the differentiation of neuronal stem cells into the astrocytic lineage (Kwak 2006).

The AICD has been shown to function similarly to the intracellular domain of Notch (NICD), a protein crucial to developmental processes due to its role in controlling cell fate decisions. Both APP and Notch are known substrates of γ-secretase, and therefore, can undergo γ-cleavage (Kimberley 2001). Due to the striking similarities of their cleavage paths, it is suggested that APP may also function as a receptor, as is the case with Notch. In fact, AICD is stabilized by the binding of the adaptor protein Fe65 in a manner similar to the association of NICD to CSL (CBF1, Suppressor of Hairless and Lag-1). Once associated to one another, the AICD-Fe65 complex translocates into the nucleus, where it is believed to affect gene transcription in the same way that the NICD-CSL complex does. Interestingly, it was reported that while in the nucleus, AICD can regulate the transcription of APP (von Rotz 2004). Additionally, it has been reported that AICD is involved in the regulation of a number of cellular processes, such as calcium and ATP levels within a cell (Hamid 2007).

1.2.2 Intracellular APP trafficking

Intracellular APP trafficking is crucial to the regulation of its processing into Aβ. APP transits first from the endoplasmic reticulum (ER), where it is synthesized, to the Golgi apparatus, where it is post-translationally modified (Figure 2). These include tyrosine sulfonation, N- and O-glycosylations, as well as ectodomain and cytoplasmic phosphorylation (Thinakaran 2008). Following modification, mature APP is presented at the cell surface where it is quickly endocytosed to the early endosome as a result of its YENPTY internalization motif near the C-terminus (Thinakaran 2008). This domain serves as a binding site for multiple cytosolic adaptors that contain a phosphotyrosine-

binding domain, such as Fe65, Mint1-3, JNK and Dab1, which allow APP to transduce signals in the nucleus or throughout the cytoplasm (Hill 2003, Pietrzik 2004). It is believed that the internalization of APP into the enclosed compartments of the endosomal/lysosomal system allows it to interact with the secretase complexes that are responsible for its processing into Aβ (Haass 1992, Koo 1994, Yamazaki 1996).

1.2.3 APP mutations and Alzheimer's disease

Thus far, more than 32 mutations in APP have been identified that have been directly linked to no less than 89 familial cases of AD (http://www.molgen.ua.ac.be/ADMutations). Some of these mutations, such as the Swedish mutation located near the β-cleavage site, can significantly increase the amount of Aβ produced by increasing the rate of β-cleavage by up to 10-fold (Mullan 1992). Other mutations located around the γ-cleavage site (ie. the London mutation) cause a preferential increase in γ-cleavage, thereby increasing the production of Aβ42 relative to Aβ40 (Eckman 1997). However, even simply increasing the expression of APP, resulting from duplication of the APP gene, has been linked to early onset AD due to the resulting increase in Aβ42 production. This has been demonstrated in Down's syndrome patients who routinely develop AD symptoms and pathology in their 40's, due to gene duplication of chromosome 21 (Walsh 2007). Some families with AD have also shown to only have duplication of the APP gene and not the entire chromosome, further supporting the role of APP in AD pathology.

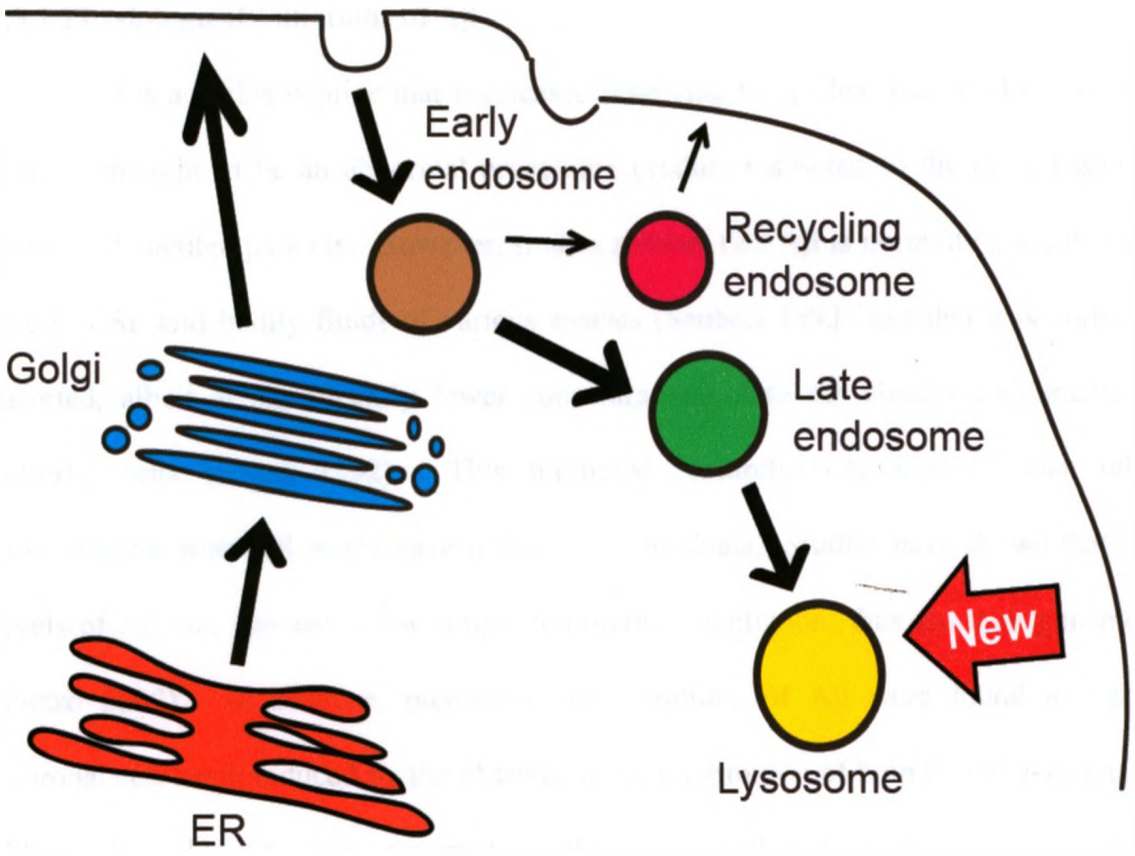


Figure 2. *Known APP trafficking pathways.* APP is first synthesized in the endoplasmic reticulum (ER), after which it is trafficked to the Golgi apparatus for post-translational modification and maturation. APP is then presented at the cell surface before being reinternalized to the early endosome. It was believed that APP was processed into Aβ in the early endosome before being excreted to the extracellular space. Remaining cleavage APP fragments would then be transported to the lysosome for further degradation. Recent studies suggest that APP processing could potentially be occurring in lysosomes. We have uncovered a novel trafficking pathway that bypasses early and late endosomes and traffics APP directly to the lysosome from the cell surface (red arrow).

1.3 Beta-Amyloid (Aβ)

1.3.1 Physiological Functions of Aβ

Aβ is a 4 kDa peptide that is released following the γ-cleavage of APP. Aβ was initially thought to be an abnormal processing product restricted to the brain tissue of aging or demented patients. However, studies showed that Aβ is normally present in the serum, CSF and bodily fluids of various species (Seubert 1992) and that it is normally secreted, albeit at significantly lower concentrations, into the conditioning media of cultured cells (Haass 1992). This prompted researchers to explore what other physiological roles Aβ might have in healthy individuals. Studies have shown that low levels of Aβ can increase hippocampal long-term potentiation, thus enhancing memory (Puzzo 2008). In addition, picomolar concentrations of Aβ were found to rescue neuronal cell death induced by the pharmacological inhibition of both β- and γ-secretases (Plant 2003). Despite these apparent benefits, studies also show that increases in the relative levels of Aβ42 enhance its ability to self-associate into neurotoxic soluble oligomers. Studies in animal models demonstrated that the presence of soluble Aβ oligomers decreases the number of synapses, thereby impairing learning performance (Shankar 2008, Cleary 2005). These oligomers have also been shown to bind directly to synapses containing NMDA receptors, effectively destroying the ability of the synapse to transduce signals to neighbouring neurons (Lacor 2007, Shankar 2007). Additionally, it has been suggested that Aβ can impair long term potentiation due to its ability to bind with prion protein (PrP) (Lauren 2009, Gimbel 2010). Despite these conflicting reports

on the apparent benefits and neurotoxicities associated with Aβ, we still do not fully understand the purpose of Aβ or its primary roles in mammalian physiology.

1.3.2 Aβ and its link to Alzheimer's disease

In accordance with the Amyloid Cascade Hypothesis, there are several lines of evidence that support the hypothesis that Aβ is responsible for the onset of AD. First was the localization of the APP gene to chromosome 21, where researchers saw that patients with Down syndrome present AD-like neuropathology (Ellis 1974). Increased APP expression increases the prevalence of Aβ in the body, thereby linking Aβ to the AD-like behaviour of Down's syndrome patients. Second, exposure of synthetic Aβ peptides to hippocampal and cortical neurons demonstrated marked neurotoxicity (Pike 1991, Busciglio 1992, Lambert 1998, Hartley 1999). Third, mutations in the regions immediately flanking or directly within the Aβ domain of APP can affect Aβ production and/or aggregation and can lead to early onset AD (Levy 1990, Goate 1991). Fourth, early onset AD has also been shown to develop as a result of mutations in PS1/PS2, which increase the ratio of Aβ42/Aβ40 (Kumar-Singh 2006, Bentahir 2006). Finally, transgenic mice with human APP and PS mutations show significant increases in extracellular Aβ and develop neuropathology and behaviour similar to AD patients (Hsiao 1998, Ashe 2005).

Aβ has been widely considered to be central to the initiation of AD (Hardy and Selkoe 2002). It is the primary component of amyloid oligomers (Gamblin 2003, Mark 1997) as well as the primary component of insoluble fibrils that deposit in plaques in the

brain. Due to the variable nature of γ-cleavage, Aβ species can range from 38-43 amino acids in length. Of these species, Aβ42 has been shown to be the most neurotoxic due to its ability to aggregate into oligomers (Kim 2005). Consequently, the majority of Familial AD mutations often result in preferential processing of APP into Aβ42 over other Aβ species (Cai 1993, Citron 1992, Haass 1994, Wolfe 2007). This would cause a shift in the Aβ42/Aβ40 ratio, with an increase in relative Aβ42 concentrations having been suggested to play a role in the initiation of AD (Selkoe 2007). Studies have shown that mice that overproduce Aβ42, but not Aβ40, develop dense plaques of insoluble Aβ that are the hallmarks of AD (McGowan 2005). As mentioned previously, AD-causing mutations can either affect APP directly, such as the case in London and Swedish mutations, or its processing machinery, such as mutations in the presenilin component of γ-secretase. Mutations in presenilin cause a preferential increase to the ratio of Aβ42 production to Aβ40 and make up the largest known group of FAD mutations, with 177 and 14 mutations described in PS1 and PS2 respectively (http://www.molgen.ua.ac.be/ADMutations). Taken together, these findings strongly suggest a link between Aβ and the onset of AD.

1.4 Endosomal-Lysosomal System

1.4.1 Endosomes

Many studies point towards a link between the trafficking of APP to the endosomal-lysosomal system and the production of Aβ. This section will provide an overview of the two main components of this system, endosomes and lysosomes, as well

as a discussion of their roles in the Amyloid Hypothesis. Endosomes are intracellular membrane-bound compartments where endocytosed and recycled materials are contained prior to entering the lysosomes. Endosomes play a key role in the sorting and subsequent trafficking of endocytosed materials. The two major classes of endosomes are the early and late endosomes. Endosomal activity and trafficking is highly regulated by the Rab membrane proteins, with specific Rabs appearing on specialized compartments. Classically, early endosomes were described to have membranes enriched with Rab5, whereas late endosomal membranes are enriched with Rabs 7 and 9. More recently, an electron microscope study by Mari *et al* (2007) was able to improve the resolution between the two major classes by combining morphological, kinetic and molecular criteria. They define early endosomes as compartments being accessible to internalized transferrin (Tf), whereas late endosomes are endocytic compartments with relatively insignificant amounts of Tf (Mari 2007).

The sorting function of endosomes was first described by Geuze *et al* in 1983, where gold particles of two different sizes were used to follow the internalization of a ligand, asialoglycoprotein (ASGP), and its receptor (ASGP-R). Once endocytosed, ASGP dissociates from its receptor is targeted to the lysosomes, whereas its receptor is quickly recycled back to the plasma membrane. Their novel immunogold labeling protocol demonstrated that after endocytosis, there is an essential protein sorting event that occurs. They called these compartments where the ligand was dissociated from its receptor the 'compartment of uncoupling receptor and ligand', or CURL. Through subsequent studies it was realized that the CURL was actually the early endosomes.

Studies have shown that in receptor-mediated endocytosis, internalized ligands dissociate from their respective receptors due to the moderately acidic pH of the early endosome lumen (~6.3-6.8) (Jovic 2010). Some proteins, such as low density lipoprotein and ASGP, are targeted to late endosomes/lysosomes for degradation while their receptors are recycled back to the cell surface (Dautry-Varsat 1983). Following sorting, endocytosed proteins are either recycled back to the plasma membrane through attached tubular extensions of the early endosome, or are targeted to the late endosome/lysosomal stages through vesicular compartments for additional processing or degradation (Geuze 1983, Mellman 1996).

Several studies have suggested a link between APP trafficking to early endosomes and the development of AD (Cossec 2010, Sapirstein 1994, Ferreira 1993). Since APP has been known to be rapidly endocytosed after being trafficked to the plasma membrane, researchers suggested that early endosomes, being the first compartment where internalized proteins are known to accumulate and to be sorted, were the key site of Aβ production (Vetrivel 2006, Selkoe 1996, Perez 1999). Additionally, the localization of BACE and presenilin to endocytic compartments further support the idea that endosomes could provide an appropriate environment for APP cleavage into Aβ (Walter 2001, Wang 2004). Finally, the secretion of Aβ cleavage fragments into the extracellular space suggested that Aβ was being produced at the plasma membrane or within compartments that are known to be in communication with the extracellular space, such as endosomes (Vitrivel 2006, Strooper 2000, Koo 1994).

1.4.2 Lysosomes

Lysosomes were first described when the labs of Christian de Duve and Albert Claude conducted studies to observe the action of insulin on the liver by measuring acid phosphatase activity (de Duve 1955). They observed that fractions obtained through gentle extraction protocols showed very little acid phosphatase activity when compared to fractions obtained through crude extraction protocols or in samples from fractions that had been stored a few days prior to assaying. They conducted similar enzyme activity assays on four other hydrolytic enzymes with acidic pH optimum. This led them to conclude these acidic proteases were housed within a separate, low pH compartment that was encased by a membrane. From these findings, de Duve *et al* (1955) first introduced the term 'lysosome' to describe this newly discovered digestive body.

Further evidence surfaced when Straus *et al* (1954) described intracellular compartments in the cells of the proximal tubules of kidneys that contained both reabsorbed material and acid phosphatase activity. These findings were the first described link between the digestive function of lysosomes and the endocytic uptake of extracellular materials. From here was born the classical concept of lysosomes being a membrane-bound compartment where the endocytosed materials are digested by enzymes in a highly acidic environment.

In addition to receiving extracellular material from endocytosis, lysosomes also receive intracellular material through autophagy (Dunn 1990). By regulating the degradation of a myriad of proteins, lysosomes play a critical role in cellular homeostasis.

Furthermore, different classes of cells can utilize lysosomes as secretory vesicles, which include the release of blood clotting agents, skin pigmentation or pulmonary surfactant (Jaiswal 2002, Stinchcombe 1999, Andrews 2000). The ruffled border of osteoclast cells responsible for bone resorption is a key docking site for lysosomes whereby enzymes involved in digesting bone matrix are secreted to the cell's exterior (Toyomura 2003). In addition, lysosomes are the major component responsible in repairing physical damage to the plasma membrane by fusing the lysosomal membrane with the cell surface (Reddy 2001). Because of their importance and roles in a number of cellular mechanisms, defects in lysosomal function have become increasingly implicated in a number of human diseases, including AD (Li 2008, LeBlanc 1999, Haass 1992, Nixon 2005). It has now been suggested that AD shares similarities with other lysosomal storage diseases, with lysosomal dysfunction being the root cause of its progression (Nixon 2007).

1.4.3 Lysosomes, Aβ and Alzheimer's Disease

Emerging evidence from the past 20 years suggests that the lysosome may play an important role in Aβ production and aggregation (LeBlanc 1999, Pasternak 2004, Haass 1992, Nixon 2005). Studies have shown that Aβ production is significantly reduced when endosomes and lysosomes are de-acidified or when endocytosis is disrupted (Ehehalt 2003, Koo 1994, Knops 1995, Schrader-Fischer 1996). It has been suggested that the four components of the APP-processing γ-secretase complex (presenilin, Aph1, Nicastrin, and Pen-2) work optimally at low pH and are highly enriched in lysosomal fractions (Pasternak 2003).

Another line of evidences which suggest a principal role of lysosomes in the pathology of AD paints them as the key site in the nucleation of Aβ fibrils commonly found in plaques. The combination of the lysosomal pH of 4.5, as well as the presence of lysosomal gangliosides (Su 2001, Yanagisawa 1995, McLaurin 1996, Soreghan 1994), comprised of carbohydrates of complex lipids, has been shown to mediate fibril nucleation (Glabe 2001, Yang 1999). Additionally, it was shown that the formation of Aβ fibrils can disrupt lipid membranes (McLaurin 199, Yip 2001) and that the culmination of such events can lead to destruction of neuronal synapses as well as lysosomal rupture (Takahashi 2002, Ji 2002), resulting in cell death (Yang 1998). Taken together, these findings suggest that lysosomes may play a principal role in Aβ production and regulation by providing the optimal working environment for APP's processing complexes.

Although these APP cleavage events have been extensively studied for more than 25 years, the subcellular compartments responsible for Aβ generation remain contentious. This presents a significant roadblock to the development of effective AD treatments. By understanding how APP is endocytosed and trafficked throughout the cell, we can begin to have a better understanding of how APP is processed and what additional functions it may have in mammalian physiology. It may be the case that by targeting the proteins which control the endocytosis of APP to these possible sites of Aβ production and fibril nucleation, we may pave the road to the development of novel therapeutic strategies against AD.

1.5 Endocytosis

There are a multitude of pathways with which cells can internalize cargo. Additionally, each cell type is capable of employing a unique combination of endocytosis mechanisms that best suits their physiological roles. Endocytosis pathways are regulated by signaling and motor proteins, which are often used to describe them. However, due to the nature of these proteins participating in more than one signaling mechanism, it is difficult to neatly classify the many pathways of endocytosis into distinct categories. The broadest distinction that can be made regarding different modes of endocytosis is the volume of material being internalized. Macroscale endocytosis refers to the internalization of material greater than 200 nm in diameter, whereas the mechanism whereby materials being internalized on a scale smaller than 200 nm is termed microscale endocytosis (Kumari 2010). Microscale endocytic pathways can be classified based upon the relative size and shapes of their endocytosing compartments, as well as their dependency on specific coating proteins, such as clathrin, and vesicular scission motor proteins, such as dynamin. See Figure 3 for a diagram summarizing different mechanisms of endocytosis based on their associated protein regulators.

1.5.1 Microscale Endocytosis

1.5.1.1 Coat proteins: Clathrin and Caveolin

Perhaps the best understood mechanism of coat-protein mediated endocytosis is the clathrin-mediated pathway. Clathrin was initially discovered in a study whereby yolk proteins were being taken up by mosquito oocytes (Pearse 1976, Roth 1964). Clathrin is

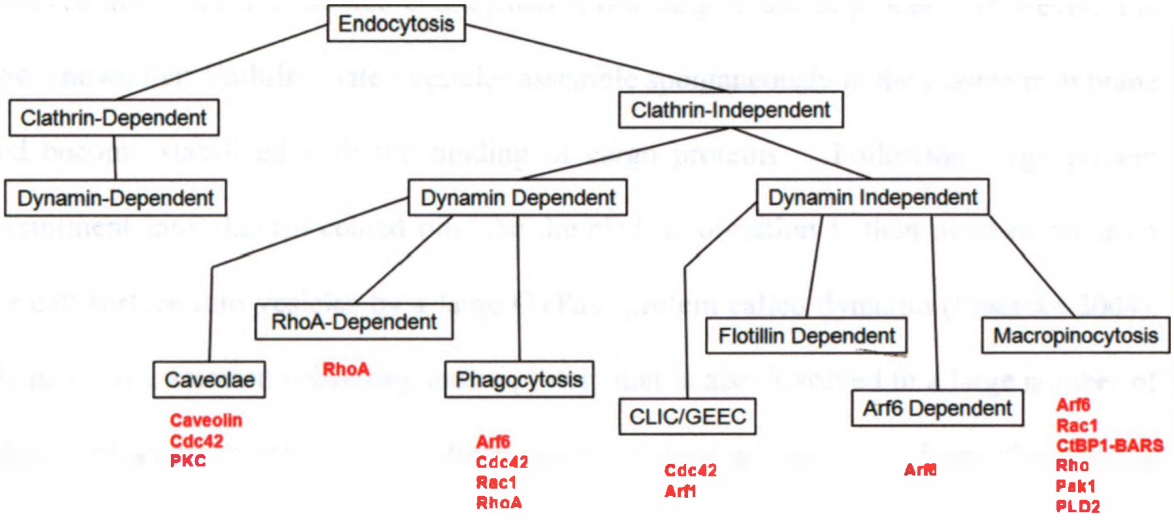


Figure 3. *Mechanisms of endocytosis.* Different mechanisms of endocytosis organized according to their dependence on key regulatory proteins. These mechanisms are broadly classified upon their dependence on the coating protein clathrin and the scission-directing GTPase dynamin. Highlighted in red under each major mechanism of clathrin-independent endocytosis are their associated regulatory GTPases and kinases.

capable of coating vesicles between 100-200 nm in diameter. Clathrin functionally assembles into a trimer of heterodimers that proceeds to form a lattice-like structure around invaginating vesicles (Kirchhausen 2000). Adaptor proteins link specific cargo to the clathrin coat, thus mediating their uptake. Approximately 150 cargo proteins have been directly linked to clathrin-mediated endocytosis (Kumari 2010). It was once believed that clathrin-mediated endocytosis was a cargo-induced process. However, it is now known that clathrin-coated vesicles assemble spontaneously at the plasma membrane and become stabilized with the binding of cargo proteins. Following cargo protein recruitment into clathrin-coated pits, the dimpled deformation is then pinched off from the cell surface into vesicles by a large GTPase protein called dynamin (Praefcke 2004). Dynamin is a scission regulating motor protein that is also involved in a large number of other endocytic mechanisms. Immediately following scission from the plasma membrane, vesicles containing internalized proteins fuse into early endosomes, where cargo can either be recycled back to the cell surface via Rab4 or Rab11-regulated recycling endosomes or be sorted and trafficked to late endosomes and finally to lysosomes by Rab9 and LAMP1 respectively (Doherty 2009). Internalization of proteins to early endosomes usually occurs within 10 minutes, whereas transport of internalized proteins to late endosomes generally occurs after 30 minutes. Proteins finally reach the lysosomes around 45 to 60 minutes, where they become degraded by proteases.

Another major coat protein-mediated pathway involves the protein caveolin, which are integral in the formation and coating of membrane invaginations called caveolae. Caveolins function by binding directly to membrane cholesterol at the cell

surface. There are 3 types of caveolin. Caveolins-1 and -2 are involved in caveolar endocytosis in non-muscular cells while caveolin-3 is only found in muscle cells (Chadda 2007). Studies have shown that not all cells are capable of caveolar endocytosis, suggesting that certain tissues specialize in utilizing caveolae. Interestingly, overexpressing caveolins in cells that traditionally do not express them can induce the formation of caveolae (Fra 1995). Due to its dependence upon cholesterol, loss of membrane cholesterol leads to the disassembly of caveolar complexes (Chadda 2007).

Caveolar endocytosis has been implicated in the internalization of a number of different ligands ranging from albumin protein to cholera and tetanus toxins to viruses, such as SV40 (Herreros 2001, Shogomori 2001, Pelkmans 2002). In most of these cases, studies have identified the ligand's corresponding receptor in the caveolar pits. However, it remains unclear as to how the ligands are recruited to their receptors. Studies have yet to explain how caveolar endocytosis is endogenously regulated in the cells that express caveolins, such as whether caveolar endocytosis occurs constitutively or if it becomes greatly up-regulated in response to specific triggers, such as ligand binding or intracellular signaling. In addition to mediating the formation of caveolae, novel findings have shown that caveolin-1 is involved in the regulation of Cdc42 activity during trafficking events at the cell membrane (Nevins 2006).

1.5.1.2 Dynamin

Dynamin is a large GTPase motor that is responsible for the scission of endocytic vesicles from the plasma membrane. As a result, dynamin has been implicated in many fission processes in eukaryotic cells (van der Bliek 1993, Koenig 1989). In mammals, there are three dynamin genes, each with multiple splice variants. Dynamin-1 is restricted to neuronal cells while Dynamin-3 expression is restricted to the brain, lung and testis. In contrast, Dynamin-2 is ubiquitously expressed in almost all tissues (Kumari 2010).

Dynamin was originally identified as a microtubule-binding homolog of the *Drosophila shibire* gene (Shpetner 1989). Further investigations using over expression of dominant negative mutants of dynamin were able to block receptor-mediated endocytosis (Lee 1999). Additional evidence for dynamin's role in endocytosis mechanisms surfaced when dynamin was found to be localized to clathrin coated pits at the plasma membrane. It appears as though dynamin is recruited to these invaginating pits at the plasma membrane by binding to proteins that have an N-BAR (N-terminal Bin-Amphiphysin-Rvs) domain, such as amphiphysin. The N-BAR domain allows these proteins to bind directly to dynamin (Slepnev 2000). At a molecular level, dynamin contains a GTPase effector domain (GED) that has been shown to be crucial to its ability to interact with the GTPase domain of neighbouring dynamin molecules. This allows for the oligomerization and the simultaneous activation of dynamin molecules in close proximity to one another (Takei 2005). Dynamins also contain a C-terminal proline/arginine-rich domain (PRD) that has been found to interact with a variety of specialized protein domains, including

proteins containing the SH3 domain. The large variety of dynamin-binding partners enables it to recruit many different proteins during coated vesicle formation.

Mechanistically, it has been hypothesized that dynamin functions as a mechanoenzyme during the scission of budding vesicles. Direct evidence of this type of behaviour was seen during *in vitro* studies which demonstrated that dynamin-coated lipid tubules were twisting in response to the addition of exogenous GTP (Roux 2006). It is this tension and torsional strain that occurs at the neck of a budding vesicle that leads to its scission from the plasma membrane. Two subsequent studies demonstrated that the polymerization and depolymerization of dynamin is the cause of these torsional changes at the plasma membrane (Pucadyil 2008, Bashkirov 2008).

In addition to being integral to clathrin-mediated endocytosis pathways, dynamin has also been implicated in a number of clathrin-independent mechanisms, which include caveolar endocytosis as well as a class of phagocytosis (Kolpak 2009). Other examples of clathrin-independent dynamin-dependent endocytosis include RhoA-dependent IL-2 receptor endocytosis (Lamaze 2001) as well as a particular APP endocytosis pathway in primary neurons (Saavedra 2007). Although dynamin appears to play an important role in vesicle formation during endocytosis, there are also a number of pathways that work independently from clathrin or dynamin, such as macropinocytosis and micropinocytosis.

1.5.2 CLIC/GEEC pathway

The CLIC/GEEC (clathrin-independent carriers/ GPI-AP enriched early endosomal compartments) pathway is one of the many clathrin-independent, dynamin-independent endocytosis pathways that also relies upon the reorganization of actin at the plasma membrane. It was first described when scientists observed the rapid and dynamin-independent endocytosis of glycosylphosphatidylinositol-anchored proteins (GPI-APs) into endocytic structures. These were aptly named GEECs. The formation of GEECs was hypothesized to be the result of the fusion of uncoated tubulovesicular CLICs that are derived directly from the cell surface (Kirkham 2005). Although it is known that CLICs are selectively enriched in GPI-APs, how these lipoproteins are sorted into this mechanism of endocytosis remains unclear. It has been suggested that the size of the extracellular domain of GPI-APs may play a role in dictating their sorting (Bhagatji 2009).

One of the key features of the CLIC/GEEC pathway is its dependence upon actin reorganization machinery (Chadda 2007). Polymerization of actin at the plasma membrane is regulated by the cycling of the Rho family GTPase Cdc42. Cdc42 cycling is, in turn, regulated upstream by Arf1, whereby Arf1 activation recruits ARHGAP10, a protein that contains a Rho-GAP domain, which plays a crucial role in initiating Cdc42 GTPase activity (Kumari 2008). Maintenance of Cdc42 cycling is important to the function of the CLIC/GEEC pathway, as locking Cdc42 in either its GTP or GDP bound state inhibited endocytosis. Another protein that was also found to regulate Cdc42

cycling is GRAF1, which also contains a Rho-GAP domain, but works independently of Arf1 activation (Kumari 2008).

1.5.3 Macroscale Endocytosis

1.5.3.1 Phagocytosis

Phagocytosis is one of two principle types of macroscale endocytosis mechanisms. It is defined as the stepwise uptake of large foreign particles by encirclement with cell membrane projections. Phagocytosis is usually receptor-triggered by recognition of the target particle (Bianco 1975). The resulting compartment is then called a phagosome. Phagocytosis is a specific mechanism of internalization that certain specialized cells can utilize, such as phagocytes and leukocytes (Kumari 2010). There are two well-described types of phagocytic entry. The first is FcR receptor mediated engulfment of immunoglobulin G-opsonized particles (FcR-mediated phagocytosis) and is dependent upon the activities of Rac1 and Cdc42 (Allen 1996). The second is complement receptor CR3-mediated encirclement of C3bi-coated particles (CR3-mediated phagocytosis) and is specifically dependent upon the activity of RhoA (Caron 1998). Interestingly, studies have shown that FcR-mediated phagocytosis requires recruitment of Arf1 and Arf6, GTPases important in regulating actin-remodeling signaling pathways, during phagocytic cup formation (Beemiller 2006). Once formed, the phagosome will undergo gradual acidification in order to degrade its contents.

1.5.3.2 Macropinocytosis

Macropinocytosis is a highly regulated, yet evolutionary ancient mechanism. Macropinocytosis is a type of macroscale endocytosis whereby large amounts of extracellular fluid is engulfed by cells through the extension, envelopment and closure of lamellopodia. Macropinosomes are characteristically large intracellular vesicles that can range in size from 0.2 μm to 10 μm in diameter (Swanson 2008). However, the size of macropinosomes has been found to be independent from the size of its enclosed cargo. The formation of these large vesicles can occur either spontaneously or as a result of stimulation from the presence of specific triggers, such as pathogens, phorbol esters or growth factors (Kerr 2009).

One of the key defining characteristics of macroscale endocytosis is its reliance upon actin polymerization machinery which allows membrane ruffles or extensions to protrude out from the cellular membrane to engulf large fluid volumes. More importantly, however, is the reliance of macropinocytosis on the regulatory proteins of actin polymerization, such as those of the Arf and Rho families of small GTPases (Nobes 2000) as well as proteins involved in regulating vesicle scission, such as CtBP1 (C-terminal Binding Protein 1)/BARS (Liberali 2008). These proteins will be discussed in greater detail below. A major link between macropinocytosis and Arf6 comes from a study whereby macropinocytosis was induced by the overexpression of EFA6, a guanine nucleotide exchange factor (GEF) that activates endogenous Arf6 (Brown 2001). The increased Arf6 activity resulted in activation of phosphatidylinositol-4-phosphate 5-kinase (PIP5K), increasing phosphatidylinositol 4,5-bisphosphate (PIP_2) levels in the

cytosol. PIP_2 is a lipid-based secondary messenger that is involved in several trafficking and actin polymerization pathways. During macropinosome maturation, PIP_2 is lost from the invaginating membrane and Rab5 is recruited. Locking Arf6 in the constitutively active form prevented recycling of macropinosomes back to the cell surface, thus causing them to become trapped within the cell (Brown 2001). In a separate study, Arf6 was also shown to affect macropinocytosis through its activation of PAK1, which then initiates the Rac GTPase signaling cascade (Dharmasardhane 2000).

1.6 Regulatory Proteins Implicated in Macropinocytosis

1.6.1 ADP-ribosylation factors (ARFs)

The ARF family of proteins is a subgroup of the Ras superfamily of small GTPases. They were initially described to function as co-factors for cholera toxin-catalyzed ADP-ribosylation of the α-subunit of heterotrimeric G proteins, but have now been recognized to play important roles in regulating cell motility and membrane trafficking pathways.

There are six known types of mammalian ARF proteins that can be classified under 3 classes (Kahn 2006). Class I includes Arfs 1, 2 and 3 which are involved in regulating the assembly of coat complexes onto budding vesicles of the secretory pathway (Bonifacino 2004). Class II includes Arfs 4 and 5, who are suggested to function

in early Golgi transport (Claude 1999, Takatsu 2002). Finally, Class III is comprised only of Arf6, which has been implicated in endosomal-membrane trafficking and actin mobilization (D'Souza-Schorey 1995, Peters 1995). ARF proteins are ubiquitously expressed in all mammalian cells and show a high degree of conserved amino acid sequences. The unique intracellular distribution of each ARF protein as well as the different molecules they interact with is essential in the determination of their cellular functions (Kahn 2006).

ARFs function similarly to other GTPases by cycling between their active GTP-bound and inactive GDP-bound molecular states (D'Souza-Schorey 2006). The hydrolysis of bound GTP, mediated by GTPase-activating proteins (GAPs), results in the release of inorganic phosphate and the energy needed for signal transduction, thereby inactivating the GTPase protein. On the other hand, guanine nucleotide-exchange factors (GEFs) are responsible for exchanging GDP with GTP on inactive GTPases in order to restore them to their active, GTP-bound states. All ARFs are myristoylated at the second Gly residue at the N-terminus, which tethers ARFs to the cell membrane (Donaldson 2011). The cycling of GDP to GTP causes a conformational change in the switch region of ARFs, the domain that allows ARF effectors to bind. Between the switch regions, a loop of β-sheet is forced away from the GTP binding site during GTP binding. This causes the N-terminus to displace from its protective hydrophobic pocket, thus promoting its insertion into the lipid bilayer of compartmental membranes (Gillingham 2007).

1.6.1.1 Arf1

Arf1 is a cytosolic GTPase with a molecular mass of 21 kDa. When activated, it becomes recruited to the membrane of the Golgi apparatus where it has been shown to be able to modulate its structure. Arf1 does so through its ability to regulate PIP_2 levels, which can then stimulate the assembly of spectrin and the actin cytoskeleton on the Golgi apparatus membranes. Additionally, Arf1 has been found to regulate clathrin-coated vesicle budding from the trans-Golgi network (TGN) and endosomal membranes through its ability to recruit the adaptor protein complexes (AP-1, AP-3 and AP-4) (reviewed in D'Souza-Schorey 2006) The actions of these adaptor proteins have also been linked to PIP_2 signaling. Other studies also report that Arf1 participates in a number of intracellular trafficking pathways. In particular, Arf1 has been known to play roles in several secretion pathways (Fensome 1996, Jones 1999, Zakharenko 1999) as well as in membrane insertion events required for phagocytosis (Braun 2007). Studies have now shown that activated Arf1 can also participate in endocytosis at the cell surface (Kumari 2008). However, the full scale of Arf1's involvement in various signaling pathways is still under detailed exploration. Some studies have pointed towards Arf6 as an upstream regulator of Arf1 activity through its interaction with the Arf-GEF, ARNO (Kumari 2008, Chadda 2007).

1.6.1.2 Arf6

Arf6 primarily resides at the plasma membrane where it can be activated or inactivated by its many regulatory proteins. It has also been implicated in clathrin-dependent and -independent internalization, actin recycling and remodeling and the

generation of macropinosomes (D'Souza-Schorey 2006, Jaworski 2007, Brown 2001). Unlike other Arfs, Arf6 is unique in that it is the only Arf to have the ability to co-localize with PIP5K *in vivo*. This unique characteristic has helped define its role in multiple signaling pathways because of the primary role of PIP5K in the generation of PIP_2, a major signaling molecule involved in actin re-arrangements and membrane traffic. Arf6 can also regulate PIP5K activity through a parallel pathway where it binds to and activates phospholipase D (PLD), an enzyme important in the hydrolysis of phosphatidylcholine to produce phosphatidic acid (PA). PA has been shown to be important in the regulation of stimulated membrane ruffling as well as PIP5K activity. A model was proposed whereby activation of ARF6 leads to the activation of both PLD and PIP5K. Activation of PLD increases PA production, which then activates PIP5K. The activation of PIP5K also causes an increase in PIP_2, which feeds back to further activate PLD. Changes in the amount of PA and PIP_2 in the membrane can help to drive both clathrin-dependent and clathrin-independent endocytosis mechanisms (Arneson 1999, Brown 2001). Additionally, there have been studies that suggest Arf6 can activate the Arf-GEF ARNO, which could then proceed to activate Arf1-Cdc42 endocytosis mechanisms. Taken together, Arf6 appears to be a major player in the regulation of a number of endocytosis and membrane trafficking mechanisms. Of particular interest is the characteristic that the active conformations of Arf1 and Arf6 are very similar. This suggests that both Arf1 and Arf6 could interact with the same downstream effectors and could have roles reminiscent of each other in similar pathways, despite the fact that Arf6 is localized to the cell membrane and Arf1 is localized to the Golgi apparatus.

A study by Kang et al (2009) showed that the clathrin-independent endocytosis of prion protein in neuroblastoma cells was found to be regulated by Arf6 activity. Prion protein has been compared to amyloid on a number of levels, and studies have shown that both appear to traffic along similar pathways (Abdulla 2001). It could be possible that Arf6 could mediate amyloid trafficking as it does with prion protein.

1.6.2 Ral

Ral belongs to the Ras super family of small GTPases, whose members function as molecular switches in several signal transduction pathways. Many of the cellular processes regulated by Ral in mammalian cells include regulating membrane transport, apoptosis, cell proliferation as well as oncogenesis. The position of Ral at the junction of several unique pathways makes it a key player in the regulation of cell signaling and homeostasis processes. It has been shown that Ral can play key roles in vesicle sorting pathways (Bielinski 1993), neurosecretion (Polzin 2002), endocytosis and the regulation of cellular morphology, gene expression and proliferation (Feig 2003).

Ral has been shown to bind to PLD, which is a well known regulator of a number of exocytosis and endocytosis mechanisms, such as macropinocytosis (Lu 2000). It is well established that Ral can bind to PLD1 and that the Ral-PLD complex can bind either Arf1 or Arf6 (van Dam 2006). To our knowledge, Ral-mediated activation of PLD is most likely mediated by Arf6, due to the fact that inactivated Arf6 inhibits epidermal growth factor (EGF)-induced PLD activity. In addition, treatment with primary alcohols,

such as 1-butanol, is able to disrupt epidermal growth factor receptor (EGFR) endocytosis by inhibiting PLD activity.

1.6.3 Ras

The Ras superfamily of signaling proteins encompasses a number of small signaling GTPases, including the Rho and Arf subfamilies. There are three main isoforms of Ras: H-Ras, N-Ras and K-Ras (Donaldson 2009). These isoforms differ in the amino acid sequences of their carboxy terminal tails as well as in their lipid modifications, which is important in dictating their subcellular localizations and activities. Apart from being localized to the plasma membrane, Ras proteins also localize to the Golgi apparatus, mitochondria as well as endosomes. Ras proteins are involved in the regulation of many cell processes that range from cell proliferation and differentiation to endocytosis, apoptosis and cell survival.

The H-Ras isoform has been implicated in initiating macropinocytosis. Activation of H-Ras via EGF stimulation induced membrane ruffling and macropinocytosis in HeLa and COS7 cells. This mechanism shares several similarities to Arf6-initiated macropinocytosis, whereby GTPase activation causes increases in cellular PIP_2 levels, resulting in macropinosome invagination. Following macropinosome maturation, PIP_2 is lost and Rab5 early endosome membrane protein is recruited. As with Arf6 initiated macropinocytosis, expression of constitutively active H-Ras causes macropinosomes to be trapped within the cell. H-Ras initiated macropinocytosis differs

from the Arf6 pathway primarily in that the H-Ras effector Akt is recruited to the macropinosomes (Porat 2008).

1.6.4 Rho family of GTPases (Rac and Rho)

The Rho family of GTPases is a large family of small 21 kDa GTP-dependent signaling proteins that play important roles in intracellular actin dynamics, cellular proliferation, apoptosis, endocytosis and membrane trafficking. It is a subfamily of the Ras super family of signaling proteins. All of the members of the Rho family, like other GTPases, act as molecular switches. The two most studied members of this family are Rac1 and RhoA.

1.6.4.1 Rac

The Rac subfamily of the Rho GTPases is comprised of four members that share great similarities in protein sequences: Rac1, Rac2, Rac3 and RhoG (Boureux 2007). The Rac proteins are important in regulating actin polymerization in most cells and have been implicated in the stimulation of lamellipodium extension, membrane ruffling, phagocytosis as well as cell motility and polarization. Despite the strong sequence similarities between the Rac members, there is a significant difference in their expression patterns throughout the human body. Rac1, the most studied member, is ubiquitously expressed, whereas Rac2 and Rac3 are restricted to cells of haematopoietic and neuronal origins, respectively (Didsbury 1989, Bolis 2003, Shirsat 1990). Most of the studies regarding Rac proteins involve expression of dominant negative isoforms in various cell

lines in order to observe their impact on actin reorganization. In studies involving neurons, it was found that dominant negative Rac1 inhibited neurite outgrowth and axonal guidance (Chen 2007). In macrophages, Rac1 appears to be important in the polymerization of actin in FcR-mediated phagocytosis (Underhill 2002). Its role in actin reorganization dynamics may implicate it in other mechanisms of clathrin- and dynamin-independent endocytosis, such as macropinocytosis and phagocytosis.

1.6.4.2 Rho

There are three highly homologous isoforms of Rho: RhoA, RhoB and RhoC. Studies have shown that they play a major role in the control of endosomal trafficking pathways as well as in cancer progression and metastasis. RhoB, in particular, has been shown to localize to endocytic vesicles and regulates their trafficking (Ellis 2000, Ridley 2006). RhoA has been implicated in the clathrin-independent internalization of the β-chain of the interleukin-2 receptor (IL-2R-β), whereby its uptake is mediated by sorting to detergent-resistant lipid rafts (Lamaze 2001). Dominant negative inhibition of clathrin polymerization or adaptor protein 180 recruitment was unable to affect the uptake of IL-2R-β. It was shown that only dominant negative inhibition of dynamin and RhoA were able to affect IL-2R-β endocytosis (Ellis 2000).

1.6.5 CtBP1/BARS

CtBP1-BARS is the short splice variant of the C-terminal Binding Protein 1 (CtBP1) gene and was first identified as a 50-kDa protein based on its ADP ribosylation in the presence of the brefeldin A toxin (Spano 1999). It belongs to a family of dual-

function proteins that has been shown to be involved in both gene transcription signaling pathways as well as membrane fission mechanisms. Its function as an alternative to dynamin-dependent scission machinery has been implicated in the fission of macropinosomes in epidermal growth factor-mediated macropinocytosis (Liberali 2008). CtBP1-BARS is activated by PAK1 (p21 activated kinase) during macropinocytosis in fibroblasts (Dharmawardhane 2000). In a study by Haga et al (2009), CtBP1-BARS was shown to be an important activator of phospholipase D1 and regulator of macropinocytosis in COS7 cells. It is unknown whether there is a direct mechanism linking CtBP1/BARS to its targets or if it plays more of a regulatory role through complex protein signaling pathways.

1.6.6 Phospholipase D (PLD)

PLD is lipid modifying enzyme responsible for the hydrolysis of phosphatidylcholine (PC) into phosphatidic acid (PA) and free choline. It is expressed in many different organisms ranging from viruses and bacteria to plants and animals. In mammals, PLD activity has been found to be present in most cell types with the exception of lymphocytes and leukocytes. There exist two main types of PLD: PLD1 and PLD2.

The catalytic activity of PLD enzymes is attributed to its two HKD motifs, defined by the key amino acids histidine (H), lysine (K) and aspartic acid (D). Point mutations in this motif disrupt PLD activity (Sung 1997). In addition to the HKD motif, PLDs also have a conserved phox sequence (PX), plekstrin domain (PH) and a PIP_2

binding site (Sciorra 2002, Sugars 2002). The PX domain was found to bind specifically with phosphatidylinosotiol phosphate lipid signaling molecules, preferably to phosphatidylinositol triphosphate (PIP_3) (Stahelin 2004). The PH domain was suggested to be important in the localization of PLD, particularly with lipid rafts during its recovery from the plasma membrane. PIP_2 binding to PLD has been suggested to be important in its activation and subsequent translocation from its inactivated pool to its activated intracellular locations. Although the current findings regarding PLD1 and PLD2 localizations remain contentious, it is has been widely suggested that PLD1 is localized to the Golgi apparatus, early and late endosomes as well as lysosomes whereas PLD2 is localized at the plasma membrane when stimulated (Jenkins 2005).

Mammalian PLD activity is highly regulated by a multitude of intracellular signaling proteins and factors. These include phosphoinositides, PKC, ARF and the Rho family of small GTPases. Phosphoinositides are a group of lipids that are related by their common base molecule phosphatidylinositol (PI). PI can become phosphorylated to increasing levels to yield PIP, PIP_2 and PIP_3, all of which play important roles in a number of signaling pathways. It has been found that PLD1 and PLD2 activity can be regulated by the binding of PIs to regulatory sequences. Along similar lines, PLD activity has also been found to be associated with PKC activation (McDermott 2004). PKC dependent regulation of PLD activity has been shown to involve the direction phosphorylation of PLD along its N-terminus. ARF GTPases have been implicated in the activation of PLD1 and PLD2. Studies have shown that ARFs can directly interact with PLD1 but might require the use of accessory factors to indirectly regulate PLD2-

mediated pathways (Hammond 1995, Sung 1999). The strongest evidence of ARF-regulated PLD activity has been identified in the transport of intracellular vesicles. Finally, members of the Rho GTPase family, which includes RhoA, Cdc42 and Rac1, have been shown to activate PLD in a number of cell types (Singer 1995, Bowman 1993). Studies have found that these GTPases directly interact with PLD in the presence of GTPγS.

1.7 Rationale and Aims

Our lab has previously demonstrated that APP and the γ-secretase complex are enriched in highly purified lysosomes (Pasternak 2003). More recently, we have uncovered a novel pathway whereby wild-type APP is rapidly and selectively internalized from the cell surface directly to lysosomes, bypassing the early and late endosomal stages of endocytosis (Lorenzen 2010). Interestingly, APP bearing either the Swedish or London mutations is excluded from this sorting pathway. The rapid dynamics of this pathway and the presence of γ-secretase in the lysosome suggest a rapid trafficking mechanism that may play a role in the production of Aβ from APP. In this project, we set out to determine what molecular mechanisms are responsible for regulating the rapid delivery of APP to lysosomes, the potential site for Aβ generation.

Imaging results from our previous study suggested that APP is internalized in a manner not characteristic of classical clathrin-dependent endocytosis, with micron-sized vesicles being formed at the cell surface that are quickly directed to fuse with

neighbouring lysosomes. Based on these observations, we suspect that neuronal cells are utilizing a mechanism reminiscent of macropinocytosis in order to accomplish this task. **Therefore, I hypothesize that APP is transported to the lysosome by macropinocytosis and that regulators of macropinocytosis control intracellular APP trafficking and Aβ production.** The two principal objectives of this project are as follows: 1) To characterize the molecular mechanisms that regulate rapid APP internalization to lysosomes in neuronal cells. 2) To determine if lysosomal transport of APP can control Aβ production and/or secretion.

CHAPTER 2: MATERIALS AND METHODS

2.1 Antibodies and Reagents

Anti-HA antibody was purchased from Roche Applied Science. Anti-Arf6 antibody was purchased from Sigma. Anti-Arf1 antibody was purchased from Epitomics. SN56 cells were obtained from Dr. Jane Rylett. Mouse cortical neurons were obtained from Dr. Sean Cregan. Fluorescently-labeled secondary antibodies and Zenon AlexaFluor 633 donkey anti-mouse were purchased from Invitrogen. Dulbecco's modified Eagle's medium, fetal bovine serum, Hank's balanced salt solution (HBSS), penicillin (P), streptomycin (S), trypsin and neurobasal media were all purchased from Gibco.

2.2 Neurons

Cortical neurons were dissected from fetal mice and kept in neurobasal media for 7 days prior to transfection. On the day of transfection, neurons were transfected with APP-CFP and LAMP1-BFP using Lipofectamine 2000. Two days following transfection, neurons were surface labeled with AlexaFluor-633 Zenon-labeled anti-HA antibodies on ice for 30 minutes. Neurons were then either fixed at 0 min or allowed to internalize at 37°C for 15 minutes, after which they were fixed in 4% paraformaldehyde (PFA) for 15 minutes. Fixed cells were then imaged using confocal microscopy. Neuronal cultures were stained with NeuN to ensure that 75% of the cultures were composed of neurons.

2.3 DNA Constructs

The βAPP construct was generated by Peiter Anbourgh. A previous study in our lab demonstrated that this βAPP construct traffics and behaves in the exact same way as full-length wild-type APP (Lorenzen 2010). A cDNA encoding APP 750- YFP was a generous gift of Dr. Bradley Hyman. The signal sequence was cloned (including N-terminal 17 amino acid signal sequence of APP as well as the L-E residues required for signal peptide cleavage (Lichtenthaler 1999) and a 3' haemagglutinin (HA) tag was added by PCR using the primers 5'GCTAGCATGCTGCCCGGTTTG3' and 5'ACGCGTAGCGTAATCTGGAACATCGTATGGGTACTCCAGCGCCCGA3'. These primers also add an 5'Nhe1 cleavage site and a 3'Mlu1 site. A shortened APP construct was generated which included the C-terminal 112 amino acids (12 amino acids upstream of the β-cleavage site) of and adding a 5'-FlAsH tag (using an optimed FlAsH sequence (Martin 2005) was amplified using the primers 3'ACGCGTTTCCTGAAC TGCTGCCCCGGCTGCTGCATGGAGCCC5', 3'ATCAAGACGGAGGAGATCT CTG5'. These primers also add a 3'Mlu1 restriction site and 5'Sal1 site. These 2 products were then ligated into pEYFP-N1 or pECFP-N1 vectors (Clontech). Similar constructs have been demonstrated to undergo both beta- and gamma-cleavage (Grimm 2008). We have previously demonstrated that this construct has the same intracellular distribution and lysosomal trafficking of full length APP (Lorenzen 2010).

Expression constructs for regulatory proteins bearing dominant negative mutations are Dyn-K44A-CFP, GFP-Arf6-T27N and GFP-Arf6-Q61L (Julie Donaldson), GFP-CTBP1/BARS-S147A (Alberto Luini), GFP-PLD2-K758R (Michael Frohman),

YFP-Arf1-T23N (Jean Gruenberg). YFP-Rac1-T17N, YFP-RhoA-T19N, YFP-Ras-S17N, YFP- RalA-S28N were generous gifts from Dr. Stephen Ferguson. LAMP1-YFP was a generous gift from Dr. Walter Mothes and recloned to use mRFP.

2.4 Cell Culture and Transfection

SN56 neuroblastoma cells were used as a cell model for this study due to the cholinergic phenotype they expressed once differentiated. Since cholinergic neurons of the forebrain have been shown to be affected in AD, we felt that the SN56 cell line would best reflect the cellular physiology of the neurons affected in AD patients.

SN56 cholinergic neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM), respectively supplemented with 5% (v/v) and 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco), and 100 μg/ml penicillin/streptomycin (Gibco). Cells were kept in culture in 75 cm^2 flasks (Falcon) and were split every 4 days. Cells were seeded at a density of 3×10^5 cells/35-mm dish (Falcon) one day prior to being transfected. Cells were then transiently transfected using Lipofectamine 2000 following manufacturer's instructions (Fermentas) in serum free media. Transfection efficiencies averaged 65% in most experiments. Following a 24 hr incubation period, cells were differentiated before imaging by the addition of 1mM dibutyryl cyclic AMP (dbcAMP; Sigma) to serum free medium (Hammond 1986, Pedersen 1996, Le 1997). All cells were kept in an incubator at 37°C in a humidified atmosphere containing 5% CO_2.

2.5 Confocal Microscopy

Imaging was performed on a Zeiss LSM-510 META laser scanning microscope using a Zeiss 63X 1.4 numerical aperture oil immersion lens. The optical section thickness was typically 1 micron. EGFP and YFP fluorescence was visualized using a 488 nm excitation laser and a 500-530-nm emission filter set. mRFP fluorescence was imaged using a 543 nm excitation laser and BP 565-615 filter set. ECFP fluorescence was imaged using 458 nm laser excitation source and a BP 475-525 filter set. AlexaFluor 633 fluorescence was imaged using 633 nm excitation laser, and a LP 650 filter.

2.6 Cell Surface Labeling

Anti-HA antibody was labeled with AlexaFluor 633 using a Zenon labeling kit (Invitrogen) according to the manufacturer's instructions. For fixed time-course studies, a freshly prepared conjugate was incubated with cells in DMEM on ice for 30 minutes. The conjugate was removed and the cells were washed twice in pre-warmed HBSS. Following the wash, warm HBSS was added and the cells were incubated at 37°C for the times indicated prior to fixation with 4% paraformaldehyde. Cells that were selected for this study showed strong expression of both the APP and compartment marker constructs in addition to normal morphology. Fixed time course experiments were repeated 3 times for each dominant negative construct/treatment, with 10 cells sampled at each time point. See Figure 4A for schematic.

For live cell imaging, the anti-HA antibody was labeled with AlexaFluor 633 using a Zenon labeling kit according to the manufacturer's instructions. Cells were

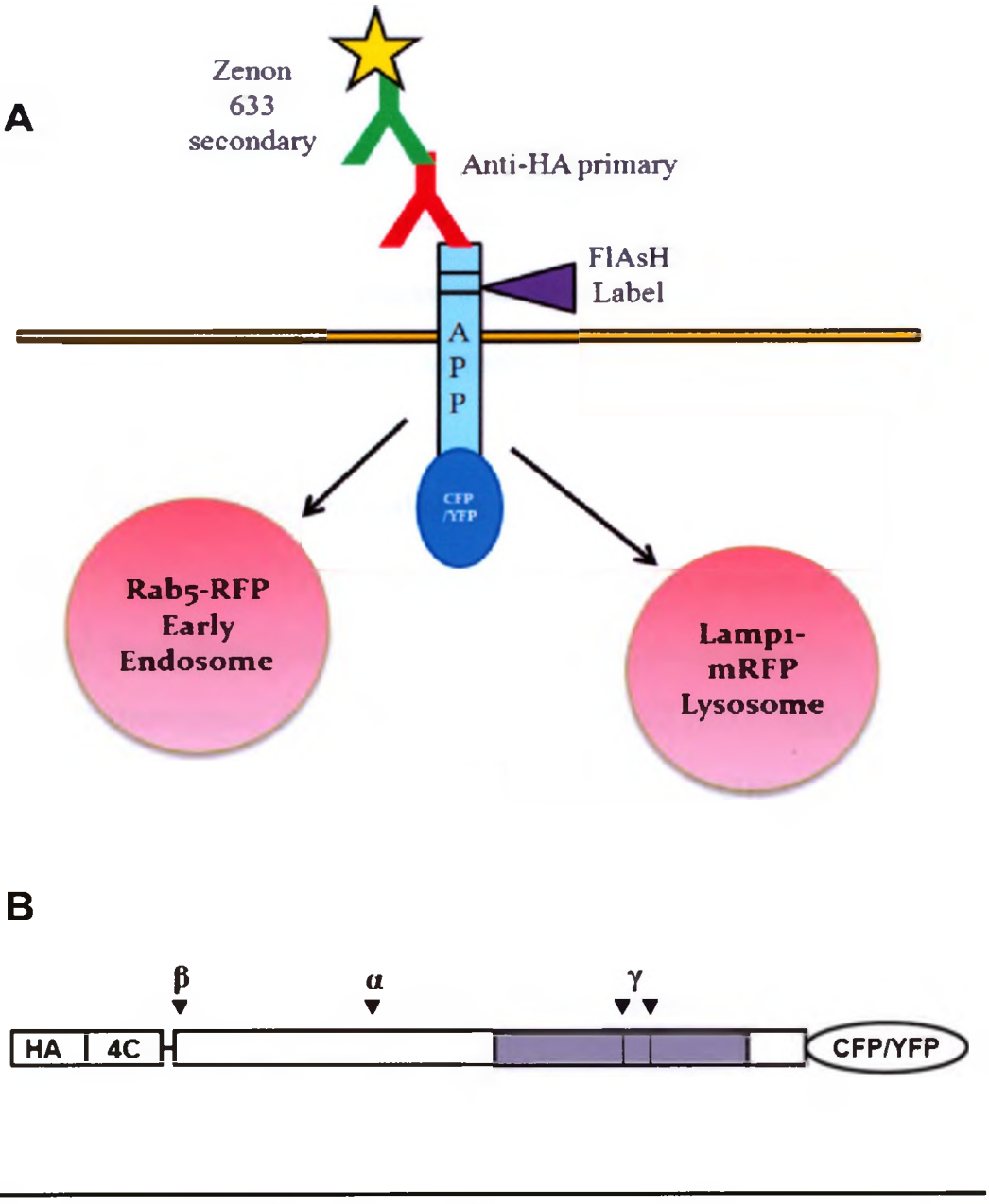


Figure 4. *Surface labeling* **(A)** APP constructs were labeled at the cell surface with either Zenon 633 secondary conjugated to anti-HA primary antibodies against the HA-epitope or FlAsH biarsenical fluorescein against the tetracysteine sequence, both of which were engineered onto our APP constructs. After surface labeling on ice, surface labeled APP was then allowed to internalize for 15 minutes to either LAMP1-mRFP labeled lysosomes or Rab5-RFP labeled early endosomes. Confocal images were then taken and colocalization was measured using Imaris analysis software. Dominant negative mutants were also cotransfected into SN56 cells to study their effect on intracellular APP trafficking. **(B)** Schematic of our βAPP construct. An HA epitope as well as a tetracysteine sequence (4C) to bind FlAsH dyes was engineered on the N-terminal. Either CFP or YFP was added to the C-terminal. β-, α-, and γ- denote the respective secretase cleavage sites. Transmembrane domain of APP is denoted by the grey region.

removed from the incubator and washed twice with warm HBSS. The conjugate was incubated with cells in HBSS for 15 minutes at room temperature. Conjugate was removed and the cells were washed twice in warm HBSS before being immediately imaged in HBSS at 37°C on a BC200 microscope stage warmer with a Bionomic BC100 controller (20/20 technologies). Images were taken using a Zeiss 510 META laser scanning confocal microscope at 2 frames/minute in 512 x 512 resolution.

2.7 FlAsH Biarsenical Fluorescein Labeling

Methods were adapted from Taguchi *et al* (2009). To prepare 400ul of labeling medium (good for 4 confocal dishes with 100 µl labeling media per dish), 8ul of 70uM FlAsH reagent (Invitrogen), 8ul of 50mM 1,2-ethanedithiol (EDT) (Sigma) and 4ul of 2M DL-Dithiothreitol (DTT) (Sigma) were combined in 380ul of HBSS and incubated in the dark at room temperature for 10 minutes. Following incubation, labeling media was kept on ice until ready to be used. SN56 cells that were plated on confocal dishes were kept on ice for 30 minutes prior to labeling. Incubation media was aspirated and the plates were washed 2x with ice cold HBSS. Each confocal dish was treated with 100ul of labeling media on ice for 3 minutes in the dark. After labeling, cells were washed 2x with room temperature 250uM EDT in HBSS to remove any excess FlAsH. The final wash was done with warm HBSS and the cells were allowed to internalize at 37°C for 15 minutes. Following internalization, cells were fixed for 15 minutes in 4% PFA. PFA was removed and the fixed cells were washed 3x in room temperature PBS and imaged on an LSM510 confocal microscope (Carl Zeiss). For FlAsH and anti-HA combination labeling, following treatment with Lumio labeling media, cells were treated with 100ul

Alexaflour-633 Zenon-tagged anti-HA antibodies at a 1:100 concentration for 30 minutes. After labeling with Zenon, cells were washed 2x in warm HBSS and allowed to internalize for 15 minutes. See figure 4A for schematic.

2.8 Aβ42 and Aβ40 ELISA

For each individual experiment, 250,000 SN56 cells were plated into each well of a 12-well plate and kept in 1 ml of pre-transfection media (+FBS; -P/S) overnight after splitting. Cells were then transfected with βAPP-CFP and one of Arf1DN, Arf6DN or Arf6CA mutant constructs in triplicate for each experimental run. Cells were incubated for 3 days following transfection and kept in 500 µl of incubation media (-FBS; +P/S). 500 µl of media was collected and assayed using an ultrasensitive Aβ42 or Aβ40 ELISA kit (Invitrogen) according to the manufacturer's instructions. This experiment was repeated 3 times and the data normalized against the control. Data was plotted and analyzed using Graphpad Prism 5.0 software.

2.9 Arf6-Arf1 siRNA

SN56 cells were split as described in cell culturing subsection. Stealth siRNA was purchased from Invitrogen with the following sequences: Arf6 (AUAAUGCGGUGCAGCUCCUGGCGGG) (adapted from Bach et al. 2010 for use in mouse) and Arf1 (GGGAAUAUCUUUGCAAACCUCUUCA). Cells were transfected with 300 pmol of Arf6 siRNA and 500 pmol of Arf1 siRNA according to Invitrogen Lipofectamine 2000 transfection protocols. Cell lysates were collected 3 days after

transfection and assayed by western blotting with a 1:1000 concentration of Anti-Arf1 (Epitomics) and Arf6 antibodies (Sigma).

For colocalization studies, cells were transfected with βAPP-CFP, LAMP1-mRFP and either 300pmol of Arf6 siRNA or 500pmol of Arf1 siRNA or a combination of both. Cells were differentiated 1 day following transfection and were then surface labeled with AlexaFluor-633 Zenon-labeled anti-HA antibodies as described above and allowed to internalize at 37°C for 15 minutes. Cells were then imaged using confocal microscopy and the percent colocalization of the brightest 2% of pixels from the APP and LAMP1 channels was measured. Measurements were compared to control cells that were surface labeled in the same way.

2.10 Protein Extraction and Western Blotting

SN56 cells (1.5×10^6) were plated on 60mm dishes (Nunclon) and transfected with the appropriate DNA constructs/siRNA transcripts. Following 3 days of incubation, cells were washed in cold PBS and lysed with 250ul NP40 lysis buffer (20mM Tris pH 8.0, 137mM NaCl, 10% glycerol, 1% IGEPAL/NP40, 3x milliQ water) for 5 minutes at 4C. Cells were then harvested using a cell scraper into 1.5ml Eppendorf tubes and centrifuged at 14,000 rpm for 15 minutes at 4°C to remove insoluble material. Protein quantification of supernatant was done using a Pierce BCA protein assay kit.

Polyacrylamide gels were made from Biorad reagents. The 12% resolving gel (5.1ml milliQ water, 6ml acrylamide, 3.75ml 1.5M Tris-HCl, 150ul 10% SDS) solution

was mixed with 150ul 10% APS (ammonium persulfate) and 6ul of TEMED (tetramethylethylenediamine) before being and allowed to set. MilliQ ultrapure water was used to smooth out the top of the resolving gel. The 5% stacking gel (3.5ml milliQ water, 1ml acrylamide, 1.5ml 0.5M Tris-HCl, 60ul 10% SDS) was then mixed with 60ul 10% APS and 6ul of TEMED before being poured on top of the resolving gel and allowed to set with a well-comb inserted. 25ug of samples were aliquoted out and mixed with 4x sample buffer (5ml glycerol, 6.25ml 0.5M Tris-HCl, 1g SDS, 2ml 0.5% bromophenol blue; pre-incubation preparation: 475ul 4x sample buffer with 25ul β-mercaptoethanol) in a 3:1 ratio of sample to buffer and heated at 60°C for 20 minutes. While samples were denaturing, 10X electrode running buffer (30.3g tris base, 144g glycine, 10g SDS, 1000ml milliQ water) was diluted to 1X and poured into the assembled gel apparatus (Biorad). Samples were then loaded with one lane loaded with SeeBlue® Plus2 prestained standard ladder (Invitrogen) and the gel was run at 110V for 1.5 hrs.

When the running front had reached the bottom of the gel, the apparatus was disassembled and the gel removed and placed in 1X transfer buffer (25ml 10X transfer buffer, 50ml methanol, 175ml milliQ water) diluted from 10X stock (30.3g Tris, 144g glycine, 1000ml milliQ water). Two pieces of filter paper and a PVDF (polyvinylidene fluoride) membrane, activated in 100% methanol for 5 minutes, were also allowed to soak in 1X transfer buffer for 15 minutes. A semidry transfer sandwich was then assembled by placing the PVDF membrane and gel between two pieces of filter paper. Semidry transfer apparatus (Thermo) was assembled and run at 15V for 1 hr. Following transfer, PVDF membrane was blocked for 1 hr in 5% skim milk solution (2.5g skim

milk powder, 50ml 0.05% TBS-T) made in 0.05% TBS-T (10X TBS, 1ml Tween-20, 1000ml milliQ water). 10X TBS was made from 24.2g Tris, 80g NaCl, 800ml milliQ water, adjusted to pH 7.6 with HCl, then volume brought up to 1000ml.

Arf1 (1:1000), Arf6 (1:1000) or α-tubulin (1:10,000) antibodies (Sigma) were added and incubated overnight at 4°C with rocking. Membranes were then washed for 5 minutes 3 times in TBS-T. Secondary antibodies (anti-mouse, anti-rabbit HRP) were diluted in 5% milk solution and incubated with the membrane for 1hr at room temperature with rocking. Membranes were washed again for 5 minutes 3 times in TBS-T. Following washes, 1ml each of chemiluminescence solution A (05ml 0.1M Tris pH 8.5, 22ul 90mM coumaric acid in DMSO (dimethyl sulfoxide), 50ul 250mM 3-aminopthalhydrazide in DMSO) and B (5ml 0.1M Tris pH 8.5, 3ul 30% H_2O_2) were mixed and incubated on the membrane for 1 minute. Membrane was stored in plastic wrap and exposed to x-ray film in a dark room. X-ray films were then developed and the bands were quantified using ImageJ analysis software.

2.11 Data Quantification and Analysis

Colocalization analysis was performed on confocal optical sections using Imaris 7.0.0 with Imaris Colocalization module (Bitplane) running on an Apple Mac Pro to examine the colocalization of the brightest 2% of pixels in each channel of interest, often between HA-tagged APP and Lamp1-mRFP or Rab5-RFP. This allows us to set a threshold for colocalization in an unbiased manner using the intrinsic properties of the

image, eliminating confounding problems caused by variations in cell-to-cell expression and image brightness/exposure thus allowing direct comparison between experiments. Graphing and statistical analysis was performed using Prism GraphPad 5.01 using one-way ANOVA with Tukey post-test with a 95% confidence.

CHAPTER 3: RESULTS

3.1 Live cell imaging of SN56 cells shows rapid endocytosis of surface labeled APP to LAMP1 positive lysosomes

To examine APP distribution in neuronal cells, we adopted the SN56 cell line. These cells are a hybrid cell line generated by fusing dissociated embryonic mouse septal neurons with N18TG2 neuroblastoma cells. SN56 cells possess neuronal morphology and cholinergic phenotype when differentiated and express APP (Hammond et al., 1986; Pedersen et al., 1996; Le et al., 1997). The construct used here was described in a previous publication in which we first demonstrated rapid internalization of APP from the cell surface to the lysosome in fixed cell time courses (Lorenzen 2010). This construct has the same trafficking pattern as a full length APP construct, and has the same distribution as immunostained APP in SN56 cells and cultured mouse neurons. To better understand this phenomenon, we performed live cell imaging of lysosomal internalization of APP in neuronal SN56 cells. Cells were transfected with HA-βAPP-CFP (green channel) and LAMP1-RFP (red channel), surface labeled with anti-HA antibodies conjugated with AlexaFluor 633 Zenon fluorescent secondary antibodies on ice for 30 minutes, and imaged while the surface label was internalized at 37°C (Fig. 5). To highlight the colocalized fluorescent signal and to quantify colocalization (in subsequent images) we used Imaris 64x 7.0.0 software (Bitplane) to create a colocalization channel by setting thresholds in each channel (in this case, the brightest 2% of pixels) in order to identify colocalized pixels which were then set to white. This technique allows for the unbiased (observer independent) settings of thresholds that is relatively independent of

imaging parameters (image brightness, exposure, transfection level) and is discussed more fully in our previous work (Lorenzen 2010) as well as in studies by Hutcheon *et al* (2000, 2004) and Holmes *et al* (2006). An example of this method demonstrating the colocalization of βAPP-CFP and LAMP1-mRFP is shown in Fig. 5A.

Figure 5B shows an internalization time course of surface labeled HA-βAPP-CFP and LAMP1-mRFP overlayed with the colocalized pixels set to white. These images show that some internalized APP is already colocalized with lysosomes at the earliest time points – the amount of time taken to place cells on the microscope stage and focus them. This process appears to begin with APP internalizing rapidly into very large vesicles. These vesicles then fuse with LAMP1 positive compartments resulting in the strong co-localization between HA-βAPP and LAMP1-mRFP. These vesicle fusion events are highlighted by white arrows in Fig. 5B, where large green vesicles containing surface-labeled APP fuse with red LAMP1-labeled lysosomes within minutes. These findings reaffirm previous studies showing APP is rapidly transported to lysosomes from the cell surface.

3.2 Mouse cortical neurons show rapid APP internalization and co-localization to LAMP1.

In order to confirm that this transport occurs in neurons and not just in immortalized cell lines, we prepared mixed cortical cultures from F14 mice. Cortical cells were transfected with HA-βAPP-YFP and LAMP1-BFP 4 days prior to the internalization assay. Cells were labeled on ice with Zenon AlexaFluor 633-labeled anti-HA antibodies for 30 minutes and then either fixed immediately or allowed to internalize

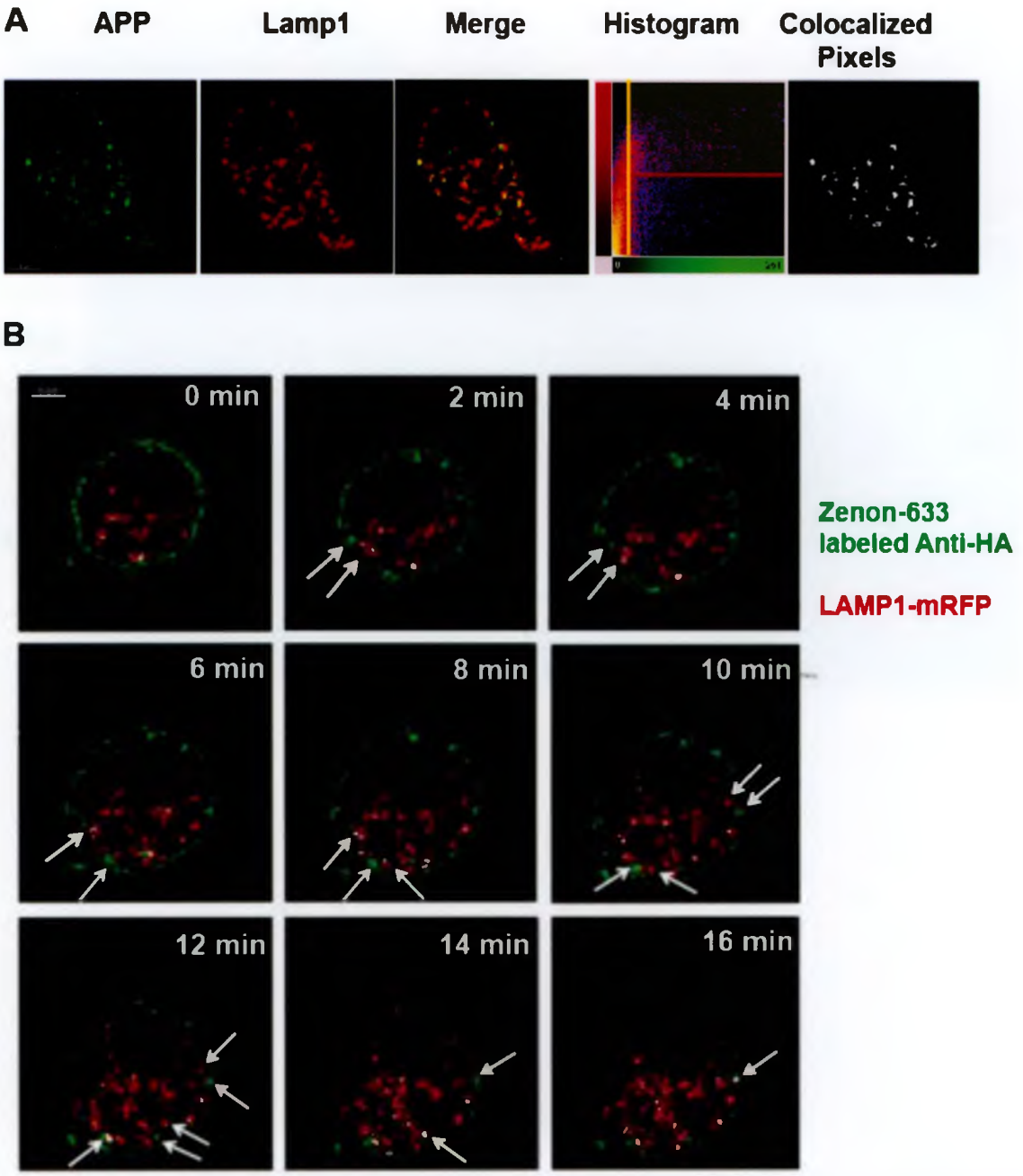


Figure 5. *Live cell APP internalization to lysosomes in SN56 cells* **(A)** SN56 Cells were transfected with HA-βAPP-CFP (green channel) and Lamp1-mRFP (red channel) and imaged by confocal microscopy. Colocalization was quantified by selecting the brightest 2% of green (right of yellow line) and red (above red line) channel pixels. Colocalized pixels are shown in white in the far right panel. **(B)** Live cell time course indicates that APP internalization begins immediately. Surface labeled APP becomes highly colocalized with LAMP1 by 16 minutes. White arrows indicate large APP containing vesicles budding from the cell surface and LAMP1 compartments in the process of fusing. Scale bar = 5 μm.

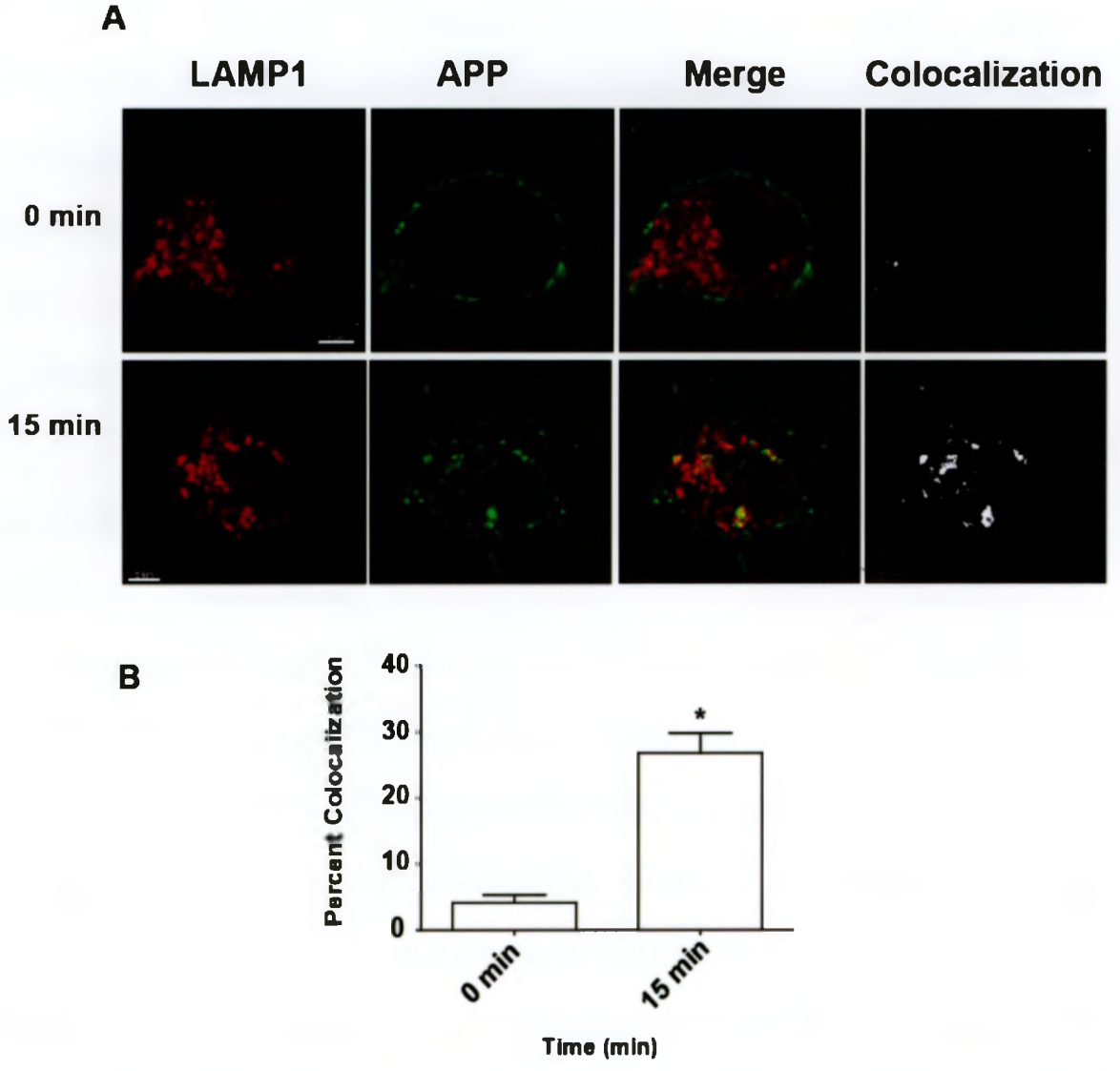


Figure 6. *Rapid transport of APP to the lysosome in mouse cortical neurons.* **(A)** Mouse cortical neurons transfected with APP-CFP and LAMP1-mRFP. Cells were surface labeled with Zenon-633 anti-HA antibodies and fixed following 0 or 15 minutes of internalization at 37 C. Colocalization of APP with LAMP1 is highlighted in white. Panels show a representative slice of the *z*-stack. Scale bar = 5 μm. **(B)** Percent colocalization of the brightest 2% of pixels between APP and LAMP1 channels. * denotes significant difference ($p<0.05$) 10 cells imaged in each of 3 separate experiments (n = 3, 30 cells total). Error bars represent the standard error of the mean (SEM).

at 37°C for 15 minutes before being fixed in 4% PFA. To demonstrate that the HA-APP had been internalized to lysosomes, we generated Z-stacks and performed 3D reconstruction of these cells. Representative images are shown in Fig. 6A. Analysis of cortical neuron Z-stacks revealed that there was a significant increase in internalized APP colocalizing with LAMP1 after 15 minutes of internalization (26.8% ± 3.1) when compared to the 0 minute control (4.0% ± 1.2) ($p<0.05$) (Fig. 6B). 10 cells were assayed in each of 3 separate experiments for each time point (n=3, 30 cells total per time point). Data shown as Mean percent colocalization ± SEM.

3.3 Internalization of APP into Lysosomes is enhanced by cell surface antibody binding and/or cross-linking.

In order to determine if the internalization of APP is driven by antibody-induced crosslinking, as demonstrated by Schneider *et al* (2008), we examined the internalization of HA-βAPP-CFP labeled with a biarsenical-fluorescein reagent (FlAsH, sold as Lumio, Invitrogen) (Gaietta 2002). This reagent binds directly to an optimized tetracysteine sequence (Martin 2005) incorporated into the HA-βAPP-CFP constructs, next to the HA epitope tag (Fig. 4) and is too small to crosslink APP proteins. SN56 cells were transfected with HA-βAPP-CFP and either Rab5-RFP or LAMP1-mRFP to mark early endosomes and lysosomes respectively. Cells were then surface labeled with the FlAsH reagent on ice for 3 minutes using the protocol published by Taguchi *et al* (2009). Cells were then allowed to internalize APP either with or without an additional antibody binding step with Zenon 633-labeled anti-HA antibody at 1:100 concentration for 30 minutes on ice. Fig. 7A shows that at time 0, FlAsH labeling appears as a ring on the cell

surface with no internalization. When allowed to internalize in the absence of anti-HA antibody, there was robust transport of FlAsH-labeled βAPP-CFP to Rab5-labeled early endosomes while there was only modest colocalization of FlAsH-labeled βAPP-CFP with lysosomes. In contrast, in the presence of 1:100 Zenon 633-labeled anti-HA antibody surface labeling, FlAsH-labeled βAPP-CFP is rapidly transported to the lysosome (Fig. 7B).

We examined the internalization of these labels in at least 3 independent experiments quantifying at least 10 cells. Colocalization was quantified using Imaris and expressed as Mean ± SEM. When cells were fixed on ice at time 0, there was almost no colocalization of FlAsH-labeled βAPP-CFP with compartment markers. In the absence of anti-HA antibody, there is robust colocalization of APP to Rab5-labeled early endosomes (43.7% ± 3.7) at 15 minutes, whereas only 7.6% ± 2.1 of FlAsH signal was colocalized with LAMP1 after the same amount of time. When FlAsH-labeled cells were treated with 1:100 anti-HA antibodies, the amount of APP translocating to the lysosome rose to 22.4% ± 1.9, tripling the amount of APP trafficked to lysosomes (Fig. 7C). These results were statistically significant ($p < 0.05$). This data demonstrates that in the absence of antibodies, APP is internalized primarily to early endosomes (Fig. 7C). While rapid lysosomal trafficking of cell surface APP does happen at rest, it is markedly stimulated by antibody binding of APP, presumably due to clustering or crosslinking. ($n = 3$).

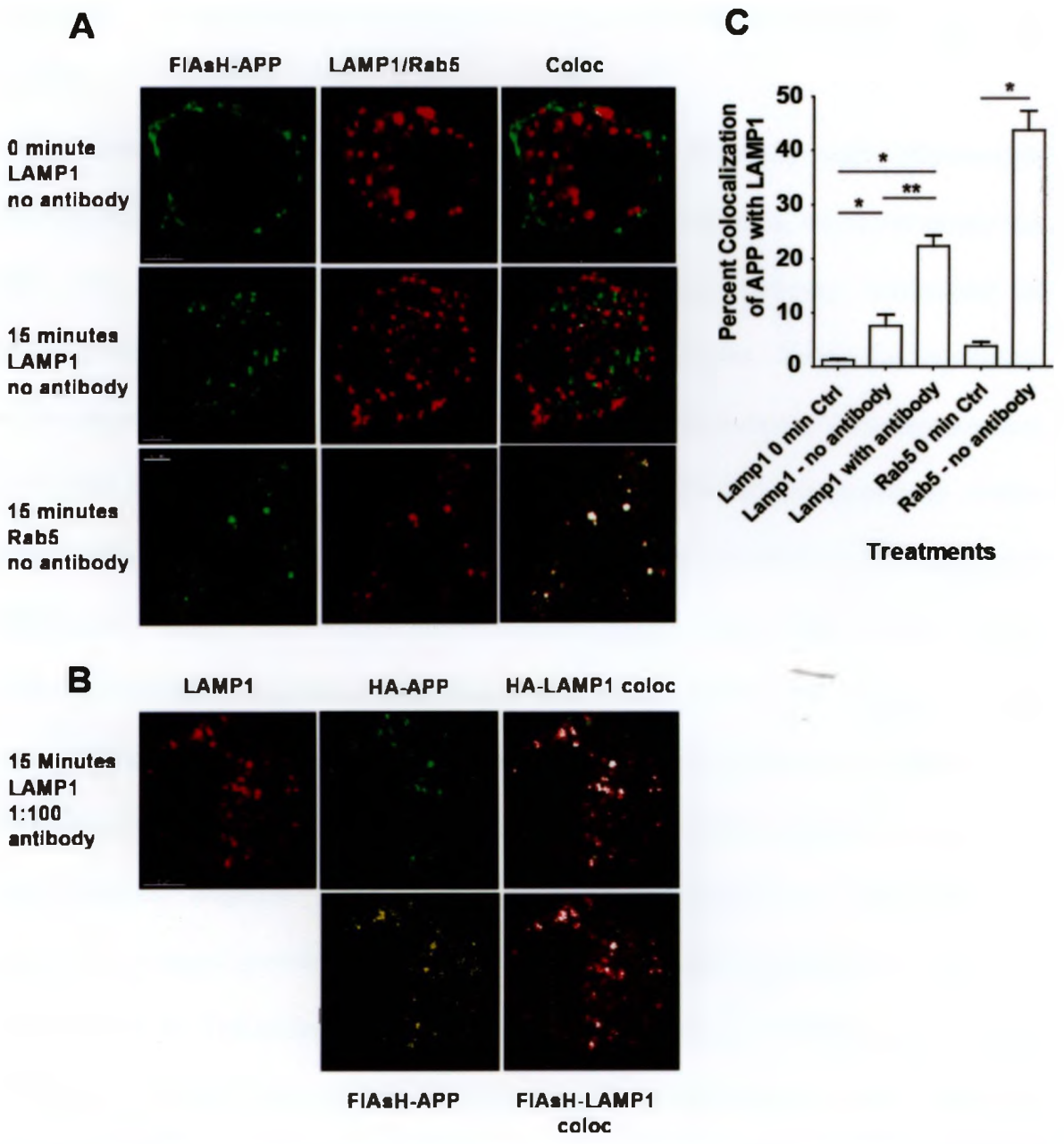


Figure 7. *Lumio FlAsH-labeled APP internalization to lysosomes in SN56 cells* **(A)** Colocalization of FlAsH biarsenical fluorescein-labeled APP (green) with LAMP1 and Rab5 (red) with no antibody present **(B)** Colocalization of FlAsH biarsenical fluorescein-labeled APP with LAMP1 with 1:100 anti-HA antibody **(C)** Percent colocalization of APP with LAMP1 and Rab5 at 0 and 15 minutes in SN56 cells. Cell surface APP was labeled using FlAsH biarsenical fluorescein reagent ('Lumio', Invitrogen) and were allowed to internalize for 15 minutes to LAMP1 and Rab5 compartments. 1:100 concentration of Zenon-labeled anti-HA antibody was used in addition to FlAsH labeling for 'with antibody' treatment group. *, ** denotes significant differences ($p<0.05$). Scale bar = 5 μm. 10 cells imaged in each of 3 separate experiments (n = 3, 30 cells total). Error bars represent the standard error of the mean.

3.4 Rapid APP trafficking to lysosomes is reduced by dominant negative mutations in Arf6.

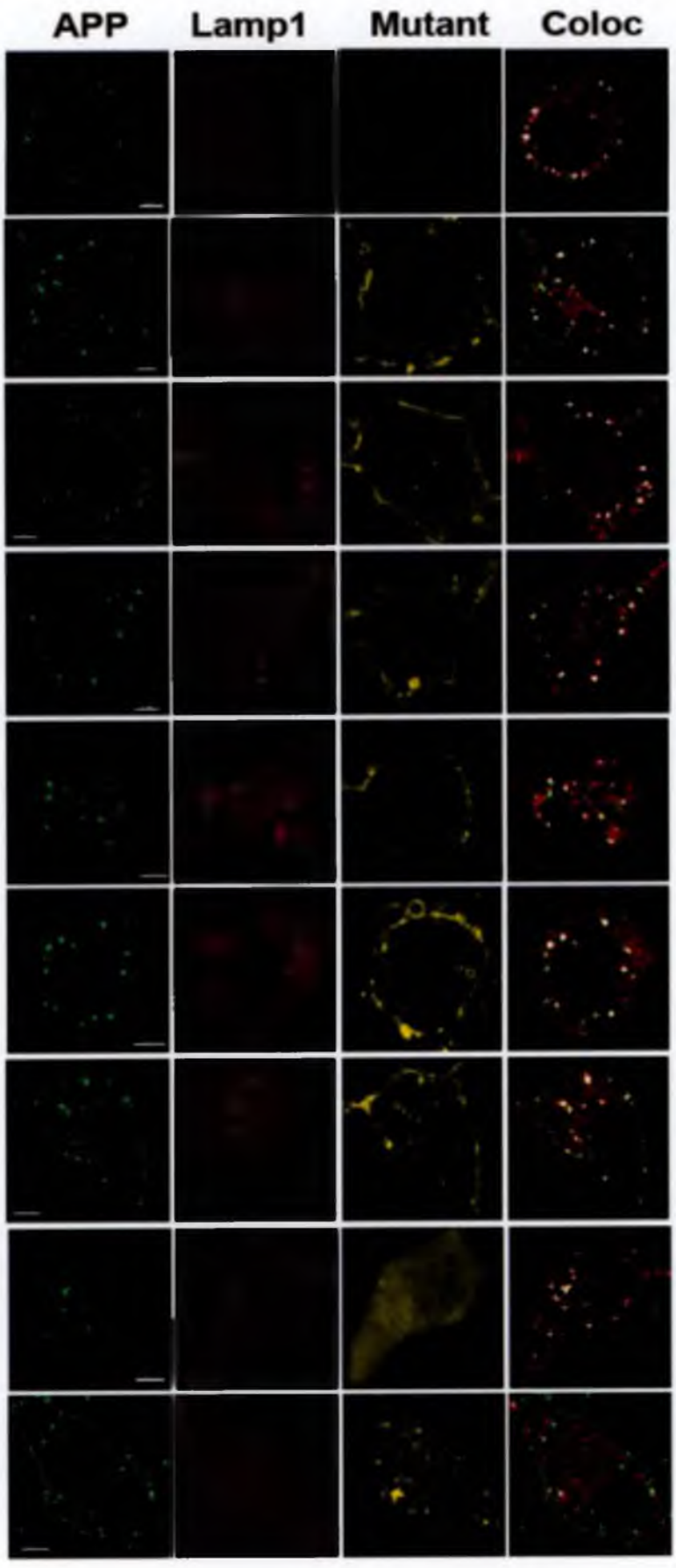
Because APP was being rapidly internalized to unexpectedly large compartments that did not have the appearance of normal clathrin-coated vesicles, we hypothesized that APP was being internalized by a clathrin-independent pathway reminiscent of macropinocytosis and might be regulated by similar mechanisms. To test this hypothesis, we examined some of the proposed regulatory proteins implicated in macropinocytosis. To do this, SN56 cells were co-transfected with HA-βAPP-CFP, a compartment marker tagged with mRFP (either LAMP1 for lysosomes or Rab5 for early endosomes), and a fluorescently-tagged trafficking regulator protein (fused to either GFP or YFP). These included dynamin, Rac1, RalA, Ras, RhoA, CtBP1-Bars, PLD2, Arf6, and Arf1. Cells were surface-labeled with Zenon-labeled anti-HA antibodies, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. Cells co-transfected with APP and dominant negative regulatory proteins were identified by visualizing the fluorescently-tagged proteins through a confocal microscope. Representative images are shown in Fig. 8. The co-localization of the brightest 2% of pixels of Zenon-633 labeled HA-APP and LAMP1 was quantified in at least 10 representative cells per experiment across at least 3 experiments for each mutant type. Data are shown as Mean percent colocalization ± SEM. APP co-localization with lysosomes in cells transfected with dominant negative forms of these proteins were 34.6% ± 2.8 (Rac1-T17N-DN), 35.5% ± 2.2 (RalA-S28N-DN), 30.6% ± 2.2 (Ras1-DN), 30.4% ± 3.5 (RhoA-T19N-DN), 35.9% ± 2.6 (CtBP1-Bars-S147A-DN), 38.8% ± 2.2 (PLD2-DN), 22.2% ± 2.2 (Arf6-T27N-DN) and 45.8% ± 2.3 (Arf1-T31N-DN), 28.1% ± 3.6 (dynamin-K44A-DN). These results

were then compared to the percent colocalization of APP with LAMP1 in control cells (35.7% ± 2.1), which were not transfected with any additional dominant negative mutants. When we performed a one-way ANOVA followed by a Tukey post-test in order to determine that of all the proteins screened, only Arf6-T27N-DN significantly reduced APP colocalization with LAMP1 ($p<0.05$). On the other hand, Arf1-T31N-DN significantly increased this colocalization ($p<0.05$) (Fig. 8B). These results indicate that Arf1 and Arf6 appear to have an effect on APP trafficking to lysosomes.

3.5 Rapid APP internalization to LAMP1 vesicles is dynamin independent and regulated by Arf6 whereas APP internalization to Rab5 vesicles requires dynamin activity and is unaffected by Arf6.

We investigated the role of Arf6 and Arf1 in lysosomal internalization. Arf6 has been implicated in a number of novel internalization pathways that work independently of clathrin, dynamin or other coating and scission proteins. Arf1 has been linked primarily to the intracellular trafficking of post-Golgi and lysosomal vesicles to the cell surface as well as in the regulation of the clathrin-independent CLIC/GEEC internalization mechanism (Jones 1999, Kumari 2008). To elucidate the role of Arf6 or Arf1 on APP lysosomal transport, SN56 cells were co-transfected with LAMP1-mRFP or Rab5-RFP, HA-βAPP-CFP and fluorescently-tagged versions of dominant-negative mutants of Arf1 and Arf6 (Fig. 8) and the constitutively active mutant of Arf6 (Fig. 9). We also compared this internalization with the effects of dominant negative dynamin, whose activity is required for the endocytosis of cell surface proteins to early endosomes. Interestingly, cells transfected with the dominant negative mutant of Arf1 showed a

8A


APP
Lamp1
Mutant
Coloc
Control
RhoA-T19N DN
CtBP1/BARS -S147A DN
PLD2 -K758R DN
Rac1-T17N DN
RalA-S28N DN
Ras1-S17N DN
Arf1-T31N DN
Arf6-T27N DN

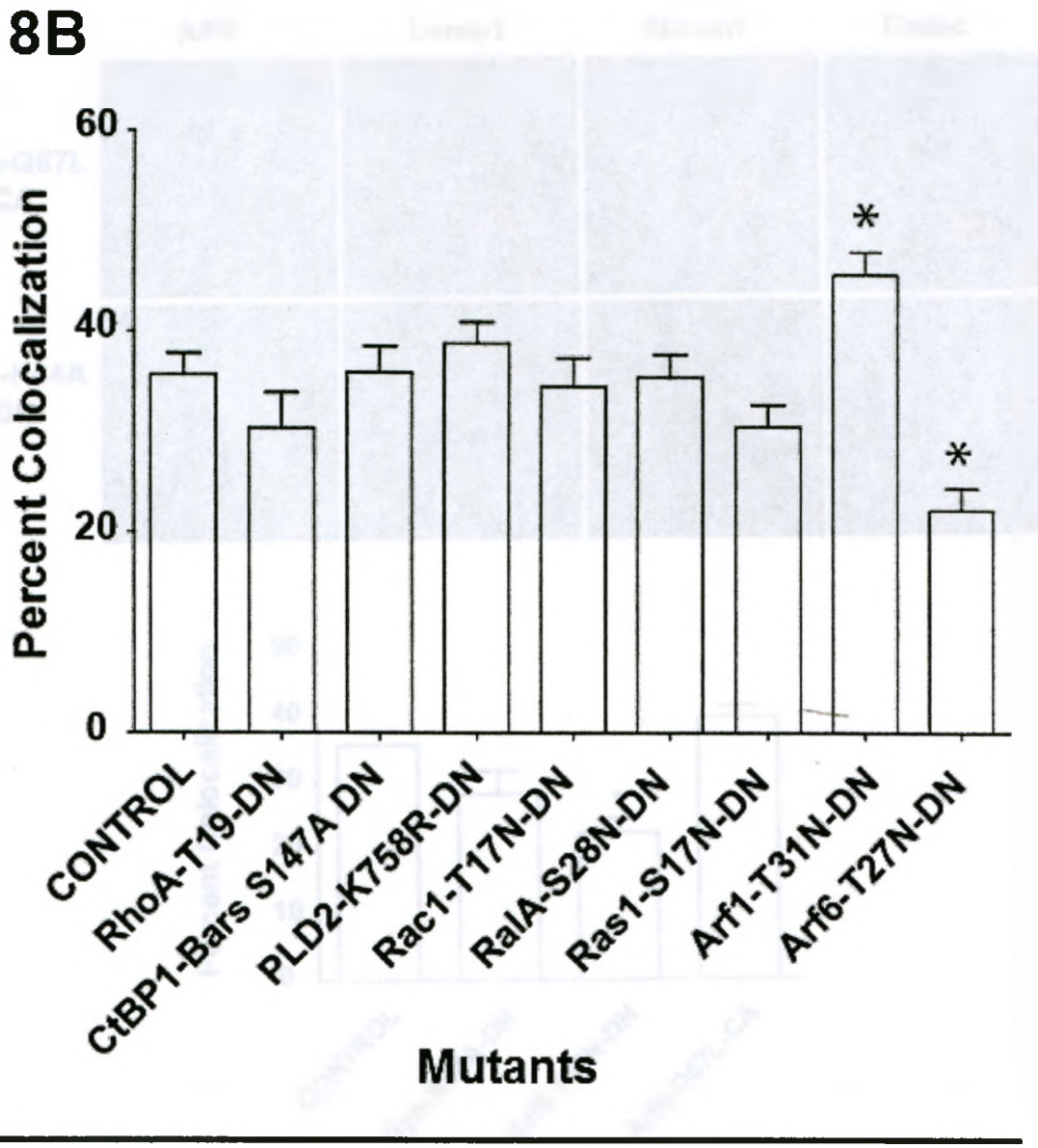


Figure 8. *Effect of dominant negative mutants on APP internalization to lysosomes in SN56 cells* **(A)** Colocalization of HA-labeled APP (green) with LAMP1 (red) in SN56 cells transfected with fluorescent protein- tagged dominant negative mutants of GTPases (yellow). The colocalized image shows the green and red channel merged, with the colocalization of the brightest 2% of pixels overlayed in white. Scale bar = 5 μm. **(B)** Quantification of APP colocalization with LAMP1 at 15 minutes in SN56 cells. Cells were transfected with APP-CFP, LAMP1-mRFP and GFP/YFP tagged dominant negative mutants of various regulators of endocytosis. Arf6-DN significantly reduced colocalization with LAMP1. Arf1-DN significantly increased APP colocalization with LAMP1. * denotes significant difference between treatment and control ($p<0.05$). Each bar represents the mean of a least 10 cells imaged in each of 3 separate experiments per dominant negative mutant. Error bars represent the standard error of the mean.

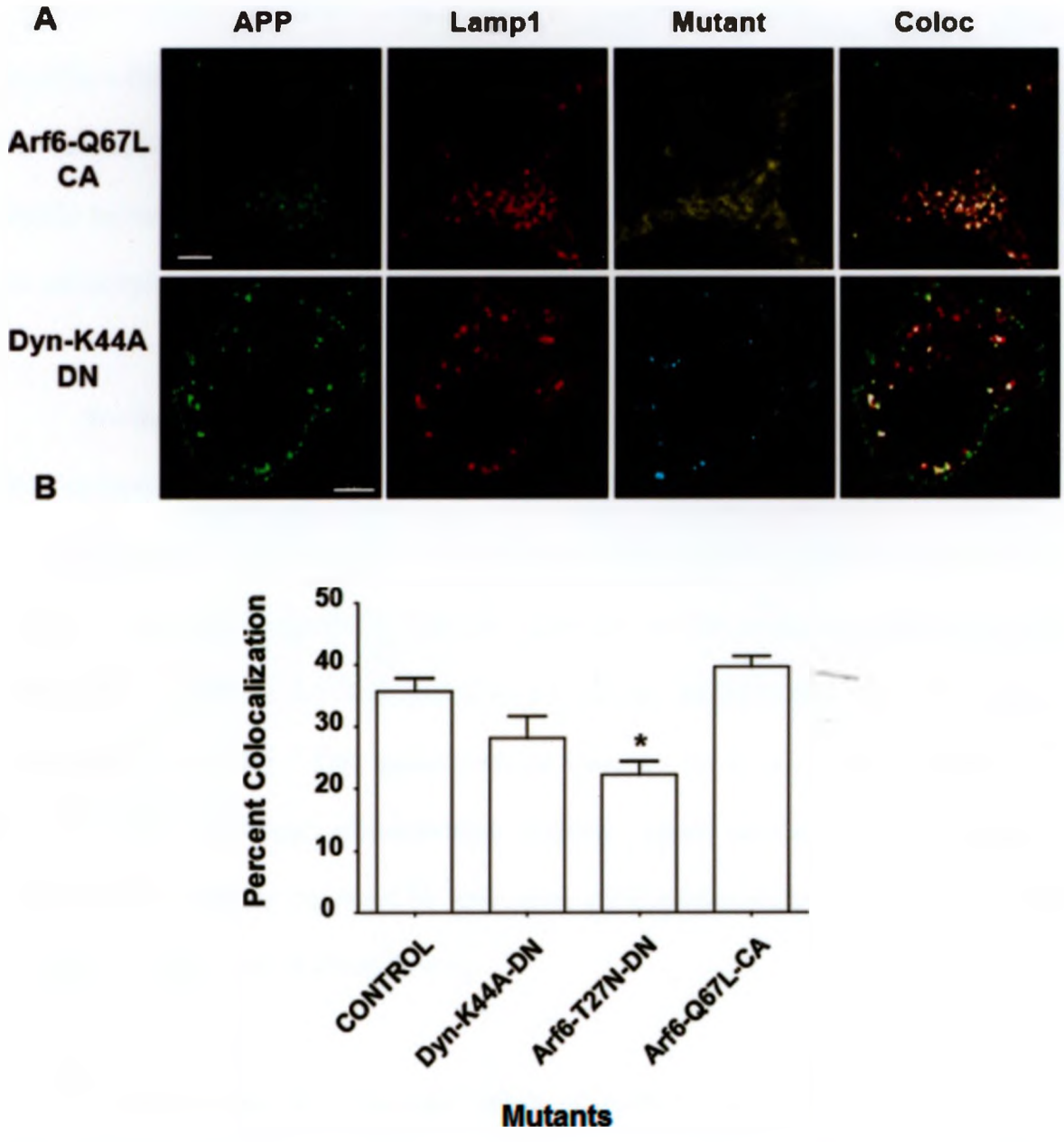


Figure 9. *Effect of Dominant Negative Arf1, Arf6 and Dynamin on APP internalization to lysosomes* **(A)** Colocalization of HA-labeled APP with LAMP1 in SN56 cells. Scale bars = 5 μm. **(B)** Percent colocalization of APP with LAMP1 at 15 minutes in SN56 cells transfected with APP-CFP, LAMP1-RFP and either dominant negative dynamin (K44A) or constitutively active Arf6 (Q67L). Neither dominant negative dynamin nor constitutively active Arf6 was able to significantly affect APP colocalization with LAMP1. * denotes statistically significant difference from control ($p<0.05$). 10 cells imaged in each of 3 separate experiments (n = 3, 30 cells total). Error bars represent the standard error of the mean.

significant increase in APP co-localization to LAMP1 to 45.8% ± 2.3 ($p < 0.05$). DynK44A-DN reduced APP transport slightly to the lysosome to 28.1% ± 3.6, but this was not statistically significant. The constitutively active form of Arf6 was able to slightly increase APP co-localization with LAMP1-mRFP (39.7% ± 1.7), but this too was not statistically significant. (n=3)

We then investigated the effects of these mutants on the internalization of APP to early endosomes marked with Rab5-RFP (Fig. 10A). In these experiments DynK44A-DN significantly reduced transport of APP to the early endosome from 32.7% ± 1.8 to 12.7% ± 2.4 (Fig. 10B) ($p<0.05$). The internalization of APP to the early endosome was unaffected at 36.4% ± 2.5 and 34.3% ± 3.0 for the Arf6DN and Arf1DN mutants respectively (Fig. 10B). This agrees with the literature by showing that, in addition to the novel rapid lysosomal internalization pathway, which we find to be a dynamin-independent mechanism regulated by Arf6 while APP is internalized to early endosomes in a dynamin-dependent manner. (n = 3)

To demonstrate the functional effect of Arf6DN on APP internalization, we performed live cell imaging on cells transfected with HA-βAPP-CFP, LAMP1-mRFP, and Arf6DN. Cells were labeled with Zenon-labeled anti-HA antibody on ice for 30 minutes and then transferred to a heated microscope stage. In these experiments, APP appears to collect in large structures that remain at the cell surface and do not fuse with LAMP1-mRFP labeled lysosomes (Fig. 11). These findings demonstrate that APP is internalized to two distinct pathways: one that is endosome-directed and dependent upon

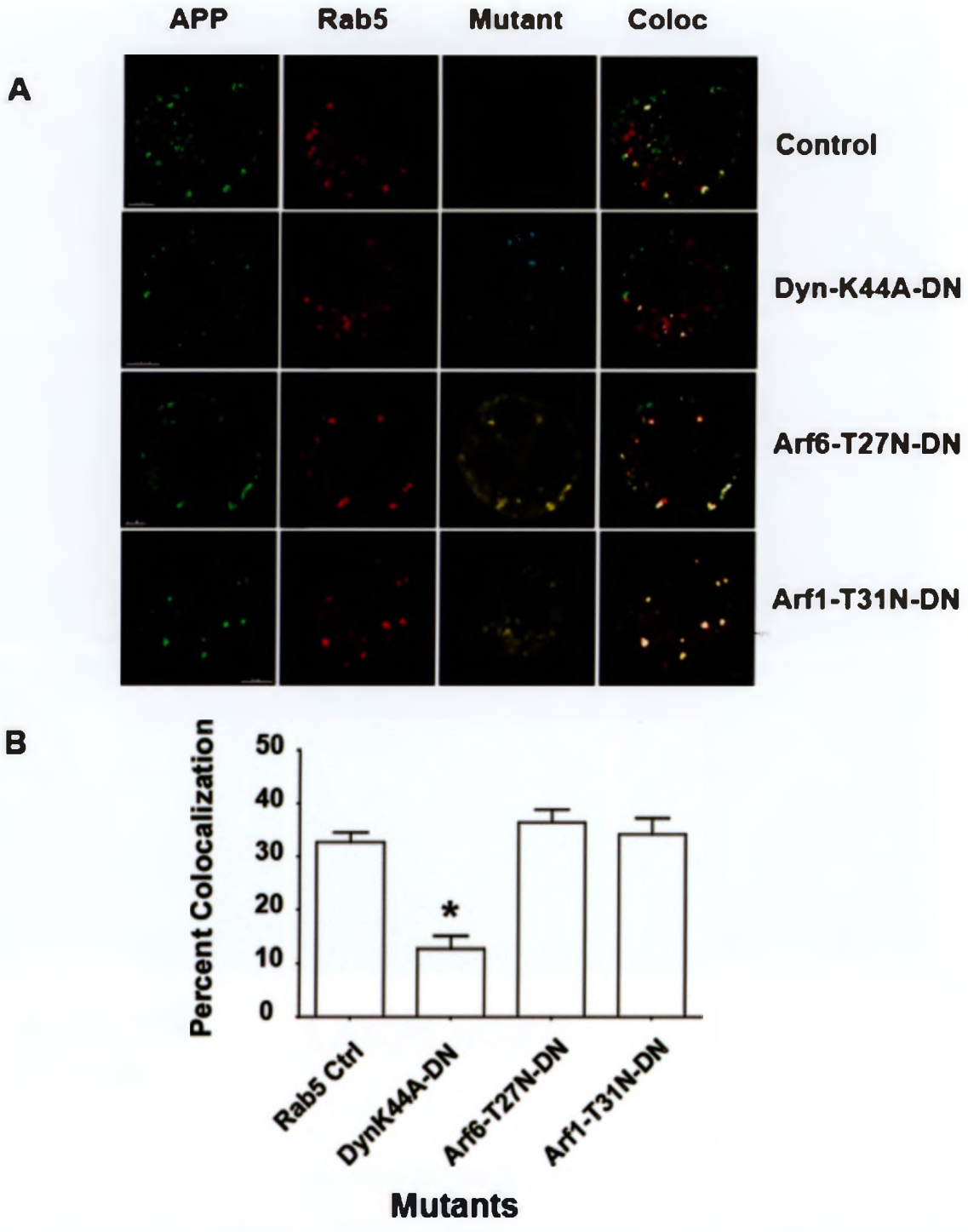


Figure 10. *APP internalization to early endosomes* **(A)** Colocalization of HA-labeled APP with Rab5 at 15 minutes in SN56 cells transfected with dominant negative mutants of Arf6, Arf1 and dynamin. Scale bar = 5 μm. **(B)** Percent colocalization of APP with Rab5 at 15 minutes. * denotes significant difference between treatment and control ($p<0.05$). Statistics are derived from imaging at least 10 cells imaged in each of 3 separate experiments. Error bars represent the standard error of the mean.

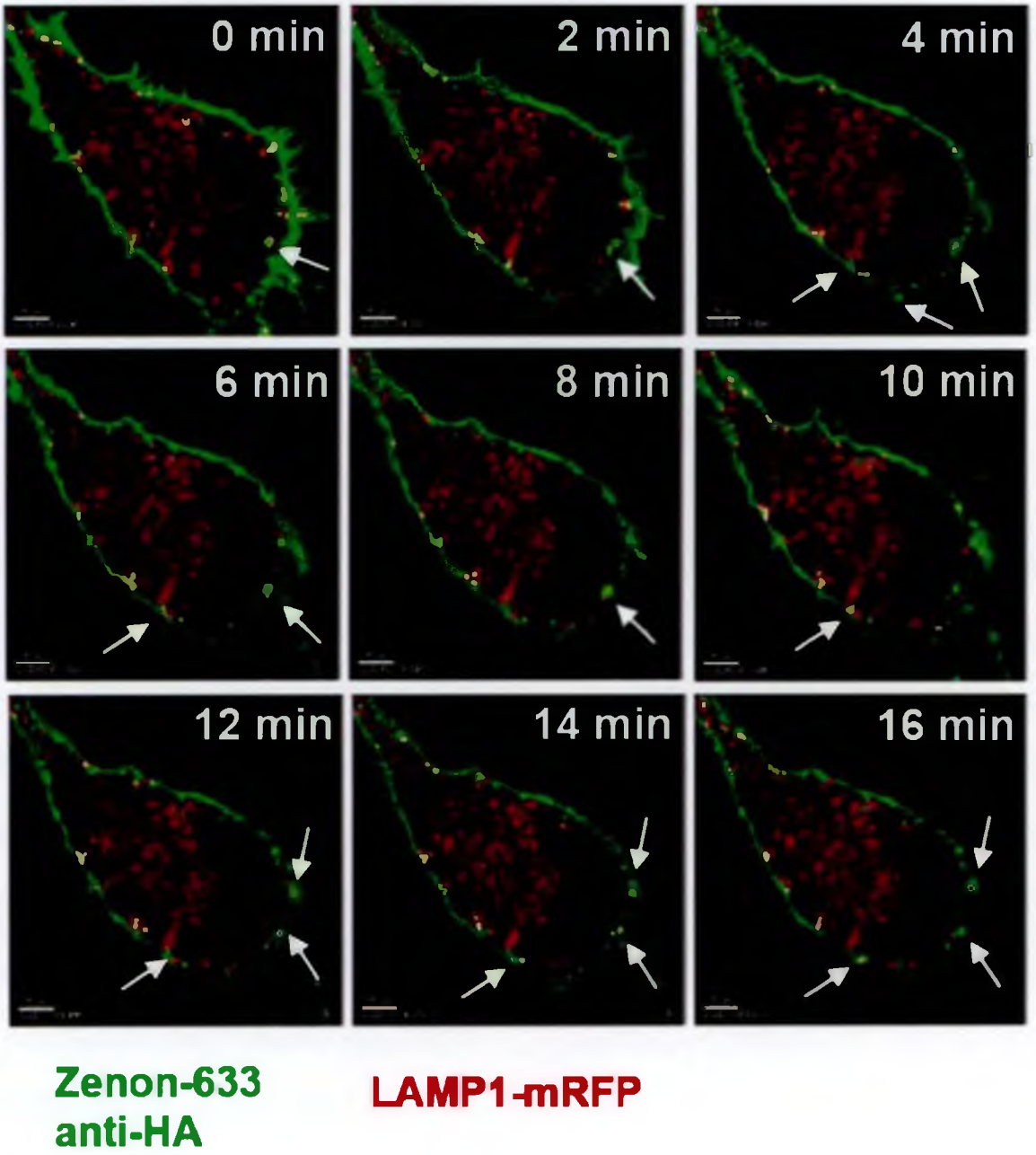


Figure 11. *Live cell imaging of SN56 cell transfected with LAMP1-mRFP (red), HA-βAPP-CFP (green) and GFP-Arf6-T27N-DN (channel not shown).* Cells transfected with Arf6-T27N-DN do not rapidly internalize APP to LAMP1 labeled lysosomes. Instead, APP remains clustered at the cell surface after 16 minutes of permitted internalization, a ring of cell surface APP remains clearly visible, which is in stark contrast to control cells (Figure 5B) where almost all APP has been internalized from the surface by the same time point. Colocalized pixels are shown in white. Scale bar = 5 μm.

Dynamin activity and another that is lysosome-direct and is dependent upon Arf6 activity. (n = 3)

3.6 SiRNA knockdown of Arf1 and Arf6 agrees with the effects of dominant negative mutants and demonstrates that Arf6 is dominant over Arf1 in regulating rapid APP internalization to lysosomes.

We wanted to confirm the effects of the dominant negative Arf1 and Arf6 mutants using siRNAs and determine, if possible, which is the upstream regulator in the trafficking of APP to lysosomes. To study this, we co-transfected SN56 cells with HA-βAPP-CFP, LAMP1-mRFP and siRNAs against Arf1 and Arf6 proteins. As shown by western blotting, siRNA concentrations at 300 pmol and 500 pmol reduced the amount of Arf6 and Arf1 proteins by 50% and 52% respectively (Fig. 12B). Representative images of siRNA-treated APP internalization studies are presented in Fig. 12A. Internalization was quantified in at least 10 representative cells in at least 3 independent experiments expressed as mean ± SEM. In these studies, the Arf6 siRNA significantly reduced APP colocalization with LAMP1 from 35.7% ± 2.1 (control) to 25.8% ± 2.2, while the Arf1 siRNA significantly increased APP colocalization to 43.6% ± 2.2 (Fig. 12C) ($p<0.05$). These results agree with those observed in cells transfected with dominant negative mutants of Arf1 and Arf6. When combined, the Arf6 and Arf1 siRNAs together reduced APP internalization to 20.9% ± 2.8. This demonstrates that Arf6 is dominant in the trafficking and delivery of APP to the lysosome from the cell surface than Arf1 (Fig. 12C).

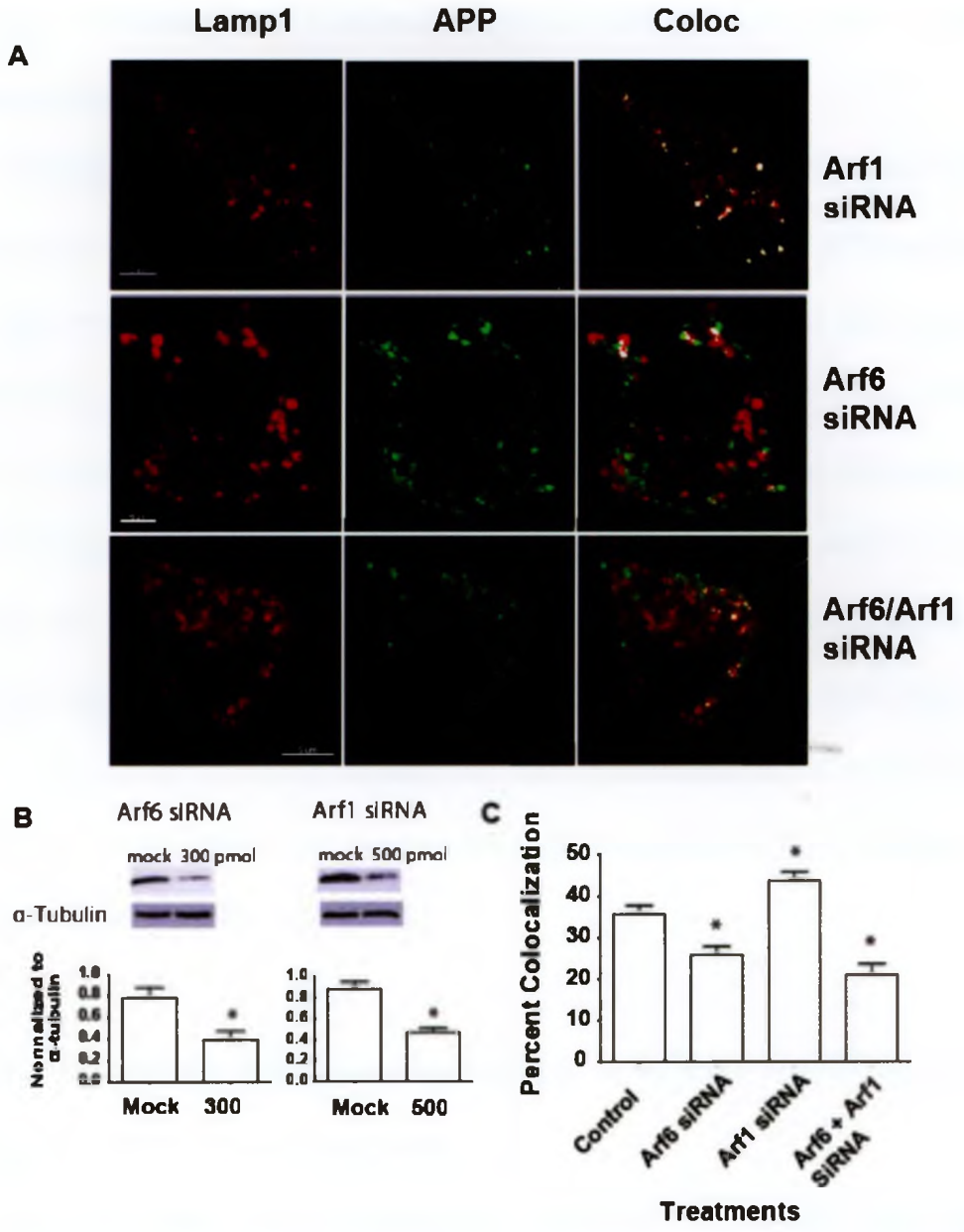


Figure 12. *SiRNA knockdown of Arf1 and Arf6 on APP internalization* **(A)** Colocalization of APP with LAMP1 at 15 minutes in SN56 cells transfected with siRNAs against Arf6 and Arf1. Cells transfected with both siRNAs show reduced APP colocalization with LAMP1 and a ring of labeled APP at the cell surface. Scale bar = 5 μm **(B)** Effective siRNA concentrations were determined through western blotting and bands were quantified using ImageJ by normalizing against α-tubulin loading controls. n = 3 **(C)** Percent colocalization of HA-labeled APP with LAMP1 at 15 minutes in SN56 cells (n = 3, 30 cells total). When both Arf6 and Arf1 were targeted by siRNAs, there was a significant reduction in APP colocalization with LAMP1, suggesting that Arf6 plays a more important role in APP internalization to LAMP1 than Arf1 does. * denotes statistically significant differences with control groups ($p<0.05$). Error bars represent the standard error of the mean.

3.7 Cells transfected with dominant negative Arf1 and Arf6 show significantly decreased secretion of Aβ42 into the culture media.

Having shown that both Arf1 and Arf6 can affect the colocalization of APP to the lysosome, we wanted to understand the effects of these mutants on APP processing into Aβ. Cells were transfected with dominant negative mutants of Arf1 and Arf6 or constitutively active mutant of Arf6 along with HA-βAPP-CFP. The media were collected following 3 days of incubation and were analyzed using an ultrasensitive Aβ42 ELISA (Invitrogen). Each experiment was performed in triplicate, and the data shown represent the Mean ± SEM of 3 independent experiments. Cells transfected with dominant negative Arf1 and Arf6 showed significant decreases in Aβ42 production to 48.0% ± 4.5 and 70.0% ± 6.9, respectively when normalized to the controls (control = 100%) (Fig. 13, $p < 0.05$). In contrast, the Arf6 constitutively active mutation had no significant effect (96.0% ± 6.1). (n = 3)

3.8 Cells transfected with dominant negative Arf1 show significantly decreased secretion of Aβ40 into the culture media.

Several studies have highlighted the importance of the Aβ42:Aβ40 ratio in the initiation and progression of Alzheimer's pathology (Selkoe 2007, Hardy and Selkoe 2002, Gorman 2008). Seeing as how both Arf6 and Arf1 mutants were able to elicit a significant decrease in Aβ42 production, we wanted to determine if they could also affect the production and secretion of Aβ40. SN56 cells were transfected with dominant negative mutants of Arf1 and Arf6 or constitutively active Arf6 along with HA-βAPP-CFP and allowed to incubate for 3 days before collecting the media for analysis using an

Aβ40 ELISA kit (Invitrogen). Each experiment was performed in triplicate, and the data shown represent the Mean ± SEM of 3 independent experiments. Surprisingly, only cells transfected with dominant negative Arf1 showed significant decreases in Aβ40 (40.0% ± 9.4) when normalized to controls (control = 100%). Neither the dominant negative (82.5% ± 5.4) or constitutively active (83.3% ± 5.4) mutants of Arf6 had any significant effect on the secretion of Aβ40 into the media (Fig. 14). These findings suggest that Arf6 primarily regulates Aβ42 production and secretion whereas Arf1 regulates the secretion of the APP cleavage products Aβ40 and Aβ42.

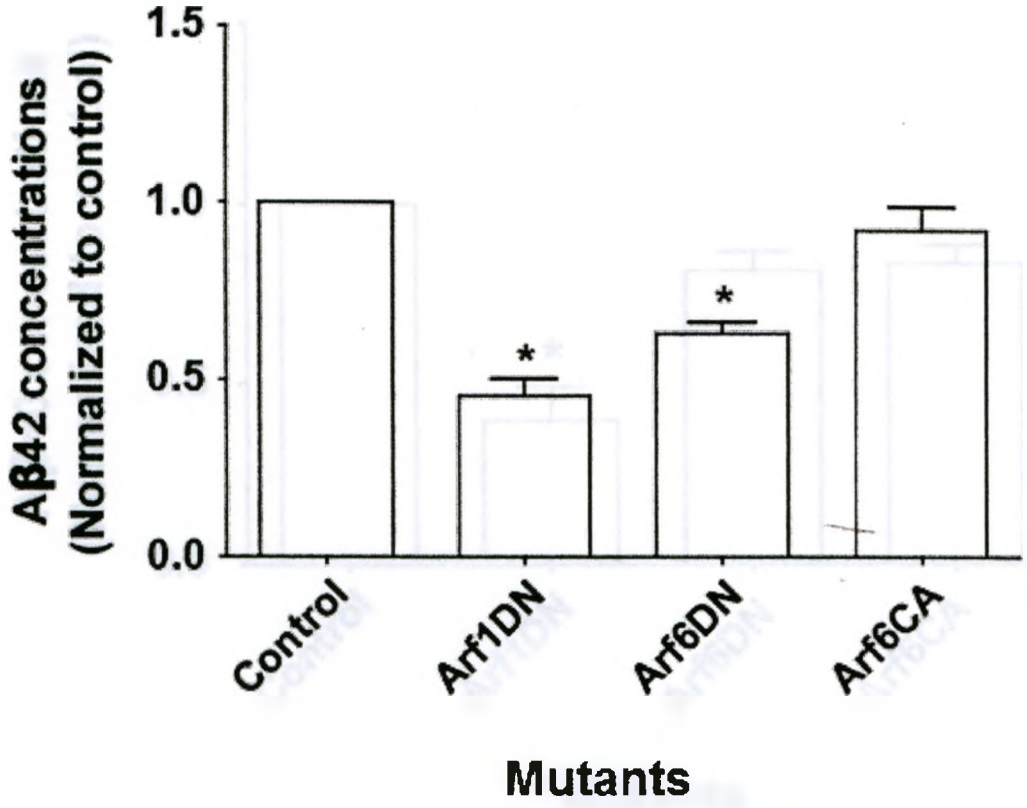


Figure 13. *Aβ42 secretion in SN56 cells.* 250,000 cells were plated in 12-well plates and transfected with wt-APP, either alone or with the construct shown. Cells were incubated for 3 days following transfection and the media was collected and assayed using an Aβ42 ELISA kit (Invitrogen). Both Arf1 and Arf6 dominant negatives significantly reduced Aβ42 secretion into the media, with Arf1 reducing to a greater extent. * denotes significant difference from the control ($p < 0.05$). Three wells were assayed per treatment in each of 3 separate experimental replicates ($n = 3$). Error bars represent the standard error of the mean.

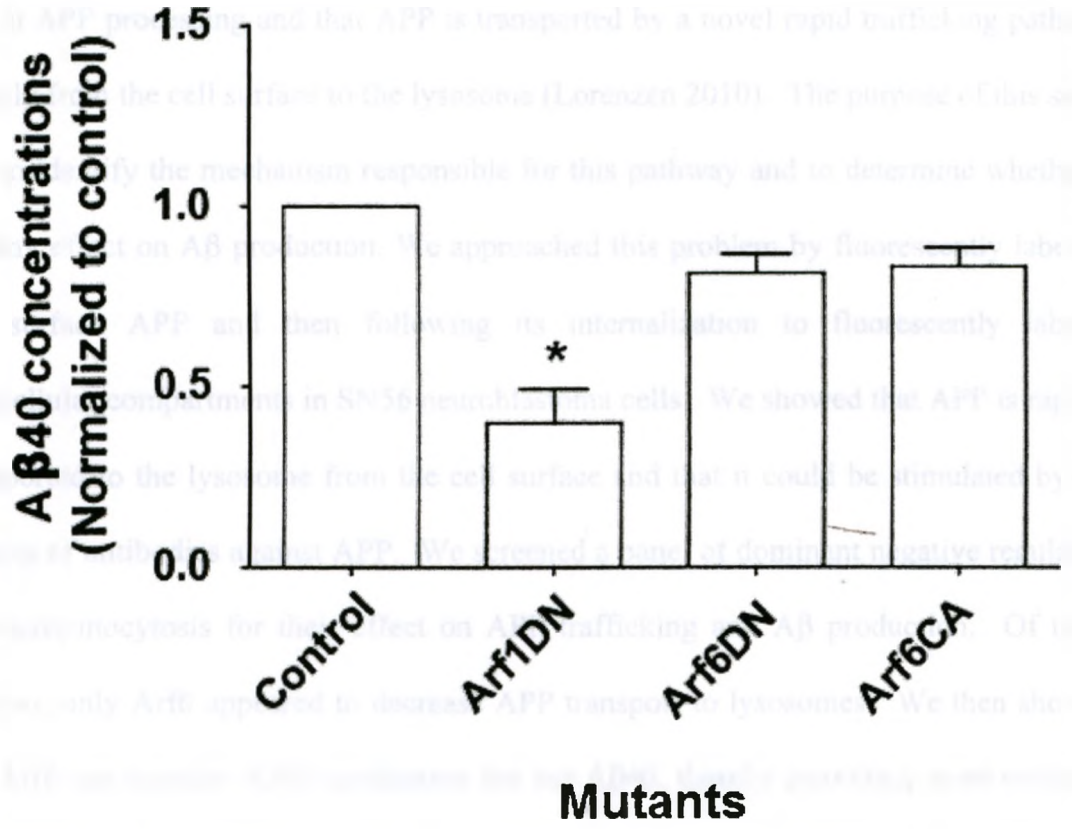


Figure 14. *Aβ40 secretion in SN56 cells.* 250,000 cells were plated in 12-well plates and transfected with wt-APP along with the construct shown. Cells were incubated for 3 days following transfection and the media was collected and assayed using an Aβ40 ELISA kit (Invitrogen). Three wells were assayed per treatment in each of 3 separate experimental replicates. Error bars represent the standard error of the mean. Only dominant negative Arf1 was able to significantly reduce Aβ40 secretion into the media. Neither constitutively active nor dominant negative Arf6 had any significant effect on Aβ40 secretion. * denotes significant difference from control ($p < 0.05$).

CHAPTER 4: DISCUSSION

Previous studies in our laboratory suggested that lysosomes may play a significant role in APP processing and that APP is transported by a novel rapid trafficking pathway directly from the cell surface to the lysosome (Lorenzen 2010). The purpose of this study was to identify the mechanism responsible for this pathway and to determine whether it has any effect on Aβ production. We approached this problem by fluorescently labeling cell surface APP and then following its internalization to fluorescently labeled intracellular compartments in SN56 neuroblastoma cells. We showed that APP is rapidly transported to the lysosome from the cell surface and that it could be stimulated by the binding of antibodies against APP. We screened a panel of dominant negative regulators of macropinocytosis for their effect on APP trafficking and Aβ production. Of these proteins, only Arf6 appeared to decrease APP transport to lysosomes. We then showed that Arf6 can regulate Aβ42 production but not Aβ40, thereby providing more evidence supporting our suggested model of lysosomes being responsible for Aβ42 generation. Looking into possible downstream targets of Arf6, we found that a possible candidate, Arf1, appeared to increase APP colocalization to lysosomes and may be important in regulating Aβ42 and Aβ40 secretion.

4.1 Mechanisms of APP Internalization

Several previous studies have demonstrated strong evidence for APP being internalized through a clathrin and dynamin dependent pathway into early endosomes (Cossec 2010, Sapirstein 1994, Ferreira 1993). As a result, it was widely believed that

early endosomes were primarily responsible for APP processing into Aβ. In this study, we show that in addition to being trafficked to early endosomes, APP is also trafficking from the cell surface directly to lysosomes at a very high rate through a pathway that bypasses endosomes entirely.

In light of these findings and those from previous studies, we suggest a model whereby APP participates in multiple trafficking pathways that allow it to be delivered to different amyloidogenic processing centers within the cell. One of these internalization pathways appears to be dependent upon both clathrin and dynamin activity. According to this pathway, cargo internalized from the cell surface via clathrin-coated pits are trafficked to the early endosomes, before being sorted and targeted to late endosomes and lysosomes for further sorting, processing or degradation (Doherty 2009). This pathway was inhibited by over expression of dominant negative dynamin-K44A as predicted. Other authors have provided evidence for lipid raft based internalization, suggesting another possible internalization pathway for APP (Schneider 2008).

Our observations suggest a third possible pathway for APP internalization. Live cell internalization studies revealed that direct lysosomal trafficking of APP occurs in vesicles that appear too large to be typical clathrin-dependent endocytic vesicles. Of the known endocytic pathways, only phagocytosis and macropinocytosis use very large (0.2μm - 10μm) compartments (Swanson 2008, Kerr 2009, Kumari 2010). Phagocytosis is responsible for the engulfment of large particles by receptor-activated membrane extensions which adhere directly to the surface of the particle (Swanson 2008, Kumari

2010). On the other hand, macropinocytosis results from the extension of membrane processes to engulf large volumes of extracellular fluid without the requirement of an extracellular article (Swanson 2008, Kerr 2009, Kumari 2010). Macropinocytosis is usually simulated by the binding a ligand onto cell surface receptor (typically growth factor receptors), but can occur constitutively (Sallusto 1995, Kerr 2009). Macropinosomes have also been previously described to preferentially fuse with lysosomes rather than endosomes (Hewlett 1994). Although often thought of as a function of immune cells, macropinocytosis has been reported in neuronal cells, where it acts as a high volume membrane retrieval system for growth cone extension/collapse and maintenance (Bonanomi 2008, Kabayama 2009). Our observation of APP transport to the lysosome appears consistent with macropinocytosis.

4.2 Intracellular APP trafficking is regulated by Arf6 and Arf1

Our observations suggest that a mechanism reminiscent of macropinocytosis could be responsible for regulating the rapid internalization of APP to lysosomes. This led us to investigate a number of small GTPases that have been found to regulate clathrin- and dynamin-independent mechanisms of endocytosis. From our screening of a panel of possible candidates, we found strong evidence suggesting that rapid APP trafficking to lysosomes is sensitive to Arf6 and Arf1 activity.

Arf6 has been implicated in a wide variety of cellular functions including clathrin-dependent and independent internalization, endosomal sorting and recycling, actin remodeling (D'Souza-Schorey 2006, Jaworski 2007) and the generation of

macropinosomes (Brown 2001). Arf6 has also been associated with clathrin-independent endocytosis of major histocompatability complex (MHC class1) M2 muscarinic acetylcholine receptors, β1 integrins (D'Souza-Schorey 2006, Jaworski 2007), and the prion protein (Kang 2009). To our knowledge, there is only one other report documenting a relationship between Arf6 and Aβ production (Sannerud 2011). In this study in HeLa cells, BACE was found to internalize into clathrin-independent compartments that eventually fused with APP-containing endosomes to accomplish β-cleavage. The trafficking of BACE was reduced by constitutively active Arf6-Q67L along with production of total Aβ, while the Arf6-T27N bearing a dominant negative mutation increased Aβ secretion. These results are in direct contrast to ours, in that we found Arf6-Q67L did not affect the levels of either Aβ40 or Aβ42, whereas Arf6-T27N was only able to reduce levels Aβ42. It is not clear why these studies differ, but macropinocytosis fulfills many cell type-specific functions, and it is likely that Arf6 might function differently in neuronal cells than in HeLa cells.

Arf6 is an interesting protein because it may act through a large number of potential downstream effectors, which may provide a number of potential therapeutic targets that can be exploited to interfere with Aβ generation and secretion. For example, Arf6 is known to regulate the production of PIP_2, a lipid cofactor that has been shown to be able to interact with actin-remodeling regulatory proteins, such as PLD, as a secondary messenger. A study by Brown *et al* (2001) highlights the ability of Arf6 to regulate macropinosome formation through PIP_2. Oliveira *et al* (2010) suggested that phospholipase D2 could be involved in the progression and pathogenesis of Alzheimer's

disease in the presence of Aβ. PLD has also been implicated in the trafficking and APP processing function of presenilin1, the functional component of the γ-secretase complex (Liu 2009, Cai 2006). Additionally, Arf GTPases can interact with PLD1 and PLD2 with the help of accessory proteins (Hammond 1995, Sung 1999). All of these studies suggest a possible link between Arf GTPases, PLD, PIP_2 and APP trafficking. However, studies in our SN56 cell model with dominant negative PLD2 did not show any significant effect on the rapid trafficking of APP to lysosomes. Our proposed future studies will examine the potential roles of PLD1 and PIP_2 in APP trafficking.

Arf1, in contrast to Arf6, is thought to primarily regulate trafficking vesicles from the Golgi apparatus to the cell surface and internal compartments. It has been suggested to play a role along with Arf6 in other types of dynamin-independent internalization mechanisms, but is not generally thought to function in macropinocytosis (Beemiller 2006, Kumari 2008, Kumari 2010). In addition, Arf1 is known to play a role in secretion (Zakharenko 1999, Fensome 1996, Jones 1999) and in membrane insertion into the plasma membrane so that phagocytosis can occur (Braun 2007). Because of its global effects on trafficking out of the Golgi apparatus, it is not surprising that Arf1 would reduce all forms of Aβ secretion by directly reducing the amount of APP leaving the Golgi apparatus. Our experiments demonstrating the decrease in Aβ42 and Aβ40 levels coupled with the apparent increase in trafficking of APP to the lysosome resulting from the inhibition of Arf1 activity suggests that Arf1 is preventing exit of APP/Aβ from the lysosome by inhibiting membrane trafficking or secretion.

A study by Jones *et al* (1999) showed that Arf1 could regulate lysosomal secretions in neutrophils through its ability to activate PLD. Several other studies have suggested a link between lysosomal and Golgi secretion pathways to the activation of Arf1, PLD and the regulation of PIP_2 production. A study by Zakharenko (1999) showed that neurotransmitter secretion along an axon is sensitive to Arf1 activity, thereby highlighting a possible role of Arf1 in regulating the trafficking of secretory vesicles to the plasma membrane in neuronal cells. Corroborating the results of Jones *et al* (1999), a previous study by Fensome *et al* (1996) showed that increasing either Arf1 or phosphatidylinositol transfer protein (PITP) activity in HL60 neutrophils was able to restore lysosomal secretion, suggesting a role for both proteins in the regulation of lysosomal secretion. Both Arf1 and PITP have been linked to PIP_2 synthesis (Jones 1999). The convergence of both Arf6 and Arf1 signaling pathways on the production and function of PIP_2 makes PIP_2 a very appealing target for future research as a possible regulator of APP trafficking and Aβ production.

4.3 The Importance of the functions of APP and Aβ

In this study, we show that there are a number of possible ways in which APP can be internalized into the cell for processing. It is interesting to speculate as to why neuronal cells have developed multiple mechanisms to ensure robust APP processing. Despite its obvious neurotoxic potential, several studies have suggested that APP and Aβ are essential to healthy neuronal physiology (Puzzo 2008). Haass *et al* (1992) demonstrated that Aβ is endogenously produced in primary neuronal cultures. Furthermore, it was shown that Aβ producing cells were morphologically normal and that

Aβ production is a normal physiological process. Their findings are consistent with the fact that Aβ can be found in the cerebrospinal fluid and plasma of normal healthy humans (Seubert 1992), suggesting that Aβ is produced throughout the body. A study by Plant *et al* (2003) demonstrated that inhibition of either γ- or β-secretase activity, and thereby inhibition of Aβ production, was toxic to neuronal cells. In addition, they show that neuronal toxicity also resulted when Aβ was bound and neutralized by anti-Aβ antibodies. Studies have shown that exogenous application of Aβ fragments of various lengths was not only able to rescue neurons from cell death (Plant 2003), but was also able to help promote neurite outgrowth and the development of hippocampal long-term potentiation (Puzzo 2008). A study by Gralle *et al* (2009) suggested that the shedding of the sAPPα ectodomain is essential to neuroprotection due to its ability to disrupt naturally occurring APP dimers. They also suggest that APP could act as an important surface receptor for signal transduction pathways. Certainly, the consensus of these studies suggests that APP must have an essential role in the regulation and maintenance of normal cellular physiology. On the other hand, the newfound physiological importance of APP and Aβ in healthy individuals may make developing an Aβ-depleting strategy against Alzheimer's disease rather difficult.

4.4 Initiation of Rapid APP Internalization to Lysosomes

In order to assess the effect of the antibodies used to label APP, we devised an experiment based on FlAsH labeling APP with a small biarsenical-fluorescein reagent that would be too small to crosslink cell surface proteins. In the absence of anti-HA antibody, APP transport to the lysosome still occurred, but APP appeared to move

preferentially to the early endosome. In contrast, antibody binding markedly stimulated APP transport to the lysosome. Several other reports have also found that binding of APP by an antibody at the cell surface triggers endocytosis. This has been suggested to result from APP cross-linking (Ehehalt 2003, Schneider 2008). Interestingly, these papers focused on lipid rafts and made use of APP bearing the Swedish mutation, which we have found to not be able to transit directly to the lysosome (Lorenzen 2010). This implies that these authors may have been examining yet another APP internalization pathway that may be specifically linked to FAD related mutations. In agreement with our studies, APP crosslinking/binding by an antibody has reported to stimulate intracellular kinases and increase Aβ42 (but not Aβ40) secretion (Sondag and Combs 2006). Another potential mechanism is that antibody binding could stabilize APP dimers, which has also been proposed to increase Aβ42 production (Munter 2007, Gorman 2008, Gralle 2009, Richter 2010). In their studies, Munter and Richter demonstrated that Aβ42 levels were increased when APP homodimerized at the cell surface via its series of GxxxG dimerization motifs. Consequently, disruption of these homodimers with chemical compounds (Richter 2010) or even with sAPPα (Gralle 2009) was neuroprotective in that it was able to significantly reduce Aβ42 production. These results support the idea that APP can respond to the binding of a ligand at the cell surface, and may function as a cell surface receptor whose stimulation could preferentially stabilize APP homodimers, resulting in increased Aβ42 production.

4.5 Effects on Aβ40 and Aβ42 Production and Secretion

Having shown that dominant negative mutations in both Arf1 and Arf6 were able to have a significant effect on APP delivery to the lysosomes, we asked whether these changes in APP trafficking could have a significant effect on the production and secretion of Aβ42 and Aβ40. Aβ42 is the most neurotoxic of the Aβ species in that it is able to form amyloid fibrils much more easily than Aβ40 (Selkoe 2007, Hardy and Selkoe 2002, Gorman 2008). A study by Murray *et al* (2009) demonstrated through mass spectrometry that Aβ40 has neuroprotective properties in that it is able to inhibit Aβ42 oligomerization into amyloid fibrils. Aβ ELISA analysis of the media of cells transfected with either dominant negative mutations of Arf1 or Arf6 revealed that disruption of Arf1 activity was able to significantly decrease secretion of both Aβ42 and Aβ40, whereas the dominant negative Arf6 only showed significant decreases in levels of Aβ42. The importance of these results is three-fold. Firstly, it demonstrates that this rapid Arf6-regulated APP trafficking pathway to the lysosome primarily controls Aβ42 production and not Aβ40. By targeting the pathway that regulates the production and secretion of the neurotoxic Aβ42 species, it may become possible to specifically reduce its production in order to slow down or even reverse the pathology of Alzheimer's disease. Secondly, because endosomal trafficking was not affected by Arf6 mutants, these results show that APP is being processed to Aβ42 in the lysosome. Lastly, because Arf1 appeared to increase APP trafficking to the lysosome, our results provide evidence that support Arf1's role in lysosome/endosome secretion pathways.

4.5.1 Importance of Lysosomes in APP Processing

The production of Aβ42 in lysosomes has broad implications for Alzheimer's disease pathogenesis as the lysosome may be an initial site where the seeding of insoluble Aβ occurs (Pasternak 2004, LaFerla 2007, Tam 2011). Aβ42 fibrilogenesis is known to be nucleated by both lysosomal gangliosides and the acidic lysosomal pH of 4.5 (Yanagisawa 1995, McLaurin, 1996, Inouye 2000, Su 2001, Waschuk 2001). These developing aggregates have been shown to disrupt the structures of neurons and synapses (Takahashi 2002) or cause lysosomal rupture leading to cell death (Yang 1998, Ji 2002). The intracellular accumulation of Aβ42 has been demonstrated in transgenic mice (Takahashi 2004, Knobloch 2007, Oakley 2006, Oddo 2006, Yan 2009, Billings 2005) and human neuropathological material where it appears in lysosomes before the appearance of plaques (LaFerla 1997, Gouras 2000, Gyure 2001, D'Andrea 2002, Cataldo 2004). The large amounts of active lysosomal enzymes in amyloid plaques also suggests that these extracellular deposits of amyloid originated in lysosomes (Cataldo 1990, Cataldo 1991, Cataldo 1994, Mach 2002).

4.5.2 Current and Future Therapeutic Strategies for Alzheimer's Disease

Several studies suggest that an equilibrium exists so that amyloid production and clearance are balanced so as to maintain healthy neuronal physiology; whilst preventing neurotoxic Aβ42 oligomers from aggregating (Hardy and Selkoe 2002). However, when the average individual ages (assuming they do not harbor a FAD mutation), the equilibrium may become offset due to either an increase in Aβ42 production or a failure in the body's capacity to efficiently clear Aβ as it is being made (Selkoe 2002). In

accordance with the amyloid cascade hypothesis, conditions which cause a surplus of Aβ42 to be produced result in an increased potential for neurotoxic Aβ oligomers to form (Hardy and Selkoe 2002). Current strategies for treating Alzheimer's disease are primarily concerned with inhibiting Aβ production or to increase its clearance. γ-secretase inhibitors disrupt amyloid production by preventing Aβ release resulting from γ-cleavage. However, as demonstrated by Plant *et al* (2003), the targeting of γ-secretase can elicit neurotoxic effects due to its influence on the processing of other essential γ-secretase substrates, such as Notch (Strooper 2003), and therefore limiting the practical usage of this strategy. Another strategy is to promote alpha cleavage, thereby inhibiting amyloid release by increasing the production of the shorter, more soluble APPα cleavage fragment. One promising approach is to increase clearance of soluble Aβ oligomers and insoluble plaque deposits from the intercellular space by targeting them with anti-Aβ antibodies that enable microglia to facilitate is removal (Bard 2000, Wilcock 2003).

Our findings suggest that Arf6 and Arf1, or their downstream signaling proteins, could potentially be key therapeutic targets for the treatment of Alzheimer's disease in the near future. Instead of completely halting the production of Aβ, we could reduce the rate at which Aβ42 is produced. We could accomplish this by interfering with the key APP trafficking pathway that controls the rate at which APP arrives to the lysosomes for specific processing into Aβ42. This would allow clearance mechanisms to stay on par with amyloid production, thereby preventing progression of AD, all the while ensuring the beneficial effects associated with maintaining low, physiologically acceptable levels of Aβ. In agreement with this strategy, studies in mice have shown that when amyloid

clearance overtakes excessive Aβ production, there was a complete reversal in Alzheimer's pathology when Aβ concentrations returned to endogenously acceptable levels (Schenk 1999, Hartman 2005, Spires-Jones 2009).

4.6 Conclusions

This study provides evidence for an important mechanism that regulates the production and secretion of Aβ42 in neuronal cells. Previous studies in our lab first revealed a completely novel trafficking pathway that delivered APP from the cell surface directly to the lysosome. Upon further investigation, we demonstrated that this rapid APP trafficking mechanism works independently from the early endosome-directed clathrin and dynamin-dependent endocytosis pathway. We also demonstrate that this rapid trafficking pathway occurs endogenously in mouse primary cortical neurons. Our observations highlight the possible role of Arf6 in regulating this rapid trafficking pathway. Disruption of Arf6 activity was able to significantly decrease secretion of the more neurotoxic Aβ42 species, but not Aβ40. We also provide evidence suggesting a role for Arf1 in regulating the secretion of Aβ. Finally, we provide evidence which suggests that the rapid internalization of APP to lysosomes is inducible through the application of high antibody concentrations during surface labeling. We suggest a model whereby factors that increase APP clustering at the cell surface causes the preferential transport of APP to the lysosome through an Arf6 regulated mechanism. This may be responsible for the production of a significant proportion of the total amount of Aβ42. Alternatively, unclustered APP could be sorted to the early endosomes through a

dynamin-dependent mechanism, where APP could be preferentially cleaved into the Aβ40 form over Aβ42. Secretion of either Aβ40/42 could be specifically regulated by Arf1 or one of its many downstream signaling partners (Fig. 15). This work reinforces the idea that APP might function as a receptor for as-of-yet undefined ligands, and provides further support for the idea of the lysosome as key a source of Aβ42. Future studies will examine the role of downstream effectors of Arf6 on Aβ production. We hope these enlightening findings will enable us to better understand the dynamics and mechanics of APP trafficking and Aβ production. Exploring this mechanism in greater detail may uncover a promising therapeutic target to be used in the treatment of Alzheimer's disease.

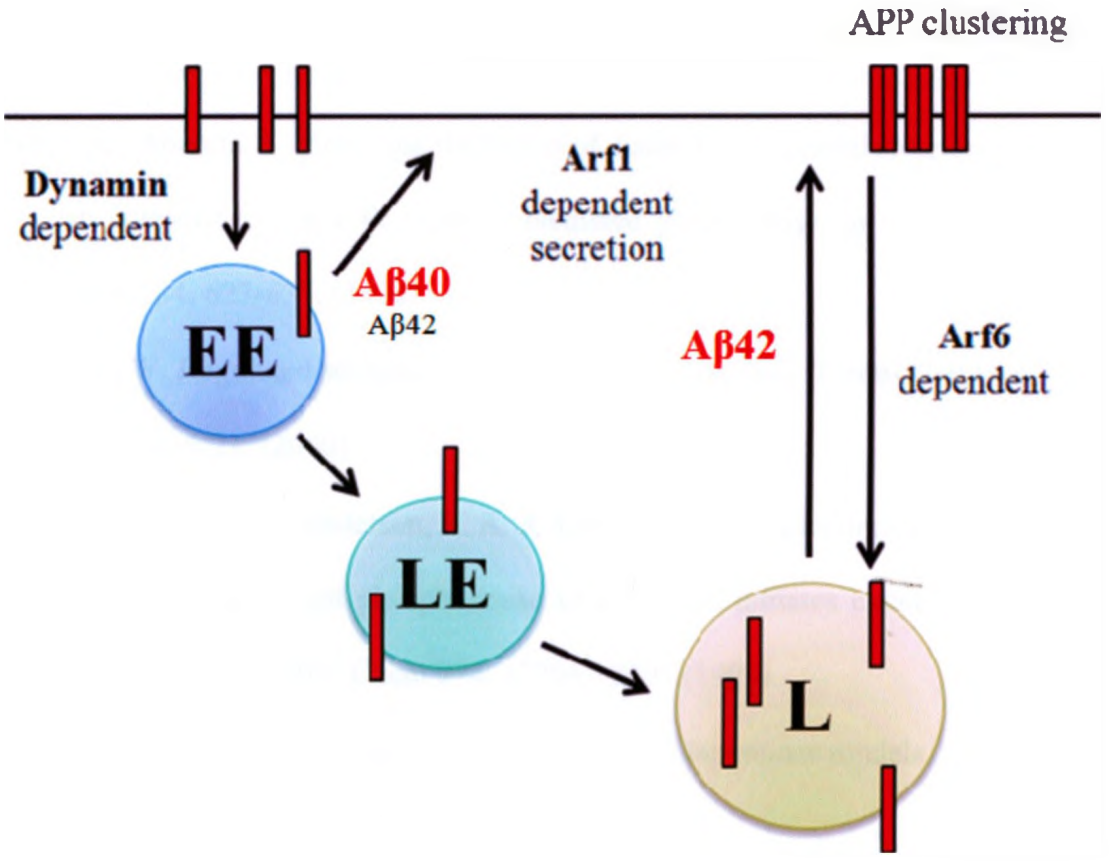


Figure 15. *Proposed model of APP trafficking and processing.* Clustering of APP induces its sorting into the Arf6-regulated rapid internalization pathway to the lysosome. Rapid delivery to lysosomes may preferentially cause APP to be processed into Aβ42. Alternatively, APP can be internalized through a dynamin-dependent pathway to early endosomes where it could be preferentially processed into Aβ40 over Aβ42. Secretion of APP cleavage products is regulated by Arf1, since inhibition of Arf1 activity locks APP and Aβ in endosomes and lysosomes. EE = early endosome. LE = late endosome. L = lysosome. Red bars = APP.

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