Function of flotillins in Alzheimer's disease and apoptosis

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LIST OF ORIGINAL PUBLICATIONS

1. **Bincy A. John**, M. Meister, A. Banning, and Ritva Tikkanen, *Flotillins bind to the Dileucine Sorting Motif of BACE1 and influence its endosomal Sorting*, **FEBS Journal**, In press February 2014

2. **Bincy A. John**, Krishna Rajalingam, Ritva Tikkanen, *Function of flotillins in survival signaling and apoptosis* (manuscript in preparation)

3. N. Kurrle, W. Ockenga, M. Meister, F. Debus, S. Kühne, **Bincy A. John**, A. Banning, and Ritva Tikkanen, *Phosphatidylinositol 3-Kinase dependent Up regulation of the Epidermal Growth Factor Receptor upon Flotillin-1 Depletion in Breast Cancer Cells*, **BMC Cancer** 2013, 13:575

4. Kurrle N., **John B.**, Meister M., Tikkanen R. 2012: *Functions of flotillins in receptor tyrosine kinase signaling and endocytosis: role of tyrosine phosphorylation and oligomerization. In C. Huang (Eds.),* **Protein phosphorylation in human health** pp 307-342 (http://dx.doi.org/10.5772/2944), Rijeka, Croatia

LIST OF ABBREVIATIONS

aa	Amino acid
Aβ	Amyloid-beta
ACDL	Acidic cluster di-leucine
AD	Alzheimer's disease
AICD	Alzheimer precursor protein intracellular domain
AIF	Apoptotic inducing factor
APP	Alzheimer precursor protein
APS	Ammoniumpersulfate
BACE	β-site APP cleaving enzyme
BSA	Bovine serum albumin
CAP	Cbl-associated protein
CARD	Caspase recruitment domains
CD95	Cluster of differentiation 95
cDNA	Copy-DNA
C-terminus	Carboxyterminus
CTL	Cytotoxic T lymphocytes
DD	Death domain
dd H_2O	Double distilled water
DED	Death effector domain
DIABLO	Direct inhibitor of apoptosis protein-binding protein with low PI
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's medium
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FADD	Fas associated protein with death domain

LIST OF ABBREVIATIONS : continued

FCS	Fetal calf serum
FGF	Fibroblast growth factor
FOXO	Fork head box
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GGA	Golgi associated, γ-ear containing, ARF binding
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IGF	Insulin like growth factor
IF	Immunofluorescence
IP	Immunoprecipitation
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MPR46/300	Mannose-6-phosphate receptor 46/300 kDa
MULE	Mcl-1 Ubiquitin E3 ligase
NLS	Nuclear localization signal
N-Terminus	Aminoterminus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK	3-phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol-3 kinase
PKB	Protein kinase B
RIP	Receptor interacting protein

LIST OF ABBREVIATIONS : continued

RPMI	Rosewell Park Memorial Institute medium
RPTK	Receptor protein tyrosine kinase
sAPPα	Secretory APP alpha
SMAC	Second mitochondria-derived activator of caspase
STAT	Signal transducers and activators of transcription
TBST	Tris buffered saline Tween
TEMED	Tetramethylethylenediamine
TGN	Trans-Golgi-network
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPA	Tissue plasminogen activator
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TRADD	Tumor necrosis factor receptor associated death domain
VEGF	Vascular endothelial growth factor
WT	Wildtype

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SUMMARY

Alzheimer's disease (AD) is a common, age associated neurodegenerative disease that manifests as progressive dementia and is characterized by accumulation of the amyloid beta (Aβ) peptide which is a processing product of a transmembrane protein termed Alzheimer Amyloid Precursor Protein (APP). The Aβ peptide is generated by a sequential proteolytic processing of APP by two distinct proteases that are termed β- and γ-secretase. The β-secretase, also called BACE-1 or memapsin 2, belongs to the family of aspartyl proteases. BACE-1 evidently cleaves APP in an acidic endosomal compartment after endocytosis of APP, thereby facilitating Aβ peptide generation.

Sorting of transmembrane proteins is generally controlled by sorting signals in the cytoplasmic domains of the cargo proteins. The short cytoplasmic tail of BACE-1 with 23 amino acids contains a sorting signal of the acidic cluster, di-leucine (ACDL) type. The two Leu residues in this determinant are important for the clathrin mediated endocytosis of BACE-1, whereas the acidic residues together with the Leu are required for the endosomal sorting and recycling of BACE-1 back to the plasma membrane. The ACDL motif binds to the members of the GGA (Golgi-localized γ ear-containg ARF-binding proteins) family (GGA1-GGA3) that are involved in the sorting of BACE-1.

One of the major aims of this study was to address the role of flotillins in the intracellular sorting of BACE-1. This study shows that flotillin-1 directly binds to the di-leucine motif in the cytoplasmic tail of BACE-1, whereas flotillin-2 only shows an association mediated by flotillin-1. Flotillin-1 competes with GGA2 for the binding to BACE-1 tail, and thus influences the endosomal sorting of BACE-1. Importantly, depletion of flotillins results in an altered localization of the wildtype BACE-1, whereas the plasma membrane resident Leu to Ala (LLAA) mutant is not affected. Flotillin knockdown results in an accumulation of BACE-1, implicating reduced degradation and enhanced stability of this protease. Thus, flotillins appear to be important for the cellular targeting of BACE-1 and also influence the amyloidogenic processing of APP, as demonstrated by an increase in the amyloidogenic C-99 processing fragments.

When flotillin depleted cells were subjected to apoptotic stresses including $A\beta_{25-35}$ synthetic peptide (inducer of the extrinsic apoptosis pathway) or several chemotherapeutic agents (staurosporine, brefeldin A, doxorubicin, carboplatin and paclitaxel: intrinsic apoptosis pathway) and cytotoxicity was determined, various apoptotic markers were activated in flotillin depleted cells. Caspase-3 and GGA3 are

well accepted apoptosis markers and an enhanced caspase-3 cleavage was detected upon STS induced apoptosis in SH-SY5Y, HeLa, and HaCaT cell lines and increased GGA3 cleavage was observed in MCF7 cell line.

One of the major reasons for the apoptotic sensitivity in the absence of flotillins was a PI3K/Akt signaling defect. Neuroblastoma cells depleted of flotillins showed diminished levels of total Akt, phospho-Akt and phospho-ERK upon STS induced apoptosis. Since PI3K/Akt was the primary survival pathway affected upon STS induced apoptosis, ectopic expression of Akt in neuroblastoma cell line reduced caspase-3 cleavage and retarded apoptosis.

The direct downstream target of Akt is FOXO3a, whose localization was investigated in flotillin depleted cells. A major proportion of FOXO3a was localized in the nucleus of flotillin knockdown cells, implicating that FOXOs are active in these cells and subsequently trigger the transcription of death genes. Strikingly, an essential anti-apoptotic molecule and a major cancer target, Mcl-1, was inherently downregulated in flotillin knockdown cells. Mcl-1 is a chief member of the Bcl-2 family as it plays a pivotal role in cell survival and it is a critical protein in cancer therapeutics as suppression of Mcl-1 protein can curtail the survival and growth of tumorous cells.

Neuroblastoma cells were rescued from undergoing permanent damage due to STS induced apoptosis by overexpression of anti-apoptotic Bcl-2. Phorbol esters are well known PKC activators, and pre-treatment of neuroblastoma cells with phorbol esters along with staurosporine reduced caspase-3 cleavage.

These results demonstrate that absence of flotillins can sensitize cellular systems to apoptosis induction. The two main characteristics of cancer cells include resistance to apoptosis and unresponsiveness to chemotherapeutic agents. It is a well established fact that impaired apoptosis is central to tumour development. This study implicates that the downregulation of flotillin function can trigger cellular susceptibility and enhances apoptosis in response to conventional chemotherapeutic agents. Therefore, flotillins can serve as vital regulators in providing a more rational approach in molecular-targeted therapies for receding cancer growth and survival.

CHAPTER 1: INTRODUCTION

1.1 Alzheimer Amyloid Precursor protein and its processing

Alzheimer's disease (AD) is the most common neurodegenerative disease, characterized by gradual erosion of the cognitive functions.[1] The chief neuropathological features include progressive formation of insoluble amyloid plaques and neurofibrillary tangles in the brain.[2] Amyloid plaques are majorly composed of Aβ, the most common form of which is 40 amino acids long and thus called $A\beta_{40}$.[2] A less abundant form is $A\beta_{42}$ peptide which differs from $A\beta_{40}$ by having two additional amino acid residues at the C-terminus and is particularly associated with this disease. Proteolytic processing of APP occurs by two alternate cleavage pathways: amyloidogenic and non-amyloidogenic pathway, which are localized in different subcellular compartments and mediate different biological effects. In the case of the non-amyloidogenic pathway, the ectodomain shedding of the APP is executed by α-secretases.[3] As this cleavage occurs within the Aβ domain, it precludes the formation of toxic Aβ peptides. Instead, it generates a soluble extracellular fragment (sAPPα) and CT83 (C-terminal fragments of APP). The main site of this pathway includes plasma membrane, endoplasmic reticulum (ER) and Golgi complex. The residual CT83 fragment generated in this pathway is processed by the γ-secretase to produce p3 fragment and the APP intracellular C-terminus domains (AICDs).[4]

Conversely, in the amyloidogenic pathway, APP is cleaved by the β-secretase or BACE-1 (β-site amyloid precursor protein cleaving enzyme) within its large extracellular domain to yield two fragments, sAPPβ (an N-terminal fragment) and CT99 or CT89.[5] Subsequently, this residual CT99 fragment is cut within the membrane by the γ-secretase, generating Aβ peptide and AICDs. Inside the cell, AICD soon undergoes a sequential cleavage at a specific caspase cleavage site to produce a CT31 fragment. AICD is a common product of both α-γ or β-γ cleavage, and is capable of translocating to the nucleus.[6]

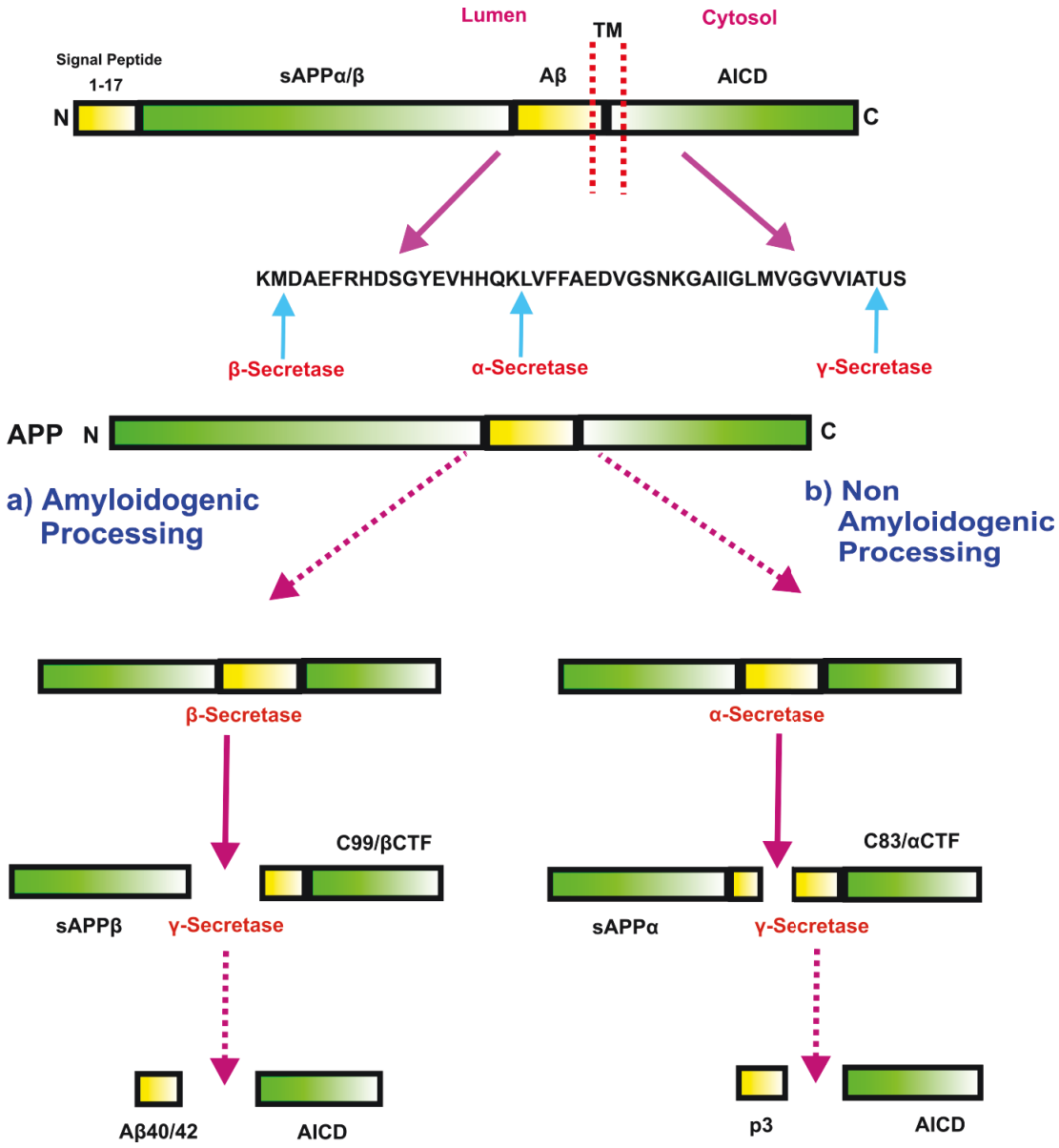


Figure 1: Schematic representation of APP processing and its proteolytic products.

The proteolytic processing of the large, transmembrane APP occurs by means of amyloidogenic and non-amyloidogenic pathways. a) The amyloidogenic pathway is initiated when the β-secretase cleaves APP at the amino terminus of the Aβ peptide and releases the sAPPβ ectodomain. Further processing of the resulting carboxy-terminal fragment by the γ-secretase results in the release of Aβ. b) In the non-amyloidogenic pathway, APP is first cleaved by α-secretase within the Aβ sequence, which releases the sAPPα ectodomain. Further processing of the resulting carboxyl terminus by the γ-secretase results in release of the p3 fragment Abbreviations: Aβ, amyloid-β; APP, amyloid precursor protein; sAPPα, soluble amyloid precursor protein-α; sAPPβ, soluble amyloid precursor protein-β; C83, carboxy-terminal fragment 83; C99, carboxy-terminal fragment 99.

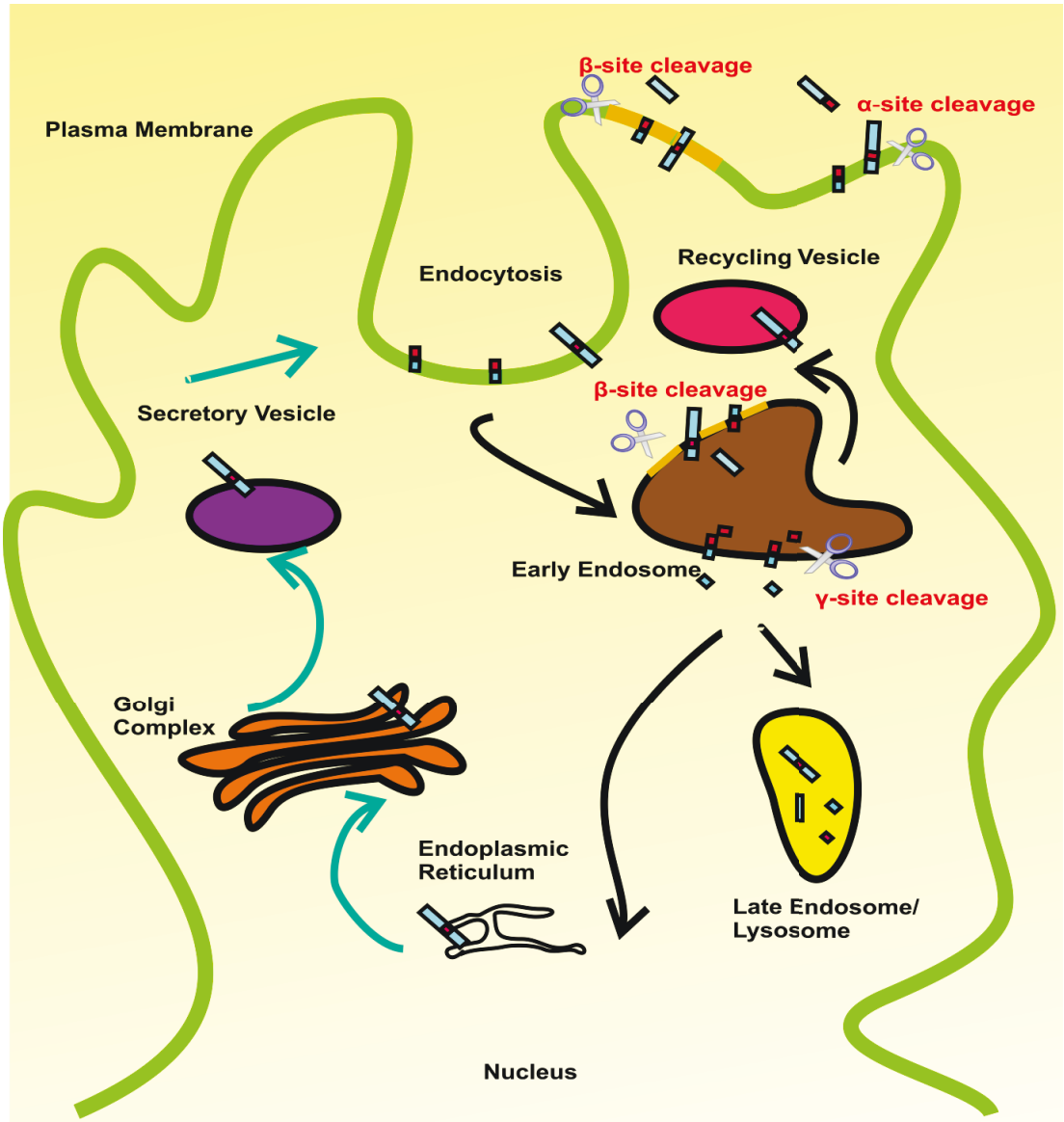


Figure 2: Schematic diagram of intracellular amyloid precursor protein trafficking.
APP present at the cell surface is rapidly internalized and subsequently trafficked through endocytic compartments and recycled back to the cell surface or degraded in lysosomes. Amyloidogenic processing occurs in endocytic organelles where β-site APP cleaving enzyme and γ-secretase process APP. Non-amyloidogenic processing occurs mainly at the cell surface, where α-secretase and β-secretase is present.

1.2 Lipid rafts

Lipid rafts are small (10-200 nm), dynamic assemblies of sphingolipids, cholesterol and proteins that associate and disassociate with them at a rapid pace.[7] Small rafts are stabilized to form larger platforms either through lipid-protein or protein-protein interactions. The long, saturated acyl chains of sphingolipids along with kinked, unsaturated acyl chains of bulk membrane phospholipids enable phase separation.[8] The sphingolipids are interspersed with cholesterol which acts as a spacer by filling the gaps and as a dynamic glue that facilitates a tight packing and keeps the raft assembly together.[9] Protein diffusion in membranes is not free but rather constrained, and this leads to the concept of organization of lipids in domains which ought to have a functional significance. These membrane lipid clusters are typically termed as lipid rafts due to their ability to act as moving platforms, and proteins can be segregated by being selectively retained or excluded from lipid rafts. Hence, lipid rafts play a significant role in determining protein-protein interactions or function as protein scaffolds.[10] Some of the features of lipid rafts such as the size, structure, and the precise composition are poorly characterized.[11] Some of the best characterized raft proteins include lipid-modified proteins containing saturated acyl chains, namely GPI-anchored proteins, double acylated proteins such as Src family kinases and the α subunits of heterotrimeric G proteins.[12,13] Therefore, lipid rafts are typically structurally and functionally diverse. Flotillins are another group of proteins which are considered as markers of lipid rafts as they are raft localized and anchored to the plasma membrane (see 1.3).

Lipid rafts are signaling platforms whose specificity and function is determined by the pattern in which they assemble various components of the biological membranes. Some of the examples include receptors, channels, recognition markers, coupling factors, signaling proteins, adaptor proteins and enzymes which are brought together at this membrane microdomain platform to facilitate various protein-protein or protein-lipid interactions and support complex signaling cascades. Additionally, it is also evident that the death receptor Fas interaction with rafts induces apoptosis in HL-60 and Jurkat cell lines.[14] Disruption of membrane raft integrity by methyl-β-cyclodextrin treatment inhibits Fas-dependent apoptosis in leukemic cells. Lipid rafts can also retard cell death signaling. In murine cell line TS1αβ stimulated with IL-4, the dephosphosphorylated Bcl-2 associated death promoter is sequestered in lipid rafts, resulting in inhibition of its pro-apoptotic function. Lipid rafts have been proposed to

play a role in axonal growth and branching[15,16], stabilization of synapses[17], cholera toxin entry via GM1 ganglioside[18], HIV-1 entry, conversion of non infectious prion protein to detrimental prion protein[19]. Moreover, lipid rafts are considered significant in cardiovascular diseases, carcinogenesis, immune system diseases[20] and also in the pathogenesis of AD [21,22].

1.3 Flotillins

Flotillins are considered to be scaffolding proteins of lipid rafts and are conventionally regarded as lipid raft marker proteins. There are two homologous members in the flotillin protein family, flotillin-1 and flotillin-2. These proteins are also called reggie proteins as they were originally discovered as regeneration proteins upregulated in regenerating axons of goldfish retinal ganglion cells after optic nerve lesion.[23] These proteins are insoluble in Triton-X 100 and found floating after sucrose gradient density centrifugation, hence termed flotillins.[24] Flotillins are evolutionarily well conserved and ubiquitously expressed from fly to man.[25] In some cell types, flotillins are present in endosomal compartments, phagosomes, Golgi complex and even exosomes.[26–29] Flotillins belong to the SPFH (stomatin, prohibitin, flotillin, HflK/C) protein family, the members of which share homology at their N-terminal region.[30] The C-terminal regions of flotillins have several short repeat motifs called flotillin repeats, which mediate the formation of homo- and hetero-oligomers.[31] Flotillin-1 contains a palmitoylation site in Cys34, which is considered to be essential for the plasma membrane localization.[32] Flotillin-2 is associated with the membranes through myristoylation in Gly2 and multiple palmitoylation at Cys4, Cys19 and Cys20.[33] Flotillins are involved in axon regeneration, neuronal differentiation, endocytosis, T-lymphocyte activation, insulin signaling, cell proliferation and tumor progression. In the case of zebrafish, knockdown of flotillins results in 70% reduction of regenerating axons and therefore confirms the role of flotillins in axon regeneration. Knockdown of flotillins also restrained axon regeneration and differentiation in hippocampal and N2a neuronal cells.[34] Flotillins are considered necessary for differentiation as downregulation of flotillins in mammalian hippocampal neurons results in failure of neurons to differentiate.[35]

Flotillins can act as signal transduction centers and they have been shown to interact with Src family kinase and fyn kinases.[36] Flotillin-1 is also considered as a

scaffolding protein in the regulation of receptor tyrosine kinase/MAP (mitogen activated protein) kinase signaling.[37] Upon flotillin-1 depletion in cultured cells, the extracellular signal regulated kinase (ERK) signaling is inhibited. On the other hand, in a flotillin-2 knockout mouse model, ERK signaling is strikingly enhanced.[38] Flotillins also play a role in insulin signaling, since they form a ternary complex with Cbl and Cbl-associated protein (CAP). Upon insulin stimulation, the hydrophobic domain of flotillin-1 interacts with SoHo (sorbin homology) domain of CAP, and this complex is subsequently recruited to lipid rafts.[39] Additionally, flotillin-1 and CAP interact with FRS2, and knockdown of flotillin-1 increases Tyr phosphorylation of FRS2.[40] Flotillin-1 can translocate to nucleus and interacts with the mitogenic protein PTOV-1. Depletion of flotillin-1 or PTOV-1 can inhibit cell proliferation, whereas overexpression of either of them induced proliferation.[41] Flotillins are also associated with tumor progression as it was found that flotillin-2 mRNA and protein levels were high in tumorigenic and metastatic melanoma cell lines.[42] Lin and co-workers reported that flotillin-1 plays an important role in promoting proliferation and tumorigenesis of human breast cancer and can be designated as a novel prognostic biomarker and therapeutic target for the disease.[43]

Flotillins have also been implicated to play important roles in pathogenesis of neurodegenerative diseases such as Prion disease, Parkinson's disease, and Alzheimer's disease. In the case of AD, Aβ has been shown to accumulate in flotillin-1 positive endocytic vesicles.[44] Furthermore, flotillin-1 is associated with extracellular Aβ plaques in AD patient brain sections.[44] In APP transgenic mice, flotillin-1 and Aβ42 colocalized in approximately 10% flotillin-1 positive rafts.[45] Additionally, studies in brain sections from AD, Down syndrome and non-demented patients with plaques showed increased expression of flotillin-1 with the progression of AD.[46] Schneider and co-workers suggested that flotillins could facilitate clustering of APP and its subsequent endocytosis.[47] They also demonstrated that flotillin-2 knockdown in mouse neuroblastoma cells impaired APP processing and resulted in reduced Aβ production. Bitsikas *et al.* investigated the role of flotillin-1 in APP trafficking and processing in both *in vitro* and *in vivo* system.[48] They generated a transgenic mouse model which overexpressed mutant form of APP as well as a mutant form of presenilin, combined with genetic ablation of either flotillin-1 alone, or both flotillin-1 and flotillin-2. According to them, a small but reproducible reduction in Aβ production and consequent decrease in amyloid plaques was observed in flotillin knockdown compared to the

control. They also suggested that flotillin-1 may not be involved in APP trafficking since no differences in cluster sizes between flotillin$^{-/-}$ and control mouse embryonic fibroblasts were detected by super-resolution microscopy. It was suggested that flotillin-1 and flotillin-2 can mediate certain cellular functions independently. Therefore, further studies using physiological representative mouse models are required to investigate the potential *in vivo* effects of flotillin ablation on APP processing and trafficking.

1.4 BACE-1: β-secretase

BACE is a 501 aa type I transmembrane aspartic protease which belongs to the pepsin and retroviral aspartic protease families. BACE has its optimum activity at low pH and is predominantly present in acidic intracellular compartments such as endosomes and trans-Golgi compartments. It was found that toxic Aβ peptide is generated by β-γ-secretase cleavage and follow-up studies in BACE-1 knockout mice[49,50] revealed that BACE-1 is the major β-secretase in the brain. In 1999, different research groups independently reported the molecular cloning of β-secretase and distinctly named it as BACE-1 [51], β-secretase [52], Asp2 [53], or memapsin 2 [54]. Soon after the discovery of BACE-1, a homologue was identified, BACE-2. Although BACE-1 and BACE-2 share 64% sequence similarity, BACE-2 has low neuronal expression compared to BACE-1 and did not possess the same cleavage activity on the substrate APP. BACE-2 cleaves APP in the middle of the Aβ domain between Phe19 and Phe20, resulting in an increased secretion of sAPPα and p3 like products and reduced Aβ species. BACE-2 mRNA is widely expressed at low levels in peripheral tissues and at high levels in colon, kidney, pancreas, placenta, prostate, stomach and trachea, but at undetectable levels in brain. Therefore, BACE-2 represents a poor β-secretase candidate.

Like many other proteases, BACE-1 is produced as a pro-enzyme in the endoplasmic reticulum. The pro-domain is cleaved by the action of furin immediately before trafficking through the Golgi complex. The mature form of the enzyme starts at Glu46 and exhibits complex N-glycosylation at four asparagine sites: Asn153, Asn172, Asn223 and Asn354. Additionally, three intramolecular disulphide bonds between cysteine 216/420, 278/443, 330/380 are present in the ectodomain of BACE-1. The short cytoplasmic tail of BACE-1 contains an acidic cluster di-leucine (ACDL) sorting motif which is a characteristic of several transmembrane proteins as it regulates endocytosis

and lysosomal targeting.[55] The mutation of the di-leucine residues L499A/L500A results in retention of BACE-1 at the plasma membrane[56,57]. The acidic di-leucine motif interacts with specialized monomeric adaptor proteins such as GGA1-3 (Golgi-localised γ-ear-containing-ARF-binding proteins), and these GGA proteins are responsible for the recruitment of receptors to the coated vesicles on the TGN for transporting to endosomes and finally to lysosomes.[58–61] The binding of GGAs increases upon phosphorylation of BACE-1 at Ser498.[62] The mutation of serine to alanine alters the distribution of BACE-1, thereby indicating that the phosphorylation plays a significant role in the transport of this enzyme.[63] It has been suggested that GGAs binds to the DXXLL motif of BACE-1 to facilitate endocytosis and/or Golgi to endosome transport of BACE-1.[64] In AD patient brains, where neuronal apoptosis occurs, GGA levels are significantly reduced.[62] As a result, GGAs are not available for efficient sorting of this protease and consequently BACE-1 is localized primarily in the early endosomes. Trafficking of BACE-1 to lysosomes for degradation is also affected, leading to stabilization of BACE-1.

Another structural feature of BACE-1 is the presence of a type I transmembrane domain Val461- Val477.[65] This transmembrane domain is responsible for the retention of BACE-1 within the late Golgi and TGN compartments, and removal of this domain results in a soluble form of BACE-1 which is secreted in the extracellular medium.

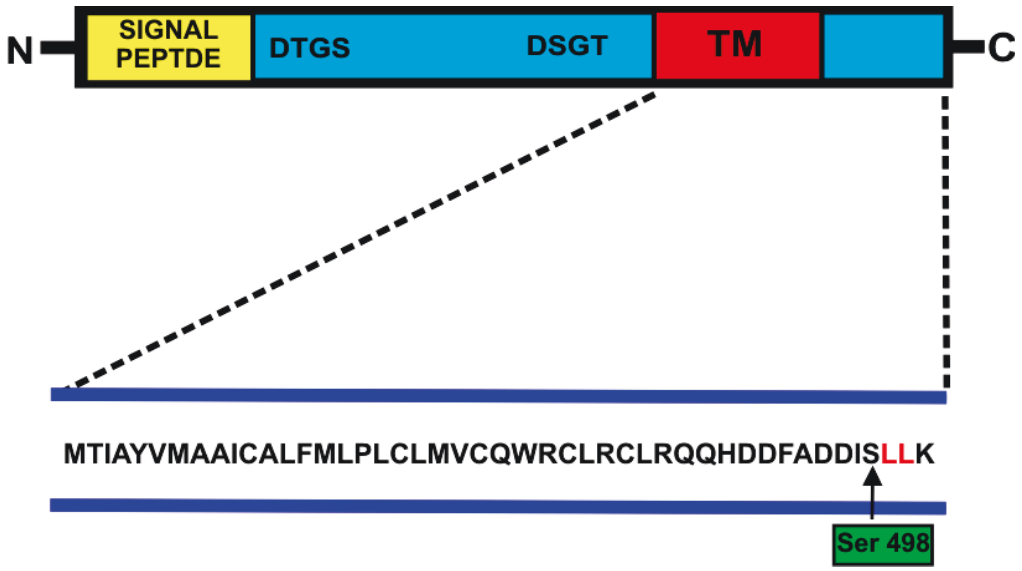


Figure 3: Schematic representation of BACE-1.

BACE-1 protein sequence comprises a short signal peptide which is removed during maturation. BACE-1 contains critical catalytic aspartic acid residues within two D-T/S-G-T/S motifs. The cytoplasmic tail of BACE-1 contains a di-leucine motif which regulates BACE-1 endocytosis and lysosomal degradation. Mutagenesis of this di-leucine to alanine residues (LLAA) results in the retention of BACE-1 at the plasma membrane.

1.5 GGAs: the adaptor proteins

GGAs were identified as trafficking molecules between the Golgi and the endosomes.[66–69] Three GGA proteins have been identified in humans (GGA1, GGA2, GGA3), and they comprise four distinct segments: a VHS (VPS-27, Hrs and STAM) domain that binds to the acidic di-leucine sorting signal, DXXLL (GGA and Tom1) domain that binds Arf:GTP; a hinge region which recruits clathrin, and a γ- adaptin ear homology domain that shows sequence similarity to the ear region of γ-adaptin and recruits various other proteins.[70,71]

GGAs are responsible for the sorting of the newly synthesized acid hydrolases to the lysosomes. These newly produced lysosomal enzymes are tagged with mannose-6-phosphate groups. Hence, these acidic hydrolases are capable of binding to the mannose-6-phosphate receptors (MPRs). MPRs also contain a DXXLL motif through which they can bind to the VHS region of GGAs.[72] GGAs are involved in the selective transport of proteins containing this sorting motif between the trans-Golgi network and the endosome and in directing endosomal cargos for lysosomal degradation. Endogenous GGA1, GGA2, and GGA3 localize predominantly to the trans-Golgi region

in NRK, HeLa, Cos7 and human embryonic skin cells[67,75–77] indicating a clear function in trans-Golgi region. GGA1 shows a highly punctuate pattern within the TGN and the late Golgi region. GGA2 shows diffuse and cytosolic staining, and GGA3 stains larger puncta in the cytosol. GGA1 is involved in retrograde transport of internalized BACE-1 from the endosomes to TGN.[73] GGA3 contains two ubiquitin binding sites in its hinge region and targets ubiquitinated cargo to the lysosomes.[74] It has been shown that ubiquitinated BACE-1 is transported to lysosomes for degradation by GGA3. Additionally, GGA3 is a substrate of caspase-3 under apoptotic conditions. As a result, cleaved GGA3 is incapable of performing its sorting function, resulting in impairment of BACE-1 degradation.[62]

1.6 Cell death: apoptosis and necrosis

There are two main types of cell death, apoptosis and necrosis, but the ultimate outcome of both them is the same (i.e destruction of affected cells) although they differ from each other significantly. In the case of apoptosis, the affected cells actively participate and orchestrate a cascade of biochemical events for a cellular suicide. In contrast, necrosis occurs when cells are subjected to adverse environmental conditions and follows an energy independent cell death. Necrosis is an uncontrolled and passive process which affects a large area of cells, whereas apoptosis is controlled, energy dependent process and affects only a cluster of cells. The two main reasons which cause necrotic cell death are interference with the nutrient supply of the cell and direct damage to cell membranes. Necrotic cell is characterized by formation of cytoplasmic vacuoles, cell swelling, distended endoplasmic reticulum, formation of cytoplasmic blebs, ruptured mitochondria, detachment of ribosomes, disrupted organelles, swollen and ruptured lysosomes, and finally disruption of the cell membrane.[75] As a result of loss of cell membrane integrity, cytoplasmic contents are released into the surrounding tissue, and dispersing chemotactic signals eventually result in an inflammatory response. In contrast, apoptotic cells do not release their cellular contents and are quickly phagocytosed by macrophages, with no inflammatory reaction.

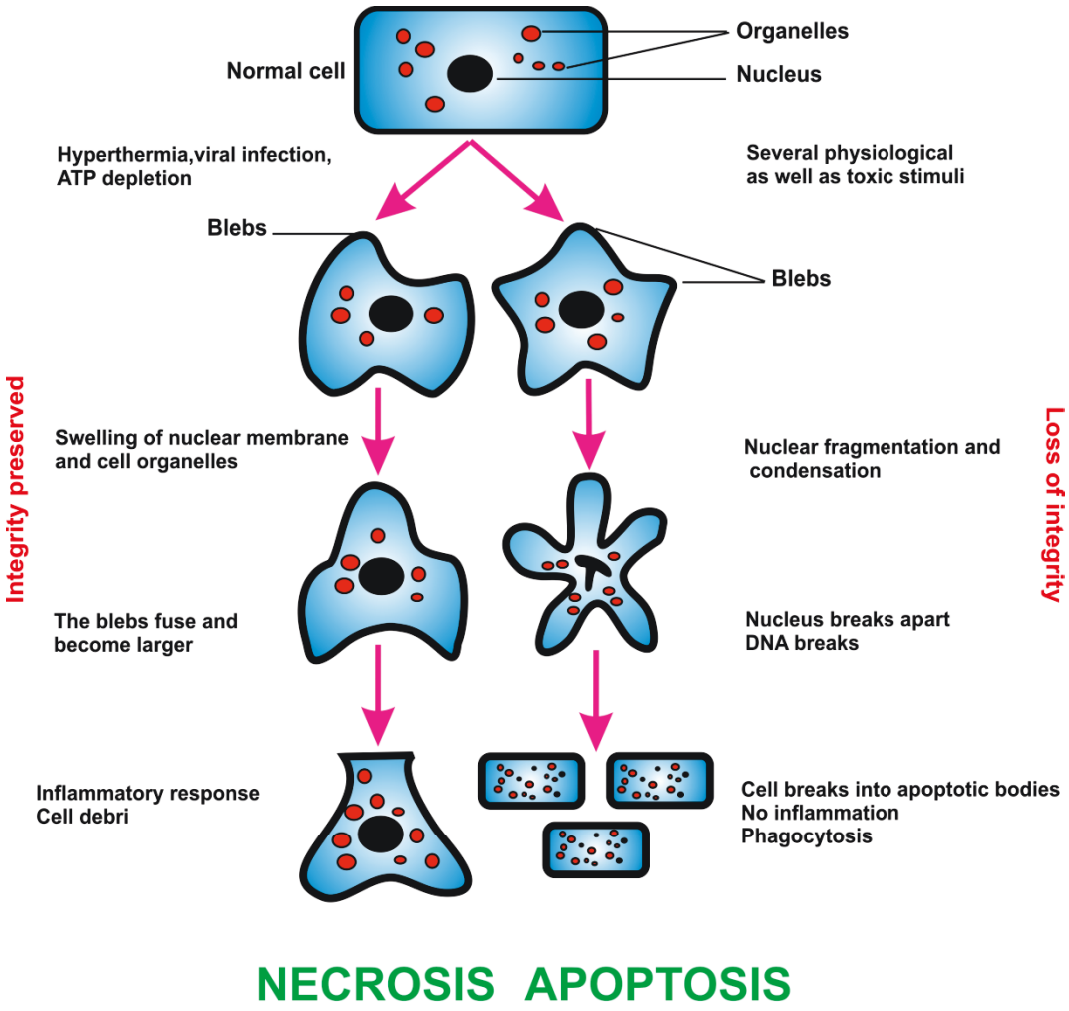


Figure 4: Morphologic differences in apoptosis and necrosis.

During apoptosis, decrease in cell volume, nuclear changes with chromatin condensation, margination and fragmentation followed by blebbing and break down of intact cell and nuclear membranes takes place. Necrosis is characterized by an increase in cell volume followed by enlargement of cell organelles including nucleus, loss of membrane integrity and release of cellular contents which contain certain enzymes such as hydrolases that influence the adjoining cells leading to inflammation reaction in the adjacent tissue.

1.7 Apoptosis

The term apoptosis was introduced by Kerr *et al*. in 1972 to describe a specific morphological process leading to the controlled cellular self destruction which was categorically distinct from necrosis.[76] Typically, an individual cell faces three possible options: proliferation (mitosis), specialization (differentiation) or death (apoptosis). During the development, many cells are produced in excess, resulting in a need for them to be eliminated through programmed cell death which thereby sculpts many tissues and

organs. Thus, apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of cell populations in tissues upon physiological and pathological conditions.

Apoptosis process is of widespread biological significance, being involved in development, differentiation, proliferation, homeostasis, regulation and removal of defect cells. Hence, dysfunction of apoptotic program is implicated in various pathological conditions such as cancer, autoimmune diseases, viral infections, neurodegenerative diseases, AIDS and ischemic diseases.[77]

1.7.1 Molecular mechanisms of apoptotic signaling pathways

Apoptosis is tightly regulated cell death program which requires the interplay of a multitude of factors. The components of the apoptotic signaling network are genetically encoded and quiescent in a nucleated cell until activated by a death inducing stimulus.[78] Primarily, apoptosis is mediated by two central pathways: the extrinsic (or death receptor) pathway and intrinsic (mitochondrial) pathway.

1.7.1.1 Extrinsic Pathway

The extrinsic pathway of apoptosis is induced by a complex including death ligands and death receptors. The death receptors are a family of type I transmembrane proteins characterized by the presence of multiple cysteine-rich repeats in the extracellular domains and the protein-protein interaction module known as the death domain (DD) in the cytoplasmic tails. The members of death receptor includes tumor necrosis factor I (TNFR I; Death Receptor 1), Fas (Death Receptor 2, CD95), TNF related apoptosis inducing ligand and receptor (TRAILR1, Death Receptor 4), TRAILR2 (Death Receptor 5, KILLER), and DR6.[79] The best defined sequence of events for extrinsic pathway are FasL/FasR and TNF-α/TNFR1 models. Upon receptor-ligand binding, cytoplasmic adaptor proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in binding of the adaptor protein FADD, and the binding of TNF ligand to TNF receptor results in the binding of the adaptor protein TRADD, with recruitment of FADD and death domain kinase called as RIP (receptor interacting protein). FADD then associates with procaspase-8 via dimerization of the death effector domain. Consequently, a death inducing signaling complex (DISC) is formed, resulting in

autocatalytic activation of procaspase-8. Caspase-8 activation triggers the execution phase of apoptosis. In the case of tumor cells, mechanism of extrinsic pathway is exploited to facilitate ligation of death receptors and modulate the cell death machinery and typically serves as an attractive target for cancer therapy.

1.7.1.2 Perforin/granzyme pathway

Cytotoxic T lymphocytes (CTLs) kill target cells via the extrinsic pathway and FasL/FasR interaction is the predominant way of CTL-induced apoptosis.[80] The target cell is often damaged due to the loss of plasma membrane homeostasis, with excessive uptake of water and loss of the cellular integrity. In perforin/granzyme pathway, granzyme enters the target cells by endocytosis, and at this stage, the target cells remain healthy as granzymes remain harmlessly sequestered in the endocytic vesicles. Rapid apoptosis ensues after transmembrane pore forming molecules termed as perforin synergizes with granzyme A and B molecules.

1.7.1.3 Intrinsic pathway

The intrinsic pathway involves a diverse array of non-receptor mediated stimuli that produce intracellular signals that directly act on the targets within the cell and are majorly mitochondrially mediated events. Negative signals include the absence of certain growth factors, hormones, cytokines, withdrawal of factors, or other factors such as radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals. Such negative stimuli on cells affect the mitochondrial membrane. Initially, the mitochondrial membrane permeability is altered, followed by the loss of mitochondrial transmembrane potential and release of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol.[81] The first set of proteins which translocate from the mitochondrial membrane to the cytosol are cytochrome C, Second mitochondria-derived activator of caspase (Smac)/ Direct inhibitor of apoptosis protein (IAP)-binding protein with low PI (DIABLO), and the serine protease HtrA2/Omi.[34,35] Cytochrome C binds and activates apoptotic peptidase activating factor 1 (Apaf-1) as well as procaspase-9, resulting in an apoptosome.[82] Smac/DIABLO and HtrA2/Omi or endonuclease G promote apoptosis by inhibiting IAP activity.[83] The second set of pro-apoptotic proteins, apoptotic inducing factor (AIF) and several endonucleases is released from the mitochondria and triggers the intrinsic apoptotic pathway.

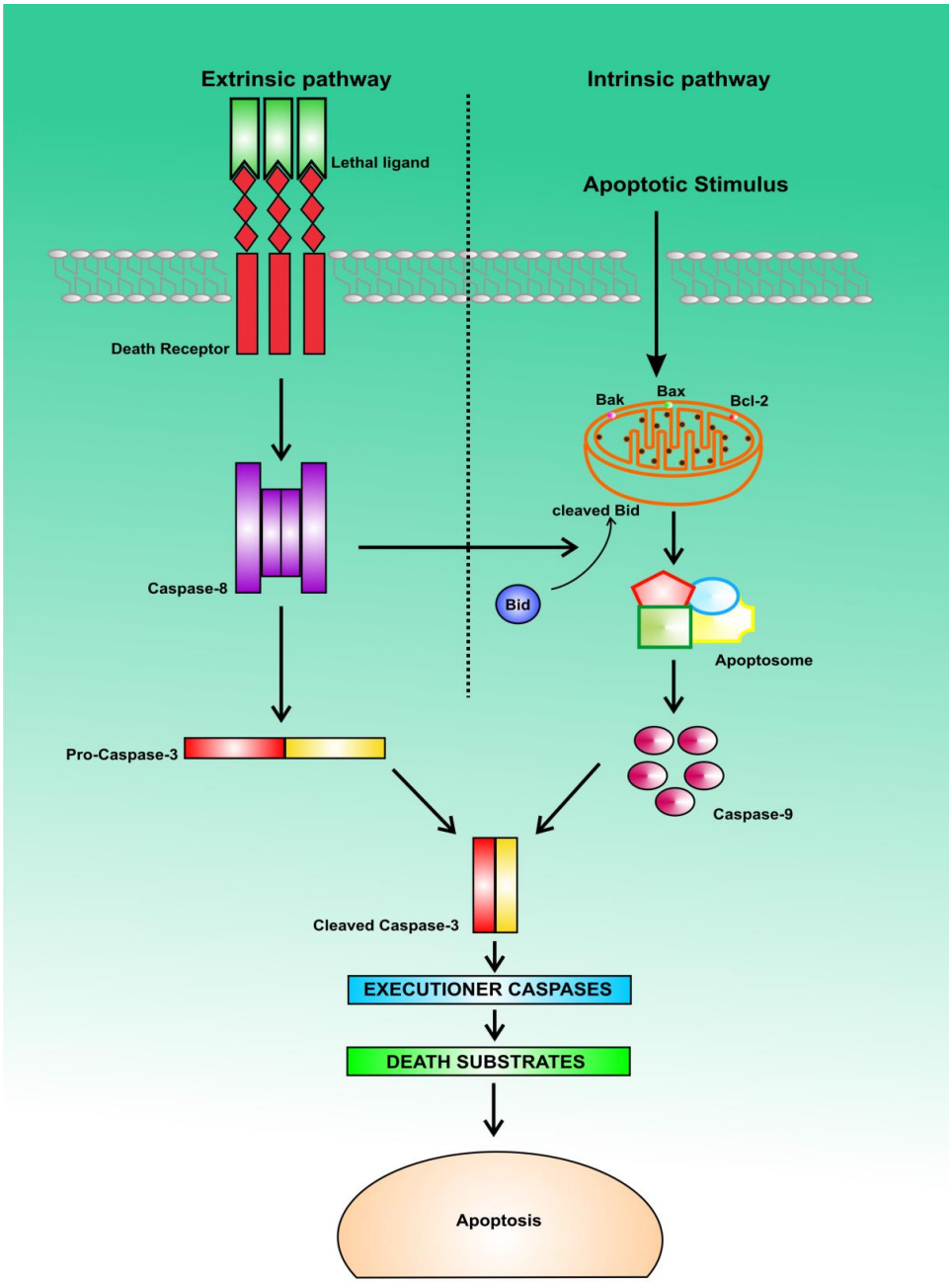


Figure 5: An overview of the extrinsic and intrinsic apoptotic pathways.

In the extrinsic pathway, death signals from the surrounding environment of the cell bind to death receptors on the surface of the cell membrane. This leads to the conversion of inactive procaspase-8 into active caspase-8. Caspase-8 activates caspase-3 which triggers the caspase cascade and leads to apoptosis. In the intrinsic pathway, as a result of negative stimuli, the pro-apoptotic protein Bax initiates the release of cytochrome C from the mitochondria. Apaf-1 and the released cytochrome C combine to form a complex known as the apoptosome. The apoptosome causes the conversion of inactive procaspase-9 into active caspase-9. Caspase-9 then goes on to activate caspase-3 in a similar manner to the extrinsic pathway. Activated caspase-3 then leads to the caspase cascade, resulting in apoptosis.

1.8 Caspases: proteases in apoptotic cell death

The name caspases derives from cysteine dependent aspartate proteases expressed as inactive zymogens in all animal cells. In the cell, caspases are synthesized as inactive zymogens which at their N-terminus possess a prodomain, followed by a large and a small subunit which sometimes are separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and the small subunit. The proapoptotic caspases can be divided into a group of initiator caspases including procaspases-2, -8, -9 and -10 and into executioner caspases including procaspases-3, -6, and -7. The initiator caspases contain protein–protein interaction motifs: death effector domains (DED) in case of procaspase-8 and -10 or caspase recruitment domains (CARD) as in the procaspase-1,-2,-3,-4,-5,-9,-11,-12. The initiator caspases are able to interact with the upstream adaptor molecules, whereas the executioner caspases perform the downstream steps in apoptosis by cleaving multiple cellular substrates.

1.9 Bcl-2 family proteins: critical checkpoints of apoptotic cell death

The Bcl-2 family of proteins comprises both anti-apoptotic and pro-apoptotic members that regulate apoptosis. Structurally, Bcl-2 family members posses up to four conserved Bcl-2 homology (BH) domains designated as BH1, BH2, BH3 and BH4 which mainly consist of α-helices and facilitate interactions with other Bcl-2 family members.[84] In mammalian cells, anti-apoptotic members include Bcl-2 and Bcl-$x_{L,}$(Bcl-x protein long isoform) and oppose two pro-apoptotic groups: the BAX group and the BH3-only proteins. The pro-apoptotic members and anti-apoptotic members reside in different subcellular locations in the absence of apoptotic stimuli. Anti-apoptotic members are primarily localized to mitochondria, endoplasmic reticulum, or nuclear membrane.[85] The pro-apoptotic proteins in turn are present in cytosol or cytoskeleton prior to apoptotic stimuli.[86–88] Upon death stimulus, the pro-apoptotic members undergo conformational changes which facilitate them to target and integrate into mitochondrial membranes.

A very important feature of Bcl-2 family is that their function is regulated by dimerization. For instance, the ability of Bcl-2 anti-apoptotic members to inhibit apoptosis can be blocked by the formation of a heterodimer with pro-apoptotic Bcl-2 member, Bax.[89] Another mode of regulation is that Bcl-2 family members can be phosphorylated such that their localization and functions are altered. For example,

phosphorylation retards the protective effect of Bcl-2 whereas deleting this target serine residue augments Bcl-2 protective activity.[90] Bcl-2 is expressed in outer mitochondrial membrane and inhibits the permeability transition of apoptotic inducers. Additionally, Bcl-2 expression retards the release of cytochrome C from mitochondria and subsequently prevents activation of caspases and preventing apoptosis.[91] The Bcl-2 family members can modulate apoptosis as they have the potential to adjust the apoptotic threshold in cells. Bcl-2 family members are also considered as targets for pharmacological intervention. For instance, the degenerative diseases and acute ischemic episodes can be prevented by using pharmacological agents directed against Bcl-2 pro-apoptotic members to retard apoptosis. Conversely, in case of cancer, Bcl-2 pro-apoptotic members can be fostered to establish apoptosis in recalcitrant cancer cells which fail to undergo normal death program.

1.10 Mcl-1: A key regulator of apoptosis

Mcl-1 (myeloid cell leukemia-1) was identified based on its increased expression during cell commitment to differentiation in human myeloid leukemia cells.[92] Structurally and functionally, Mcl-1 resembles Bcl-2 as they show sequence similarity and can delay apoptosis.[93,94] The carboxyl terminus of Mcl-1 is homologous to Bcl-2, with the extreme carboxyl terminus containing a transmembrane domain, and the cytosolic region contains BH1, BH2, BH3 domains. Mcl-1 has a non-Bcl-2 homologous N-terminal portion of the protein, and this part is much longer than in other pro-survival Bcl-2 family members such as Bcl-2, Bcl-xl and Bcl-w. The N-terminus region of the protein is enriched with proline (P), glutamic acid (E), serine (S), and threonine (T) residues, hence abbreviated as PEST region.[92] Such a PEST region and presence of arginine pairs is a characteristic feature of short lived proteins.[92,95] The protein turnover of Mcl-1 may be shortened or lengthened depending on the cellular conditions and is cell type dependent. Gene ablation studies have revealed that physiological functions of Mcl-1 are distinct from the pro-survival Bcl-2 relatives. Mcl-1 deficient mice show peri-implantation lethality. This was not due to increased apoptosis, implicating that Mcl-1 has a distinct role in implantation. Conditional knockout mice are also available and show that Mcl-1 is essential for both development and maintenance of B and T lymphocytes. Conditional deletion of Mcl-1 in early hematopoietic progenitors results in a severe loss of bone marrow, implicating a significant role for Mcl-1 in

hematopoietic stem cell survival. Mcl-1 has been shown to be overexpressed in a variety of human hematopoietic and lymphoid cancers, including B cell lymphoma, chronic lymphocytic leukemia, chronic myeloid leukemia, mantle cell lymphoma and multiple myeloma.[96–101]

Mcl-1 levels are strictly regulated at transcriptional, post-transcriptional and post-translational levels. Mcl-1 expression is growth factor dependent, and it is able to protect cells upon growth factor withdrawal-induced apoptosis. Various trophic factors such as cytokines, namely IL-3, IL-5, IL-6, granulocyte–macrophage colony stimulation factor, and growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) can induce Mcl-1 expression. Tissue plasminogen activator (TPA) induces an increase in the mRNA levels of Mcl-1 in monocyte/macrophage differentiation. The promoter region of Mcl-1 contains consensus signal transducers and activators of transcription (STAT) response elements, cAMP response elements (CRE), and nuclear factor kappa B binding sites. Conversely, Mcl-1 is transcriptionally down regulated under various stress conditions such as growth factor withdrawal, UV radiation, or apoptotic induction by staurosporine. Mcl-1 is subjected to post-transcriptional regulation, where the mRNA of Mcl-1 is alternatively spliced to remove exon 2, and thus produces a shortened form of Mcl-1, designated as Mcl-2 or short isoform of Mcl-1(Mcl-1_s). Mcl-1_s lacks BH1, BH2, BH3 and the transmembrane region. Another much shorter splice variant has also been identified, designated as Mcl-1_{ES} or Mcl-3, which is a result of a lack of exon 1 region. This isoform lacks 53 amino acids from the PEST region, but contains all three BH regions and the transmembrane domain. These shorter isoforms also promote apoptosis by sequestering the pro-apoptotic Bcl-2 members and by inhibiting full length Mcl-1 by binding to them. At post-translational levels, Mcl-1 is polyubiquinated by a novel E3 ubiquitin-ligase named as Mcl-1 Ubiquitin E3 ligase (MULE) and targeted for proteasome dependent degradation. Under apoptotic conditions, Mcl-1 is cleaved by caspase-3 at Asp127 and Asp157 sites. As a result, the large N-terminus portion of Mcl-1 is removed, leaving behind BH1-3 and the C-terminal transmembrane domain intact. Although the precise role for this caspase mediated cleavage is unknown, the cleavage products are speculated to have a function in either pro-survival, pro-death, or act as neutral components in apoptosis.

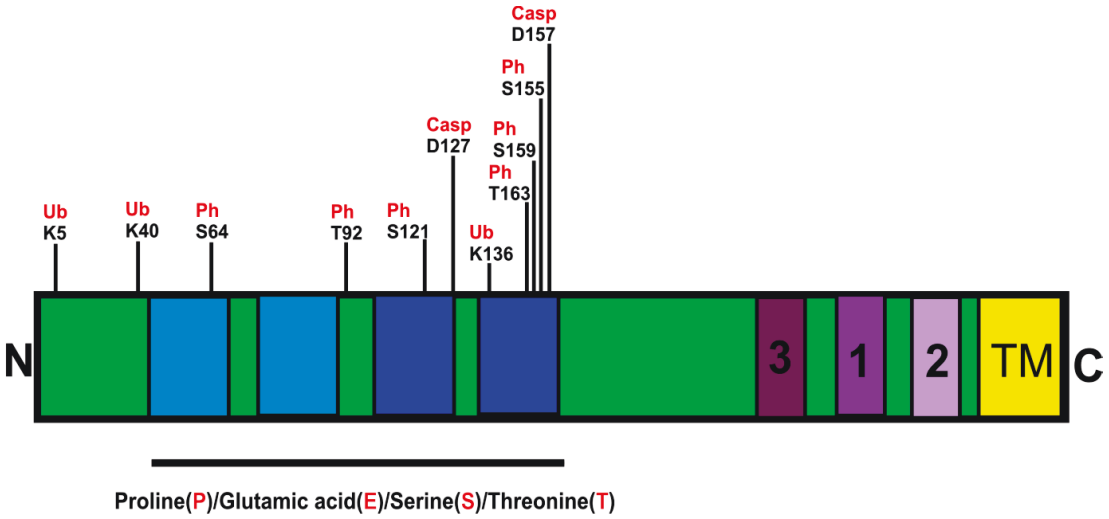


Figure 6: Molecular organization of Mcl-1.

Mcl-1 contains a transmembrane domain at the extreme C-terminal region. It contains three Bcl-2 homology domains numbered (1-3). Two weak PEST sequences (indicated in light blue) and two strong PEST sequences (indicated in dark blue). Sites of post-translational modifications are also shown: ubiquitination (Ub), caspase cleavage (Casp), and phosphorylation (Phos).

1.11 Akt (Protein kinase B): a major determinant in cell survival signaling

Akt protein is a 59 kDa serine/threonine kinase which contains an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a regulatory domain that contains a hydrophobic motif (HM). The PH domain and the catalytic domain bear significant homology to protein kinase C (PKC) and protein kinase A (PKA) family members. Hence, Akt is also named Protein kinase B (PKB) or RAC (related to A and C) protein kinase. In mammalian cells, Akt has three closely related and highly conserved cellular isoforms termed as Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ. The three Akt isoforms show different tissue and organ expression. Akt1 exhibits high expression in brain, heart, testis, and thymus. Akt2 shows high tissue expression in brown fat, cerebellum (purkinje cells), heart, and skeletal muscle. Akt3 is mostly expressed in the brain and testis. Gene knockout studies in mice indicate that Akt1, Akt2 and Akt3 are functionally distinct. Mice ablated of Akt1 are reduced in size and undergo spontaneous apoptosis in testis and thymus gland. Mice lacking Akt2 are of normal size but impaired in the ability to maintain glucose homeostasis.[102–104] Akt3 deficient mice have no observable phenotype but show a reduced brain size.[105] All three kinases contain similar phosphorylation/activation sites: threonine 308 (Akt1), 309 (Akt2), and 305 (Akt3) and

serine 473 (Akt1), 474 (Akt2), and 472 (Akt3). Phosphorylated or activated Akt regulates many of its cellular functions through the phosphorylation of a series of downstream proteins. Each isoform differentially regulates cellular function as it is confined to distinct subcellular localization. Akt1 is present in the cytoplasm, Akt2 in mitochondria, and Akt3 in the nucleus. The subcellular localization of a specific Akt isoform is not significantly altered by the presence or absence of other Akt isoforms, ionizing radiation, or epidermal growth factor. [106]

Akt is activated through receptor tyrosine kinase pathways, such as platelet derived growth (PDGF-R), insulin, EGF, basic fibroblast growth factor (b-FGF), and insulin like growth factor I (IGF-I). In the absence of growth factor stimulation in quiescent cells, all three catalytic forms of Akt are catalytically inactive. Upon stimulation, Akt is activated through a phosphatidylinositol-3 kinase (PI3K) dependent process.[107] PI3K proteins are characterized by their ability to phosphorylate inositol ring 3'-OH group in inositol phospholipids.[108] Class I PI3K comprises a catalytic domain (p85). This class is further divided into subclass 1A, with protein tyrosine kinase activity (Receptor Protein Tyrosine kinase, RPTK), and the subclass IB, which is activated by receptors coupled with G proteins. RPTK activation results in the generation of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3). Subsequently, PI-3,4,5-P_3 recruits PI3K downstream players such as Akt which is then translocated to the inner side of plasma membrane where 3-phosphoinositide-dependent kinase (PDK1) is present. PDK1 interacts with Akt via its PH domain and instigates Akt to undergo conformational changes.[109–112] As a result, the two main regulatory sites of Akt are exposed and their phosphorylation culminates in Akt activation. PDK1 which is thought to be constitutively active, phosphorylates Akt at Thr308 present within its catalytic domain, and stabilizes the Akt molecule. Subsequently, PDK2 phosphorylates Akt at Ser473 present within the hydrophobic motif. The phosphorylation at these two sites is necessary and sufficient for the full activation of Akt.

In the case of serum starved cells, Akt is constitutively phosphorylated at Ser124 and Thr450, which are independent of PI3K, and neither serum starvation nor treatment of cells with PI3K inhibitor wortmannin interferes with the phosphorylation of these sites. Phosphorylation of Akt may either positively or negatively alter the subcellular localization, or modify protein stability or the functions of the downstream substrates.[113] Akt is activated in cancers through mutations or amplifications of upstream genes like

PIK3CA.[114] Loss of the lipid phosphatase PTEN can lead to constitutive activation of Akt.[115] Akt1 is very rarely amplified in cancers as compared to Akt2 which is altered in most cancers.[116] A somatic mutation in the PH domain results in substitution of glutamic acid at codon 17 of Akt1 with lysine (E17K) and affects the lipid binding specificity of Akt, resulting in pathological membrane association and constitutive signaling.[117] Under physiological and pathological conditions, Akt protein can be degraded by ubiquitin proteasome dependent pathway, caspase-mediated cleavage, and caspase induced ubiquitination.

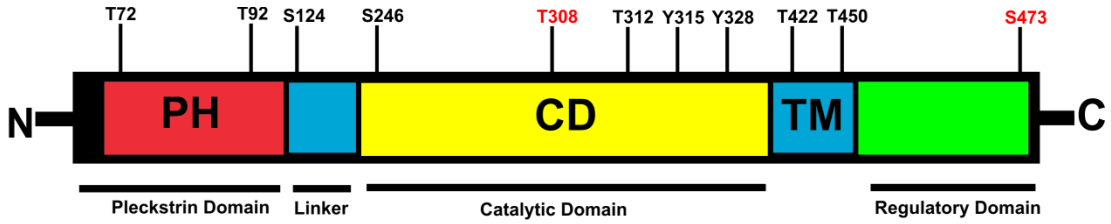


Figure 7: Diagram of the Akt1 protein.

The domains are PH (Pleckstrin Homology) 30% identical to pleckstrin and other PH domains: a short helical region (Linker), no significant homology to other proteins; Kinase (Catalytic Domain) homologous to all kinases, 50% identical to PKA, PKC, SGK and S6 families; transmembrane region (TM), and Regulatory Domain. Indicated in red are the two phosphorylation sites shown to be essential for activation of Akt1: Threonine 308 and Serine 473. C: Carboxyl-terminal, N: Amino-terminal

1.12 FOXOs (Fork head box proteins): an essential element for cellular homeostasis

Fork head box (FOXO) proteins, are a subgroup of transcription factors characterized by a conserved DNA-binding forkhead domain. FOXO has been classified on the basis of the sequence homology within this domain. FOXO3a is one of the members of this subfamily which activate or repress multiple genes involved in cell cycle regulation, apoptosis, DNA damage repair, protection against oxidative stress, and metabolism. Transcriptional activity of forkhead transcription factors is regulated at three levels: i) cellular translocation ii) DNA binding, and iii) protein degradation.[118] FOXOs rely on active transport mechanisms for their nuclear entry. FOXO1, 3, 4, 6 contain nonclassical nuclear localization signal (NLS) consisting of three arginines next to the forkhead box PKB site (Ser253), and three lysine residues located 19 residues downstream of the arginines. The biological activity of FOXO proteins is regulated by

post-translational modifications, namely phosphorylation, acetylation, ubiquitination, and protein interactions.[119] The transcription factors of the forkhead family are the best characterized downstream targets of Akt. In a state where Akt is inactive or dephosphorylated, FOXOs also remain dephosphorylated in the nucleus, and are capable of transcriptional activity.[120] In contrast, upon growth factor stimulation, Akt is active and phosphorylates FOXO at three key regulatory phosphorylation sites (Thr32, Ser253, and Ser315 in FOXO3a). The phosphorylation of FOXOs creates a 14.3.3 binding site. The binding of 14.3.3 to FOXO masks the nuclear localization signal and promotes the exit of FOXO from the nucleus, resulting in suspension of all transcriptional activity of FOXO.[121] In summary, FOXOs are regulated at different levels and in different cellular compartments. FOXOs have a role in cell cycle regulation, cell death, metabolism, protection from oxidative stress and survival. Moreover, FOXO family plays a significant role in development of organs, such as pancreas[122] and the ovaries [123], and in complex diseases, such as diabetes [124].

1.13 Aims of the present study

The major aim of this study was to address the role of flotillins in the intracellular sorting of β-secretase, BACE-1. The BACE-1 harbours an ACDL motif within its cytoplasmic tail and provides sorting signals for intracellular trafficking. Until now the significance of this ACDL motif in mediating BACE-1 and flotillin interaction was not known and this was one of the objectives of this study. As several studies have revealed that flotillins can function independently, we investigated the role of each of the flotillins in BACE-1 binding. Additionally, the significance of flotillins in APP processing was determined.

Secondly, the association between flotillins and cytoxicity was investigated. In cancer therapeutics, one of the means of targeting tumorous cells is to programme them to undergo apoptosis and retard cancer growth and survival. In this study, we evaluated the role of flotillins in sensitizing cells to various extrinsic and intrinsic apoptotic inducers and determined novel means by which interfering with flotillin function could affect apoptosis pathways and enhance cancer therapy efficacy.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and reagents

Table 1: Chemicals and reagents (alphabetical order)

Name	Company
Ampicillin sodium salt	Roth, Karlsruhe, Germany
Aprotinin	Roth, Karlsruhe, Germany
Biorad Protein Assay	Biorad, Munich, Germany
Brefeldin A	Sigma,Taufkirchen, Germany
Bromophenol blue sodium salt	Roth, Karlsruhe, Germany
Bovine serum albumin (BSA)	PAA laboratories, Pasching, Austria
BSA (protease free)	PAA laboratories, Pasching, Austria
Chloramphenicol	Roth, Karlsruhe, Germany
Coomassie brilliant blue G-250	Applichem, Darmstadt, Germany
1,4-diazabicyclo(2,2,2) octane (DABCO)	Fluka, Neu-Ulm, Germany
4,6-Diamino-2-phenylindoledihydrochloride (DAPI)	Merck, Darmstadt, Germany
DMEM (high glucose)	Gibco (Invitrogen), Karlsruhe, Germany
Dithiothreitol (DTT)	Applichem, Darmstadt, Germany
Dyanabeads Protein A	Dyanl, Oslo, Norway
ECL Western Blot Reagents	GE Healthcare, Buckinghamshire, UK; Perkin Elmer, Waltham, MA, USA
Epidermal Growth Factor (EGF) human recombinant	Sigma, Taufkirchen, Germany
Fetal bovine serum (FBS)	Gibco (Invitrogen), Karlsruhe, Germany
Gelmount mounting medium	Biomeda, Foster City, CA, USA
Glutathione Sepharose 4B	GE Healthcare, Uppsala, Sweden
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Roth, Karlsruhe, Germany
Kanamycin sulphate	Roth, Karlsruhe, Germany
Lipofectamine 2000 transfection reagent	Invitrogen, Karlsruhe, Germany

Table 1: Chemicals and reagents (alphabetical order) : Continued

Name	Company
Leupeptin hemisulphate	Roth, Karlsruhe, Germany
Luminol (3-Aminophthalhydrazide)	Applichem, Darmstadt, Germany
Lysozyme	Roth, Karlsruhe, Germany
MACSfectine	Miltenyi Biotech, Finland
Metafectine EASY	Biontex, Munich, Germany
Octyl- β-D-glucopyranoside (NOG)	Sigma- Aldrich, Taufkirchen, Germany
Optimem+GlutaMEX	Gibco (Invitrogen), Karlsruhe, Germany
Pansorbin Cells	Calbiochem, Nottingham,UK
p-Coumaric acid	Sigma,Taufkirchen, Germany
Pencillin/Streptomycin (100X)	PAA laboratories, Pasching, Austria
Pepstatin A	Roth, Karlsruhe, Germany
Phenylmethanesulfonyl fluoride	Roth, Karlsruhe, Germany
Ponceau S	Applichem , Darmstadt, Germany
Precision Protein Standard	Biorad, Richmond, CA, USA
Proteinase Inhibitor Cocktail	Sigma -Aldrich, Taufkirchen,
Puromycin (10mg/ml)	Gibco, Invitrogen, Karlsruhe, Germany
Rosewell Park Memorial Institute (RPMI)	Gibco, Invitrogen, Karlsruhe, Germany
Sodium fluoride	Fluka, Neu-Ulm, Germany
Sodium orthovanadate	Sigma, Taufkirchen, Germany
Sodium pyruvate	PAA laboratories GmbH, Austria
Staurosporine	LC labs, USA
TrpLE	Gibco, Invitrogen, Karlsruhe, Germany
Trypsin (10X)	PAA laboratories, Pasching, Austria

All chemicals not listed above were purchased from Applichem (Darmstadt), Sigma Taufkirchen), Fluka (Neu-Ulm), Merck Darmstadt or Roth (Karlsruhe)

Table 2: Primary Antibodies (alphabetical order)

Antibody	Source	WB	IF	Reference
Alpha-tubulin	Rat	-	1:100	LabGen
APP	Rb	1:1000	-	In house preparation
Akt	Rb	1:1000	-	Cell Signaling Technology Danvers, MA, USA
Caspase-3	Rb	1:500	-	Cell Signaling Technology Danvers, MA, USA
Caveolin-1	M	1:500	-	BD transduction Laboratories, NJ,USA
C-myc	Rb	1:1000	1:20	Santa Cruz Biotechnology, Santa Cruz, CA, USA
C-myc	M	1:1000	-	Cell Signaling Technology Danvers, MA, USA
BACE-1	M	1:5000	-	Gift from Bard De Strooper's Lab
BAD (Y203)	Rb	1:500	-	Abcam via Biozol, Esching, Germany
ERK2 (C-14)	Rb	1:1000	-	Santa Cruz Biotechnology ,Santa Cruz, CA, USA
Flotillin-1	M	1:1000	-	BD transduction Laboratories, NJ,USA
Flotillin-2	M	1:1000	-	BD transduction Laboratories, NJ,USA
Flotillin-1	Rb	1:1000	1:50	Sigma-Aldrich, Taufkirchen, Germany
Flotillin-2 C	Rb	1:1000	1:50	Sigma-Aldrich, Taufkirchen, Germany
FOXO3a (75D8)	Rb	1:500	-	Cell Signaling Technology, Danvers, MA, USA
14.3.3 (H-8)	M	1:10,000	-	Santa Cruz Biotechnology, CA, USA
GAPDH	M	1:30,000	-	Abcam via Biozol, Esching, Germany
GFP	Rb	1:1000	-	Clonetech, Mountainview, CA,USA
GGA2	M	1:1000	-	BD transduction Laboratories, NJ,USA

Table 2: Primary Antibodies (alphabetical order) : Continued

Antibody	Source	WB	IF	Reference
GGA3	M	1:1000	-	BD transduction Laboratories, NJ,USA
Mcl-1	M	1:1000	-	BD transduction Laboratories, NJ,USA
Phospho Akt	Rb	1:1000	-	Cell Signaling Technology, Danvers, MA, USA
Phospho ERK1/2	M	1:1000	-	Santa Cruz Biotechnology Santa Cruz, CA, USA
Phospho FOXO3a	Rb	1:500	-	Cell Signaling Technology, Danvers, MA,USA
PARP	Rb	1:1000	-	Cell Signaling Technology, Danvers, MA,USA

(Abbreviations: M=Mouse, Rb=Rabbit, WB=Western blotting, IF=Immunofluorescence)

Table 3: Secondary Antibodies : HRP and fluorochrome conjugates

Antibody	Dilution	Reference
Goat anti-Mouse HRP	1:10,000 (WB)	Dako,Glostrup,Denmark
Goat anti-Rabbit HRP	1:10,000 (WB)	Dako,Glostrup ,Denmark
Goat anti-Mouse Cy3	1:300 (IF)	Jackson Immunoresearch, West Grove, PA, USA
Goat anti-Rabbit Cy3	1: 300 (IF)	Molecular Probes, Invitrogen, Karlsruhe, Germany
Goat anti-Rabbit Alexa fluor 488	1:300 (IF)	Molecular Probes, Invitrogen, Karlsruhe, Germany

(Abbreviations: HRP=Horse Raddish Peroxidase, WB= Western Blotting, IF=Immunoflorescence)

Table 4: Buffers (alphabetical order) :

Buffer	Composition
APP CTF extraction buffer	10 mM Tris, pH 8.0, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100 and add fresh 60 mM n-octyl-β-d-glucopyranoside
Blocking buffer (WB)	5% non-fat milk powder in TBS-T
Coomassie blue staining solution	0.1 % Coomassie brilliant blue 42.5% ethanol 5% methanol 10% acetic acid
Destaining buffer (Coomassie)	20% methanol 7.5% acetic acid
Direct GST pulldown buffer	50 mM Tris-HCl pH 7.5 150 mM NaCl 1 mM EDTA 1 mM DTT 0.01% Triton X-100
ECL solution	1250 µM 3-aminophtalhydrazine (Luminol) 200 µM p-Coumaric acid 100 mM Tris –HCl pH 8.5, add fresh 10% H_20_2 (1:1000)
Glycerol stock buffer	65% glycerol 0.1 M $MgS0_4$, 25 mM Tris-HCl pH 8
LB agar	15 g agar powder pure in 1 L LB medium
LB (Luria Miller) medium	20 g LB broth In 1 L dd H_2O
Lysis buffer (Stuermer buffer)	50 mM Tris-HCl pH 7.5 150 mM NaCl 2 mM EDTA pH8 1% NP 40

Table 4: Buffers (alphabetical order) : Continued

Buffer	Composition
Phospho Lysis bufer	10 mM Tris-HCl pH 8 150 mM NaCl 5 mM EDTA pH 8 0.5% Triton X-100 Add fresh 60 mM N-Octyl-β-glucopyranoside 1 mM NAF (for checking phosphorylation) 1 mM NA_3VO_4 (for checking phosphorylation) Protease Inhibitor Cocktail
Lysis buffer for GST protein purification	50 mM HEPES pH 7.5 150 mM NaCl 1 mM EDTA pH 8 5% glycerol 0.1% NP- 40 Add fresh 1.5 µM aprotinin 23 µM leupeptin 1.5 µM pepstatin A (in 100%EtOH) 1 mM PMSF (Phenylmethanesulfonyl fluoride in isopropanol 1 mM DTT
Lysis Buffer for nuclei isolation	10 mM HEPES(pH 7.9) 1.5 mM $MgCl_2$ 10 mM KCl 0.5 mM DTT 1% (v/v) NP-40 (add freshly before use)
Nuclei lysis buffer	40 mM HEPES (pH 7.9) 400 mM KCl 10% (v/v) glycerol
Phosphate buffered saline (PBS)	150 mM NaCl 20 mM NaH_2P0_4

Table 4: Buffers (alphabetical order) : Continued

Buffer	Composition
Ponceau S staining solution	0.1% Ponceau 5% acetic acid
SDS electrophoresis buffer	192 mM glycine 25 mM Tris-Base 0.1% SDS
Sample buffer (4X) loading dye	250 mM Tris-HCl pH 6.8 8% SDS 40% glycerol 0.2% bromophenol blue
Stripping solution acidic	0.1 M glycine pH 2.5
Stripping solution basic	0.1% NaOH
TAE buffer	40 mM Tris-Acetate pH 8.5 1 mM EDTA pH 8
TBS-T	100 mM Tris-HCl pH 7.4 150 mM NaCL 0.05% Tween 20%
Transfer buffer	192 mM glycine 25 mM Tris-Base 10% methanol

Table 5: Bacterial strains

Name	Description	Reference
E. *coli* XL-1 Blue	DNA amplification	Stratagene, LA Jolla, USA
E. *coli* Rosetta (DE3) pLysS	Protein expression	Novagen, Darmstadt, Germany

Table 6: Cell lines (alphabetical order)

Name	Description	Reference
HaCaT	Human immortalized keratinocytes	Boukamp *et al.* 1988
HeLa	Human cervix adenocarcinoma cells	ATCC, Rockville, MD, USA
SH-SY5Y	Human neuroblastoma cells	ATCC, Rockville, MD, USA
MCF 7	Human breast cancer cells	Soule *et al.* 1973

2.2 DNA Analysis

2.2.1 Polymerse chain reaction, restriction and ligation

Polymerse chain reaction (PCR) was performed to generate GST-fusion constructs of BACE-1 and MPR proteins. To expand these constructs the Expand High Fidelity PCR System (Roche) and the One Taq DNA Polymerse system (NEB) were used according the manufacture's protocol using the primers listed in the table 7 and 8.

Expand High Fidelity PCR System (Roche)

Template	10 ng
Primer forward	0.5 µl
Primer reverse	0.5 µl
dNTP (10mM)	1 µl
10X High Fidelity Buffer with $MgCl_2$	5 µl
Polymerase	1µL
dd H_2O	Fill up to 50 µL

Temperature	Time	
94°C	2 min	
94°C	15 sec	30x
T_m-4°C	30 sec	
72°C	30 sec-3 min (1min/kb)	
72°C	5 min	

One Taq Polymerase (NEB)

Template	10 ng
Primer forward (100µM)	0.5 µl
Primer reverse ((100µM)	0.5 µl
dNTP (10mM)	1 µl
5X One Taq Standard Reaction buffer	10 µl
One Taq DNA Polymerse	0.25 µl
dd H_2O	Fill up to 50 µl

Temperature	Time	
94°C	30 sec	
94°C	15 sec	30x
T_m-4°C	30 sec	
72°C	30 sec-3 min (1min/kb)	
68°C	5 min	

PCR products were visualized on 1% agarose gels in TAE buffer containing ethidium bromide (10µg/ml). They were purified using the High Pure PCR Purification Kit (Roche) or the Ilustra GFX PCR DNA DNA and Gel Band Purification Kit (GE Healthcare). Digestions were performed using restriction enzymes purchased from New England Biolabs (NEB) according to the manufacture's protocol. Dephosphorylation of the vector was done using shrimp alkaline phosphatase (Roche). Ligation of the vector with the insert was done using T4 DNA ligase (NEB) for 12 h at 16°C. The ligation product was transformed in E.*coli.* strain XL-1 Blue (see plasmid DNA amplification and purification). The sequence of newly created constructs was checked using the sequencing service of MWG Biotech AG (Ebersberg, Germany.)

2.2.2 Plasmid DNA amplification and purification

Plasmid DNA was transformed by heat shock in the E.*coli.* strain XL1-Blue. The transformed bacteria were grown overnight at 37°C on the selective LB plates containing the appropriate antibiotic. A single colony was inoculated and grown in 5ml LB media with antibiotic overnight at 37°C for preparation of glycerol stocks and/or plasmid DNA purification using a GenElute Miniprep Kit (Sigma) followed the manufacturer's instructions. Higher amounts of plasmid DNA were obtained from 100-200 ml of bacterial culture using NucleoBond Xtra MIDI (EF) Midiprep Kit (Machery - Nagel).

2.2.3 Plasmid constructs

Human full length BACE1-myc (WT and LLAA, GenBank: NM_012104) expression constructs were kindly provided by G. Tesco. The cytoplasmic domain of BACE-1 was PCR amplified from these constructs using the indicated primers (table 7) and cloned into pGEX-4T1 (GE Healthcare) for the expression of GST fusions of BACE1 tail. The cytoplasmic tail of the CD-MPR (GenBank: NM_002355.3) was cloned with a similar strategy in pGEX-4T1. Rat flotillin-1-GST (GenBank: U60976) and flotillin-2-GST (GenBank: AF023302) coding regions were cloned into pET41a expression vector (Novagen).[37]

Table 7: Primers for BACE-1 and MPR fusion proteins

Primer description	Primer sequence
BACE-1 CT fwd BamHI	CTATA GGATCC CAGTGGCGCTGCCTCCG
BACE-1 WT rev EcoR1	CTATA GAATTC TCACTTCAGCAGGGAGATGTC
BACE-1 LLAA rev EcoR1	CTATA GAATTC TCACTT CGCCGC GGAGATGTCATC
MPR46 CT fwd BamHI	CTATA GGATCC CAGCGACTGGTAGTGGGAG
MPR46 WT rev EcoR1	CTATA GAATTC CTA CATTGGTAATAAATGGTCATCCC
MPR46 LLAA rev EcoR1	CTATA GAATTC CTA CATTGGTGCTGCATGGTCATCCC

The pCDNA3 myr Akt delta PH (constitutively active Akt1: Plasmid 9009) and pCDNA3 Akt T308A 473A (dominant negative Akt1: Plasmid 9030) were purchased from Addgene. The full length WT Akt1 (GenBank accession number: NM_001014431) was amplified from HaCaT cDNA by standard PCR using the primers listed in table 8 and cloned into pCDNA3 vector (Addgene).

Table 8: Akt1 constructs and corresponding primers

Primer description	Primer sequence
WT human Akt1 fwd BamH1	CTATA GGATCC ATGAGCGACG TGGCTATTGTG
WT human Akt1 rev EcoR1	CTATA GAATTC TCA GGCCGTGCCGCTGGCC

2.2.4 GST Protein expression and purification

Bacterial expression strains BL21, transformed with either pGEX4T1, pGEX4T1 BACE-1 WT CT GST, pGEX4T1 BACE-1 LLAA CT GST, pGEX4T1 CD-MPR WT GST, pGEX4T1 CD-MPR LLAA CT GST, flotillin-1 GST and flotillin-2 GST, were grown at 37°C with shaking to reach OD_{600} 0.4-0.6. Bacteria were then induced with 1 mM IPTG for 6 h at 37°C. The bacterial cells were pelleted and lysed in GST-lysis buffer supplemented with 100 µg/ml lysozyme, 1.5 µM aprotinin, 23 µM leupeptin , 1.5 µM pepstatin A, 1 mM PMSF and 1 mM dithriothreitol (DTT). GST proteins from the lysates were allowed to bind to glutathione-sepharose beads (GE Healthcare) overnight at 4°C on a rotating wheel.

2.3 Cell culture studies and biochemical methods

2.3.1 Cell culture, transfection and RNA interference

SH-SY5Y cells were cultured in Rosewell Park Memorial Institute Medium (RPMI, GIBCO, Invitrogen) supplemented with 10% fetal calf serum (FCS, GIBCO, Invitrogen) and 1% penicillin/streptomycin (PAA Laboratories) at 37°C under 5% CO_2. MCF-7 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture (DMEM, GIBCO, Invitrogen) supplemented with 10% fetal calf serum (FCS, GIBCO, Invitrogen) and 1% penicillin/streptomycin (PAA Laboratories) at 37°C under 8 % CO_2. For HaCaT cells, DMEM was additionally supplemented with 1% sodium pyruvate (PAA Laboratories) and 1% Non-essential amino acids (Biochrom AG). Transient plasmid transfection (1 µg of plasmid DNA) in HeLa cell line was performed using lipofectime 2000 (Invitrogen) or MACSfectine (miltenyi biotech) according to the manufacturer's protocol. All transient transfections in SH-SY5Y cell line were performed using MACSfectine according to the manufacturer's protocol.

2.3.2 Immunofluorescence

HeLa cells were grown on coverslips and transiently transfected with BACE-1 WT, BACE-1 LLAA or pCDNA3 for 24 h. Cells were fixed with methanol, blocked with 1% BSA in PBS, labeled with primary antibodies for 1 h and then stained with Cy3 and Alexa Fluor 488 conjugated secondary antibodies for 45 min at room temperature. SH-SY5Y cells were grown on coverslips, treated with 10 µM retinoic acid for 4 days to attain differentiated neuroblastoma cells. These differentiated cells were treated with $A\beta_{25-35}$ for 24 h or 72 h, fixed with methanol, blocked with 1% BSA in PBS, incubated with primary antibodies for 1 h and stained with secondary antibodies for 45 min. All the samples were embedded in Gelmount (Biomeda; Foster city, CA, USA) supplemented with 1,4-diazadicyclo(2,2,2) octane (DABCO, Fluka, Neu-Ulm, Germany). Images were taken with a confocal laser scanning microscope (Zeiss LSM510 Meta)

2.3.3 Cell lysis, gel electrophoresis and Western blot

Cell pellets were lysed in Stuermer lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) for 30 min on ice and lysates were cleared by centrifugation. In case of lysate preparation for coimmunoprecipitation or GST pull

down assays, phospho lysis buffer supplemented with protease inhibitor cocktail and 60 mM NOG was used. Cells subjected to growth factor stimulation or apoptotic induction were lysed in phospho lysis buffer supplemented with protease inhibitor cocktail, 1 mM NaF, 1 mM Na_3VO_4 and 60 mM NOG. For APP C-terminal fragment preparation, APP CTF extraction buffer supplemented with protease inbitor cocktail and 60 mM NOG was used. Protein concentration was determined with Biorad protein assay reagent. Equal protein amounts of the lysates were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) for all purposes. NuPAGE Novex Bis–Tris Mini Gel system with MES buffer was used for the detection of APP C-terminal fragments and followed by Western blot.

2.3.4 Coimmunoprecipitation

Transfected cells were lysed for 30 min on ice in phospho lysis buffer, supplemented with protease inhibitor cocktail and 60 mM NOG. Lysates were precleared three times with 50µl Pansorbin beads (Calbiochem, Nottingham, UK). Precipitation was performed using antibody-precoupled Protein A Dynabeads (Invitrogen) overnight at 4°C on a spinning wheel. The next day, the beads were washed three times with phospho lysis buffer. Precipitated proteins were solubilized in 2x SDS sample buffer containing 50 mM DTT by heating at 94°C for 5 min. Proteins were separated by SDS-PAGE and subjected to immunoblotting with specific antibodies.

2.3.5 Indirect GST pull down assay

HeLa cells in which flotillin-1 or flotillin-2 were transiently knocked down or stably knocked down were lysed for 30 min on ice in phospho lysis buffer supplemented with protease inhibitor cocktail and cleared by centrifugation. The cell lysates were incubated with either 5 µg of GST or GST tagged proteins immobilized on glutathione sepharose beads (GE healthcare) and protease free BSA (final concentration: 1%) overnight at 4°C. The beads were washed three times with 1 ml phospho lysis buffer, resuspended in loading buffer, heated for 5 min at 94°C and separated by SDS-PAGE.

2.3.6 GST pull down assays using purified proteins

For direct GST pulldown experiments, the GST tag was removed with 2U of thrombin for 20 µg of purified flotillin-1 GST and flotillin-2 GST bound to sepharose

beads. The beads were then washed three times with cleavage buffer (20 mM Tris-HCl, pH 8.4, 0.15 mM NaCl, 2.5 mM $CaCl_2$) freshly supplemented with 1 mM DTT. The reaction was carried out overnight at 19°C. Next day, thrombin was inactivated with 1 mM PMSF, and the GST bound beads were removed with centrifugation. The supernatant containing flotillin-1 or flotillin-2 were mixed with 5 µg of each of the GST fusion proteins (GST, BACE-1 WT GST, BACE-1 LLAA GST) and rotated at 4°C for 3 h. The beads were then washed three times with precision protease buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100), heated for 5 min at 94°C and separated by SDS-PAGE.

2.3.7 Differentiation of neuroblastoma cells

The SH-SY5Y cells were plated onto the 12 well tissue culture plate and the following day treated with 10 µM retinoic acid (RA). The cells were treated with RA for 7 days until neurites were visible and the medium was replaced after every second day of treatment.

2.3.8 MTT Assay

SH-SY5Y cells were seeded in 12-well plates at an initial density of $0.5x10^5$ cells/well. The following day, cells were treated with $A\beta_{25-35}$ for 72 h or 1 µM STS for 2 h or 4 h time periods. At the end of the apoptotic treatment, cells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (0.5 mg/ml, Sigma-Aldrich) at 37°C for 4 h. Thereafter, 600 µl DMSO was added to the cells to dissolve the formazan crystals, and the absorbance was measured at 570 nm, with reference at 690 nm.

2.3.9 Growth factor treatment

SH-SY5Y cells were serum starved for 16 h prior to treatment with 100 ng/ml EGF for the indicated times. After growth factor stimulation, cells were lysed for 30 min on ice in phospho lysis buffer supplemented with protease inhibitor cocktail, 1 mM NaF, 1 mM Na_3VO_4 and 60 mM NOG.

2.3.10 Treatment with apoptotic inducers

SH-SY5Y cells were seeded at an initial density of 0.5 x 10^6 cells/well onto a 12 well tissue culture plate. The next day, SH-SY5Y cells were treated with 1 µm STS, 1 µg/ml brefeldin A, 24 nM paclitaxel, 500µM carboplatin or 0.5 µM doxorubicin for the indicated times. At the end of the treatment, cells were lysed for 30 min on ice in phospho lysis buffer supplemented with protease inhibitor cocktail, 1 mM NaF, 1 mM Na_3VO_4 and 60 mM NOG.

2.3.11 Nuclear Fractionation

SH-SY5Y cells were seeded at an initial density of 2x10^6 cells onto 6 cm tissue culture plate. The next day, these cells were treated with 1 µM STS for 2 h. At the end of the treatment, cells were harvested by scraping in cold PBS and centrifuged at 1000 g for 2 min at 4°C. The pellet was gently resuspended in 500 µl cold lysis buffer and rotated on spinning wheel for 10 min at 4°C. After 10 min, a 20 µl aliquot of the cell suspension was mixed with trypan blue to check if intact cells were present. Separation of the cytosol was achieved by centrifugation at 7100 g for 1 min at 4°C. The supernatant contained the cytosol fraction and the residual pellet was gently resuspended in 500 µl of cold lysis buffer. The resuspension was centrifugated at 7100 g for 1 min at 4°C and the pellet was retained. The resulting pellet was then gently resuspended in 500 µl of cold nuclear lysis buffer and 6.25 µl of 5 M NaCl. After incubation on ice for 30 min, the suspension was centrifugated at 20, 800g for 20 min at 4°C and the supernatant contained the nuclear fraction. The cytosolic and nuclear fractions were solubilized in 4x loading buffer and heated at 94°C for 5 min.

2.4 Statistical Analysis

Unless otherwise stated, all experiments were performed at least three times for the statistical analysis. Western blotting bands of proteins were quantified by scanning densitometry using Quantity One software (Biorad) and normalized to GAPDH. Data are shown as the mean ± S.D. Statistical comparisons were made using one-way analysis of variance (ANOVA) or two-way ANOVA with Dunnet's or Bonferroni's multiple comparison test as appropriate using GraphPad Prism 6 software. Values of $p < 0.05$ were considered significant (*), whereas values of $p < 0.01$ and $p < 0.001$ were defined very significant (**) and highly significant (***), respectively.

2.5 Electronic Manipulation of Images

The images have in some cases as a whole been subjected to contrast or brightness adjustment. No other manipulations have been performed unless otherwise stated.

CHAPTER 3: RESULTS

3.1 Interaction of flotillins with BACE-1 is mediated by the acidic di-leucine motif

Previous reports have established that flotillin-1 associates with BACE-1, and flotillin-1 overexpression increases the association of BACE-1 with detergent insoluble microdomains.[125] In most cell types, flotillin-1 and flotillin-2 are associated as hetero-oligomers, but in the study of Hattori *et al.,* the role of flotillin-2 binding to BACE-1 was not taken into consideration. Therefore, to analyze the interaction of flotillins with the BACE-1 cytoplasmic tail containing the di-leucine sorting motif, two BACE-1 GST fusion proteins were generated, the sequence of which is shown in figure 8. BACE-1 GST peptide encoding for the short cytoplasmic tail (23 amino acids) and BACE-1 LLAA GST containing a mutated tail in which the two leucine residues within the ACDL were mutated into alanine (LLAA) were generated, expressed, purified, and analysed by SDS-PAGE. As a negative control for the GST pulldown experiments, GST alone was expressed.

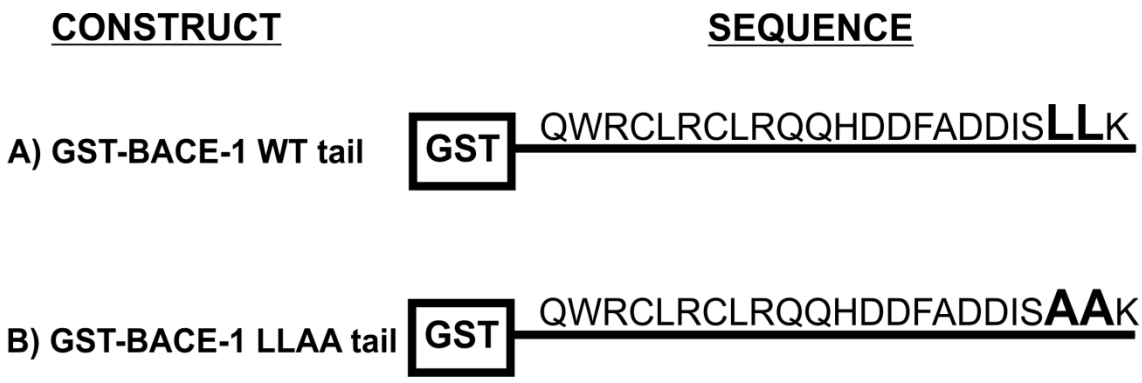


Figure 8: BACE-1 fusion proteins

(A) Human BACE-1 cytoplasmic tail, N-terminally fused to GST with intact di-Leu sorting motif (B) Human BACE-1 cytoplasmic tail, N-terminally fused to GST with di-Leu mutated to alanines.

3.2 Interaction of BACE-1 cytoplasmic tail with flotillins is primarily mediated by flotillin-1

In order to study the role of each flotillin in the binding to BACE-1, HeLa cells were depleted of flotillins by means of siRNA as previously described.[126] In HeLa cell line, flotillin-1 depleted cells express almost unchanged amounts of flotillin-2, whereas siRNA mediated knockdown of flotillin-2 results in profound depletion of flotillin-1 due to the de-stabilization of the protein.[127] For the GST pull down experiments, lysates from siRNA flotillin-1, siRNA flotillin-2, and control HeLa cells were incubated with equal amounts of purified BACE-1 WT GST, BACE-1 LLAA GST or GST for a control and the precipitates were analyzed by SDS-PAGE. As shown in figure 9, in control cells, both flotillins bound specifically and strongly with the WT tail, whereas the LLAA mutant showed only trace binding to flotillins. Having found that both flotillins show more binding to the BACE-1 WT tail compared to the mutant tail, the next step was to find out the significance of each of the flotillins in mediating this interaction. Interestingly, in the absence of flotillin-1, the binding of flotillin-2 to the WT tail of BACE-1 was reduced to the same level as with LLAA tail in these and control cells. This indicated that flotillin-1 binds specifically to the di-leucine motif, whereas the binding of flotillin-2 is almost exclusively mediated by flotillin-1.

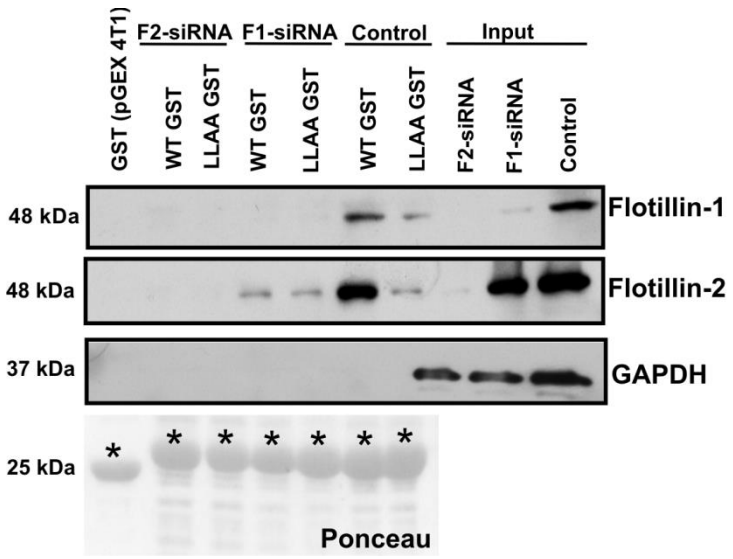


Figure 9: Flotillins bind to the di-Leu motif in BACE-1

Purified GST fusion proteins of BACE-1 WT or BACE-1 LLAA coupled to glutathione sepharose beads were incubated with transient knockdown HeLa lysates. After SDS-PAGE, the respective proteins were detected by means of Western blot using specific antibodies. Ponceau staining was used to visualize the fusion proteins used in the assay.

3.3 Direct interaction of the flotillins with BACE-1 is mediated by the dileucine sorting signal in the cytoplasmic tail of BACE-1

In order to analyze if the interaction of flotillins with BACE-1 is a direct one, direct GST pull down experiments were conducted using bacterially expressed and purified proteins. Flotillin-1 GST, Flotillin-2 GST, BACE-1 WT GST, BACE-1 LLAA GST, and GST alone as a control were generated, expressed, purified and analyzed on SDS-PAGE. Flotillin-1 GST and flotillin-2 GST were subjected to thrombin cleavage to remove the GST tag. Equal amounts of BACE-1 WT, BACE-1 LLAA or GST were incubated with the purified flotillin-1 or flotillin-2 proteins and the precipitate was analyzed by SDS-PAGE (figure 10). In line with the previous findings, flotillin-1 strongly bound to the BACE-1 WT GST tail, whereas no binding was detected with the LLAA tail. In case of flotillin-2, only a trace binding was observed close to the background level.

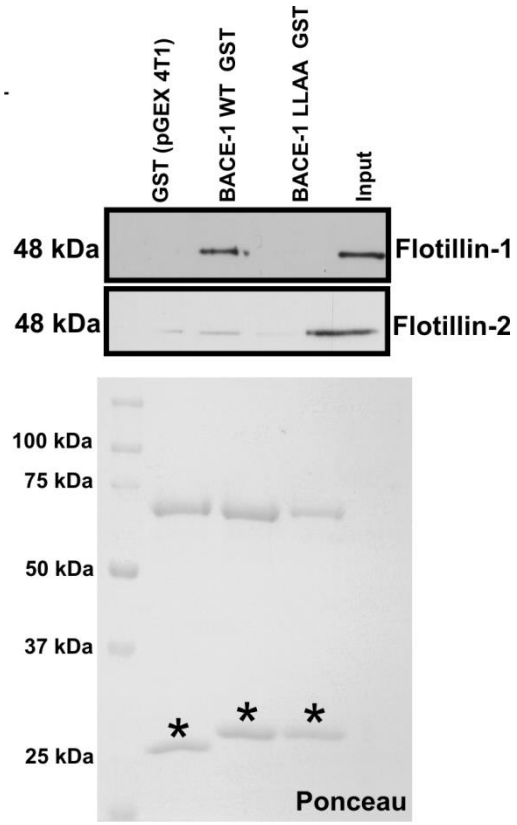


Figure 10: Flotillin-1 directly interacts with BACE-1 via the di-Leu sorting motif.
Binding of purified recombinant flotillin-1 and flotillin-2 to BACE-1 cytoplasmic tail (WT or LLAA) was tested. After SDS-PAGE, the respective proteins were detected by means of Western blot using specific antibodies. Ponceau staining was used to visualize the fusion proteins used in the assay.

3.4 Flotillins and GGA proteins compete for the di-leucine binding motif in the cytoplasmic tail of BACE-1

The di-leucine motif present in BACE-1 WT tail is a binding site for GGA proteins which have been shown to affect the endosomal trafficking of BACE-1.[128] Therefore, there is a possibility that flotillins and GGA proteins share this binding motif. In order to test if flotillins and GGA proteins cooperatively bind to BACE-1 or compete for this binding site, GST pull down experiments were conducted. Equal amounts of BACE-1 GST, BACE-1 LLAA GST or GST alone were incubated with lysates from siRNA flotillin-1, siRNA flotillin-2, and control HeLa cells and the precipitates were analyzed by SDS-PAGE. In accordance with previous results[128], BACE-1 WT tail bound to GGA2, whereas the binding was abrogated by the LLAA mutation (figure 11). In the

absence of flotillins, WT BACE-1 tail binds strongly to GGA2, implicating that GGA2 and flotillins compete for the same binding site.

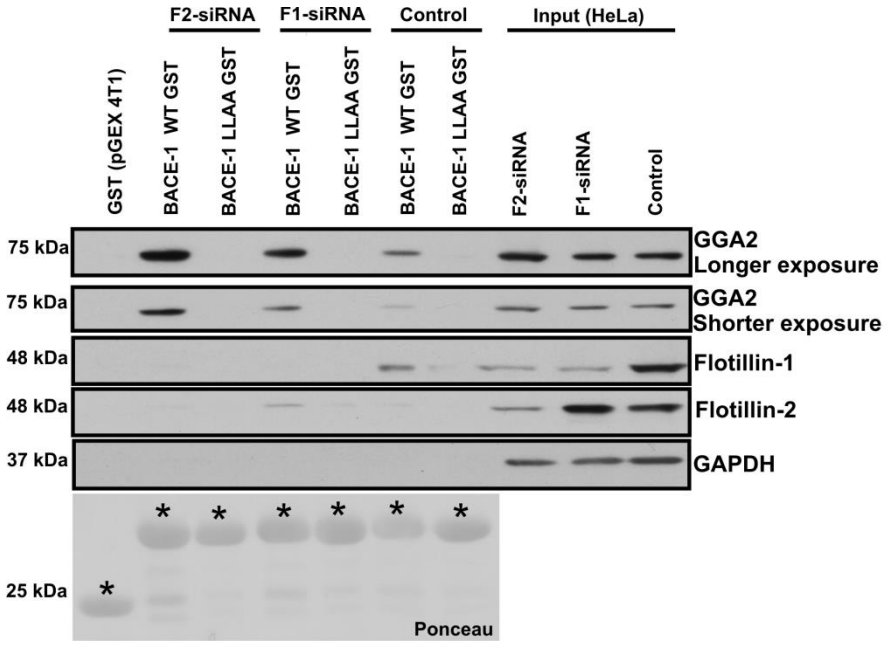


Figure 11: Flotillins compete for the binding to BACE-1 cytoplasmic tail with GGA2.
Purified GST fusion proteins of the BACE-1 cytoplasmic tail (WT or LLAA) coupled to glutathione sepharose beads were incubated with lysates from transient flotillin knockdown HeLa cells. The binding of GGA2 and flotillins was tested using specific antibodies after SDS-PAGE and Western blot. Ponceau staining was used to visualize the fusion proteins used in the assay.

3.5 Flotillins specifically bind to the di-leucine sorting motif of BACE-1

In order to find out if flotillins generally bind to the acidic di-leucine motifs in other class of proteins, two CD-MPR GST fusion proteins, MPR WT GST and MPR LLAA GST, were generated, expressed, purified and analyzed by SDS-PAGE. Coomassie staining showed that the expressed proteins have the expected molecular weight of approximately 25 kDa for GST and 30 kDa for CD-MPR WT GST and CD-MPR LLAA GST. For the GST pull down experiments, lysates from control HeLa cells were incubated with equal amounts of purified MPR WT GST, MPR LLAA GST or GST alone for a control and followed by SDS-PAGE. In this assay, neither of the flotillins bound to the CD-MPR tail (figure 12). On the other hand, GGA2 showed a clear interaction with CD-MPR WT tail and this interaction was abrogated upon

mutation of di-leucine motif to alanine residues. GST pull down experiments were performed using lysates from shRNA flotillin-1, shRNA flotillin-2 and control HeLa cells and incubated with equal amounts of CD-MPR WT GST, CD-MPR LLAA GST or GST control alone. Subsequently, an enhanced GGA2 binding to the CD-MPR WT tail was detected in the absence of flotillins. Although flotillins did not bind to MPR, the absence of flotillins enhanced GGA2 binding to MPR. It is possible that absence of flotillins might facilitate some other intermediatery protein to aid in strengthening GGA2 and MPR interaction. It is also possible that in the absence of flotillins the GGA2 protein is free from flotillin binding and thereby available to mediate an interaction with MPR proteins.

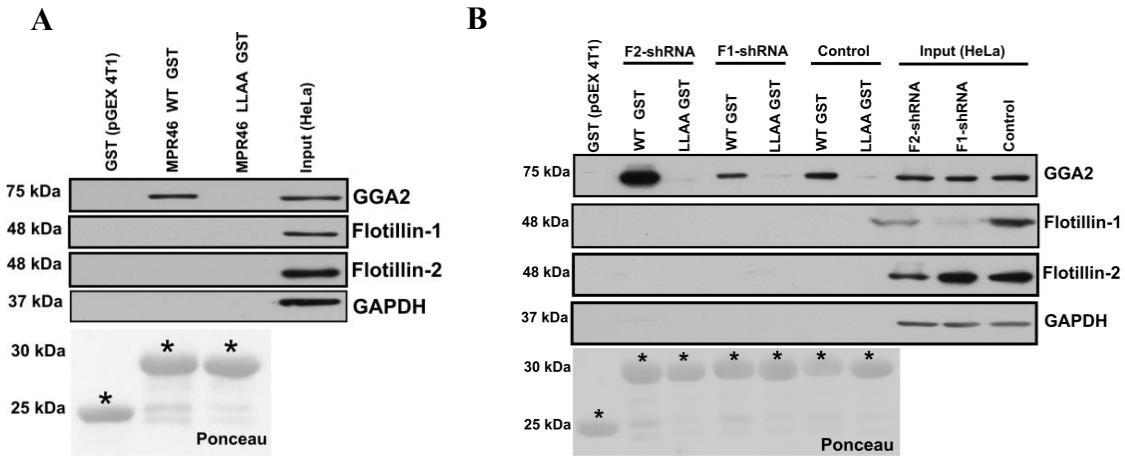


Figure 12: Flotillins specifically bind to the di-Leu motif in BACE-1.
(A) Purified GST fusion proteins of CD-MPR46 WT or CD-MPR46 LLAA coupled to glutathione sepharose beads were incubated with HeLa WT lysates or (B) flotillin depleted stable knockdown HeLa cells.

3.6 Coimmunoprecipitation of BACE-1 WT and BACE-1 LLAA with flotillins

Full length, myc-tagged BACE-1 WT and LLAA mutant were expressed in HeLa cells and coimmunoprecipitation experiments were performed. For this purpose, either c-myc (see figure 13 A) or flotillin-1/flotillin-2 was immunoprecipitated (see figure 13 B). The presence of overexpressed or coprecipitated BACE-1 WT myc and BACE-1 LLAA myc was probed with specific antibody against myc tag while expressed or coprecipitated flotillin-1/2 was detected using flotillin mouse monoclonal antibodies. An interaction of BACE-1 WT was observed with flotillin-1, whereas the

LLAA mutant showed only weak binding. These findings indicate that flotillin-1 interacts specifically with the di-leucine sorting motif of BACE-1.

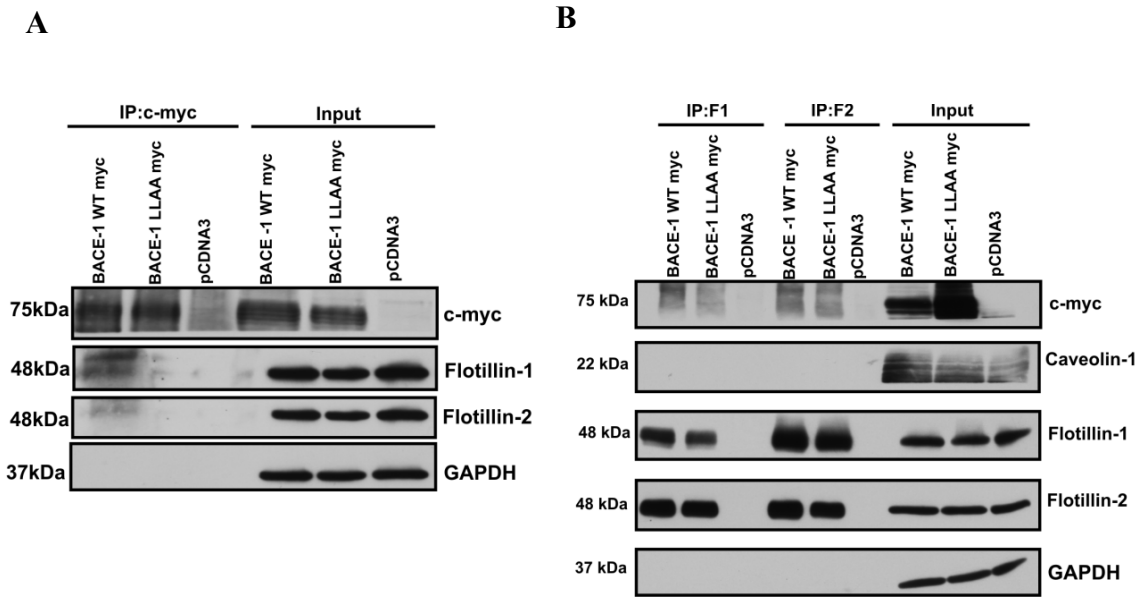


Figure 13: Flotillins interact with ectopically expressed BACE-1 WT in HeLa cells.
(A) A myc tagged BACE-1 WT or BACE-1 LLAA was immunoprecipitated from HeLa cells and the coprecipitation of endogenous flotillin-1 or flotillin-2 was detected by Western blot.
(B) Endogenous flotillin-1 or flotillin-2 was immunoprecipitated from HeLa cells and the coprecipitation of myc tagged BACE-1 WT or BACE-1 LLAA was detected by Western blot. Caveolin-1 was used to control the specificity of this coimmunoprecipitation.

3.7 Flotillin knockdown leads to endosomal accumulation of BACE-1 WT but not BACE-1 LLAA mutant

Since the ACDL motif is relevant for both endocytosis and recycling of BACE-1, the next step was to check if the depletion of the cellular flotillin pool could affect the localization of BACE-1. For this purpose, HeLa cell lines in which flotillin expression was stably knocked down by means of lentivirus mediated shRNA were used. Flotillin-1 and flotillin-2 stable knockdown HeLa cell lines were transiently transfected with BACE-1 WT-myc, BACE-1 LLAA-myc or an empty vector pCDNA3 and the localization was studied by immunostaining. In control shRNA cells, WT BACE-1 myc was localized to a large extent at the plasma membrane and faintly at the perinuclear region. Upon flotillin depletion, a more intense staining of the perinuclear region was

observed, indicating that flotillin depletion affects the cellular localization of BACE-1. In the absence of flotillins, the staining also appeared brighter, implicating that the expression of BACE-1 might be enhanced as well due to an increased protein accumulation (see figure 14). BACE-1 LLAA mutant was primarily localized on the plasma membrane in case of both control and knockdown cells (see figure 15)

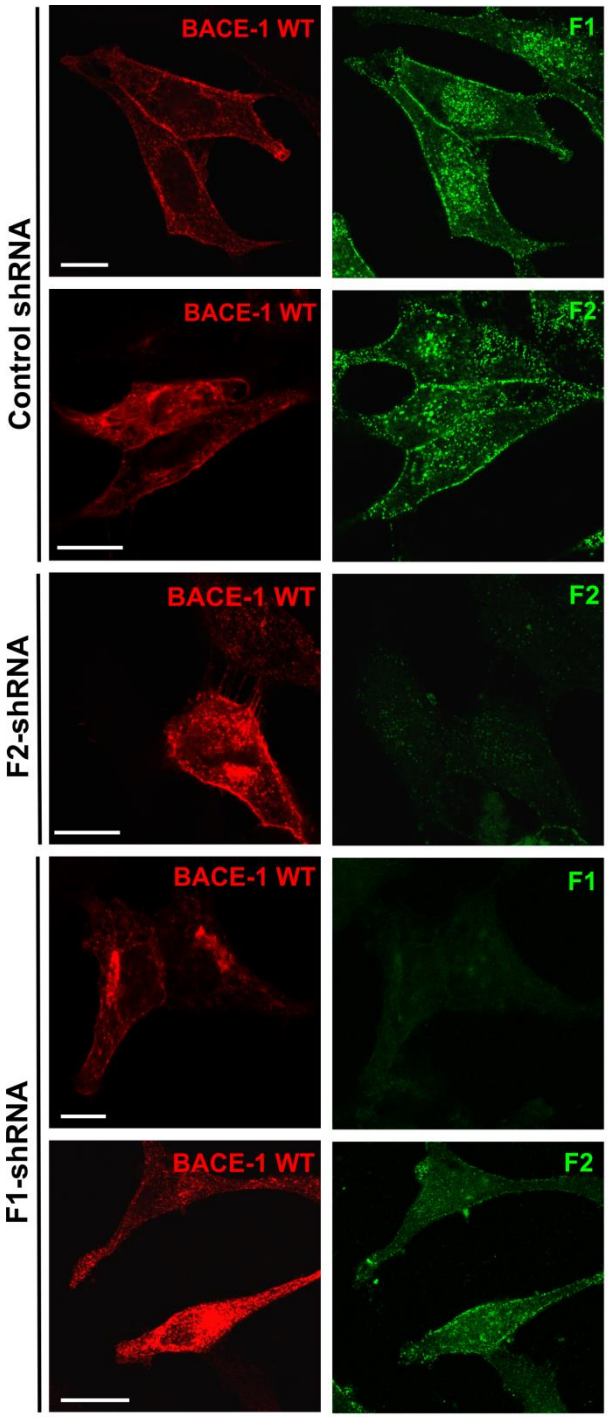


Figure 14: Subcellular localization of BACE1-myc in HeLa cells.
Full-length, myc-tagged BACE-1 was expressed in control, flotillin-1 and flotillin-2 knockdown HeLa cells. Cells were fixed in methanol and immunofluorescently labeled for myc-tag (red) and either flotillin-1 or -2 (green). Scale bar: 10 µm.

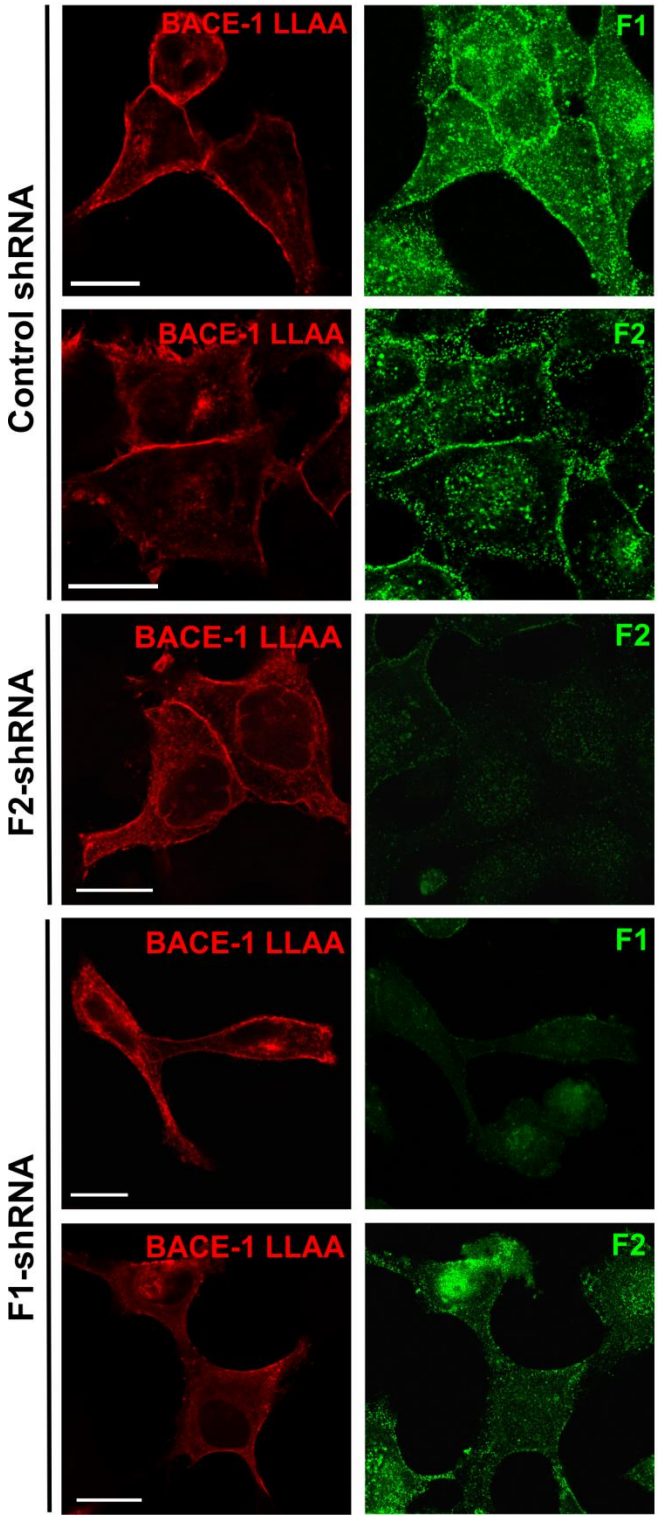


Figure 15: Flotillin depletion influences the subcellular localization of BACE1 LLAA-myc Full-length, myc-tagged BACE-1 LLAA was expressed in stable flotillin-1 and -2 knockdown HeLa cells. Cells were fixed in methanol and immunofluorescently labeled for myc-tag (red) and either flotillin-1 or flotillin-2 (green). Scale bar: 10 µm.

3.8 Flotillin depletion results in increased processing of APP

According to Tesco and co-workers, knockdown of GGA3 resulted in an increase in BACE-1, C-99 and Aβ.[62] Since in this study an accumulation of BACE-1 was observed so we sought to check APP processing and for this we used an antibody that detects the C-terminus of APP in stable knockdown HeLa cells. The amount of full-length APP was not significantly changed in these cells, whereas the C-terminal BACE-1 processing fragment (designated as C99) was significantly increased upon flotillin-2 knockdown (figure 16A-B). Flotillin-1 knockdown cells also showed a tendency to increased APP processing. However, these data did not reach significance due to the variation of the C99 amount between different experiments. Interestingly, a shift in molecular weight of the C-terminal fragments was detected in flotillin-2 knockdown cells. We speculated whether the molecular weight differences of the C-terminal fragments between flotillin-1/2 knockdown and control cell line was an outcome of a differential APP processing by the α- or β secretases. For this purpose, we used 6E10 antibody which detects the first half of Aβ fragment that is produced by β-γ cleavage and does not detect the α-secretase cleavage products (data not shown). Since 6E10 antibody detected C-terminal fragments generated by all cell lines, it was ensured that the detected fragment was C-99 fragment generated by β-γ secretase cleavage. We suggest that the difference in molecular weight of C-99 fragments that appears in flotillin-2 knockdown cells may be due to post-translational modifications such as phosphorylation or ubiquitination of APP.

The increase in C-99 fragment upon flotillin depletion was also tested in human neuroblastoma SH-SY5Y cell line (figure 16 C-D). For this purpose, lysates from flotillin-1, flotillin-2 stable knockdown and control SH-SY5Y cell lines were prepared and C-terminal fragments were detected using the APP antibody. In SH-SY5Y cells, knockdown of flotillin-1 resulted in profound depletion of both flotillin-1 and flotillin-2. Therefore, both shRNA flotillin-1 and shRNA flotillin-2 SH-SY5Y cells showed an increase of C-99 fragment compared to the control cells. In sum, these data show that flotillin knockdown increases the expression and endosomal localization of BACE-1, which in turn results in increased amyloidogenic processing of APP.

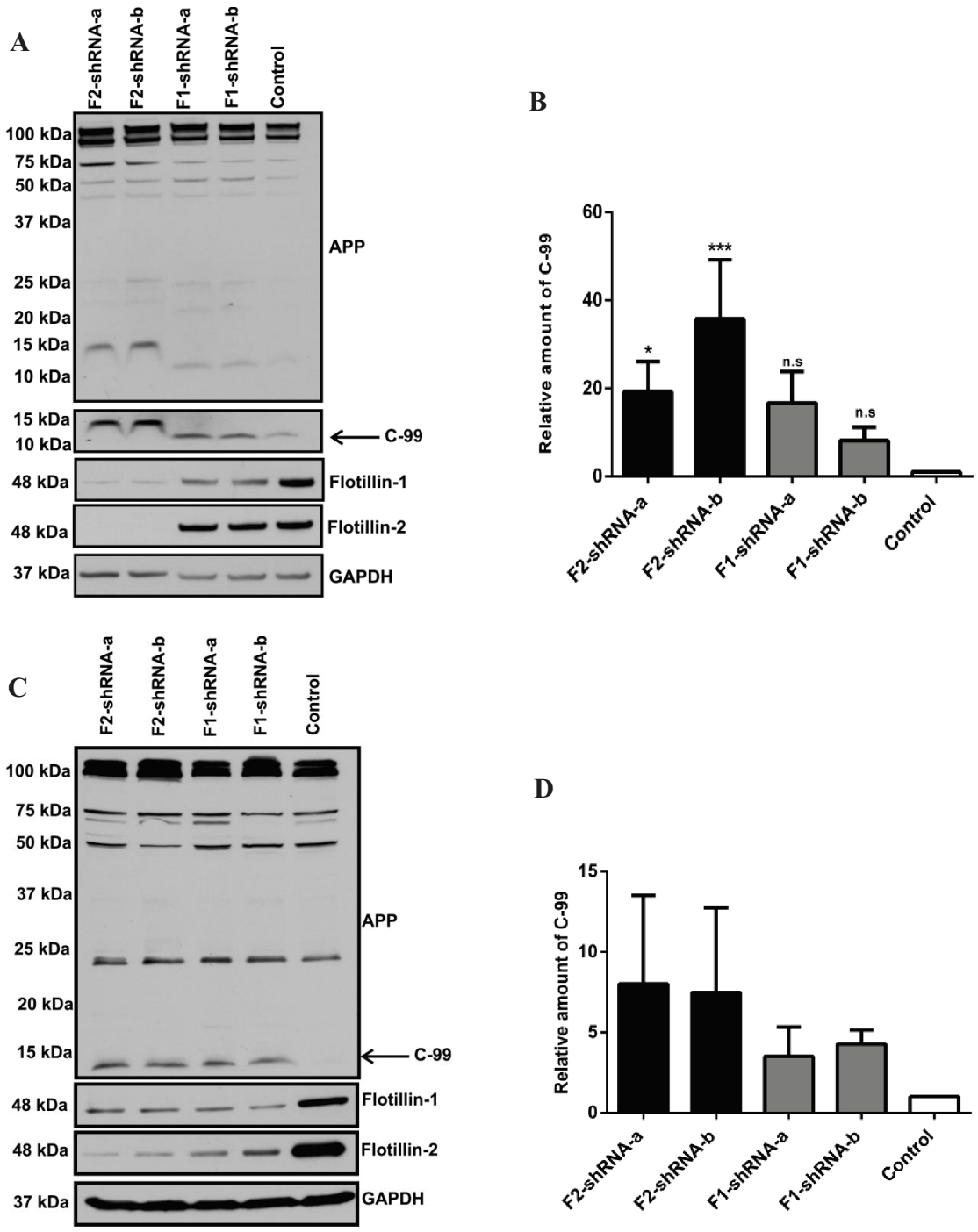


Figure 16: Flotillin depletion results in increased APP processing

(A-D) Processing of APP in flotillin knockdown HeLa or SH-SY5Y cells was measured using APP antibody and quantification of the C-99 APP processing fragment was normalized against GAPDH. Bars represent the mean ± S.D. of three independent experiments. One-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.9 Flotillin proteins in the regulation of apoptosis

3.9.1 Retinoic acid mediated neuronal cell differentiation in human neuroblastoma cells

To attain a typical neuronal phenotype in SH-SY5Y cells, flotillin knockdown and control cells were differentiated by retinoic acid (RA) and extension of neurites was observed 24 h after application of RA and this phenomenon was retained for 7 days. In order to test if flotillin knockdown altered this differentiation process in neuronal cells, immunostaining was performed using α-tubulin antibody to detect neurite extensions (figure 17). At the end of a 7 day RA treatment, both flotillin knockdown and control cells exhibited neurite extensions in contrast to the undifferentiated SH-SY5Y cells.

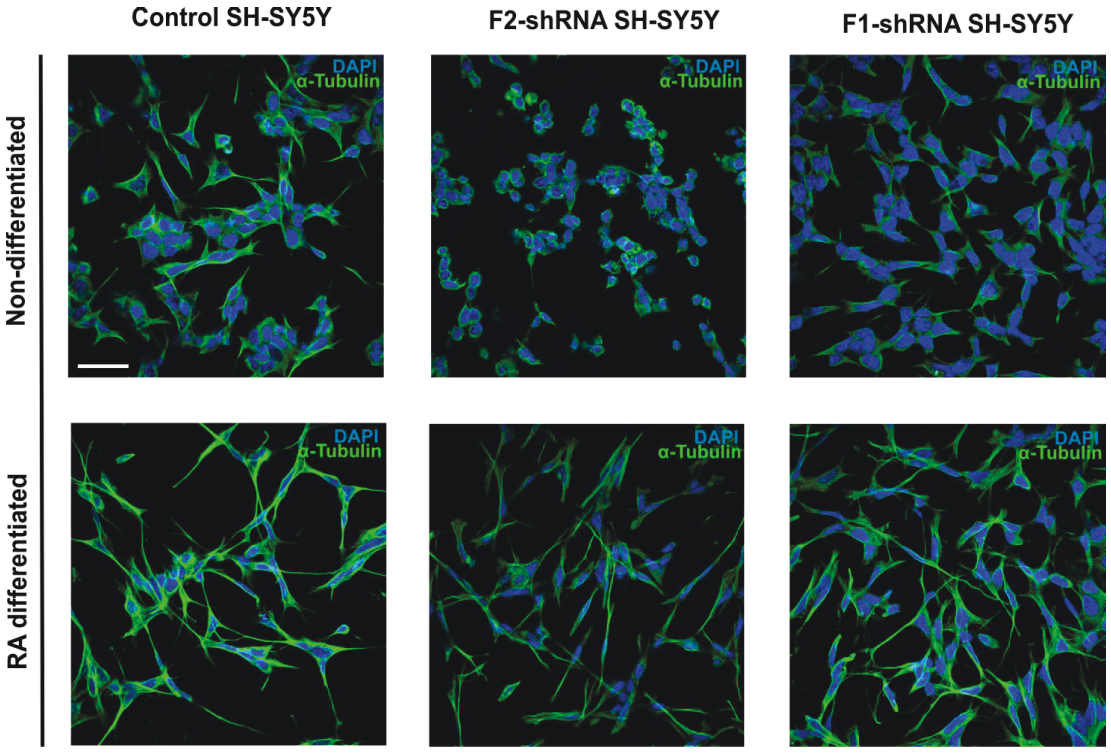


Figure 17: Neuronal differentiation in SH-SY5Y cells induced by retinoic acid.
(A) Human neuroblastoma cells grown on coverslips were treated with 10 µM retinoic acid for 7 days. Cells were immunofluorescently labeled for α-tubulin (green). Scale bar: 10 µm

3.9.2 Differentiated neuroblastoma cells depleted of flotillins are sensitive to $A\beta_{25-35}$ neurotoxic peptide

To test whether the neurotoxic peptide $A\beta_{25-35}$ induced cytotoxicity in RA mediated differentiated neuronal cells, shRNA flotillin-1, shRNA flotillin-2 and control SH-SY5Y cells were incubated with 25 µM $A\beta_{25-35}$ for 24 h, 48 h and 72 h. DNA fragmentation is a hallmark of apoptosis and usually a late event in apoptosis. DNA fragmentation was clearly visible as early as 48 h treatment of $A\beta_{25-35}$ treatment mainly in shRNA flotillin-1 SH-SY5Y cells (figure 18). At this time point, neither visible vacuole formation nor DNA fragmentation was detected in shRNA flotillin-2 or control cells. Taken together, $A\beta_{25-35}$ was confirmed to be effective at a concentration as low as 25 µM in the observation of nuclear fragmentation upon flotillin-1 depletion.

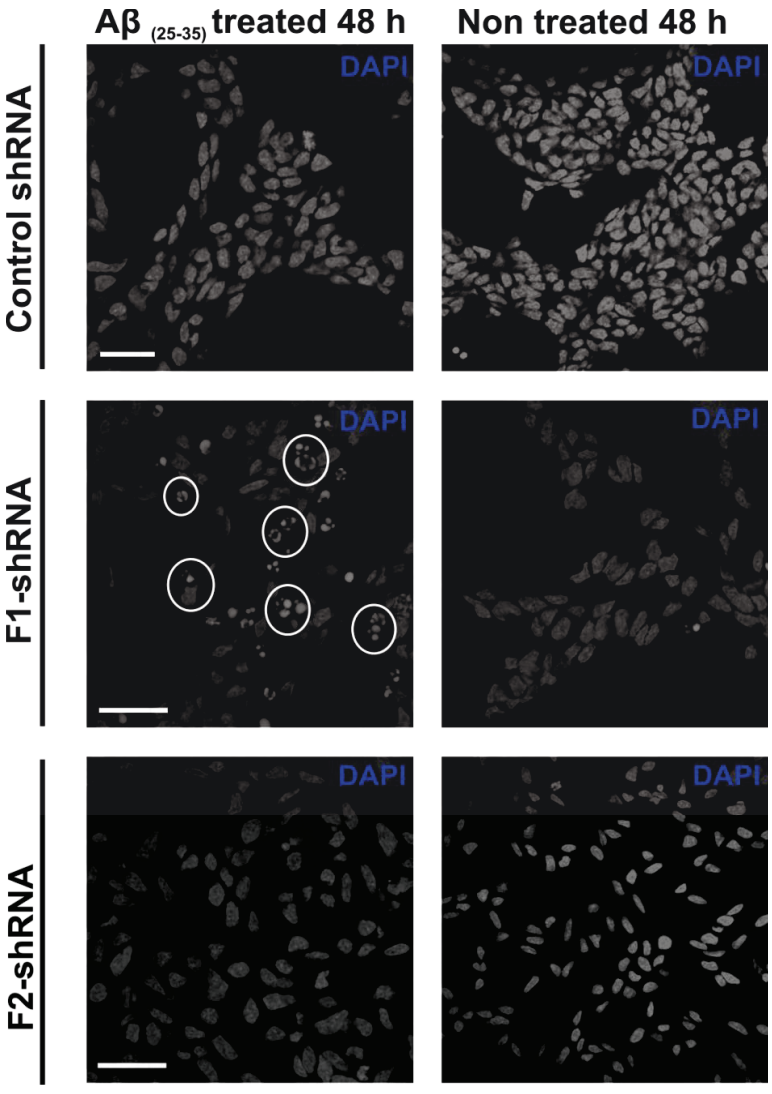


Figure 18: Nuclear fragmentation upon $A\beta_{25-35}$ treatment in differentiated SH-SY5Y cells

F2-shRNA, F1-shRNA and control SH-SY5Y cells were treated with $A\beta_{25-35}$. After 48 h, DAPI staining for nuclear fragmentation was performed.

We next sought to confirm these findings by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT) reduction assay as it is a widely employed tool to determine cell viability. In this assay, MTT dye added to the medium of the cell is taken up via endocytosis only by metabolically live cells and reduced to purple formazan crystals in the cell via mitochondrial nicotinamide adenine dinucleotide phosphate (NADH) dependent dehydrogenases.[129] The amount of formazan crystals precipitated in the cells was quantitated after dissolving the crystals by addition of dimethyl sulphoxide (DMSO).

MTT assay was performed after $A\beta_{25-35}$ treatment in shRNA flotillin-1, shRNA flotillin-2, and control SH-SY5Y cells. MTT was added to the cells at a concentration of 0.5 mg/ml and the relative amount of metabolized MTT formazan was then determined at the end of 72 h. Flotillin-1/2 knockdown SH-SY5Y cells pretreated with the neurotoxic peptide resulted in decreased MTT metabolism compared to the control cells (figure 19). Taken together, fully differentiated flotillin-1/2 knockdown cells are sensitive to $A\beta_{25-35}$ mediated neuronal toxicity.

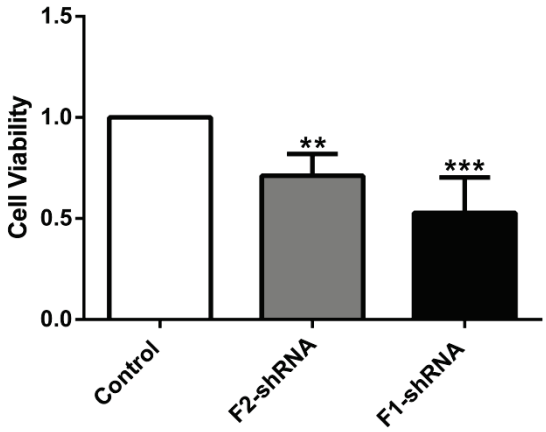


Figure 19: MTT assay in $A\beta_{25-35}$ treated SH-SY5Y cells

(A) Control, flotillin-1 or flotillin-2 depleted cells were cultured in 24-well plate and treated with 25 μM $A\beta_{25-35}$. Cell viability was determined by MTT assay as described in the materials and method section. Bars represent the mean ± S.D. of three independent experiments. One-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.9.3 Flotillin-1 and flotillin-2 depletion sensitizes cells to STS induced apoptotic cell death

Staurosporine (STS), a protein kinase inhibitor, has been shown to induce apoptosis in all mammalian cells studied so far and is a valuable tool in apoptosis studies. STS, at a concentration of 1 μM, induced cell death with apoptotic features in SH-SY5Y cells. As shown in figure 20, the incubation of flotillin-1 and flotillin-2 depleted SH-SY5Y cells with 1 μM STS caused a decrease in cell viability, as determined by MTT assay. A marked decrease in the number of viable cells upon depletion of both flotillin-1 and flotillin-2 was apparent with STS treatment at the end of 4 h.

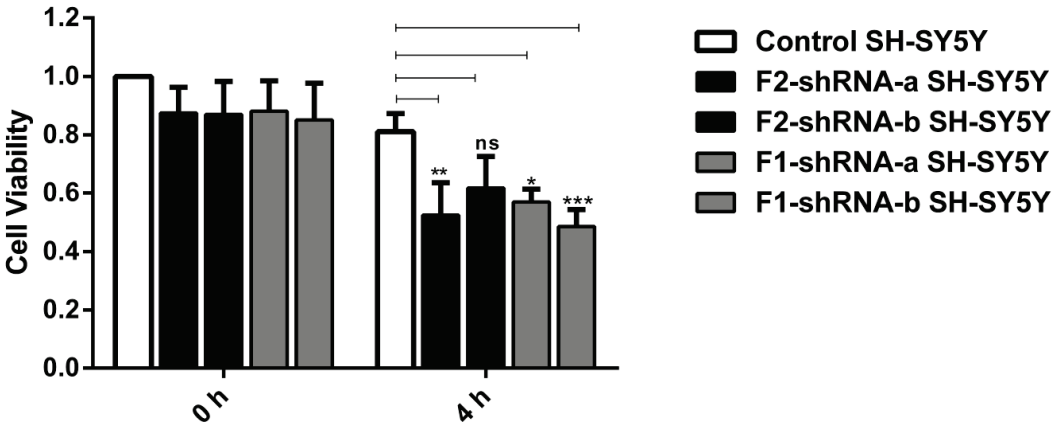
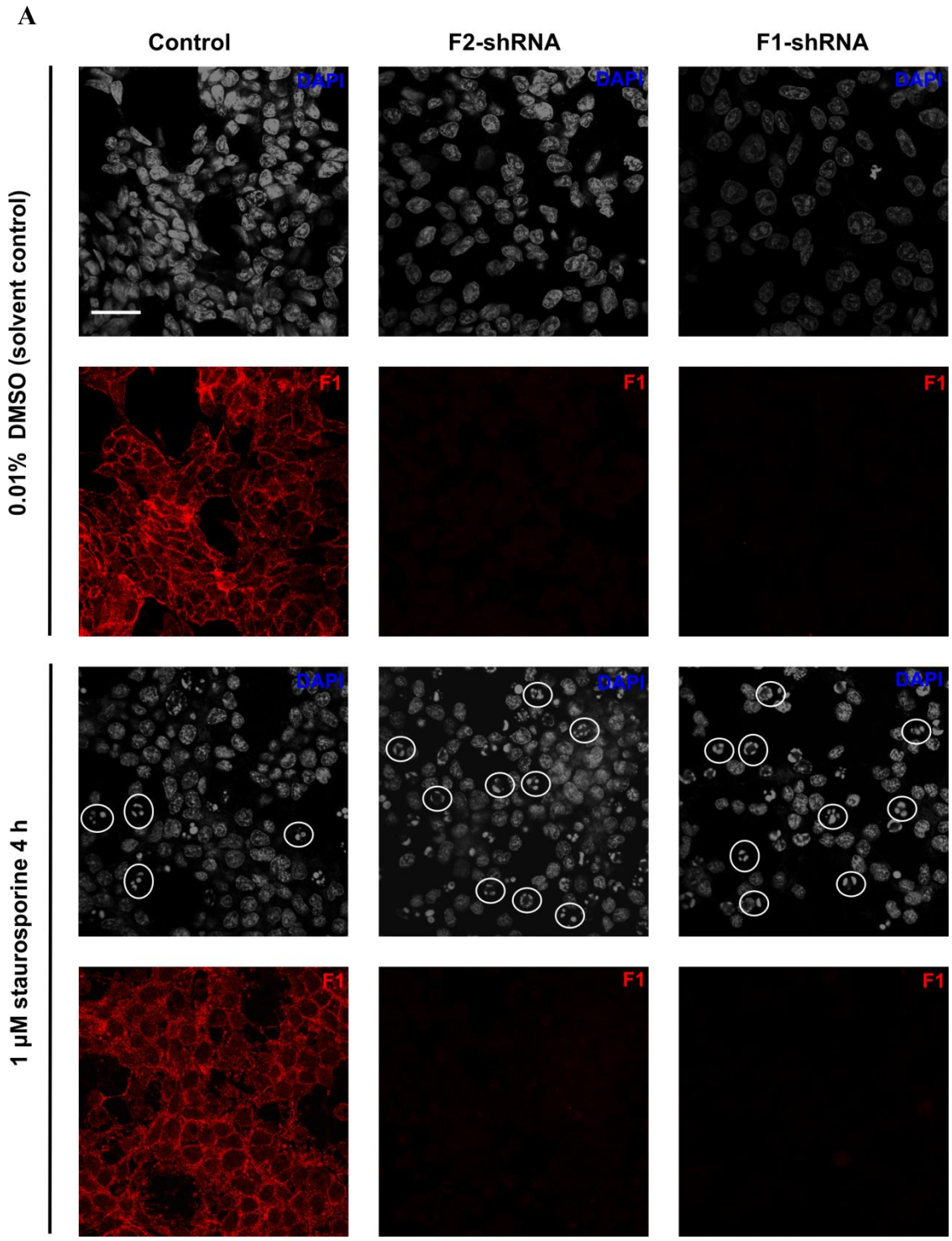


Figure 20: MTT assay in STS treated SH-SY5Y cells

Control, flotillin-1 or flotillin-2 depleted cells were cultured in 24-well plate and treated with 1 μM STS, followed by MTT assay as described in the materials and method section. Bars represent the mean ± S.D. of three independent experiments. Two-way ANOVA test. * $p < 0.05$, ** $p < 0.01$.

Nuclear fragmentation upon STS treatment in flotillin depleted cells was evaluated to confirm the above findings (figure 21). Nuclear fragmentation of the STS treated flotillin depleted cells was first evident after 2 h of incubation and was more intense after 4 h. Nuclear staining with DAPI revealed morphologic changes indicative of apoptosis, such as chromatin condensing and nuclear fragmentation. Although sporadic apoptotic nuclei could be seen at earlier time points, they became frequent after 4 h of STS incubation. Taken together, flotillin-1 and flotillin-2 depleted cells appeared to be the most sensitive and control cells the most resistant to the STS induced cell death.


A
Control
F2-shRNA
F1-shRNA
0.01% DMSO (solvent control)
1 µM staurosporine 4 h
DAPI
F1

B

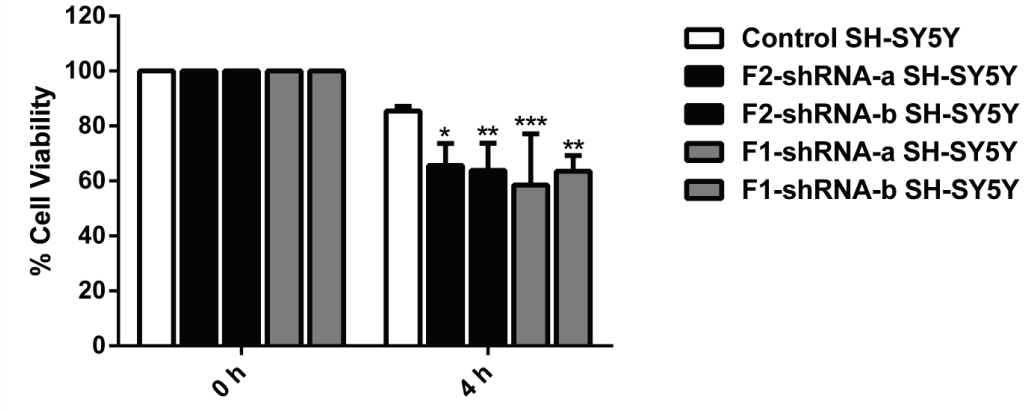


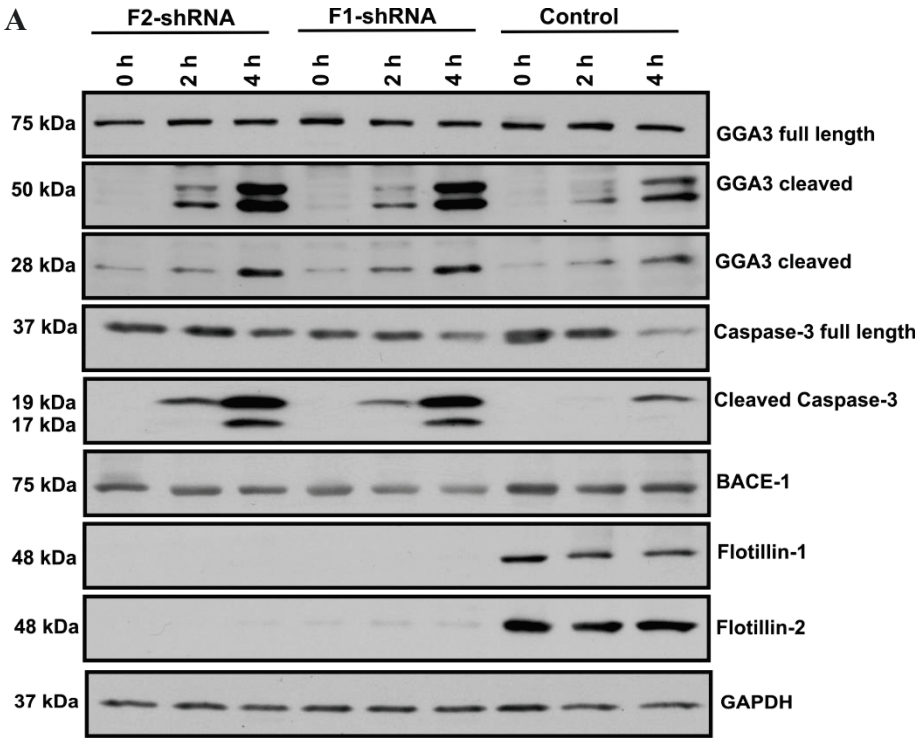
Figure 21: Nuclear fragmentation in STS treated SH-SY5Y cells.
(A) F2-shRNA, F1-shRNA and control SH-SY5Y cells were treated with 0.01% DMSO (solvent control) or 1 µM staurosporine. At 0 h and 4 h, DAPI staining for nuclear fragmentation was performed. Nuclei exhibiting apoptotic like morphology such as irregular-shaped nuclei and numerous apoptotic bodies (white circles) strongly stained with DAPI were observed after treatment. (B) Apoptotic cells were counted and percentage of cell viability was determined.

3.9.4 Characterization of apoptotic profile and signaling pathways in neuroblastoma cells upon STS induced apoptosis.

3.9.4.1 Flotillin depletion activates apoptotic markers

Caspase-3 is synthesized as a 32 kDa pro-form that is cleaved during the activation into a large fragment of 19 kDa[134], depending on the apoptotic signal, and a small fragment of 17 kDa[135]. To determine whether caspase-3 is activated upon STS treatment in flotillin depleted SH-SY5Y cells, we examined the presence of caspase-3 cleavage products using Western blotting. For this purpose, shRNA flotillin-1, shRNA flotillin-2 and control SH-SY5Y cells were treated with 1 µM STS for 2 h or 4 h time periods and we examined the activation of caspase-3 using immunoblot analysis. Treatment of flotillin depleted cells with STS readily resulted in cleaved caspase-3, with the appearance of small fragments (19 and 17 kDa), indicating that increased cleavage of the pro-caspase was coupled with the observed increase in cell death (figure 22). Since it has been previously shown by Tesco *et al*. that the BACE trafficking molecule GGA3 is cleaved by caspase-3 upon induction of apoptosis in H4 cells[62], we sought to check the expression of GGA3 by immunoblot in flotillin-1 or flotillin-2 knockdown and control SH-SY5Y cells. GGA3 was extensively cleaved in flotillin-1 or flotillin-2

depleted cells generating two major fragments, 48 kDa and 37 kDa, evidentiating that the absence of flotillins results in cellular susceptiblity to apoptotic damage. According to Tesco *et al*. BACE-1 is a stress-related protease that is upregulated in cerebral ischemia, and BACE-1 shows an inverse relationship to the levels of GGA3, an adaptor protein that is involved in BACE-1 trafficking.[62] Therefore, we tested the expression of BACE-1 in flotillin depleted and control neuronal cells, subjected to solvent vehicle treatment and STS treated settings. There was no apparent increase in BACE-1 levels/total cell protein during apoptotic time course.



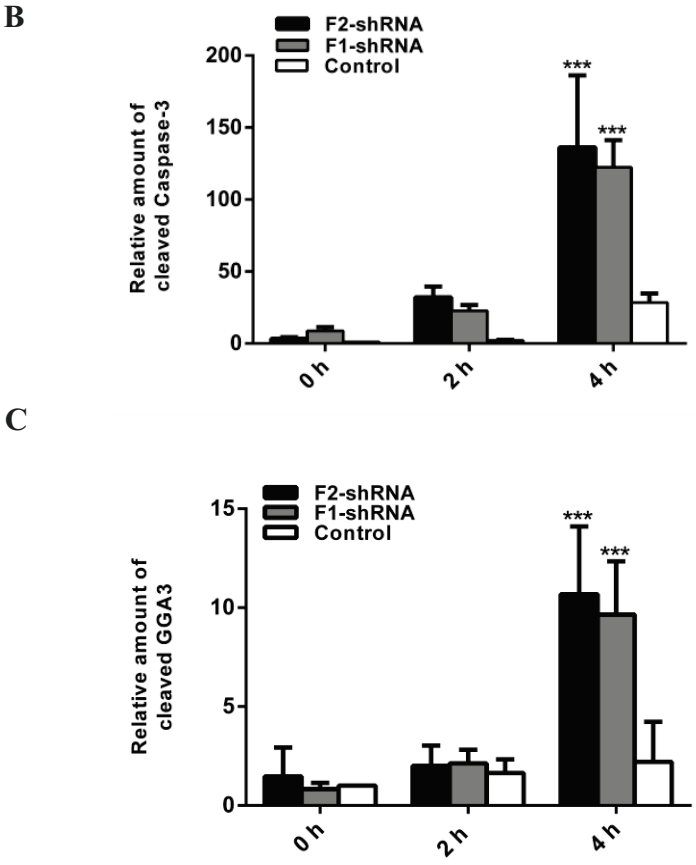


Figure 22: Activation of apoptotic markers upon STS induced apoptosis in shRNA flotillin-1, shRNA flotillin-2 or control SH-SY5Y cells.

(A) F2-shRNA, F1-shRNA or control SH-SY5Y cells were subjected to treatment with 0.01% DMSO (solvent control) or 1 µM STS for 2 h or 4 h, and Western blot analysis for expression of GGA3, caspase-3, BACE-1, flotillin-1, flotillin-2 and GAPDH were performed using specific antibodies.

(B, C) Quantification of cleaved caspase-3 and cleaved GGA3 was normalized to GAPDH.

3.9.4.2 Flotillin depletion upon STS treatment leads to apoptotic sensitivity in other cell lines

In order to test apoptotic sensitivity upon flotillin depletion in other cell lines, we used HeLa, HaCaT and MCF7 cells. A transient flotillin knockdown was mediated by siRNA in HeLa cell line and followed by treatment with STS for 2 h and 4 h. Additionally, flotillin stable knockdown HaCaT and MCF7 cell lines were subjected to STS treatment for 2 h and 4 h. At the end of the treatment, lysates were prepared and cleaved caspase-3 was determined for HeLa and HaCaT cell lines. Since MCF7 cells do not express caspase-3[130], another apoptosis marker, the cleaved GGA3 was detected by Western blotting. As shown in figure 23, enhanced cleaved caspase-3 was observed when both flotillin-1 and flotillin-2 were depleted in HeLa and HaCaT cells. In HeLa and HaCaT cells, an unchanged amount of flotillin-2 is expressed upon flotillin-1 depletion. Therefore, flotillin-2 depletion resulted in a higher caspase-3 cleavage as compared to the flotillin-1 depleted state. In MCF7 cells, faint GGA3 cleaved bands were visible in flotillin depleted cells as compared to the control cells. Taken together, these results indicate that flotillins are indispensible for survival as lack of their expression render different cell systems to apoptotic induction.

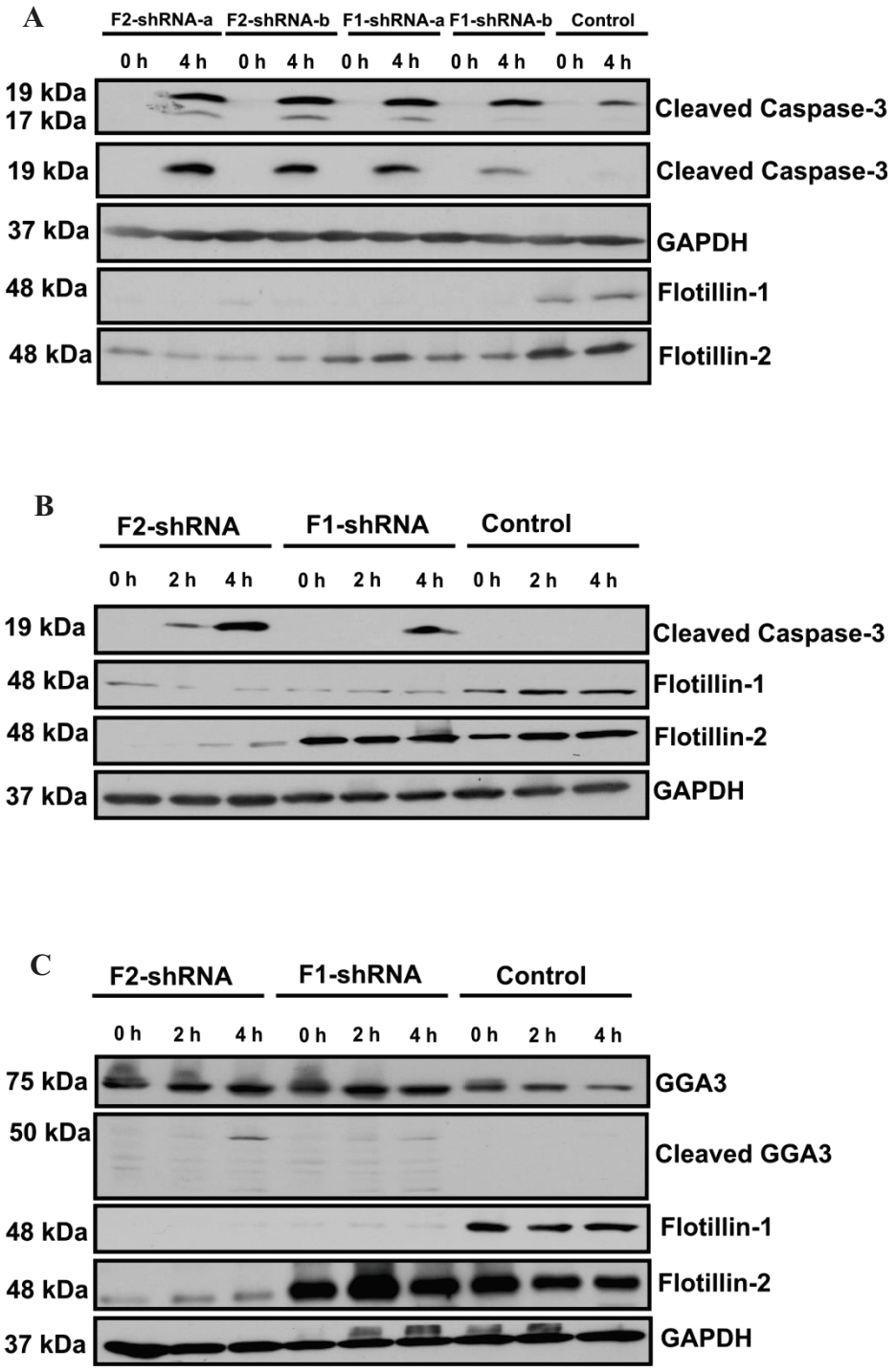


Figure 23: STS induced apoptosis in HeLa, HaCaT, MCF7 cell lines.

(A, B) Flotillin depleted and control HeLa and HaCaT cells were subjected to 1 µM STS treatment for 2 h or 4 h and lysates were prepared. Induction of apoptosis in HeLa and HaCaT was detected using caspase-3 antibody. (C) Flotillin depleted and control MCF7 cells were subjected to 2 µM STS treatment for 2 h or 4 h and lysates were prepared. Induction of apoptosis in MCF7 was detected by GGA3 antibody.

3.9.4.3 Flotillin depleted cells are sensitized to apoptosis induced by ER stressor, mitotic inhibitor and chemotherapy drugs

Endoplasmic reticulum (ER) is critically involved in protein metabolism. Normal ER function is required for the correct folding of many proteins and their post-translational modifications, such as glycosylation and disulfide bond formation. ER stress is induced by the disturbance of the environment in the ER lumen, such as the calcium homeostasis or the redox status, or by the disturbance of ER function, such as glycosylation and transportation to Golgi complex. One of the typical chemicals that induce ER stress is brefeldin A, which inhibits protein transport to the Golgi complex.[131,132] Thus, this chemical causes protein folding dysfunction, and the accumulated misfolded/unfolded proteins induce ER stress. To investigate the role of flotillins in the relationship between ER stress and apoptosis, shRNA flotillin-1, shRNA flotillin-2 and control SH-SY5Y cells were treated with brefeldin A for 24 h and cleaved caspase-3 was determined by Western blotting (figure 24A). Treatment with brefeldin A resulted in an accelerated caspase-3 cleavage in flotillin depleted cells as compared to the control cells. This led to the idea that flotillins are necessary for cell survival and their absence may result in cellular susceptibility to any apoptotic insult. In order to verify this hypothesis, flotillin-1 and flotillin-2 knockdown cells were exposed to various other apoptotic inducers and the extent of cellular death was determined by caspase-3 cleavage by Western blotting.

Compelling evidence indicates that paclitaxel kills cancer cells through induction of apoptosis by binding to the microtubules and causes kinetic suppression of microtubule dynamics. Although the biochemical events downstream from paclitaxel binding to the microtubules that lead to apoptosis are not well defined, it is apparent that this agent induces arrest of cell cycle at the mitotic phase and accentuates cytotoxicity.[133] Therefore, to examine whether flotillin depletion in general sensitizes cells to other forms of stress agents, shRNA flotillin-1, shRNA flotillin-2 and control cells were treated with paclitaxel for 24 h and the expression of cleaved caspase-3 was determined by Western blotting (figure 24 B). In accordance with the previous findings, flotillin-1 and flotillin-2 knockdown cells were the most sensitive to paclitaxel induced cytotoxicity, as indicated by increased caspase-3 cleavage upon apoptotic induction. Carboplatin and doxorubicin are common cancer chemotherapeutic drugs that induce apoptosis in various cell systems. To further confirm the findings that flotillins depletion result in apoptotic sensitivity, shRNA flotillin-1, shRNA flotillin-2 and

control SH-SY5Y cells were treated with carboplatin for 24 h or doxorubicin for 20 h followed by cleaved caspase-3 detection by Western blotting. Consistent with previous observations, flotillin depleted cells were severely affected by both carboplatin (figure 24C) and doxorubicin (figure 24D) chemotherapy cancer drugs in contrast to control cells. Taken together, these results indicate that flotillins are relavant for survival and inevitable for mediating resistance against various forms of apoptotic stimuli.

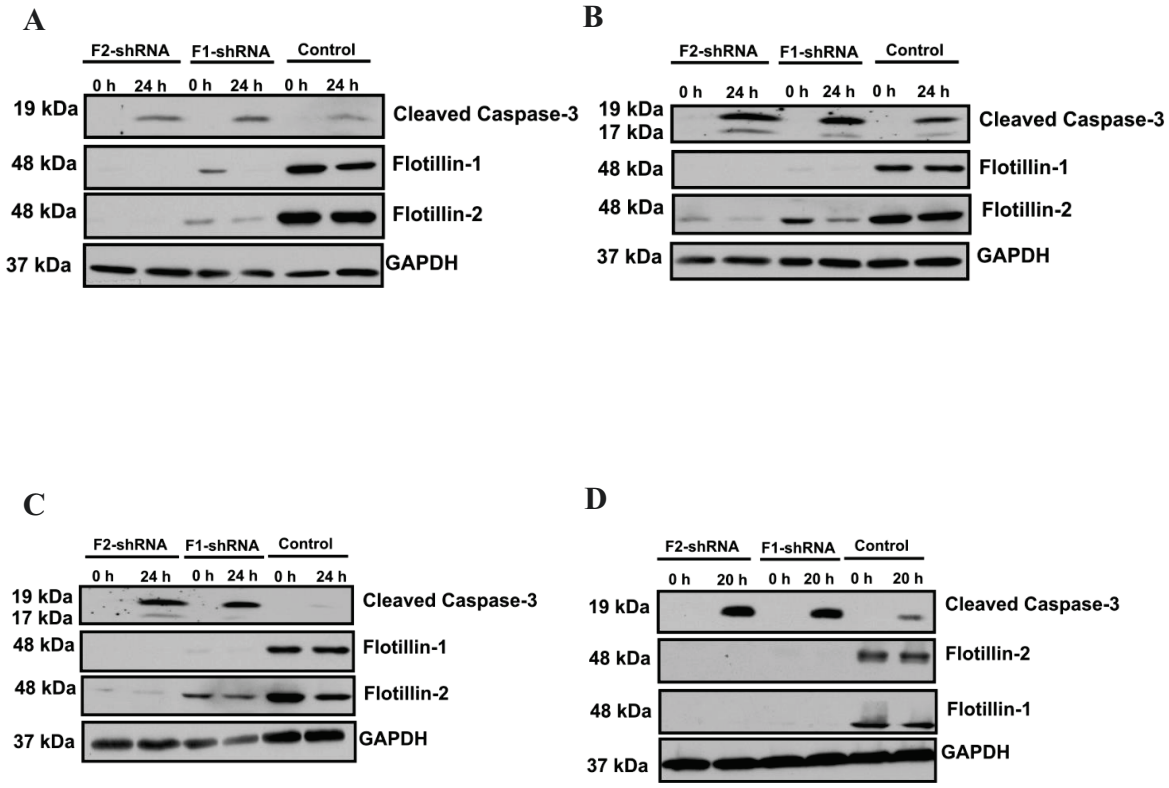


Figure 24: Various apoptotic agents induce apoptosis in flotillin-1 and flotillin-2 knockdown SH-SY5Y cells.

F2-shRNA, F1-shRNA or control SH-SY5Y cells were treated with (A) 1µg/ml of Brefeldin A for 24 h (B) 24 nM Paclitaxel for 24 h, (C) 500 µM Carboplatin for 24 h and (D) 0.5 µM Doxorubicin for 20 h, and Western blot analysis for the expression of caspase-3, flotillin-1, flotillin-2 and GAPDH was performed using specific antibodies.

3.9.4.4 Flotillin depletion affects ERK and Akt signaling pathway

It has been recently suggested that ERK is an important effector in a pathway that mediates cell survival.[136,137] Therefore, we investigated whether the inhibition of ERK signaling pathway would affect STS induced apoptosis in flotillin depleted cells. Untreated flotillin depleted cells displayed a significant decrease in the minimal basal levels of phospho ERK as compared to the non treated control cell (figure 25). Upon STS induced apoptosis, the phospho ERK was completely inhibited in shRNA flotillin-1, shRNA flotillin-2 and control cells. Next, in order to determine the involvement of PI3K/Akt pathway, we assessed the total Akt expression and checked the Akt activity by immunoblotting with specific antibody against phosphorylation of Ser473 under normal and STS induced apoptosis in flotillin-1 or -2 knockdown and control cells. As shown in figure 25, untreated flotillin depleted cells displayed a significant decrease in the mininal basal levels of phospho Akt Ser473 as compared to the non treated control cells. Upon STS induced apoptosis, the phospho Akt was completely inhibited in shRNA flotillin-1, shRNA flotillin-2 and control cells supporting the inhibition of Akt activity upon STS induced apoptosis. Intrestingly, STS treatment in flotillin knockdown cells resulted in reduction of total Akt levels in contrast to the control cells. The quantification of total Akt levels normalised to GAPDH in flotillin depleted cells compared to control cells showed a tendency to decreased total Akt levels but did not reach a significant value due to variations between the experiments.

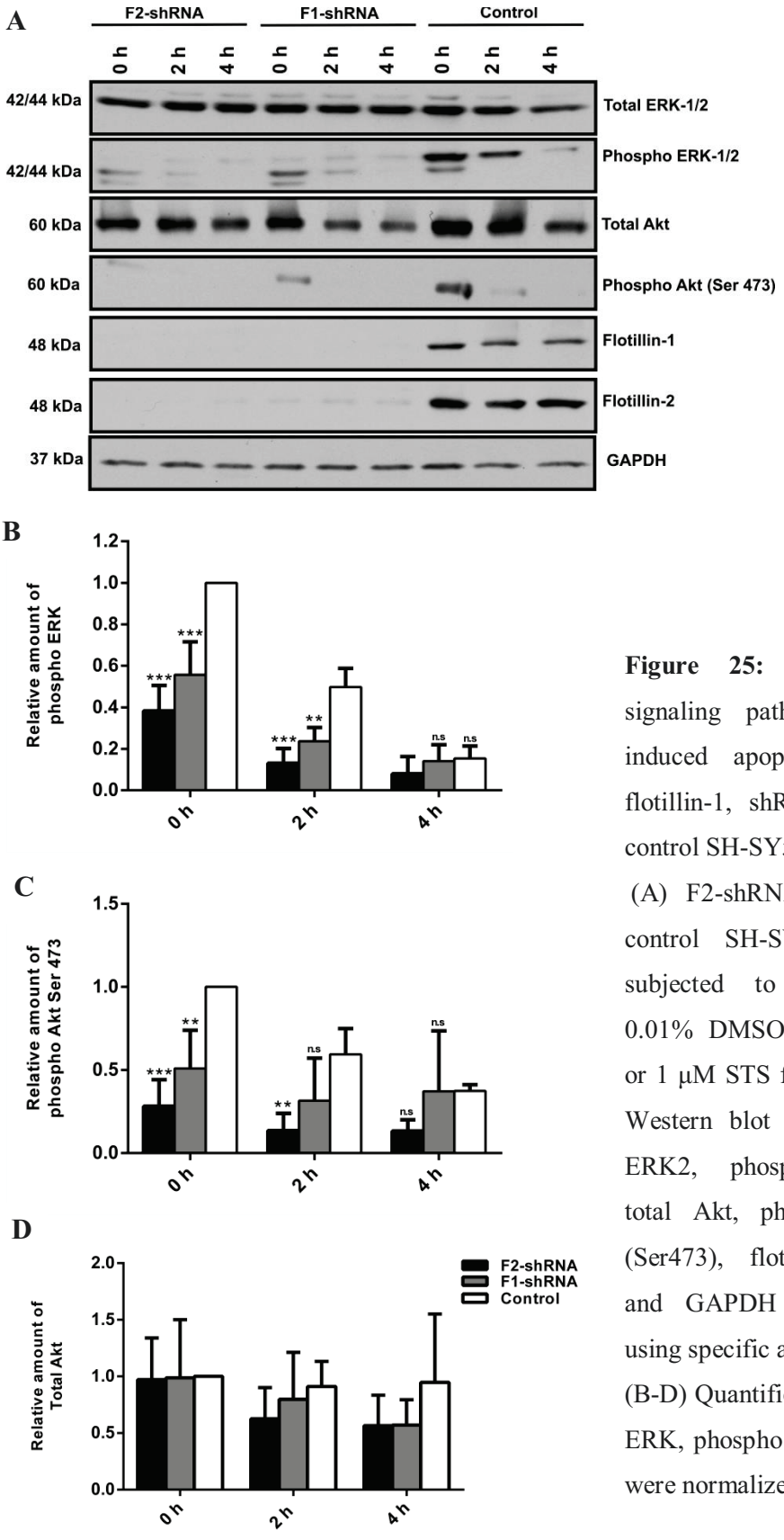


Figure 25: ERK and Akt signaling pathway upon STS induced apoptosis in shRNA flotillin-1, shRNA flotillin-2 or control SH-SY5Y cells.

(A) F2-shRNA, F1-shRNA or control SH-SY5Y cells were subjected to treatment with 0.01% DMSO (solvent control) or 1 μM STS for 2 h or 4 h, and Western blot analysis for total ERK2, phosphorylated ERK, total Akt, phosphorylated Akt (Ser473), flotillin-1, flotillin-2 and GAPDH were performed using specific antibodies.

(B-D) Quantifications of phospho ERK, phospho Akt and total Akt were normalized to GAPDH.

3.9.4.5 Ectopic expression of Akt promotes PI3K signaling and retards apoptosis

To test whether the sensitization of flotillin-1 and flotillin-2 knockdown cells to STS induced apoptosis was due to impairment in Akt activity, a vector encoding for wild-type AKT cDNA was employed in this study. It has been previously demonstrated that an overexpression of wild-type Akt leads to protection of cells from a variety of apoptotic stimuli, including treatment with DNA-damaging agents, PI3K inhibitors, Fas-crosslinking agents, UV (or γ) irradiation, c-myc overexpression, growth factor withdrawal, TGF β treatment, matrix detachment, or cell cycle perturbation.[138] Additionally, a mutant of Akt that has a dominant negative phenotype was employed to verify the involvement of Akt in mediating survival of flotillin depleted cells. A constitutively active Akt, myr Akt, which has been previously shown to enhance viability in other apoptotic systems,[139,140] was also tested.

To elucidate the cellular mechanisms by which Akt inhibits STS induced apoptosis, shRNA flotillin-1, shRNA flotillin-2 and control SH-SY5Y cells were transiently transfected with WT Akt, CA Akt, DN Akt and empty vector, and the expression of these constructs was verified by immunoblotting. WT Akt expression protected flotillin-1 or flotillin-2 depleted cells from STS induced apoptosis, as evidenced by reduction in cleaved caspase-3 levels by Western blotting (figure 26). On the other hand, DN Akt kinase or vector transfected cells continued to exhibit an accelerated caspase-3 cleavage upon apoptosis induction. Following the treatment with STS, the flotillin-1 or flotillin-2 deprived cells and control cells expressing CA Akt exhibited poor survival as compared to the cells expressing WT Akt. However, the initial hypothesis was that the expression of CA Akt would render apoptotic resistance to these cells as it has been previously reported by several research group, that an ectopic expression of CA Akt retards the rate of cell death when subjected to stress conditions. One of the explanations for this inconsistency is based on the recent reports by Nogueira and co-workers that strong activation of Akt increases oxidative stress and sensitizes cells to reactive oxygen species (ROS) mediated cell death.[141] Moreover, rapamycin, an mTOR inhibitor strongly sensitizes Akt-expressing cells to apoptosis by an inhibitory negative feed back mechanism as mTOR can inhibit Akt activity.[142] In line with this, flotillin-1 and flotillin-2 knockdown cells expressing CA Akt do not show significant reduction in apoptotic cell death as prolonged activation of Akt negatively influences the survival and cannot delay the caspase-3 cleavage upon STS induced

apoptosis. Taken together, these results indicate that the maximal effect on survival was demonstrated upon overexpression of WT Akt, and loss and/or hyperactivation of Akt function fails to inhibit ongoing apoptosis.

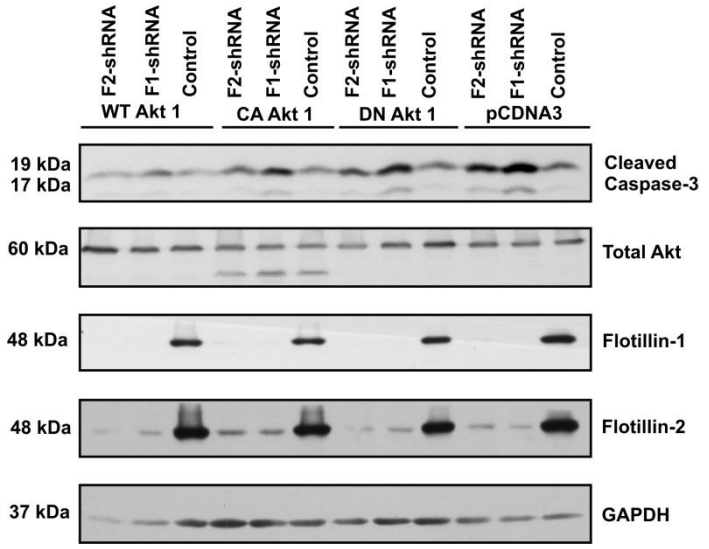


Figure 26: Overexpression of WT Akt 1 rescues STS induced apoptosis in SH-SY5Y cells. F2-shRNA, F1-shRNA or control SH-SY5Y cells were transfected with control empty vector, WT Akt1, CA Akt1 or DN Akt1 construct and cultured in the presence 1 μM STS for 4 h.

3.9.4.6 Effect of abnormal Akt activity on its downstream targets upon flotillin depletion

In hindsight, activation of Akt, catalysed by PDK1, results in phosphorylation of its downstream target FOXO proteins at serine and threonine sites. Consequently, their DNA binding is impaired, and increased binding to the chaperone protein 14.3.3 takes place. In order to determine the expression levels, FOXO3a and Ser253 phospho specific FOXO3a antibodies were used in this experiment. Typically, FOXO3a is dephosphorylated upon apoptotic stimuli and it is evident from figure 27 that 4 h incubation with STS triggered dephosphorylation of FOXO3a at Ser253 site. Usually, the profile of phosphorylation of FOXO3a at Ser 253 is indicative of its inactive status, and is a result of Akt phosphorylation at Ser473 site. The reason for this is that the activated Akt rapidly phosphorylates FOXO3a and restricts FOXO3a to cytosol and curtails the activation of death genes. Therefore, we postulated that flotillin depleted cells that are sensitive to apoptotic stress should have low basal phosphorylation of FOXO3a compared to the control cells, and by this means dephosphorylated FOXO3a

continues to remain in an active state and triggers the transcription of death genes. Strikingly, the results obtained were inverse to our hypothesis, as control cells showed a lesser phosphorylation of FOXO3a at Ser253 compared to the flotillin-1 and flotillin-2 depleted cells. At this point, a plausible reason for this discrepancy is not available but we speculate that phosphorylation of FOXO3a may be less due to declining levels of total FOXO3a in control cells.

In the next step, we sought to examine the expression of pro-apoptotic proteins whose unrestrained activity would otherwise consign cells to death. Akt/PKB is a major survival kinase that is capable of inactivating pro-apoptotic proteins, and one of the potential targets of this survival kinase includes regulators of mitochondrial function such as the Bcl-2 family member, Bad. The proapoptotic protein, Bad has been suggested to link survival signals to the mitochondrial cell death machinery.[143] Henceforth, Western blot analysis was performed to evaluate the expression of Bad in STS treated flotillin-1 and flotillin-2 knockdown cells compared to the control cells. Corroborating with the previous findings, STS treated, flotillin depleted cells showed a high expression of Bad protein compared to the control cells. Furthermore, it has been previously described that Akt-phosphorylated Bad binds to 14.3.3 proteins and becomes consequently sequestered in cytosol. This interaction prevents 14.3.3 from homodimerizing and inactivating anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-X) at the mitochondrial membrane.[144,145] Moreover, a recent report from Clapp and coworkers demonstrated that cell death induced by multiple stresses can be counteracted by 14.3.3 proteins.[146] Therefore, the expression levels of 14.3.3 in shRNA flotillin-1, shRNA flotillin-2 and control cells were detected using a pan 14.3.3 antibody which identifies all 14.3.3 human isoforms. After 4 h STS treatment of flotillin depleted cells, two bands were detected by pan 14.3.3 antibody and they appeared to be the strongest in flotillin-1 and flotillin-2 depleted cells in contrast to control cells. These results indicate that 14.3.3 protein is cleaved by active caspase-3 upon STS induced apoptosis and this effect is apparent in flotillin depleted cells.

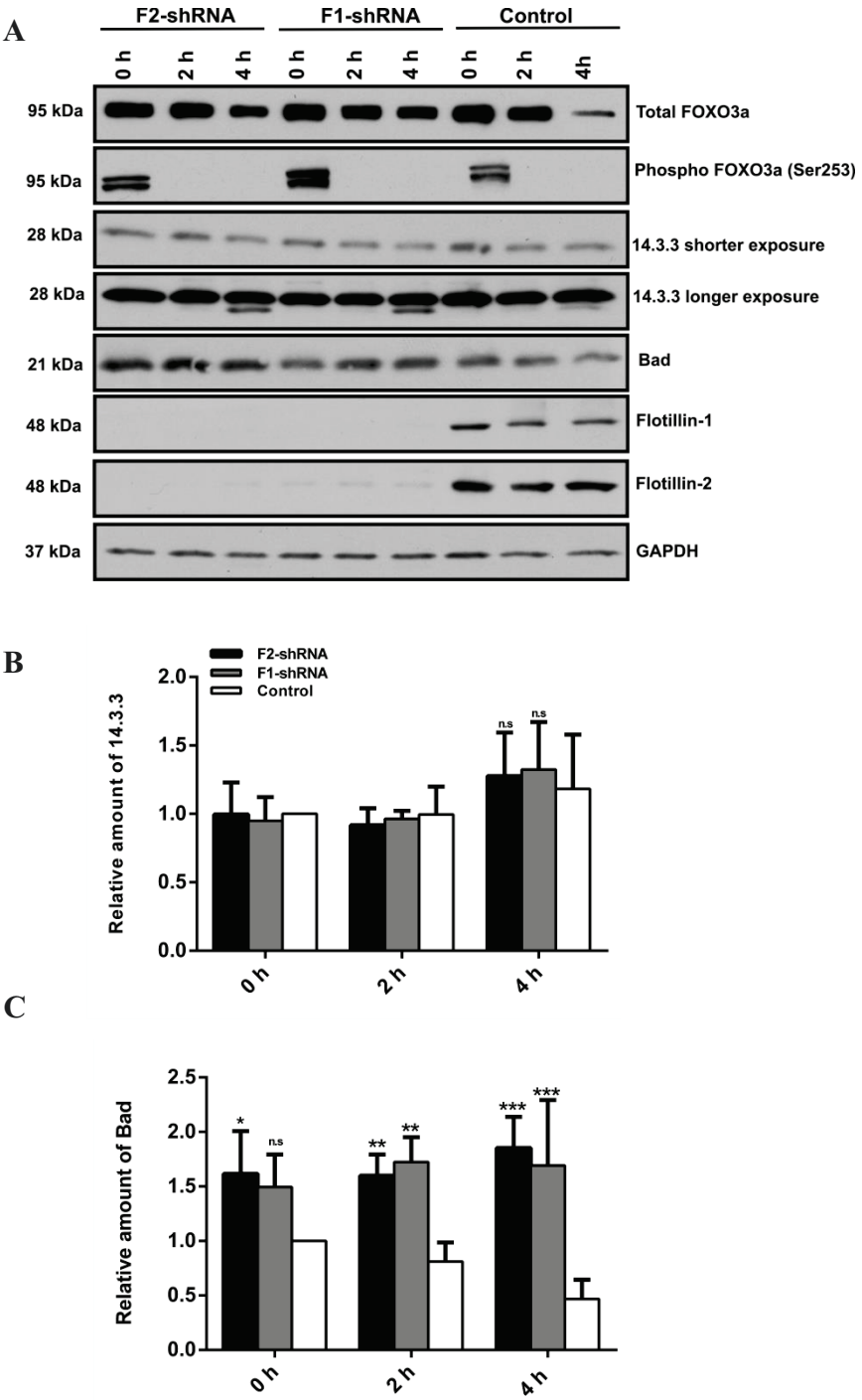


Figure 27: Effect of STS on shRNA flotillin-1, shRNA flotillin-2 or control SH-SY5Y cells. (A) F2-shRNA, F1-shRNA or control SH-SY5Y cells were subjected to treatment with 0.01% DMSO (solvent control) or 1 μM STS for 2 h or 4 h, and Western blot analysis for expression of total FOXO3a, phosphorylated FOXO3a (Ser253), 14.3.3, Bad, flotillin-1, flotillin-2 and GAPDH were performed using specific antibodies. (B,C) Quantification of 14.3.3 and Bad was normalized to GAPDH. Two way ANOVA test was performed and comparisons were made with respect to the control in each group. The data corresponding to figure 22, 25 and 27 are part of the same Western blotting experiment.

3.9.5 Staurosporine causes nuclear translocation of FOXO3a in flotillin depleted cells

In order to activate the apoptotic machinery, FOXO3a has to reside in the nucleus, indicating that STS may also affect the subcellular localization of this transcription factor in the flotillin depleted SH-SY5Y cells. To test this, we performed nuclei fractionation on shRNA flotillin-1, shRNA flotillin-2, and control cells after treatment with STS for 2 h that preceded the induction of FOXO3a expression. As shown in figure 28, endogenous FOXO3a resided primarily in the cytosol of the untreated cells, whereas nuclear localisation was negligible. Poly ADP ribose polymerase (PARP) is a classic nuclear as well as apoptotic marker that is present in the nuclear fraction only, and upon STS induced apoptosis is cleaved into two fragments. FOXO3a was equally distributed between cytosol and nuclei in STS treated control cells, whereas the flotillin depleted cells showed an increased FOXO3a translocation from the cytosol to nucleus upon STS treatment, although a negligible amount of FOXO3a was present in cytosol as well. These results support our previous findings that flotillin depletion sensitizes the cells to undergo accelerated apoptosis and the translocation of FOXO3a from cytosol to nucleus upon STS induced apoptosis triggers the downstream apoptosis machinery.

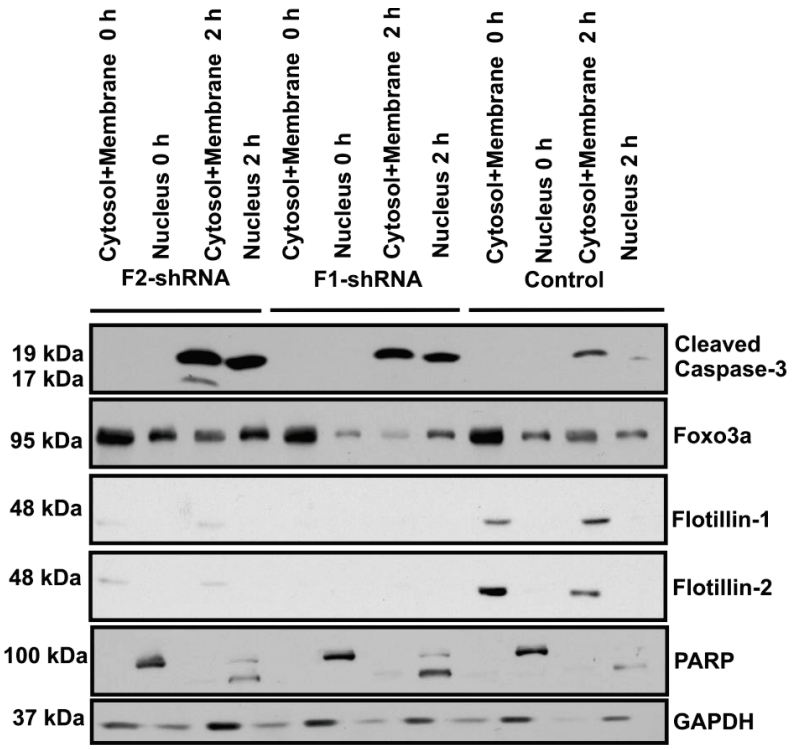


Figure 28: Nuclear localisation of FOXO3a in SH-SY5Y cells upon STS induced apoptosis. F2 shRNA, F1 shRNA and control SH-SY5Y cells were treated with 1 µM STS. Cytoplasmic/membrane and nuclear protein lysates were prepared at 0 h and 2 h. Protein expression levels were analyzed by Western blotting using antibodies against specific antibodies against FOXO3a, caspase-3, flotillin-1, flotillin-2, PARP and GAPDH.

3.9.6 Apoptotic sensitivity due to flotillin depletion correlates with decreased levels of Mcl-1

Mcl-1 is an anti-apoptotic member of Bcl-2 family of proteins that has a critical role in regulating the delicate balance between survival and death signals.[147] Moreover, Mcl-1 has a short half life and consequently rapid fluctuations occur in the level of Mcl-1 protein levels. The presence of growth factors such as EGF can enhance Mcl-1 protein levels and stress agents can enhance degradation of Mcl-1. Therefore, to assess whether Mcl-1 expression is altered in flotillin depleted and control neuroblastoma cells, we studied Mcl-1 protein levels in shRNA flotillin-1, shRNA flotillin-2, and control cells at steady state, EGF treatment for 10 min and STS treatment for 4 h (figure 29). In stark contrast to control cells, a marked reduction of Mcl-1 protein levels was observed in flotillin-1 and flotillin-2 knockdown cells in steady state. In the same experiment, pretreatment with EGF for 10 mins demonstated low level of Mcl-1 protein in flotillin-1

or -2 depleted cells compared with control cells. Upon STS induced apoptosis, Mcl-1 protein underwent degradation after 4 h in shRNA flotillin-1, shRNA flotillin-2, and control cells. Corroborating with all previous findings, an accelerated caspase-3 cleavage was detected only in flotillin-1 and flotillin-2 knockdown cells. Herein, we determined that a minimal expression level of Mcl-1 is critical for cell survival and combating apoptotic stimulus. Flotillin-1 and flotillin-2 knockdown cells exhibit low expression of basal Mcl-1 protein levels and may contribute to apoptosis upon flotillin depletion.

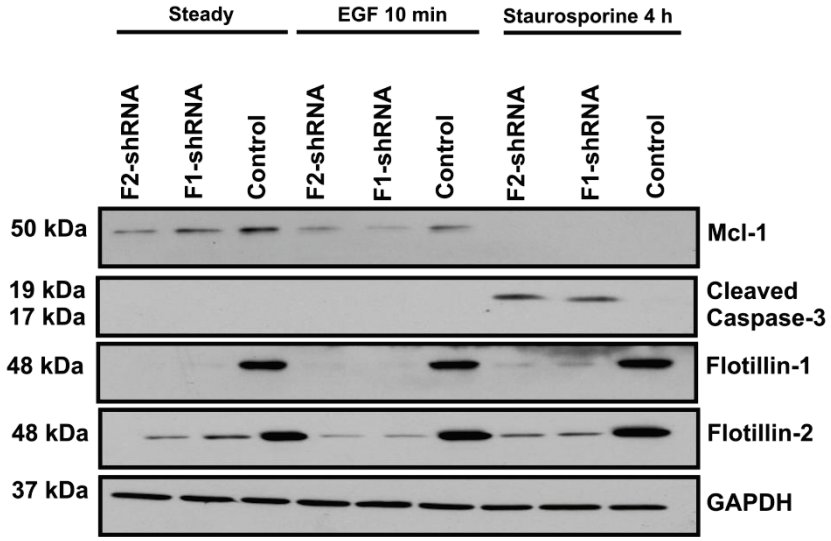


Figure 29: Mcl-1 degradation and caspase activation in STS treated SH-SY5Y cells.
F2-shRNA, F1-shRNA and control SH-SY5Y cells were treated with 0.01% DMSO (solvent control) or with 100 ng/ml EGF for 10 min or 1 μM STS for 4 h. Total cell lysate was analysed by Western blot with Mcl-1, caspase-3, flotillin-1, flotillin-2 and GAPDH antibodies.

As shown in figure 29, a short EGF treatment or STS treatment for 4 h did not result in major changes in Mcl-1 protein levels. Therefore, Mcl-1 protein expression levels were tested upon different settings, including a prolonged EGF treatment for 2 h and a short STS treatment for 30 min as these settings could facilitate more stablity of Mcl-1 protein. For this purpose, Mcl-1 protein levels were checked in shRNA flotillin-1, shRNA flotillin-2 and control SH-SY5Y cells under steady state, stavation for 16 h, EGF stimulation for 2 h, and STS treatment for 30 min. As shown in figure 30, flotillin depleted cells show low expression of Mcl-1 (indicated by arrow sign) as compared to

the control cells. These results indicate that flotillin depletion show reduced Mcl-1 protein levels under different settings. Although, a slight increase in Mcl-1 protein level was observed in control cells upon EGF stimulation but STS induced apoptosis resulted in degradation of Mcl-1 primarily in flotillin knockdown cells followed by the control cells.

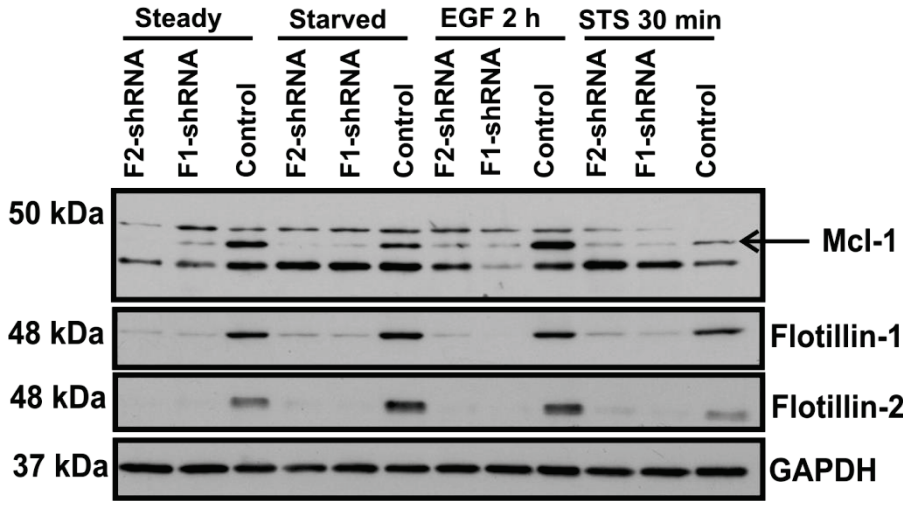


Figure 30: Mcl-1 protein levels remain less in flotillin depleted cells upon prolonged EGF treatment and restrained STS treatment

F2-shRNA, F1-shRNA and control SH-SY5Y cells were treated with 0.01% DMSO (solvent control) or with 100 ng/ml EGF for 2 h or 1 µM STS for 30 min. Total cellular lysate was analysed by Western blot analysis with Mcl-1, flotillin-1, flotillin-2 and GAPDH antibodies.

3.9.7 Bcl-2 overexpression in flotillin depleted cells decreases caspase-3 activation and cell death.

Upon STS treatment, flotillin-1 and flotillin-2 depleted cells shows enhanced cellular pyknosis, nuclei fragmentation, and cell death. In order to evaluate the functional role played by Bcl-2 in preventing apoptosis induced by STS, shRNA flotillin-1, shRNA flotillin-2, and control cells were transiently transfected with pEGFP vector encoding Bcl-2. Next step was to determine if the expression of this anti-apoptotic protein could reverse the cytotoxic effects observed after STS treatment. The proto-oncogene Bcl-2 protects the cells from apoptotic cell death in several lesion models by inactivating Bax via heterodimerization and/or preventing the release of cytochrome C from mitochondria and further caspase activation. Overexpression of Bcl-2 in flotillin-1 or flotillin-2 depleted SH-SY5Y cells followed by STS treatment for 4 h

significantly reduced the caspase-3 cleavage as compared to the vector transfected flotillin depleted cells. Consistent with the previous results, the cleaved caspase-3 levels in flotillin depleted cells expressing Bcl-2 or pEGFP was always high in comparison to the control cells (see figure 31). Taken together, STS induced caspase-3 activation was strongly inhibited by Bcl-2 overexpression and consequently provided resistance to apoptosis.

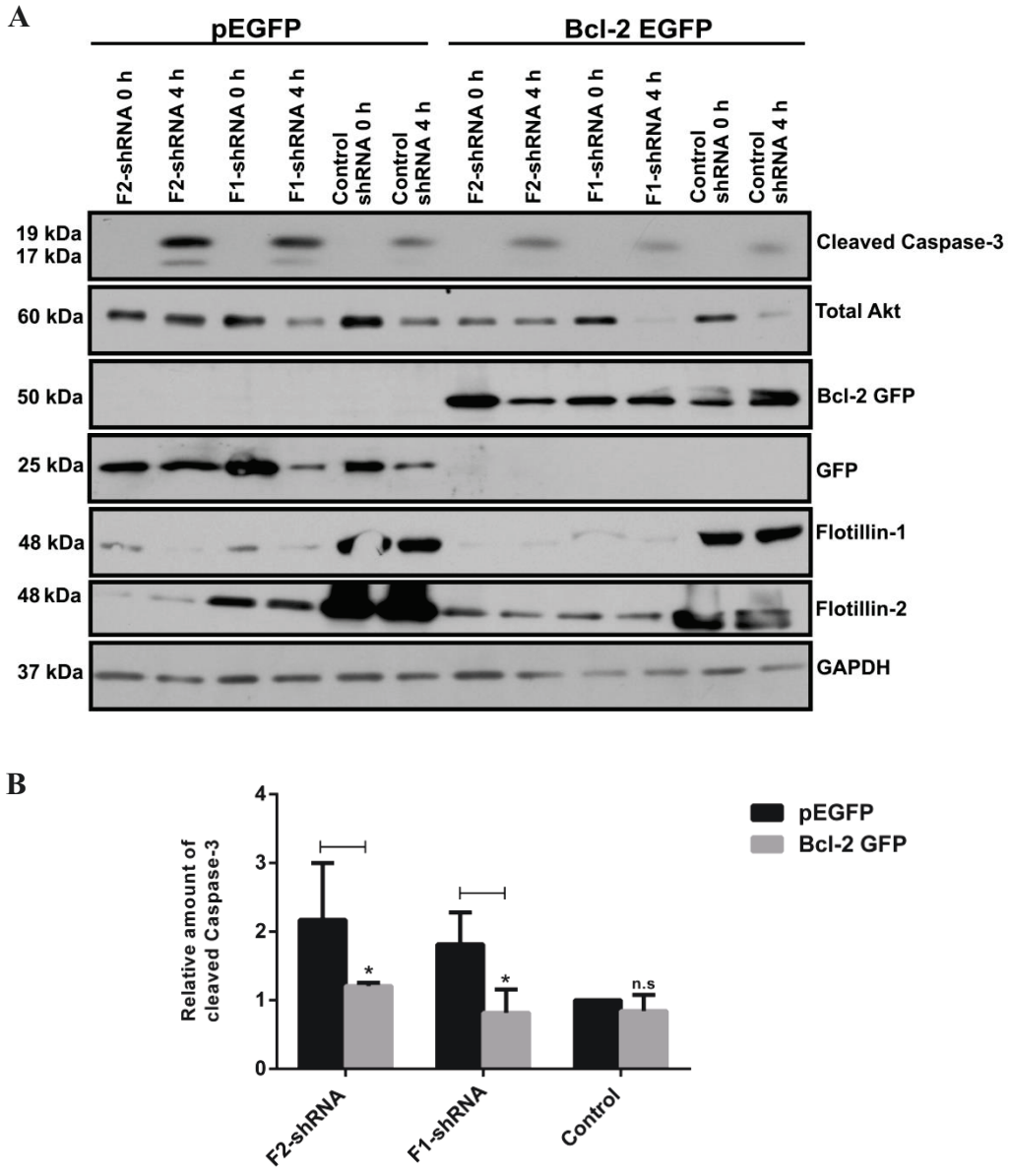


Figure 31: Overexpression of Bcl-2 rescues staurosporine induced apoptosis in SH-SY5Y cells. F2-shRNA, F1-shRNA or control SH-SY5Y cells were transiently transfected with control pEGFP and Bcl-2 GFP and cultured in the presence 0.01% DMSO (solvent control) or 1 μM staurosporine for 4 h. Total cell lysates were analysed by Western blot with caspase-3, total Akt, flotillin-1, flotillin-2, GFP, GAPDH.

3.9.8 Phorbol esters protect flotillin depleted cells from STS induced apoptosis

Phorbol-12-myristate-13-acetate (PMA) activates members of the PKC family and transduces signals that regulate diverse biological functions. PMA can substitute for diacylgylycerol, the endogenous activator of PKC, and stimulate the downstream signaling pathway of PKC. Various studies have suggested that PMA activates the Raf/MEK/ERK pathway efficiently via PKC activation in many cell types.[148] To evaluate if PMA treatment conferred resistance to apoptotic stimulus, we examined the effect of PMA on STS induced caspase-3 cleavage in flotillin-1 and flotillin-2 knockdown cells. Consistent with the previous findings, treatment of shRNA flotillin-1, shRNA flotillin-2, and control cells with STS alone resulted in apoptosis as indicated by the enhanced caspase-3 cleavage in flotillin depleted cells compared to the control cells. When PMA was added along with STS for 4 h, cleaved caspase-3 was relatively reduced in flotillin depleted and control cells compared to the STS alone induced apoptosis. Phospho ERK 42/44 signals were observed in flotillin-1/-2 knockdown and control cells upon PMA treatment alone. STS treatment completely inhibited phospho ERK signals and a combination of STS and PMA could not restore phospho ERK 42/44. As shown in figure 32, after 4 h the PMA induced survival effect was most effective in control cells whereas the cells depleted of flotillins were severely affected upon STS treatment and PMA treatment could only partially prevent apoptosis, as indicated by the reduction in caspase-3 cleavage.

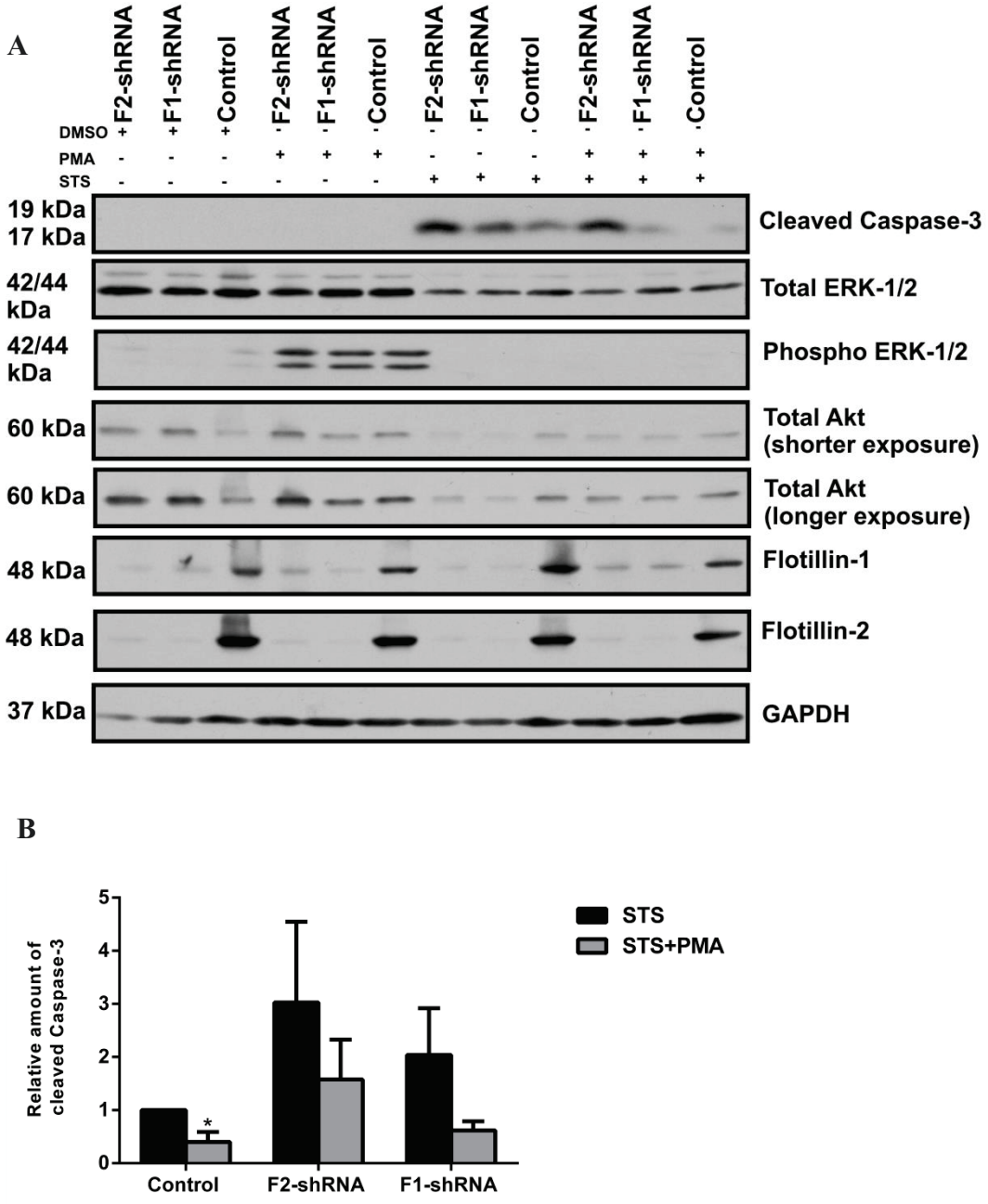


Figure 32: Reduced staurosporine induced apoptosis upon PMA pre-treatment in SH-SY5Y cells.

Flotillin-1 shRNA, flotillin-2 shRNA or control SH-SY5Y cells were treated with either 0.01% DMSO (solvent control), 50 ng/ml PMA alone for 10 min, 1 µM staurosporine for 4 h or a combination of PMA and staurosporine for 4 h. Total cell lysates were prepared and Western blotting analysis for the the expression of caspase-3, total ERK, phospho ERK, total Akt, flotillin-1, flotillin-2 and GAPDH was performed using specific antibodies.

CHAPTER 4: DISCUSSION

4.1 Flotillins bind to BACE-1 via ACDL motif

A previous report from Hattori *et al.* showed that flotillin could be coprecipitated in overexpressed BACE-1 from HEK293 cells, and flotillin-1 overexpression resulted in an increased association of BACE-1 with detergent resistant membrane domains.[125] In the aforementioned study, three major aspects were not addressed: a) the role of flotillin-2 binding to BACE-1, b) whether the binding of flotillin-1 to BACE-1 is direct or indirect, and c) the significance of the ACDL motif in mediating BACE-1 and flotillin interaction. Our results indicate that flotillin-1 binds strongly and specifically to the BACE-1 WT tail, whereas the LLAA mutant shows only trace binding to flotillins. The ACDL motif within the BACE-1 cytoplasmic tails provides sorting signals that are essential for proper intracellular trafficking.[149] Newly synthesized pro-BACE-1 is transported from ER to Golgi. In Golgi complex, the pro-peptide is removed from BACE-1 and BACE-1 is subjected to post-translational modifications.[149] The trafficking and the localization of BACE-1 are largely dependent on the ACDL motif, composed of DISLL residues, in its cytoplasmic tail.[149] Among the post-translational modifications of BACE-1, phosphorylation of the serine 498 residue (Ser498) is crucial for binding of BACE-1 to the GGA monomeric adaptor proteins which are involved in the sorting of cargo between the trans-Golgi network and endosomes and vice versa.[64,150]

Deletion of the ACDL motif[151] or mutation of the leucines to alanines[56] affects the subcellular distribution of BACE-1, such that a large extend of this protease is localized at the cell surface. The ACDL of BACE-1 binds to the members of the GGA protein family, which are involved in the sorting of cargo proteins between TGN and endosomes. A similar cluster of acidic amino acids followed by a di-leucine motif is present in the cytoplasmic tail of CD-MPR proteins, and this motif is required for the sorting of lysosomal enzymes by the receptor. Typically, a mutant of MPR46, in which the leucine pair was replaced by alanines (MPR46 LL/AA) shows an altered intracellular distribution and trafficking.[152] As with BACE-1, the di-leucine motif in MPR46 tail is required for a sorting event in endosomes.

Another example of a di-leucine motif consisting protein is the EGFR C-terminus which contains a sequence located between amino acid residues 675 and 697, encompassing a

di-leucine motif at residues 679 and 680. This di-leucine motif is necessary for efficient lysosomal transport of ligand-occupied full-length EGF receptors.[153] We questioned whether flotillins exhibit a general binding to any protein harboring this motif or if flotillins are cargo specific. Our protein interaction studies revealed that flotillins bind specifically to the ACDL motif of BACE-1, but fail to bind to the ACDL motif in CD-MPR (in this study) or to the ACDL motif in EGFR tail (M.Meister thesis).

4.2 Flotillin-1 depletion influences the localization and expression of BACE-1

Our study suggests a novel role for flotillins in intracellular/endosomal trafficking of transmembrane proteins. Our results show that flotillin knockdown resulted in an increase in BACE-1 staining and accumulation of BACE-1 in intracellular structures. These result hints towards a direct role of flotillins in endosomal trafficking of a transmembrane protein such as BACE-1. Earlier studies associated with flotillins and endocytosis suggests that flotillins are involved in internalization of GPI-anchored proteins such as CD95 and of gycosphingolipid GM1.[154] Flotillins are known to regulate the endocytosis of cholera toxin which is mediated by its binding to GM1[155], but a direct role of flotillins in the endocytosis of the Shiga toxin[156] or plant toxin ricin[156] is not known. Flotillin depletion increases the toxicity of Shiga toxin and ricin and it might be that flotillins are involved in the retrograde transport of these toxins. These three toxins are transported via the retrograde pathway directed towards Golgi/TGN and the ER. Similarly, it has been suggested that retrograde transport of internalized BACE-1 from endosomal compartments to the plasma membrane occurs via the Golgi/TGN route.[157] Henceforth, our results for the first time implicate a role of flotillins in endosome-Golgi transport of a transmembrane protein, as all previously reported flotillin dependent cargo molecules are transported by lipids. It has been shown that the protease activity of BACE-1 is influenced by various post–translational and cell biological events.[158] Mature BACE-1 primarily localizes within the cholesterol-rich lipid rafts and replacing the BACE-1 transmembrane domain with a GPI anchor exclusively targets BACE-1 to lipid rafts and substantially increases Aβ production.[159] Indeed, various types of lipids accentuate BACE-1 activity and three cysteine residues within the cytoplasmic tail of BACE-1 are palmitoylated,[160] which promotes raft localization. There is a possibility that specific lipids might be required for BACE-1

transport to Golgi and this may involve specific membrane microdomains characterized yet again by flotillins.

4.3 Flotillins and GGAs compete for the same sorting motif in BACE-1

It is a well established idea that BACE-1 acidic di-leucine motif interacts with specialized monomeric adaptor proteins such as GGA1-3 and is responsible for the recruitment of receptors to the coated vesicles on the TGN for transport to endosomes and finally to lysosomes.[64]All three GGA proteins appear to be involved in the trafficking of BACE-1 as depletion of any of the three caused a significant BACE-1 re-distribution. It has been suggested that GGA3 controls the transport of BACE-1 towards lysosomes for degradation whereas GGA1 and GGA2 mediate endosome TGN trafficking.

In this study, we took cue from three known aspects of GGAs with respect to BACE-1 trafficking: a) amongst the GGAs, depletion of GGA3 results in stabilization and increase of BACE-1 levels due to delayed lysosomal degradation[62,161], b) GGAs bind to the tail of other proteins containing similar ACDL motifs, such as CD-MPR[162], and c) depletion of GGAs affects BACE-1 trafficking.[62,64] We verified that flotillins also used a similar mechanism in regulating BACE-1 trafficking. Both GGA proteins and flotillin-1 bind to the same sorting signal in BACE-1. Therefore, we checked if the binding of GGAs and flotillin-1 is cooperative or competitive. Interestingly, absence of flotillins resulted in an increased binding of GGA2 to the BACE-1 ACDL motif, implicating that GGA2 and flotillin-1 compete for the same binding site.

At present, we can speculate three important aspects of GGA-Flotillin-BACE-1 interplay: a) flotillin and GGA2 compete for the same binding motif in BACE-1, b) presence of flotillin-1 reduces the binding of GGA2 , and c) depletion of either flotillins or GGA proteins results in endosomal accumulation of BACE-1. It is apparent that flotillin depletion does not prevent but enhances binding of GGA2 to the ACDL motif. This hints towards a binding mechanism in which GGA2 binds to BACE-1 first and later flotillins displace GGA2, culminating in an effective and stronger flotillin-1 binding to the ACDL motif. These results indicate that binding of flotillin-1 to BACE-1 might be a pre-requisite for sorting of BACE-1 into endosomal compartments. Taken together, flotillin depletion produces a BACE-1 trafficking phenotype which is also

apparent upon depletion of GGAs. This confirms that flotillin-1 depletion phenocopies the depletion of GGAs in BACE-1 accumulation and stabilization of this protease.

4.4 Role of flotillins in Alzheimer's disease: Revisited

It has been shown that depletion of flotillin-2 results in an altered trafficking and processing of APP. Our results show that depletion of flotillins results in an accumulation of BACE-1 and this can influence APP processing. It is known that amyloidogenic processing pathway mediated by a concerted action of β-secretase and γ-secretase occurs in lipid rafts. Our results indicate that flotillin depletion result in enhanced BACE-1 levels and eventually in increase of C-99 fragments. Our results fit well with the findings of Tesco and colleagues, who demonstrated that an inhibition of GGA3 through siRNA led to an elevation of BACE-1, C-99, and Aβ.[62] On the other hand, Schneider *et al*. suggested that flotillin-2 depletion results in decreased amyloidogenic processing of APP, due to plasma membrane retention and inefficient endocytosis of APP.[47] However, these data cannot be directly co-related with our findings as they used N2a cell line which was subjected to enforced expression of a mutant APP (so-called Swedish mutant) whose processing is profoundly different from the endogenous, wildtype protein. Additionally, they used a mouse neuroblastoma N2a cell line which might not provide appropriate information pertaining to a human APP processing mechanism. In contrast, we have used HeLa cell line and human neuroblastoma SH-SY5Y cell line which can be used as a suitable cellular system for studying APP processing. Moreover, Very recently, Nichols group generated a mouse model for Aβ-dependent cerebral amyloidosis, APPS1 mice, combined with ablation of either flotillin-1 alone, or both flotillin-1 and flotillin-2.[48] According to them, reduced Aβ and amyloid plaques were observed in the flotillin-$1^{-/-}$ and flotillin-$1^{-/-}$, flotillin-$2^{-/-}$ mice expressing the mutant APP and a mutant human presenilin-1 (PS1). However, their results provide only limited information regarding the molecular basis of flotillin function in APP trafficking and its processing. Firstly, Nichols group used a very severe and artificial model encompassing an enforced expression of both APP and presenilin. Therefore, this system cannot mirror the precise role of flotillins in physiological APP processing. They state that only a minor reduction in Aβ production was observed compared to the controls. Typically, Aβ production is accelerated upon exogenous expression of APP or PS1 and it is most likely that an enforced expression of

both these proteins might have masked any significant changes in Aβ level upon flotillin-1 or both flotillin-1 and flotillin-2 loss. Therefore, further studies need to be carried out using physiological representative mouse models to investigate the potential *in vivo* effects of flotillin ablation on APP processing.

4.5 Flotillins are essential in mediating resistance to cytoxicity

In recent years, possible implications of lipid rafts in cell physiology and in the maintenance of cell homeostasis have been sought with immense curiosity. Specifically, the possibility of lipid rafts to be involved in the complex framework instructing the apoptotic cascade has been investigated in a series of research work carried out in different cell systems.[163,164] Lipid rafts are enriched in glycosphingolipids, and several studies have accentuated that these structures act as a clutch for propagating receptor-mediated cell death process. It has been validated that CD95/Fas receptor-mediated clustering occurs in lipid rafts; thereby, controlling both the fate and the activation of T cells.[165,166] These findings indicate that lipid rafts play an active role in making life and death decisions. For this reason, we addressed the significance of flotillins in maintaining a delicate balance between life and death since flotillin proteins define these membrane microdomains. The above mentioned idea also stems from a finding from our group indicating that the central survival pathways such as MAP kinase and Akt signaling were affected upon flotillin-1 knockdown.[37]

4.6 Flotillin depletion sensitizes neuroblastoma cells to various apoptotic inducers

In order to determine cytotoxicity, preliminary experiments were conducted using a synthetic amyloid beta peptide, and we opted for $A\beta_{25-35}$ as it is the shortest fragment that exhibits large β-sheet fibrils and retains toxicity of the full-length peptide. It has been shown that extrinsic apoptotic pathway involving TNF-related-apoptosis inducing ligand (TRAIL) mediates neuronal cell death invoked by $\beta AP_{25-35,}$ and inhibition of TRAIL pathway results in neuroprotection.[167] Therefore, we checked for the *in vitro* effect of the exogenously applied synthetic $A\beta_{25-35}$ on differentiated flotillin depleted or control neuroblastoma cells and determined the cytotoxicity with MTT assay and nuclear fragmentation assay. According to the MTT assay, flotillin-1 depleted cells were the most sensitive to $A\beta_{25-35,}$ followed by flotillin-2 knockdown cells. In contrast, control cells were more resistant to $A\beta_{25-35}$ synthetic peptide. DNA

fragmentation is a late event in apoptosis and typically evident at the end of a coordinated cell death event. Therefore, visible fragmented nuclei were primarily observed in flotillin-1 knockdown SH-SY5Y cells.

Next, we used STS which has been long used *in vitro* as an initiator of apoptosis in many different cell types, and is a widely used stress inducer. We show in the present study that STS induces relatively high levels of apoptosis, as indicated by caspase-3 cleavage upon flotillin depletion in SH-SY5Y, HeLa, and HaCaT cell lines. In neuroblastoma cells, STS results in apoptotic sensitivity in flotillin depleted cells, as evidenced by caspase-3 cleavage, GGA3 cleavage, 14.3.3 cleavage, and upregulation of Bad. Additionally, flotillin depleted SH-SY5Y cells were sensitized by various other apoptotic inducers including PKC inhibitor- STS, ER stress inducer- brefeldin A, cancer chemotherapeutic drugs- doxorubicin, paclitaxel, and carboplatin. One of the major reasons for the apoptotic sensitivity in the absence of flotillins was a PI3K/Akt signaling defect. Flotillin depleted cells showed diminished levels of total Akt, phospho-Akt and phospho-ERK upon STS induced apoptosis. The direct downstream target of Akt is FOXO3a and a major proportion of FOXO3a was localized in the nucleus of flotillin knockdown cells, implicating that FOXOs are active in these cells and trigger the transcription of death genes. Additionally, an essential anti-apoptotic molecule and a major cancer target, Mcl-1, was inherently downregulated in flotillin knockdown cells. The neuroblastoma cells were rescued from undergoing permanent damage due to STS induced apoptosis by overexpression of anti-apoptotic Bcl-2. Phorbol esters are well known PKC activators, and pre-treatment of neuroblastoma cells with phorbol esters along with apoptotic agent also reduced caspase-3 cleavage. According to our study, the two main reasons for increased apoptotic sensitivity upon flotillin depletion are de-regulation of anti-apoptotic proteins and a defect in survival signaling pathway (see below).

4.6.1 De-regulation of anti-apoptotic proteins in flotillin depleted cells

A number of proteins can directly or indirectly influence apoptosis, and the best characterized apoptotic regulators include Bcl-2 family of proteins. According to the very popular, “primed for death” hypothesis, cancer cells are continuously exposed to death signals and they largely depend on the composition and the amount of anti-apoptotic Bcl-2 proteins for survival. Many studies have highlighted that de-regulation of Bcl-2 and other anti-apoptotic family members is one of the key defining features of

cancer cells in comparison to normal cells. Bcl-2 gene and protein amplification has been discovered in various malignancies, including chronic lymphocytic leukaemias[168], small cell lung cancers[169], and breast carcinomas[170]. Pertinently, it has been demonstrated that not only does the overexpression of the anti-apoptotic members of Bcl-2 family play a role in cancer development, but their elevated expression can also be associated with resistance to cancer therapeutics, including chemotherapy and radiotherapy. Among the many Bcl-2 inhibitors, obatoclax in combination with carboplatin and etoposide in patients with extensive-stage small cell lung cancer produced promising outcomes.[171] It is seen that when other anti-apoptotic Bcl-2 family members are ectopically expressed, the loss of Mcl-1 can be tolerated in cancer cells.[172] Our results show that Mcl-1 expression is reduced in flotillin depleted neuroblastoma cells. Therefore, we overexpressed Bcl-2 in flotillin depleted neuroblastoma cells to verify if the enforced expression of another anti-apoptotic molecule could compensate for the loss of Mcl-1. Pertinently, the ectopic expression of Bcl-2 rescued the neuroblastama cells from undergoing concomitant apoptosis.

Mcl-1 is unique among pro-survival Bcl-2 molecules in that it is essential for early (lethal at embryonic day 3.5) embryonic development[173] as well as for the survival of multiple cell lineages including lymphocytes[174], hematopoietic stem cells[175], neutrophils[176], and neurons[177]. Interestingly, germline ablation of Mcl-1 leads to peri-implantation embryonic lethality, at such an early embryonic developmental stage, no other anti-apoptotic molecule is similarly required.[173] This study underscores the fact that Mcl-1 is undoubtedly an essential and indispensible molecule for survival of several cell types. In the developing nervous system, ablation of Mcl-1 leads to widespread neuronal apoptosis and embryonic lethality.[177] Mcl-1 is one of the most highly amplified genes in a variety of human cancers and its expression is often associated with chemotherapeutic resistance and relapse.

There has been an increasing interest in developing Bcl-2 and Mcl-1 inhibitors as a cancer therapeutic strategy, focusing on impeding anti-apoptotic activity to promote cell death. Very recently, ligands and modified peptides that bind specifically to the Mcl-1 BH3 binding groove have been developed, but so far appear to be effective only when combined with chemotherapeutic agents.[178] According to our study, ablation of flotillins in neuroblastoma cells can impair Mcl-1 expression, and this can probably retard cancer cell survival and improve sensitivity to chemotherapeutic agents. Henceforth, cellular

systems deprived of flotillins can inhibit Mcl-1 that typically assists in tumor growth and survival.

4.6.2 Enhanced apoptosis in flotillin depleted cells is a consequence of survival signaling defect

Akt is implicated in antagonizing neuronal apoptosis, and it is well accepted that Akt regulates apoptosis by impinging on the activity of proteins and gene expression both directly and indirectly. Additionally, it has been shown that flotillin-1 depletion impairs phosphorylation of Akt at Ser473 and flotillin-1 directly interacts with proteins of MAPK cascade.[37] Therefore, in this study, we investigated the PI3K/Akt and MAPK signaling pathway in response to STS induced apoptosis. We found that Phospho Akt Ser473 and Phospho ERK 42/44 were significantly inhibited in flotillin depleted neuroblastoma cells when subjected to STS induced apoptosis. STS treatment for 4 h resulted in reduction of total Akt in the absence of flotillins. Ectopic expression of Akt inhibits apoptosis as exemplified by reduced caspase-3 cleavage and rescues flotillin depleted cells from undergoing apoptosis.

Akt activation induces different cell survival mechanisms and constitutes the central pathway for survival upon death inducing stimuli. Akt inactivates by phosphorylation the pro-apoptotic factors Bad and caspase-9, as well as the Forkhead family of transcription factors that induce the expression of pro-apoptotic factors such as Fas ligand.[138] Moreover, Akt activation has been related in cancer cells with increased resistance to apoptosis induced by TRAIL/APO-2L (TNF related Apoptosis Inducing Ligand), a member of the TNF superfamily that has been shown to have selective anti-tumor activity.[179] A more recently identified substrate of Akt is the apoptosis signal regulating kinase 1 (Ask1), which stimulates MAP kinase kinases that eventually activate the JNK and p38 MAP kinases.[180] Typically, Akt activation inhibits apoptosis by promoting the increased expression of survival molecules or the degradation of pro-apoptotic molecules. Additionally, P13K/Akt/PTEN pathway has become an attractive target for drug development as such agents might inhibit proliferation and reverse the repression of apoptosis and the resistance to cytotoxic therapy in cancer cells.

In this study, we also investigated the downstream target of Akt which is FOXO proteins. FOXO family proteins have emerged as master regulators that control a plethora of cellular activities through regulation of different patterns of gene expression

in response to diverse stimuli.[181] Here, we focused on the effect of Akt on FOXO3a because total Akt was inhibited in flotillin depleted cells upon STS induced apoptosis. FOXO3a is phosphorylated by Akt at Ser253, and phosphorylation of these amino acids provides binding sites for 14.3.3 proteins, resulting in the retention of FOXO3a by 14.3.3 in the cytoplasm. We had postulated that flotillin depleted cells that are sensitive to apoptotic stress should have low basal phosphorylation of FOXO3a compared to the control cells. By this means, dephosphorylated FOXO3a continues to remain in an active state and triggers the transcription of death genes. However, we observed that control cells showed a lesser phosphorylation of FOXO3a at Ser253 compared to the flotillin-1 and flotillin-2 depleted cells. At this point, a plausible reason for this discrepancy is not available but we propose that phosphorylation of FOXO3a may be less due to declining levels of total FOXO3a levels in control cells. In fact, proteosome pathway is involved in the degradation of FOXO3a, and apoptotic stimulus results in reduction of total FOXO3a levels in a time dependent manner. Additionally, at the end of 4 h STS treatment, control cells showed a degradation of FOXO3a, whereas flotillin depleted cells exhibited inefficient degradation of FOXO3a. This effect can be explained based on the findings by Lin *et al*. that flotillin-1 depleted MCF7 cells showed a marked upregulation of FOXO3a compared to control cells.[43] Therefore, an upregulation of FOXO3a upon flotillin depletion could result in an increased accumulation compared to the control cells. In this study, we have shown that treatment of flotillin knockdown SH-SY5Y cells with STS also resulted in an accumulation of FOXO3a in the nucleus, whereas FOXO3a is equally distributed between cytosol/nuclei in control SH-SY5Y cells. This observation would suggest that there is a threshold for the amount of FOXO3a present in nucleus that is required to induce apoptosis.[182–184] A resonable explanation would be that the apoptosis induced by STS is in part mediated by increased nuclear translocation of FOXO3a in flotillin knockdown cells, thereby allowing transcription of FOXO target genes that promote apoptosis.

Besides Akt signaling pathway, another important pathway in regulating survival signals is the MAPK/ERK signaling pathway. Activation of ERK 1/2 has been shown to inhibit apoptosis in response to a wide range of stimuli, such as tumor necrosis factor (TNF), Fas ligand, TRAIL, hypoxia, growth factor withdrawal, nitric oxide, hydrogen peroxide, and chemotherapeutic agents.[185] In response to most of these stimuli, ERK 1/2 undergoes either transient or prolonged activation, fostering an anti-apoptotic effect. In contrast, inhibition of ERK 1/2 promotes apoptosis. In mammalian

cells, ERK 1/2 signaling can block apoptosis at levels upstream, downstream or induce changes in mitochondrial transmembrane potential and cytochrome C release. ERK 1/2 is capable of inactivating pro-apoptotic Bcl-2 family member Bad through phosphorylation at Ser112. Apart from suppressing the functions of pro-apoptotic proteins, ERK 1/2 can promote cell survival by enhancing the activity of anti-apoptotic molecules, such as Mcl-1, which is phosphorylated at Thr163 by ERK1/2, thus increasing its stability and anti-apoptotic activity.[185] More direct evidence hails from the fact that caspase-9 is regulated by ERK 1/2. Therefore, ERK1/2 plays a significant role in facilitating anti-apoptotic effects by downregulating pro-apoptotic molecules via a decrease in their activity or a reduction of their protein expression by transcriptional repression. It has been recently shown that flotillins are downstream transcriptional targets of ERK signaling.[186] Flotillin transcription is not only regulated by ERK, but also by the retinoic X receptor complexes. The roles of flotillins are implicated in MAPK signaling pathways, including the EGF receptor, insulin receptor, and TrkA receptor. Furthermore, with respect to the fibroblast growth factor (FGFR) mediated MAPK signaling, flotillin-1 interacts with both FRS2 and FRS3 scaffolding proteins.[40] Our group has reported the dual role of flotillin-1 in EGFR activation and MAPK downstream signaling.[37] An EGF stimulation in siRNA mediated flotillin-1 knockdown HeLa cells results in diminished activation of the receptor tyrosine kinase. Flotillin-1 is able to bind to three primary MAPK components, including C-Raf, MEK1 and ERK2 simultaneously and consequently modulate ERK activation and regulate transcriptional regulation. In the above mentioned study, it has been shown that phospho ERK 42/44 is reduced in flotillin-1 knockdown cells and PMA pre-treatment could not restore the phosphorylation status of ERK. ERK 1/2 activation or inactivation largely varies depending on the cell type or type of stimuli received by the cellular system. For instance, a flotillin-2 knockout mouse model or flotillin-2 knockdown cultured cells showed an increase in ERK activation.[187] In this system, the downstream targets of ERK and p53 were upregulated at both mRNA and protein levels. These studies indicate that differential outcomes can be obtained depending on the ablation of one of the two flotillins. As flotillin-2 knockout or knockdown can result in enhanced ERK signaling, whereas flotillin-1 knockdown can impair ERK signaling. According to our findings, SH-SY5Y cells which show profound depletion of flotillin-2 upon flotillin-1 knockdown also results in impaired ERK signaling and consequently can invoke cellular sensitivity to apoptotic agents.

It is a dominant idea that activation of PKC can block programmed cell death, and this was first suggested by studies in non-neuronal cells using phorbol esters, a class of tumor promoters that bind to the diacylglycerol–binding site of PKC and promote its activation. Support for an anti-apoptotic PKC activity was also provided by studies demonstrating that direct inhibition of PKC with non-specific[188,189] or specific inhibitors[190,191] induced apoptosis in a variety of non-neuronal cell types: human glioma, Burkitt lymphoma cells, and small cell lung lung carcinoma. In our study, we used PMA treatment prior to STS induced apoptosis in flotillin depleted and control neuroblastoma cells. Our results support the general hypothesis that PKC regulates neuronal apoptosis, demonstrating that PMA treatment is protective since caspase-3 cleavage was significantly reduced in control cells and a partial reduction in caspase-3 cleavage was observed in flotillin knockdown SH-SY5Y cells. However, the phospho ERK 42/44 was profoundly inhibited upon STS treatment, and PMA pre-treatment could not rescue the phosphorylation status of ERK, corroborating with the findings of Amaddii *et al*.[37]

Accumulating evidences show that flotillins are important regulators of cellular signaling and their overexpression is associated with various types of cancers, such as melanoma, breast cancer, head and neck cancer, and gastric cancer.[42,43,192–194] Consequently, flotillins can regulate cell proliferation, and flotillins may be considered as promising targets for cancer therapy. In light of flotillin-2 knockout mouse model, we need to be cautious while interfering with flotillin function as it might result in unwarranted effects such as downregulation of growth associated genes. In line with this, another study shows that stable knockdown of flotillin-1 in the human breast adenocarcinoma MCF7 cell line results in upregulation of EGFR mRNA and protein expression and resulted in hyperactivation of MAPK signaling.[195] According to this report, it is important to pay special attention when flotillin expression is targeted in cancer cells, as unexpected events may promote cancer cell growth and proliferation.[195] Typically, impaired cell death is a ubiquitous characteristic of cancer cells, determining their resistance to apoptotic signals, and can be largely refractory to many of the available chemotherapeutic drugs. Certain studies indicate that interfering with flotillin function can result in undesired effects in cancer.[38,195] At the same time, our findings suggest that cancer cells, which are often recalcitrant to apoptotic cell death or chemotherapeutic agents, can be sensitized to these agents by interfering with flotillin

function. By this means, flotillins can be employed in strategizing an effective cancer therapy, as flotillin deficiency renders various cell types to apoptotic cell death induced by currently used chemotherapeutic agents including doxorubicin, paclitaxel and carboplatin.

4.7 Future aims

4.7.1 Flotillins and GGA: A friend or foe

This study provides evidence for the role of GGA-flotillin-BACE-1 interplay in cargo sorting. Flotillins and GGAs are capable of binding to the same sorting motif in BACE-1 and our findings show that flotillin-1 and GGA2 compete for the same binding site. Therefore, to further elucidate upon the tri-protein interplay, we should investigate the order in which flotillin-1 and GGAs bind to BACE-1. This will provide a clear understanding regarding which of the two proteins, GGAs or flotillins, plays a dominant role in BACE-1 recruitment into endosomal microdomains and provide additional evidences in determining that flotillins are capable of interacting with cargo molecule (BACE-1) as well as the adaptor proteins (GGAs). Since this study demonstrates that flotillins and GGA2 competes for the same binding site and at the same time flotillins interact with GGA proteins (M.Meister thesis), thereby GGAs and flotillins can function competitively or co-operatively with each other.

Additionally, GGAs comprise of four distinct segments such as a VHS, DXXLL, a hinge region, and a γ-adaptin domain and each of them perform specific functions in cargo transport. Therefore, flotillin binding to each of these domains can be tested and by this means the exact binding motif can be determined.

It is known that the phosphorylation of BACE-1 at Ser498 is important for its distribution and plays a significant role in the transport of this enzyme. The phosphorylation at Ser498 in BACE-1 results in an enhanced binding to GGAs.[150] This observation gives rise to various questions with respect to flotillins. Does phosphorylation of BACE-1 at Ser498 affect flotillin distribution and sorting function? Will enhanced binding of GGA to BACE-1 in a phosphorylated state positively or negatively influences the flotillin interaction with BACE-1? How will the localization and function of flotillins be affected if the Ser498 site in BACE-1 is mutated? Further studies in this direction can provide an answer to the above mentioned questions.

4.7.2 Flotillins: Novel means to target apoptosis pathways and enhance cancer therapy efficacy

One of the major advances in cancer research in recent years is the recognition that cell death primarily mediated by apoptosis is crucially involved in the tumor formation and also determines the treatment efficacy. The most common method used in clinical oncology involves killing of tumor cells by chemotherapeutic agents, irradiation or immunotherapy. All of these anticancer strategies have been associated with activation of apoptosis signal transduction pathways in cancer cells such as the intrinsic and/or the extrinsic pathway. Thus, failure to undergo apoptosis might result in resistance to anti-cancer methodologies. Therefore, it has been imperative to identify new molecules and possible strategies to regulate apoptosis in response to anti-cancer chemotherapy. The novel understanding that flotillin deficiency can increase sensitivity of cancer cells to chemotherapeutic agents provides better opportunities for a more rational approach in developing molecular-targeted therapies for combating cancer in future.

CHAPTER 5: REFERENCES

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ZUSAMMENFASSUNG

Einleitung

Die Alzheimer‘sche Erkrankung und β-Sekretase

Die Alzheimer’sche Erkrankung ist eine neurodegenerative Erkrankung, die hauptsächlich ältere Menschen betrifft. Im Verlauf der Erkrankung kommt es zu einem fortschreitenden Gedächtnisverlust und zum Verlust kognitiver Fähigkeiten. In den Gehirnen der Betroffenen findet man eine charakteristische Anhäufung von sogenannten „amyloiden Plaques“, deren Hauptbestandteil das Amyloid-Beta-Peptid (Aβ) ausmacht. Das Aβ-Peptid entsteht durch enzymatische Spaltung aus einem größeren Vorläuferprotein, dem *Amyloid Precursor Protein* (APP). APP, welches an der Zelloberfläche vorliegt, kann internalisiert werden und durch endosomale Kompartimente entweder an die Plasmamembran zurückgeführt oder im Lysosom degradiert werden. Für die amyloidogene Prozessierung des APP sind die als β- and γ-Sekretasen bezeichneten Proteasen verantwortlich. Diese Prozessierung erfolgt in endosomalen Kompartimenten, wohingegen die nicht-amyloidogene Prozessierung von APP an der Plasmamembran stattfindet, da sich hier auch die α-Sekretase befindet. Die β-Sekretase wird auch als BACE-1 oder Memapsin 2 bezeichnet und gehört zur Gruppe der Aspartatproteasen. BACE-1 ist ein Typ-1-Transmembranprotein und liegt überwiegend in sauren intrazellulären Kompartimenten, wie beispielsweise Endosomen und dem trans-Golgi-Komplex vor. Der zytoplasmatische Teil des BACE-1-Proteins befindet sich am C-Terminus und enthält ein kurzes saures Dileucinmotiv (*acidic cluster di-leucine*, ACDL). Dieses DXXLL Sortierungssignal, wobei X für eine nicht-konservierte Aminosäure steht, ist ein Merkmal vieler Transmembranproteine und reguliert deren Endozytose und lysosomalen Transport. Das ACDL-Motiv von BACE-1 interagiert mit spezialisierten monomeren Adapterproteinen, wie beispielsweise den *Golgi-localized, gamma ear-containing, ARF binding* (GGA) Proteinen 1, 2 und 3. Diese Adapterproteine sind sowohl für die Rekrutierung von Rezeptoren in Vesikel des trans-Golgi-Netzwerkes als auch für die Aufnahme von Frachtproteinen in Endosomen beziehungsweise Lysosomen verantwortlich.

Flotilline

Proteine der Flotillin-Familie werden ubiquitär exprimiert, sind hoch konserviert und assoziieren mit Lipid Rafts, bei welchen es sich um Membranmikrodomänen, die mit Cholesterol und Sphingolipiden angereichert sind, handelt. Die Assoziation von

Flotillinen mit Lipid Rafts wird über Acylierungen, Homo- sowie Hetero-Oligomerisierung und hydrophobe Bereiche im C-Terminus vermittelt. Sie werden daher häufig auch als Markerproteine für Lipid Rafts verwendet. Zur Flotillin-Familie gehören zwei homologe Mitglieder, Flotillin-1 und Flotillin-2. Sie sind an der Regeneration von Axonen und an neuronalen Differenzierungsprozessen beteiligt. Außerdem erfüllen sie Funktionen bei verschiedenen zellulären Prozessen, wie zum Beispiel bei der Endozytose, bei der Aktivierung von T-Lymphozyten, beim Insulinsignalweg, bei der Zellproliferation und beim Tumorwachstum. Flotillin-1 spielt eine wichtige Rolle bei der Regulation von Rezeptortyrosin- und Mitogen- aktivierten Proteinkinase (MAPK) Signalwegen. Flotilline kommen vermehrt in verschiedenen Tumoren vor (z.B. Melanomen, Brustkrebs, Kopf- und Hals-Tumoren, Magentumoren). Außerdem wurde Flotillinen eine Rolle während der Pathogenese von neurodegenerativen Erkrankungen, wie beispielsweise Prionenerkrankungen, Morbus Parkinson und der Alzheimer'schen Erkrankung, zugesprochen.

Apoptose: Extrinsische und intrinsische Wege

Der programmierte Zelltod (Apoptose) ist ein streng regulierter Vorgang und erfordert das Zusammenspiel vieler Faktoren. Die Apoptose kann verschiedene Auslöser innerhalb und außerhalb von Zellen haben. Grundsätzlich unterscheidet man deshalb zwei zentrale Wege: den extrinsischen Todesrezeptorweg und den intrinsischen mitochondrialen Weg.

Der extrinsische Weg wird durch einen Komplex aus sogenannten Todesliganden und Todesrezeptoren ausgelöst. Nachdem der Rezeptor von einem Liganden gebunden wurde, werden zytoplasmatische Adapterproteine mittels ihrer Todesdomänen an den Rezeptor rekrutiert. Nachfolgend bildet sich der als DISC (*death inducing signaling complex*) bezeichnete Signalkomplex aus. Dies führt zur Aktivierung von Pro-Caspasen und leitet die aktive Phase der Apoptose ein.

Der intrinsische Weg wird ohne Beteiligung von Rezeptoren durch verschiedene Stimuli, die intrazelluläre Signale generieren und sich hauptsächlich auf die Mitochondrien auswirken, ausgelöst.

Ziele der Arbeit

Das Hauptziel der vorliegenden Arbeit war, die Funktion von Flotillinen bei der endosomalen Sortierung von BACE-1 aufzuklären. Außerdem sollte der

Zusammenhang zwischen Flotillinen und zytotoxischen Ereignissen untersucht werden. Hierbei lag der Schwerpunkt bei Ereignissen die die Apoptose auslösen. Dabei sollte geklärt werden, ob es möglich ist, Krebszellen durch Unterdrückung der Flotillinexpression eine höhere Sensitivität gegenüber Chemotherapeutika zu verleihen.

Ergebnisse und Diskussion

Flotilline binden BACE-1 am ACDL-Motiv

Die Interaktion von Flotillinen mit BACE-1 wurde mit Hilfe von GST-Präzipitationen untersucht. Erstmals konnte hiermit bewiesen werden, dass Flotillin-1 eine starke, spezifische Bindung mit der BACE-1 Wildtypsequenz eingeht, wohingegen mit einer LLAA-Mutante nur eine schwache Bindung zu sehen war. Während Flotillin-1 direkt an das Dileucinmotiv im cytoplasmatischen Bereich von BACE-1 binden kann, scheint die Bindung zwischen Flotillin-2 und BACE-1 indirekter Natur zu sein. Flotilline binden spezifisch an das ACDL-Motiv von BACE-1, nicht aber an den Kation-abhängigen Mannose-6-phosphat-Rezeptor (*cation-dependent mannose 6-phosphate receptor*, CD-MPR), welcher auch ein zu BACE-1 ähnliches ACDL-Motiv beinhaltet. Dies zeigt, dass es sich bei der Bindung um eine Fracht-spezifische Interaktion handelt und dass Flotilline nicht an alle Proteine, die dieses Motiv besitzen, binden können. Da Flotilline und GGA-Proteine an das gleiche Sortierungssignal von BACE-1 binden, stellte sich die Frage, ob es sich bei der Bindung von GGA-Proteinen und Flotillinen um eine kooperative oder um eine konkurrierende Bindung handelt. Interessanterweise war in Abwesenheit von Flotillinen die Bindung von GGA2 an das ACDL-Motiv von BACE-1 erhöht, was dafür spricht, dass Flotillin-1 und GGA2 um die Bindung an das ACDL-Motiv in BACE-1 konkurrieren. In Flotillin-depletierten Zellen konnte mittels Immunfluoreszenzfärbungen eine verstärkte BACE-1-Färbung nachgewiesen werden, was ein Hinweis für eine erhöhte Stabilität des BACE-1 Proteins ist. Eine ähnlich erhöhte Stabilität des BACE-1 kennt man bereits von Zellen in denen GGA3 depletiert wurde, da hier die lysosomale Degradation von BACE-1 beeinträchtigt ist. Diese Beobachtung ist vergleichbar mit der hier beschriebenen Anreicherung von BACE-1 nach Depletion von Flotillinen. Zusätzlich konnte in der vorliegenden Arbeit gezeigt werden, dass Flotilline auch einen indirekten Einfluss auf die Prozessierung von APP haben, da sie den subzellulären Transport und die Menge an vorhandenem BACE-1 beeinflussen. In Flotillin-depletierten Zellen ließ sich eine vermehrte amyloidogene

Prozessierung von APP beobachten, was durch eine erhöhte Menge des C-99-Fragmentes zum Ausdruck kam. Eine mögliche Erklärung für diesen Effekt ist die Akkumulation von BACE-1 und eine somit begünstigte APP-Prozessierung.

Die Depletion von Flotillinen sensitiviert Zellen für die Induktion von Apoptose

Der Zusammenhang zwischen Flotillinen und Zytotoxizität wurde mit Hilfe verschiedener apoptotischer Stressauslöser, wie z.B. $A\beta_{25-35}$, ER-Stress (Brefeldin A), Mitoseinhibitoren (Paclitaxel) und Chemotherapeutika (Carboplatin, Doxorubicin), in Flotillin-depletierten Neuroblastomzellen untersucht. Da die Verwendung der obengenannten Inhibitoren insgesamt in der Aktivierung von Caspase-3 und der Einleitung der Apoptose resultiert, werden im Folgenden die Ergebnisse die mit dem Proteinkinase C (PKC)-Inhibitor und Chemotherapeutikum Staurosporin (STS) erhalten wurden, repräsentativ aufgeführt. Insgesamt zeigten Flotillin-depletierte SH-SY5Y-Zellen sowie weitere stabile Flotillin-depletierte Zelllinien (z.B. HeLa, MCF7, HaCaT) eine stark erhöhte Anfälligkeit gegenüber STS-induzierter Apoptose. Für die Messung der Apoptose in Staurosporin-behandelten Flotillin-depletierten SH-SY5Y-Zellen wurden typische Apoptosemarker verwendet. In Flotillin-depletierten Neuroblastomzellen führte STS zu einer erhöhten Spaltung von Caspase-3, GGA3 und 14.3.3, einem Adapterprotein, welches Protein-Proteininteraktionen unterstützt und eine Rolle in der Signaltransduktion, bei der Stressantwort und der Apoptose spielt. Weiterhin war eine Hochregulation des *Bcl-2 associated death promotor*-Proteins (Bad) zu beobachten. Der Phosphoinositid 3-Kinase (PI3K)/Proteinkinase B (Akt)-Signalweg ist eine der zentralen Signaltransduktionskaskaden, die das zelluläre Überleben regulieren. Im Folgenden sollte die Auswirkung der STS-induzierten Apoptose in Flotillin-depletierten Zellen auf den PI3K/Akt und auf den MAP-Kinase-Signalweg untersucht werden. Diese Analyse ergab Folgendes: 1) Die Phosphorylierungen von Akt an Serin 473 und von Tyrosin 204 bzw. 187 der *extracellularly regulated kinases* (ERK1/2), auch bekannt als p44/p42 MAP-Kinasen waren in Flotillin-depletierten Neuroblastomzellen nach STS-induzierter Apoptose im Vergleich zu Flotillin-exprimierenden Zellen vollständig unterdrückt, 2) eine vierstündige STS-Behandlung führte in Flotillin-depletierten Zellen zu einer signifikanten Abnahme der Gesamtmenge von unphophosphorylierten Akt, 3) eine ektopische Expression von Akt hemmt die Spaltung von Caspase-3 und schützt die Flotillin-depletierten Zellen vor der Apoptose.

Zusätzlich zu Akt wurde dessen Zielprotein, Forkhead-Box-Protein 3a (FOXO3a) untersucht. FOXO3a ist ein dem Akt-Signalweg nachgeschaltetes Zielgen. Aktiviertes Akt phosphoryliert FOXO3a an Serin 253, wodurch sich die sonst normalerweise gleichmäßige Verteilung von FOXO3a zwischen Zytosol und Kern auf das Zytosol beschränkt und es dadurch zur Einschränkung der Transkription von Todesgenen kommt. In STS-behandelten Flotillin-depletierten Zellen war jedoch eine Akkumulation von FOXO3a im Zellkern zu beobachten. Die Apoptose lässt sich mit Hilfe von anti-apoptotischen Proteinen hemmen. Ein Prototyp eines solchen Zelltodregulators ist das Proto-Oncogen Bcl-2. Durch ektopische Expression von Bcl-2 ließ sich eine apoptotische Schädigung von Flotillin-depletierten Zellen hemmen. Weiterhin wurden die Zellen vor Behandlung mit STS mit dem Proteinkinase C-(PKC)-aktivierenden Phorbol-12-myristat-13-acetat (PMA) behandelt um zu untersuchen, ob PMA seinen bereits für neuronale Zellen postulierten anti-apoptotischen Effekt auch in Flotillin-depletierten Zellen ausüben kann. In Kontrollzellen führte eine PMA-Vorbehandlung zu einer signifikanten Hemmung der Caspase-3-Spaltung, wohingegen in Flotillin-depletierten Zellen noch eine schwache Caspase-3-Spaltung nachgewiesen wurde. Als weiteres anti-apoptotisches Molekül wurde das *induced myeloid leukemia cell differentiation* (Mcl-1)-Protein, welches in vielen Tumoren überexprimiert wird, untersucht. In Flotillin-depletierten SH-SY5Y-Zellen war die Mcl-1-Expression vermindert.

Zusammenfassend konnte gezeigt werden, dass eine Hemmung der Flotilline die Empfindlichkeit von Zellen gegenüber konventionellen chemotherapeutischen Agenzien erhöht und es somit möglich ist, den apoptotischen Zelltod in Krebszellen herbeizuführen.

Fazit

Die Ergebnisse der vorliegenden Arbeit zeigen, dass Flotilline für die endosomale Sortierung von BACE-1 benötigt werden und somit einen Einfluss auf die amyloidogene Prozessierung von APP haben. Die hier gezeigten Ergebnisse tragen zum Verständnis der Funktion von Flotillinen während der Pathogenese der Alzheimer'schen Erkrankung bei. Des Weiteren erhöht das Fehlen von Flotillinen die Sensitivität von Krebszellen gegenüber chemotherapeutischen Substanzen. Diese Erkenntnis könnte bei der zukünftigen Entwicklung molekularer Krebstherapien von Nutzen sein.

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