COMPARATIVE INTERACTOME INVESTIGATION OF γ-SECRETASE COMPLEX IN ALZHEIMER’S DISEASE

by

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A thesis submitted in conformity with the requirements
For the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Amy Hye Won Jeon
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ABSTRACT

γ-Secretase plays a pivotal role in the production of neurotoxic amyloid β-peptide (Aβ), the principal component of amyloid plaques present in Alzheimer’s disease. It consists of a core complex of presenilin (PS), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2) proteins. PS harbors the catalytic aspartates required for regulated intramembrane proteolysis and the paralogs (PS1 and PS2) contribute to the assembly of distinct subpopulations of γ-secretases that may fulfill distinct roles. To characterize the molecular environments of distinct γ-secretases complexes in-depth quantitative comparisons were performed on 1) wild-type PS1 and its derivative carrying point mutations known to cause heritable early-onset AD in mice, and 2) PS1- or PS2-containing γ-secretase complexes equipped with N-terminal tandem-affinity purification (TAP) tags on PS paralogs in HEK293 cells. Isobaric labeling of co-purifying peptides for quantitative mass spectrometry revealed that γ-secretase complexes interact with other protein networks, including the cellular catenin-cadherin network, the molecular machinery that targets and fuses synaptic vesicles to cellular membranes, and the H+-transporting lysosomal ATPase macro-complex. The study revealed mature γ-secretase complexes containing PS1 or mutant PS1 to be indistinguishable in their protein composition, confirmed several previously proposed γ-secretase interactors, identified many
novel interactors and uncovered a subset of proteins which can engage in robust interactions with
\(\gamma\)-secretase complexes in individual cell types but may escape detection when whole brains are used as biological source materials. Interestingly, signal peptide peptidase (SPP), a Type II TM cleaving aspartyl protease, was pre-dominantly found to co-purify with PS2-containing \(\gamma\)-secretase complexes and could be shown not to influence their maturation but to affect cleavage or release of cellular A\(\beta\). A model emerged from this work that suggests PS1 and PS2 paralogs may divide up the task of handling a broad range of membrane stubs at least in part by associating with different molecular environments.
DEDICATION

To my grandmother, father and mother, Joanna and Joseph:
I can do all things through Christ who strengthens me (Philippians 4:13)
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Gerold Schmitt-Ulms for giving me the wonderful opportunity to learn in his laboratory. Throughout the past four and a half years, his patience, motivation, continuous support and guidance have supported me every step of my PhD study. He was always accessible and willing to help and I can’t thank him enough for everything he has done for me. I am honored to have had this opportunity to study as his student and hope to provide the same patience and generosity to others, as he has shown me, in my future science career.

I would like to thank my PhD thesis advisory committee members, Drs. Janice Robertson, Jeromy Mogridge, and Thomas Kislinger for extraordinary advice and encouragement. Because of their helpful guidance, my perspectives in critical thinking and scientific understandings have been greatly influenced. I also would like to especially thank Drs. Peter St.George-Hyslop and Christopher Böhm for their exquisite collaboration and guidance. I feel privileged to have been able to work with these world-renowned Alzheimer’s Disease researchers. Dr. Böhm’s wisdom, enthusiasm and friendship have provided me with the striving force in completion of my PhD thesis.

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I wish to acknowledge the amazing staff of the Tanz Center for research in Neurodegenerative Diseases at the University of Toronto. A special thank you to Gerry Smith, Lina Calomino, Sheik Ali, Scott MacDonald, Elaine Banks and Maria Balahura; I will always remember the kindness and support that I received from each of you.

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-VP</td>
<td>4-Vinylpyridine</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
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<tr>
<td>Aph-1</td>
<td>Anterior Pharynx Defective-1</td>
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<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>BACE</td>
<td>β-secretase</td>
</tr>
<tr>
<td>BIN1</td>
<td>Bridging integrator 1</td>
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<td>BNG</td>
<td>Blue native gel</td>
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<tr>
<td>[C-11]PiB</td>
<td>[C-11]-labelled Pittsburgh Compound B</td>
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<td>CAD</td>
<td>Collision-activated dissociation</td>
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<td>CBP</td>
<td>Calmodulin binding peptide</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<td>CHAPSO</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>CID</td>
<td>Collision-induced dissociation</td>
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<td>CLU</td>
<td>Clusterin</td>
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<td>co-IP</td>
<td>Co-immunoprecipitation</td>
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<td>COX1</td>
<td>Cyclo-oxygenases I</td>
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<td>Complement Component (3b/4b) receptor 1</td>
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<td>CSF</td>
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<td>CT</td>
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<td>CTF</td>
<td>C-terminal fragment</td>
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<td>DAPT</td>
<td>N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s Disease</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GSAP</td>
<td>γ-Secretase activating protein</td>
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<td>GSI</td>
<td>γ-Secretase inhibitor</td>
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<td>GSM</td>
<td>γ-Secretase modulator</td>
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<tr>
<td>GS-TEV</td>
<td>Protein G, Streptavidin binding peptide, TEV cleavage site</td>
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<td>HEK</td>
<td>Human embryonic kidney</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ICAT</td>
<td>Isotope-coded affinity tag</td>
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<tr>
<td>ICIP</td>
<td>Integrated cell interrogation platform</td>
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<tr>
<td>iCLIPs</td>
<td>Intramembrane cleaving proteases</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantitation</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning sites</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>m/z</td>
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<tr>
<td>Nac-GlcN</td>
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<td>Nct</td>
<td>Nicastrin</td>
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<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NTF</td>
<td>N-terminal fragment</td>
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<tr>
<td>p3</td>
<td>3 kDa product</td>
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<tr>
<td>PAL</td>
<td>Proline-alanine-leucine</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>Pen-2</td>
<td>Presenilin enhancer protein 2</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PICALM</td>
<td>Phosphatidylinositol binding clathrin assembly protein</td>
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<td>qTOF</td>
<td>Quadrupole-TOF</td>
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<td>RCR</td>
<td>Replication-competent recombinants</td>
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<td>RIP</td>
<td>Regulated intramembrane proteolysis</td>
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<td>S2P</td>
<td>Site 2 protease</td>
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<td>Streptavidin binding peptide</td>
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<td>Strong cation exchange</td>
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<td>SILAC</td>
<td>Stable isotope labelling with the amino acids in cell culture</td>
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<td>Self-inactivating</td>
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<td>Selected reaction monitoring</td>
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<td>TAP</td>
<td>Tandem affinity purification</td>
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<td>Tobacco etch virus</td>
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<tr>
<td>TCEP</td>
<td>Tris-(2-carboxyethyl)-phosphine</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
</tbody>
</table>
v/v Volume per volume
WGA Wheat Germ Agglutinin
WT Wild-type
w/v Weight per volume
Chapter 1: Literature Review
1.1 An Overview of Alzheimer’s Disease

1.1.1 Introduction to Alzheimer’s Disease

Alzheimer’s disease (AD) is a complex, progressive neurodegenerative disease that is the most common cause of dementia. AD was first identified by a German psychiatrist and neuropathologist Alois Alzheimer in 1907. He described a novel brain disorder affecting late middle-aged and older humans, which results in a progressive and ultimately fatal loss of intellectual capabilities, particularly with short-term memory (Dries and Yu, 2008; Grigorenko and Rogaev, 2007; Wolfe, 2007). Since then, continuous efforts have been made to gain a better understanding of the disease. A recent report, “2011 Alzheimer’s Disease Facts and Figures” published by the Alzheimer Association states that AD is the 6th leading cause of death in the US for those 65 years of age or older; costing 183 billion dollars annually with limited treatment options available (Alzheimer Association, 2011). The cost of treating AD will continually grow unless an effective and economical treatment is found. Importantly, with the baby boomers now reaching the critical age of 65, an age when one in eight people have AD, the need to find a treatment is becoming more apparent (Figure 1.1.1A, B).

One of the biggest obstacles in AD research is the fact that its cause is still only poorly understood. With the initiating molecular events remaining unclear, it is not only impossible to define the onset into this progressive disease but can also be difficult to distinguish between mild or early AD and normal aging processes. Epidemiologically and genetically, AD can be divided into:

1. familial AD (FAD) with Mendelian inheritance of predominantly early-onset (<65 years), which makes up ~5% of AD cases, and
2. sporadic AD with less apparent or no familial aggregation and of late onset (>65 years), which makes up the vast majority of AD cases (Figure 1.1.1C).

In last few decades, several genes were identified to contain disease-causing mutations in FAD including amyloid precursor protein (APP) on chromosome 21q, presenilin 1 (PS1) on chromosome 14q, and presenilin 2 (PS2) on chromosome 1q (Bertram et al., 2010; Levy-Lahad et al., 1995; Robakis et al., 1987; Rogaev et al., 1995; Sherrington et al., 1995). In addition, with the development of genome-wide association studies (GWAS), more genes were identified as
A. 

Alzheimer's Disease
Vascular Dementia (a.k.a. Post-Stroke Dementia)
Mixed Dementia
Dementia with Lewy bodies
Frontotemporal Dementia (FTD)
Parkinson's Disease
Others

B. 

(\%) 

50

65-74
75-84
85+

White
African-American
Hispanic

C. 

Total AD Cases

Familial AD (FAD)
Early-onset (< 65 yrs)
~ 5-10% of AD cases

AD-Causing Genes
Amyloid precursor protein (APP)
Presenilin 1 (PS1)
Presenilin 2 (PS2)

Sporadic AD
Late-onset (> 65 yrs)
~ 95% of AD cases

AD Risk Genes
Apolipoprotein E (APOE)
Clusterin
CR1, PICALM, BIN1
Figure 1.1.1 Alzheimer’s Disease (AD) Prevalence. (a) Pie chart showing the most prevalent dementias, with AD representing the most common disease. (b) Bar graph depicting estimated AD prevalence in people of Caucasian, African-American and Hispanic origins in the US from the Washington Heights-Inwood Columbia Aging Project (WHICAP). No known racial genetic difference exists, but rather the differences in socioeconomic characteristics such as the levels of education, and access to health care may play a role in prevalence. For people aged 65 and over, approximately 1 in 8 have AD and for people aged 85 and over, 1 in 2 have AD (adapted from Alzheimer Association Facts and Figures 2011). (c) AD can be categorized into familiar AD (FAD) with early-onset (<65 years) and sporadic AD with late-onset (>65 years). Genetic linkage studies of FAD identified the Amyloid precursor protein (APP), Presenilin 1 (PS1), and Presenilin 2 (PS2) as the AD-causing genes. Genome-wide association studies (GWAS) of late-onset AD identified apolipoprotein E (APOE), clusterin (CLU), complement component (3b/4b) receptor 1 (CR1), phosphatidylinositol binding clathrin assembly protein (PICALM), bridging integrator 1 (BIN1) as AD risk genes.

1.1.2 Amyloidogenic Pathway of Beta Amyloid (Aβ) Peptide Production

AD is pathophysiologically characterized by extensive neuronal loss and the presence of two hallmark features, intracellular neurofibrillary tangles and extracellular amyloid plaques, which have been shown to comprise hyperphosphorylated Tau protein and aggregated beta amyloid (Aβ) peptides, respectively (Figure 1.1.2) (Wolfe, 2006). These two features appear to synergistically trigger molecular events which lead to the progression of AD. For example, the biogenesis and accumulation of Aβ peptides is thought to contribute to the generation of free radicals, mitochondrial oxidative damage and inflammatory processes that characterizes AD (Verdile et al., 2007). The primary event that results in the accumulation of neurotoxic Aβ peptides is thought to be the dysregulated proteolytic processing of its parent protein, APP. APP is a Type 1
Intracellular neurofibrillary tangles and extracellular amyloid plaques are the key hallmarks in AD pathology. Tangles are aggregates of the hyperphosphorylated tau protein and plaques are dense, mostly insoluble deposits of amyloid-beta peptide (adapted from Alzheimer Association, 2011).

transmembrane (TM) glycoprotein that is encoded by a gene on human chromosome 21 (Kang et al., 1987; Li et al., 2009; Robakis et al., 1987). APP isoforms range in length from 365 to 770 amino acids, with the predominant splice-isoforms being 695, 751, and 770 amino acids long (Dries and Yu, 2008; Zheng and Koo, 2006). Not surprisingly, people with Down’s syndrome, also known as Trisomy 21 individuals, carry three copies of APP genes. Of note, these individuals develop signs of neuropathological perturbations resembling AD relatively early in their life (Isacson et al., 2002).

APP can undergo series of proteolytic cleavages by two competing pathways, the non-amyloidogenic and amyloidogenic pathways. Three major secretases that are postulated to be involved in the proteolytic cleavage of APP are: α-secretase, BACE (formally known as β-secretase), and γ-secretase (Haass and Selkoe, 1993). The initial cleavages at α- and β-sites result in “ectodomain shedding” (Lichtenthaler, 2006). This ectodomain shedding is a prerequisite for γ-secretase cleavage (Dries and Yu, 2008; Kopan and Ilagan, 2004). In the non-amyloidogenic pathways, the α-secretase cleaves within the Aβ domain of APP molecules (Figure 1.1.3A) thereby precluding the formation of Aβ and generating a non-amyloidogenic membrane-bound
C-terminal fragment, C83, as well as a secreted form of APP (sAPPα). C83 can be further processed by γ-secretase into an APP intracellular domain (AICD) C-terminal fragment and a 3 kDa product (p3) (Laudon et al., 2007; Zheng and Koo, 2006). The functions of AICD and p3 are still only partially understood. AICD has been shown to be involved in the transcriptional regulation within nuclei, which in turn may influence the degradation of Aβ in the extracellular space (Zheng and Koo, 2006).

In the amyloidogenic pathway, BACE cleaves APP at the N-terminal boundary of the Aβ domain, liberating another soluble form of APP, sAPPβ, and a C-terminal fragment, C99, containing the whole Aβ domain (Figure 1.1.3B). The subsequent processing causes the intramembrane cleavage of C99 fragment by γ-secretase to shed the Aβ peptide (Li et al., 2009; Verdile et al., 2007). This γ-secretase cleavage appears to proceed via two distinct cleavages: the first targeting a site close to the cytosolic boundary of the TM domain (ε-site) generating AICD, and the second cleavage targeting a site located approximately in the middle of the APP TMD (γ-site) generating Aβ40 and Aβ42 (Lundkvist and Naslund, 2007). Thus, the ε-cleavage site resides 7-9 residues distal to γ-secretase cleavage sites (Krishnaswamy et al., 2009; Lundkvist and Naslund, 2007). It has been proposed that the ε-cleavage and γ-cleavage are hosted by distinct subcellular environments, with ε-cleavage depending on endocytic function and being induced by the acidification of the pH (Fukumori et al., 2006).

In the amyloidogenic pathway, various amyloid peptide fragments ranging from 37 to 42 amino acids are generated by cleavages at multiple alternative γ-secretase sites – the most abundant form comprising 40 amino acid residues (Aβ40) (Figure 1.1.3C) (Fukumori et al., 2006; Woo et al., 2009). Aβ peptides containing 42 amino acid residues (Aβ42) have been shown to represent the most neurotoxic, aggregation-prone form, and have been found to represent the predominant peptide increased in AD brains (Butterfield, 2002; Butterfield and Boyd-Kimball, 2004; Lambert et al., 1998). Recently, it has been proposed that the absolute quantity of Aβ peptides produced in the brain may be less important than the molecular nature and ratio of Aβ peptides, which is reflected in a changed Aβ42/Aβ40 ratio (Blennow et al., 2009; Graff-Radford et al., 2007; Hansson et al., 2007; Kuperstein et al., 2010; Wolfe, 2007). Therefore, the Aβ42/Aβ40 ratio has been accepted as a diagnostic tool for AD. For example, in a healthy normal brain, Aβ42 is detected at about 10-fold lower levels than Aβ40; and in FAD brain the Aβ42/Aβ40 peptide ratio
Figure 1.1.3 Amyloid Precursor Protein (APP) proteolytic pathways. APP is processed by two pathways, the amyloidogenic and non-amyloidogenic pathway. (a) In the non-amyloidogenic pathway (left arrow), APP undergoes ectodomain shedding by α-secretase to release the soluble APP ectodomain (sAPPα). This cleavage generates the APP C-terminal fragment C83, which serves as a substrate for γ-secretase cleavage. The subsequent γ-secretase cleavage generates the p3 peptide and the APP intracellular domain (AICD) fragment. This pathway does not produce Aβ peptide. (b) In the amyloidogenic pathway (right arrow), APP undergoes ectodomain shedding by β-secretase to release the soluble APP ectodomain (sAPPβ). This cleavage further generates the APP C-terminal fragment C99, which serves as a substrate for γ-secretase cleavage. Dependent on the precise γ-secretase cleavage sites, its endoproteolytic action generates various alternative Aβ peptide fragments, including the neurotoxic Aβ42 peptide (adapted from Dries and Yu, 2008). (c) Aβ peptide fragments generated by γ-secretase range from 37 to 42 amino acids in length (adapted with permission from Fukumori et al., 2006. Copyright (2006) American Chemical Society).
has been shown to approximate 3:7 (Kuperstein et al., 2010; Uemura et al., 2010). This slight shift in ratio has been proposed to dramatically affect the production of synaptotoxic intermediates that bind to synapses and inhibit their spontaneous activities, thereby interfering with memory formation in mice (Kuperstein et al., 2010). This suggests that therapeutically, lowering the absolute amount of Aβ in AD patients would be less beneficial than the ability to influence or restore the physiological ratio of Aβ peptides.

The fact that changes in the Aβ_{42}/Aβ_{40} peptide ratio affect AD progression lends support to the argument for a causal role of Aβ peptides in the pathogenesis of AD, commonly referred to as “amyloid cascade hypothesis” (Hardy and Selkoe, 2002). This hypothesis postulates that the abnormal production of Aβ is the initial step in triggering the pathophysiological cascade that eventually leads to AD, and that other hallmarks of AD – hyperphosphorylated tau protein, neurofibrillary tangles, vascular damage, synaptic loss, and inflammation – are consequences, rather than causes of the disease process (Bertram et al., 2010; Hardy and Selkoe, 2002; Karran et al., 2011; Li et al., 2009; Woo et al., 2009). A more recent variation of the amyloid hypothesis proposes that neuronal injury is associated not so much with the presence of insoluble plaques but rather correlates with levels of soluble oligomeric aggregates of Aβ (Lambert et al., 1998; Picone et al., 2009; Townsend et al., 2006; Walsh et al., 2002; Walsh et al., 2005). A conformational change of Aβ from a partial α-helical fold into a well-organized β-sheet structure is a widely-accepted characteristic of Aβ aggregation, where oligomers represent an intermediate stage of the processes that lead to fibril formation (Grasso, 2010; Santos et al., 2012; Tabaton et al., 2010). In one AD mouse model study, 56 kDa dodecameric Aβ oligomers levels were shown to be correlated with the degree of memory impairment (Lesne et al., 2006). In addition, it has been shown that the production of total Aβ did not significantly change in some AD cases, whereas oligomeric Aβ production was found to significantly decrease (DaRocha-Souto et al., 2011). Finally, a paper by Farfara and colleagues has suggested that γ-secretase may play a role in regulating microglia activity, known to be important for clearing neurotoxic Aβ deposits (Farfara et al., 2010). From the above, it is apparent that the further study of γ-secretase complexes may reveal insights that can help us to better understand the molecular etiology of AD.
1.1.3 \(\gamma\)-Secretase Complex Proteins

\(\gamma\)-Secretase is a unique intramembrane proteolytic enzyme that comprises a tetrameric core complex consisting of presenilin (PS), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2) proteins (Table 1.1.1) (Figure 1.1.4) (Francis et al., 2002; Goutte et al., 2002; Kimberly et al., 2003; Sato et al., 2007; Wolfe, 2007; Yu et al., 2000). \(\gamma\)-Secretase was initially characterized as a proteolytic enzyme capable of cleaving the C-terminus of A\(\beta\) in the membrane (Haass and Selkoe, 1993). Proteolytic enzymes, which can cleave substrate proteins within the membrane, are referred to as intramembrane cleaving proteases (iCLIPs) and the process of cleaving is commonly referred to as regulated intramembrane proteolysis (RIP). RIP is considered a pivotal step for many physiological functions that rely on integral membrane proteins being separated into their extracellular and intracellular domains (De Strooper and Annaert, 2010). This process confers directionality to the respective physiological events, due to the irreversible nature of these cleavages. There are four known classes of iCLIPs:

![Figure 1.1.4 The schematic of \(\gamma\)-secretase complex proteins. \(\gamma\)-Secretase complex consists of four core proteins – presenilin (PS), anterior pharynx defective-1 (Aph-1), nicastrin, presenilin enhancer-2 (Pen-2). Arrow indicates the PS endoproteolysis site.](image-url)
Table 1.1.1 Theoretical molecular weights of γ-secretase core proteins without glycosylation.

<table>
<thead>
<tr>
<th></th>
<th>Nicastrin</th>
<th>PS1-NTF</th>
<th>PS1-CTF</th>
<th>Aph-1</th>
<th>Pen-2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.a</td>
<td>709</td>
<td>298</td>
<td>169</td>
<td>257</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>MW (Kda)</td>
<td>78.5</td>
<td>35</td>
<td>20</td>
<td>28.5</td>
<td>12</td>
<td>174</td>
</tr>
</tbody>
</table>

metalloprotease site 2 protease (S2P), serine protease rhomboids, aspartyl protease signal peptide peptidases (SPPs) and PSs (Spasic and Annaert, 2008; Wakabayashi and De Strooper, 2008; Wolfe et al., 1999a). The iCLIPs are thought to consist of a hydrophobic core that is built from multiple TMDs, with the catalytic center buried in the core, allowing access to hydrophobic membrane proteins while they remain inserted in the membrane. It is not yet fully understood how this cleavage process within the hydrophobic environment can provide access to a water molecule, a critical requirement for catalysis. In order for the cleavage to occur at least a transient hydrophilic environment needs to be created.

The recently resolved crystal structures of rhomboid and S2P proteases provided insights into how peptide bond hydrolysis can take place within the lipid environment (Ben-Shem et al., 2007; Erez et al., 2009; Feng et al., 2007; Urban and Shi, 2008; Wang et al., 2006). Structural analysis of an S2P homolog from the archaeabacterial species Methanocaldococcus jannaschii (mjS2P) has revealed two distinct active site conformations (open and closed) and a channel that leads to the active site from the cytosolic side (Figure 1.1.5) (Erez et al., 2009; Feng et al., 2007). The inner surface of the channel contains a number of polar groups and charged amino acids including histidine, arginine, and glycine that may facilitate the constant water access during the closed conformation. During the open conformation, TM helices separate from each other, acting as a lateral gate, and allow the active site-substrate binding and proteolytic cleavage. With the knowledge gained from these studies, a model of the γ-secretase complex has been suggested. It proposes TM domains to be involved in the lateral gating of substrates and access of water molecules to the active site through a hydrophilic path within the otherwise hydrophobic membrane, a mechanism which might be shared amongst iCLIPs (Sato et al., 2006; Sato et al., 2008; Tolia et al., 2006).
Figure 1.1.5 Ribbon diagram of S2P homolog from the archaebacterial species *Methanocaldococcus jannaschii* (mjS2P) structure. The two observed conformations, open and closed, show different distances between TM1 and TM5-TM6 pair. Substrate peptide gains access to the catalytic zinc atom only during the open conformation. The inset displays the active site region of the water-soluble metalloenzyme thermolysin with histidine (H) and glycine (E) residues (adapted with permission from Erez et al., 2009. Copyright (2009) Nature Publishing Group).

Several studies have attempted to solve the structure of the γ-secretase complex, but its exact conformation or architecture remains unknown. The technical difficulties in purifying, crystallizing, and analyzing a protein complex consisting of at least four core-proteins with 19 TMDs are formidable. Some limitations in studying the crystal structure of the complex are:

1. its complex maturation pathway (Spasic and Annaert, 2008);
2. the general requirement of certain lipids for activity (Fraering et al., 2004; Osenkowski et al., 2008);
3. the multiple N-glycosylations on nicastrin;
4. the tendency of the active complex to break into subcomplexes when exposed to detergents that are more appropriate for structural studies (Fraering et al., 2004);
One of the first attempts at determining the structure of the \(\gamma\)-secretase complex relied on negative stain electron microscopy (EM) and 3D image reconstruction of the core complex purified from Chinese hamster ovary (CHO) cells which had been engineered to express tagged components (Lazarov et al., 2006). This 15Å resolution study showed a large, cylindrical interior chamber of about 20-40Å in length, with a low density interior (~2 nm) and apical and basal pores, that was suggested to be a water-accessible proteolytic channel within the lipid bilayer (Figure 1.1.6A). The study provided valuable information on the rough structure of \(\gamma\)-secretase complex.

![Figure 1.1.6 3D structure of \(\gamma\)-secretase complex.](image)

(a) 15Å resolution study display the potential transmembrane segment of 60 Å apart with a large central chamber and one opening (H1) at the top and one at the bottom (H2) (adapted from Lazarov et al., 2006). (b) 12Å resolution study display the potential transmembrane segment of 40 Å in left, right and front view (adapted with permission from Osenkowski et al., 2009. Copyright (2009) Elsevier).
complex. However, the resolution achieved in this study was not sufficient to accurately reveal the details of the structure. Since then, a few similar negative-stain EM based studies captured 3D structural images of γ-secretase and achieved marginally improved resolutions but still exhibited technical limitations (Cacquevel et al., 2008; Ogura et al., 2006). Recently, a cryo-EM based single-particle analysis of the core complex resulted in a model structure with an estimated ~12Å resolution. Cryo-EM offers relative to negative-stain EM the advantage that structural features are not inferred but directly observed (Osenkowski et al., 2009). Indeed, the cryo-EM study revealed additional details including an overall spherical structure with a diameter of ~8-9 nm, which suggested that approximately half of the sphere is embedded in the membrane and the other half is exposed mostly in the extracellular milieu (Figure 1.1.6B). However, cryo-EM suffers from low-contrast and the resolution achieved was still insufficient to detect fine details such as the localization of individual α-helices, and the exact spatial arrangement of the γ-secretase core proteins. A resolution of less than 10Å may be needed for these additional features to come into focus and provide more reliable information about the stoichiometry of the complex that may lead to more comprehensive insights into the forces that keep the complex together. Osenkowski and colleagues reported the absolute mass of the purified γ-secretase complex as 230 kDa. This result corroborates the previously reported possible 1:1:1:1 stoichiometry of four γ-secretase core complexes (Sato et al., 2007). Nevertheless, previously reported molecular weight estimates of the γ-secretase ranging from ~250 kDa to ~2000 kDa still raise the possibility of transient interactions with other protein complexes or dimer/multimer formations (Krishnaswamy et al., 2009).

Overall γ-secretase complex formation

The assembly of the γ-secretase complex requires a series of partially-understood steps and occurs during its passage through the secretory pathway. Distinct pools of γ-secretase may exist in the cell – at any time a majority of γ-secretase complex (~95%) may undergo maturation steps and therefore shuttle between the ER and Golgi. Once matured, γ-secretase complex (~5%) resides at the plasma membrane and within endosomal compartments (Dries and Yu, 2008). In the ER, all core proteins of the γ-secretase complex are synthesized, and are incorporated into sub-complexes. Initially, nicastrin and Aph-1 form a dimeric subcomplex (Figure 1.1.7A). Next, PS holoprotein is incorporated into the nicastrin:Aph-1 subcomplex, a step that appears to be essential for nicastrin to undergo full maturation of its N-linked glycans (Figure 1.1.7B).
Subsequently, the complex is transported to the cell surface as a trimeric pre-complex. Lastly, Pen-2 is incorporated into the complex. When Pen-2 binds to PS, the previously inactive PS becomes biologically active and the endoproteolytic cleavage of the PS holoprotein into a 30 kDa N-terminal and a 20 kDa C-terminal fragment (NTF and CTF, respectively) between residues N292 and V293 can be observed (Figure 1.1.7C). This complex is thought to represent the fully matured, active γ-secretase complex (Figure 1.1.7D) (Dries and Yu, 2008; Li et al., 2009; Shirotani et al., 2004).

**Figure 1.1.7 Assembly of the active γ-secretase core complex.** (a) First, nicastrin and Aph-1 form a dimer subcomplex. (b) Second, PS holoprotein is incorporated by the nicastrin:Aph-1 subcomplex. (c) Then, Pen-2 is added to the trimer complex. This triggers PS endoproteolysis, which is depicted by an arrow. (d) The generation of fully mature, active γ-secretase core complexes requires PS endoproteolysis.
Presenilin: a catalytic component

Presenilin (PS), an aspartyl protease that comprises 9-transmembrane domains (TMD), is the catalytic component of γ-secretase (Laudon et al., 2005; Spasic et al., 2006; Wolfe et al., 1999b). As noted previously, PS is synthesized within the ER as an immature 50 kDa holoprotein. (Laudon et al., 2007; Martoglio and Golde, 2003; Spasic et al., 2006; Wolfe, 2006). This endoproteolysis has been proposed to allow PS to adapt its active conformation through the removal of steric barriers posed by the large cytoplasmic loop connecting TMDs 6 and 7, (Thinakaran et al., 1996). In addition, this cleavage step may affect the spatial orientation of the two catalytic aspartyl residues, which are embedded within NTF and CTF fragments (Esler et al., 2000; Wolfe, 2006; Wolfe et al., 1999b).

PS first attracted wide interest in 1995, when Sherrington and colleagues identified the PS1 gene, originally called S182, as a major contributor to early-onset genetic AD (Sherrington et al., 1995). The finding that PS1 mutations impair or alter γ-secretase-dependant activities including the production of Aβ40, Aβ42, intracellular domains of APP (AICD) and Notch (NICD) in early-onset AD provided evidence that PS facilitates γ-secretase activity (Shen and Kelleher, 2007; Wolfe, 2007). Early on, it was understood that the human genome codes for two PS genes which are highly homologous, sharing 67% sequence homology. PS1 is encoded on chromosome 14 (14q24.3) and PS2 on chromosome 1 (1q42.2) (Table 1.1.2) (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995; Tandon and Fraser, 2002). PS is evolutionally conserved in a large range of species including D. Malanogaster, C. elegans, H. lucorum, M. musculus, and H. sapiens (Grigorenko and Rogaev, 2007). The most conserved sequences within PS orthologs are found in the TM domains that surround the two aspartic residues. These are embedded in the short YD and GxGD motifs (with positions of aspartic residues at D257 and D385 in PS1, and at D263 and D366 in PS2), and a conserved C-terminal proline-alanine-leucine (PAL) motif. PS are not typical, conventional aspartyl proteases, since they lack the common D(T/S)G motif commonly present in aspartyl protease active sites. 185 disease-causing mutations for PS1 and over 13 mutations for PS2 have been reported to date. For a current overview and listing of all known mutations, see the AD Mutation Database (www.molgen.ua.ac.be/admutations). Interestingly, PS mutations are scattered throughout the entire length of the protein, including extracellular, cytosolic and TM domains, and the alteration of a single amino acid residue is sufficient to cause AD (Shen and Kelleher, 2007). In addition to functioning as an enzyme that is
Table 1.1.2 PS1 and PS2 comparison table

<table>
<thead>
<tr>
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<th>PS1</th>
<th>PS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>14 (14q24.3)</td>
<td>1 (1q42.2)</td>
</tr>
<tr>
<td>Protein Length</td>
<td>497 aa</td>
<td>446 aa</td>
</tr>
<tr>
<td>Aspartyl Residues</td>
<td>D257 (YDLG)</td>
<td>D263 (YDLG)</td>
</tr>
<tr>
<td></td>
<td>D385 (LGLGDFI)</td>
<td>D366 (LGLGDFI)</td>
</tr>
<tr>
<td>Highest Expression</td>
<td>Testis, lung</td>
<td>Heart, pancreas, brain</td>
</tr>
<tr>
<td>AD mutation</td>
<td>185</td>
<td>13</td>
</tr>
<tr>
<td>Knockout Mice</td>
<td>Late embryonic lethality, disturbed somitogenesis, cranial hemorrhage, underdevelopment of the subventricular zone of the brain, midline closure deficiencies and neuronal migration disorder</td>
<td>Viable, fertile mice with mild pulmonary fibrosis and hemorrhage with age</td>
</tr>
<tr>
<td>Sequence Homology</td>
<td>~67%</td>
<td></td>
</tr>
</tbody>
</table>

involved in the shedding of the Aβ peptide, PS has been shown to fulfill functions that are independent of the γ-secretase complex, including in vesicular trafficking, calcium homeostasis, beta-catenin stabilization and cell adhesion (Coen and Annaert, 2010; Li et al., 2009; Tu et al., 2006; Wakabayashi and De Strooper, 2008).

Moreover, there is much evidence to suggest that PS1 and PS2 may exhibit non-redundant properties and functions. First, PS1 and PS2 exhibit differences in the lengths of their primary structures and hydrophobicity profiles, isoelectric points, aspartyl residue locations (Bojarski et al., 2007; Lundkvist and Naslund, 2007; Martoglio and Golde, 2003; Verdile et al., 2007). The full-length PS2 protein is shorter (448 amino acids) than full-length PS1 (467 amino acids), partly because its CTF lacks some hydrophillic motifs present in PS1 (residue 350 to 371), which also results in PS2 being more hydrophobic than PS1 (Bojarski et al., 2007). In addition, whereas the predicted isoelectric point of PS1 is 5.18, the corresponding value for PS2 is 4.51. This results in significantly different elution profiles during anion-exchange chromatography (Bojarski et al., 2007). Second, although both genes are predominantly expressed in neurons and
exhibit approximately equivalent mRNA expression levels in all tissues, there is some preferential expression in a subset of tissues: Whereas PS1 expression is highest in testis and lung, PS2 expression is highest in heart, pancreas and brain (Hutton and Hardy, 1997). Third, knockout mice of PS1 and PS2 genes result in phenotypic differences. PS1 knockout mice exhibit late embryonic lethality, disturbed somitogenesis, cranial hemorrhage, underdevelopment of the subventricular zone of the brain, midline closure deficiencies and neuronal migration disorder, whereas PS2 knockout results in mild pulmonary fibrosis and hemorrhage with age in otherwise viable and fertile mice (Hutton and Hardy, 1997). On the other hand, whereas PS1 knockout seemed to spare mitochondria, PS2 knockout mice showed diminished mitochondrial function and perturbed mitochondrial membrane potentials (Behbahani et al., 2006). Of note, levels of PS2 are approximately 2.6-fold greater in PS1 knockout cells than in wild-type cells (Lai et al., 2003). This increase in PS2 levels may contribute to the increase in the PS2-containing active, mature γ-secretase complex and may compensate for the loss of PS1-containing γ-secretase complexes. Fourth, PS1 and PS2 differ in their APP processing and γ-secretase activity: PS1 knockout mice have been characterized to exhibit a close to 80% decrease in γ-secretase activity, while PS2 knockout mice displayed little or no effect in γ-secretase activity (Martoglio and Golde, 2003). A double knockout of PS1/PS2 showed completely abolished γ-secretase activity (Martoglio and Golde, 2003; Zhang et al., 2000). Fifth, PS1 and PS2 form molecularly distinct γ-secretase complexes in humans (Franberg et al., 2010; Sato et al., 2007). It has been suggested that Aβ secreted from brain-derived cell lines is distinct for PS1- or PS2-containing γ-secretase complexes and that Aβ is largely produced by PS1-mediated γ-secretase activity (Lai et al., 2003; Mastrangelo et al., 2005). Additionally, PS2-containing γ-secretase complexes show lower proteolytic activity than PS1-containing γ-secretase complexes (Ghidoni et al., 2007; Lai et al., 2003; Shirotani et al., 2007). These results demonstrate that PS2 may play a minor role in the overall γ-secretase activity which leads to the shedding of Aβ. Sixth, PS1 and PS2 may be part of similar (or overlapping) but, nonetheless, distinct signalling pathways. Knockout of both isoforms inhibit P13K/Akt signalling pathways, increase tau phosphorylation, suppress MEK/ERK pathways, and cause a defect in platelet derived growth factor (PDGF) signalling pathways (Kang et al., 2005). However, PDGF signalling is selectively restored by PS2 but not by PS1, a cellular biology that might be facilitated by FHL2, a PS2-interacting transcriptional co-activator (Kang et al., 2005). All of the
above suggests that a comparative analysis of interaction partners of PS proteins may provide insights which in turn may reveal a strategy for selectively inhibiting the production of neurotoxic Aβ peptide.

The hydrophilic, catalytic components within γ-secretase are composed of TMDs 6, 7, and 9 (Sato et al., 2006; Sato et al., 2008). Specifically, TMD9 is a structural part of the catalytic cavity of PS1, directly interacting with the catalytic TMD6 (Tolia et al., 2008). It contains the conserved PAL motif on the cytoplasmic face of TMD9 and appears to be crucial for conformational changes leading to the activation of the catalytic site (Tolia et al., 2008). The PAL motif is completely conserved in all PS and their homologues, including SPP. Interestingly, TMD9 has been proposed to allow the hydrophobic lipid environment to be accessed by water (Tolia et al., 2008). It has been indicated that the presence of a proline at position 433 (proline within PAL motif) may induce a flexible kink also in nearby TM helices, allowing the conformational changes that bring the two catalytic aspartates in close proximity to each other.
during catalysis (Tolia et al., 2008). Not surprisingly, mutations within TMD9 are known to interfere with this biology and have been shown to be causative for early onset AD.

Furthermore, there have been evidences that PS may function independent of γ-secretase complexes in: (1) ER calcium regulation, (2) catenin-cadherin signaling, (3) protein turnover and transport (reviewed in Bezprozvanny and Mattson, 2008; De Strooper and Annaert, 2010; Ho and Shen, 2011; Mattson 2010). The correlation between AD and defects in calcium signalling has been described even before the identification of PS genes (Ito et al., 1994). Since then, studies have shown biochemical evidences of PS functioning in calcium regulation both in vivo and in vitro; yet, the molecular mechanisms behind this interaction are still controversial. One consistent observation reported in these studies is that PS mutations can result in increased Ca\(^{2+}\) release from the ER. Several reports have revealed that mutant PS1 (L286V) increases Ca\(^{2+}\) release by activating ER inositol trisphosphate (IP3) and ryanodine receptors (RyR) and exhibit enhanced Ca\(^{2+}\) responses to glutamate receptor stimulation (Chan et al., 2000; Guo et al., 1996; Guo et al., 1999). Furthermore, several studies on other FAD mutants, including PS1-M146V and PS2-N141I, have corroborated effects on IP3 and RyR suggesting that PS mutations may alter functional properties of RyR-sensitive Ca\(^{2+}\) pools by disrupting a physiological role of PS as a low-conductance, passive Ca\(^{2+}\) leak channels in the ER membrane (Chan et al., 2000; Lee et al., 2006; Leissring et al., 1999; Tu et al., 2006). Moreover, it has been shown that upregulated sarco-/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) activity, which was increased in PS1-M146V mutant Xenopus laevis oocytes, may contribute to excessive ER Ca\(^{2+}\) levels (Green et al., 2008). Taken together, it is evident that PS plays a role in intracellular Ca\(^{2+}\) dynamics and it has been proposed that the disruption of this role may lead to a cascade of changes that may ultimately result in neurodegeneration.

PS also contributes to a γ-secretase-independent function in the regulation of the catenin-cadherin signalling pathway. The interaction between PS and catenin-cadherin signalling pathways has been investigated since it may influence both γ-secretase-dependent proteolysis of cadherin substrates and γ-secretase-independent functions of PS. PS1 interacts directly with catenins and cadherins at the cell surface, possibly contributing to the biology of adherens junctions (Baki et al., 2001; Georgakopoulos et al. 1999; Kouchi et al., 2009). Specifically, it has been shown that PS1 functions as a negative regulator of the Wnt/β-catenin signalling pathway
PS1 mutations reduce its ability to stabilize β-catenin and lead to increased degradation, the loss of β-catenin signalling, in turn, increases neuronal vulnerability to AD pathogenesis (Zhang et al., 1998). Nevertheless, it appears as if the interaction between PS1 and β-catenin concerns only a small pool of membrane-associated PS. Consistent with these observations, the decrease or knockout of PS1 may only exhibit a small effect on the soluble cytoplasmic pool of β-catenin.

Third, PS plays an important role in protein turnover and transport and has been shown to localize to presynaptic and postsynaptic membranes and contribute to synaptic functions (De Strooper and Annaert, 2010). Inactivation of PS by conditional double knockout in either presynaptic (CA3) or postsynaptic (CA1) neurons of the hippocampal Schaeffer-collateral pathway resulted in presynaptic impairments in early short-term plasticity prior to postsynaptic N-methyl-D-aspartic acid receptor (NMDAR) dysfunction that resembles the progression in AD pathogenesis (Saura et al., 2004; Zhang et al., 2009; Zhang et al., 2010). In addition, overexpression of human mutant PS1-L286V in transgenic mice induced a transient increase in NMDAR-mediated response, LTP, and spine density at 4- to 5-months of age, without neurodegeneration (Auffret et al., 2009). This synaptic dysfunction decreased by 13 months of age in an age-dependent manner. PS has also been reported to play a role during membrane trafficking and vesicle transport through interacting with Rab11, a small GTPase (Dumanchin et al., 1999). Lee and colleagues (2010) reported that in PS1-null blastocysts and brains of mice with reduced PS1 expressions, the degradation of proteins was impaired, and the clearance of autophagosomes was prevented. These observations were attributed to defects in lysosomal acidification of cellular degradation compartment. Causative for this failure to acidify lysosomes appeared to be a disruption of oligosaccharide maturation and subsequent transport of the proton translocating V0a1 subunit of the vacuolar (H+)-ATPase (V-ATPase). However, more recently, Zhang and colleagues (2012) re-examined this hypothesis and was unable to confirm effects of PS1 knockout on the turnover of autophagic substrates, lysosomal vesicle pH, V0a1 maturation, or lysosome function. Hence, the notion of PS1 regulating cellular autophagosomal or lysosomal functions is still controversial.

For a while, scientists have debated whether PS mutations cause gain-of-function or loss-of-function phenotypes. At this time, biochemical evidences have shown that PS mutations cause
not only loss-of-function effects, such as reduced proteolytic activity, but also a gain-of-function changes, manifest in an increased shedding of Aβ42 peptide leading to an increase in the Aβ42/Aβ40 ratio (Bergmans and De Strooper, 2010; Shen and Kelleher, 2007; Wolfe, 2007). Thus, the two seemingly mutual exclusive possibilities have been united by data which document that a loss of function can concomitantly lead to a gain of function. A concept which emerged from these studies is the notion that γ-secretase cleavages proceed in two or more steps. Initially, γ-secretase cleaves substrates at their ε-site, thereby producing longer Aβ peptides of 48- or 49-residues, followed by subsequent cleavages which proceed with a spacing of 3-4 residues to eventually produce 39-43 residue Aβ peptides (Selkoe and Wolfe, 2007; Wolfe, 2007). Therefore, when PS mutations lead to a partial impairment of proteolytic activity, a larger proportion of longer Aβ peptides such as Aβ42 may result. Not surprisingly, this debate has much broader implications, beyond their role for Aβ cleavages.

**Nicastrin: a gate keeper**

Nicastrin is a Type I TM protein with a 670 amino acid long hydrophilic N-terminal domain, a transmembrane domain, and a relatively short cytoplasmic C-terminus of twenty amino acid residues (Yu et al., 2000). It was first identified to be the part of the γ-secretase complex in a co-immunoprecipitation study with PS1 using mass spectrometry in human embryonic kidney 293 (HEK293) cells – it was originally designated as anterior pharynx defective 2 (Aph-2) (Yu et al., 2000). Nicastrin is a critical factor for normal development such that knockout mice die before the embryonic age of 10.5 days (Li et al., 2003). In addition, it has been suggested that nicastrin is critical for the correct assembly of the γ-secretase complex within ER and for intracellular trafficking of the complex to the cell surface (Morais et al., 2003; Shah et al., 2005). The large extracellular domain of nicastrin is also essential for the stoichiometric interaction between the complex and APP-C99 and thus may act as a receptor for γ-secretase substrates (Shah et al., 2005).

Similar to PS, nicastrin also undergoes a series of highly-regulated maturation processes that are necessary for the generation of active γ-secretase complexes. Like other γ-secretase core proteins, nicastrin is co-translationally channelled into the ER. Subsequently, the 78 kDa holoprotein is N-glycosylated in the ER to yield an endo-H-sensitive ~110 kDa immature form (imNct). This imNct has a half-life of less than 6 hours, and will rapidly degrade unless it undergoes further
glycosylation to a highly stable ~130 kDa mature form (mNct) (Dries and Yu, 2008; Kaether et al., 2002; Kimberly et al., 2002; Yang et al., 2002). The only form that can be incorporated into the γ-secretase complex is mNct. The N-glycosylated imNct produced in the ER leaves the ER, but may be recycled from the intermediate compartment and cis-golgi complex back to ER via its interaction with Rer1p (retention to endoplasmic reticulum protein 1) (Spasic et al., 2007). Rer1p is a small TM cargo receptor that operates in the golgi complex-to-ER retrieval of protein subunits of protein complexes (Sato et al., 2004). Interestingly, Aph-1 and Rer1p recognize the same polar residues within the nicastrin TMD. They compete for nicastrin binding in the ER, and the balance of the two proteins may act as a negative regulator of the assembly process of γ-secretase (Spasic et al., 2007). Binding to Rer1p could, therefore, cause the selective retrograde transport of imNic from intracellular compartments or cis-golgi back to the ER. This step may serve a role in preventing nicastrin from prematurely escaping and with the result that the probability of it encountering Aph-1 is enhanced (Figure 1.1.9) (Spasic and Annaert, 2008). One study has suggested that the conserved glutamate residue (Glu-332) within nicastrin is important in this process, facilitating γ-secretase maturation (Chavez-Gutierrez et al., 2008).

It has been suggested that nicastrin may function as a possible substrate docking site and a gatekeeper. More specifically, nicastrin may function as a receptor for the N-terminal stubs that are generated by ectodomain shedding of Type I TM proteins, and may hence recognize γ-secretase substrates and present them to the γ-secretase complex (De Strooper, 2005; Shah et al., 2005). Type I TM proteins that have not undergone ectodomain shedding cannot interact properly with nicastrin and do not gain access to the active site of the γ-secretase complex (Dries and Yu, 2008; Wolfe, 2006; Wolfe, 2009).

Previously, nicastrin has been considered as a critical component of the γ-secretase proteolytic function, since inhibition of nicastrin resulted in a complete loss of APP and notch cleavage (Yu et al., 2000). However, Zhao and colleagues reported that nicastrin deficient γ-secretase complexes in mouse embryonic fibroblast (MEF) cells were still able to cleave APP and Notch (Zhao et al., 2010). The authors conclude that the complex without nicastrin was highly unstable and nicastrin may still represent an indispensible component of the γ-secretase complex.
Figure 1.1.9 γ-Secretase maturation pathway from ER to Golgi. γ-Secretase proteins are newly synthesized in the ER. There are two pools of γ-secretase complexes – the large majority of γ-secretase complexes (~95%) is inactive and shuttle between the ER and Golgi, and a small fraction of active γ-secretase (~5%) resides at the plasma membrane and within endosomal compartments. Immature nicastrin co-translationally inserted into the ER interacts with Aph-1 and Rer1p. Only the nicastrin pool bound to Aph-1 will progress to join PS and Pen-2 and form active γ-secretase complex. Once the complex is fully formed, its interaction with ER retrieval components is masked, allowing the transportation to their destinations in the plasma membrane (adapted with permission from Spasic et al., 2008. Copyright (2008) Journal of Cell Science).

Anterior pharynx defective-1 (Aph-1): a possible regulator for proteolytic processing of PS
Another constituent of γ-secretase, Aph-1 is a ~30 kDa, 308 amino acid, 7 TMD protein with both N- and C-termini located in the lumen (Shirotani et al., 2004). Aph-1 was first identified in 2002 by two separate groups looking for loci that genetically interact with Notch, PS and nicastrin in C. elegans (Francis et al., 2002; Goutte et al., 2002). There are two Aph-1 genes in...
human encoding three variants; Aph-1aS and Aph-1aL on chromosome 1 and Aph-1b on chromosome 15 (Shirotani et al., 2004; Verdile et al., 2007). It has been shown that there is less Aph-1b mRNA in human tissues, as compared to Aph-1a, suggesting that the Aph-1b transcript is not as widely expressed as Aph-1a (Kimberly et al., 2003). In addition, Aph-1 variants do not engage in self-dimerization or multimerization but interact individually with other γ-secretase constituents that contain either PS1 or PS2 to form a proteolytically active γ-secretase complex (Shirotani et al., 2004). Specifically, Aph-1 binds preferentially to immature nicastrin in the ER/cis-golgi in a stable 1:1 ratio to form a ~140 kDa sub-complex (Kimberly et al., 2003). More specifically, the fourth TMD of Aph-1, which contains a GXXXG helix-helix interaction motif with three conserved glycine motifs (Gly-122, Gly-126, and Gly-130) plays a critical role in the stabilization of PS, nicastrin and Pen-2 (Laudon et al., 2007; Lee et al., 2004; Verdile et al., 2007). Similar to PS gene products, Aph-1 variants appear to display distinct functions. Whereas the Aph-1a specific γ-secretase complex is shown to be essential for Notch signalling, inactivation of Aph-1b containing complexes may primarily contribute to AD-relevant phenotypes and to a lesser extent to Notch-related signalling (Serneels et al., 2005; Serneels et al., 2009). Another study in Aph-1 knockout mice showed that the Aph-1a isoform is the only murine Aph-1 homolog that is required for proper embryonic development (Ma et al., 2005). This finding augmented the idea that distinct with different homologue combinations may behave differently reflecting functional differences in their cellular biology. The exact role of Aph-1 both within γ-secretase complexes and elsewhere still remains unknown. More comprehensive studies of Aph-1 proteins and their interacting partners are required to elucidate their functions.

Presenilin Enhancer-2 (Pen-2)

Presenilin enhance protein 2 (Pen-2) is a small hairpin-like ~12 kDa, 101 amino acid protein containing two TM domains with both N- and C-terminals facing the lumen/cytoplasm (Crystal et al., 2003; Francis et al., 2002). Pen-2 was identified together with Aph-1 in C. elegans studies. A few years after it was first discovered, Pen-2 was recognized to function as a regulator of PS endoproteolysis, a critical step during maturation and for generating γ-secretase activity (Francis et al., 2002; Goutte et al., 2002). Pen-2 is encoded by a single gene on chromosome 19, and other orthologues were identified in several species including in D. melanogaster and M. musculus (Dries and Yu, 2008). Pen-2 has been shown to interact with nicastrin, Aph-1 and PS, and
without this interaction, Pen-2 is rapidly degraded by the proteosome (Crystal et al., 2004; Steiner et al., 2002). In addition, it has been shown that the DYLSF domain of Pen-2 and a conserved Trp-Asn-Phe (WNF) motif within TMD4 of PS are critical for the interaction between these proteins (Hasegawa et al., 2004; Kim and Sisodia, 2005; Watanabe et al., 2005). When the trimeric intermediate pre-complex consisting of nicastrin:Aph-1:PS leaves the ER it is not yet fully active. Instead, a final maturation step initiated by Pen-2 incorporation is required to cause endoproteolysis of PS and to allow the complex to exit from the ER to Golgi compartments (De Strooper and Annaert, ; Spasic and Annaert, 2008). This maturation process appears to depend on slightly more acidic environment and has been suggested to involve a conformational change within the complex (Dries and Yu, 2008; Shirotani et al., 2003). Furthermore, the C-terminal half of Pen-2 appears to contain an ER retention signal, which allows Rer1p to play a role in retrieving unincorporated Pen-2 back to the ER for future incorporation into the γ-secretase complex. The possible role of Pen-2 within the mature γ-secretase complex may be to function as a ‘molecular clamp’ that holds together PS fragments (Kaether et al., 2004; Prokop et al., 2005; Prokop et al., 2004).

1.1.4 Current Diagnostics and Clinical Treatment Options

In addition to a somewhat unclear molecular etiology, the unavailability of a single diagnostic test that can accurately diagnose AD, poses major challenges for the early detection of AD. Currently, the diagnostic options include initial cognitive testing, cerebrospinal fluid (CSF) biomarker detections, genotyping, and brain imaging scans including positron emission tomography (PET), magnetic resonance imaging (MRI), and computer tomography (CT) scans (Ikonomovic et al., 2012; Mattsson et al., 2012; Woo et al., 2009). Previously, the initial cognitive testing was performed on the basis of two criteria, Diagnostic and Statistical Manual 4th Edition (DSM IV) clinical criteria and National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINDS-ADRSA) criteria (American Psychiatric Association, 2000; Dubois et al., 2007; Fita et al., 2011). These criteria described several domains that may be impaired in AD including memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving and functional abilities. More recently, the National Institute on Aging (NIA) and the Alzheimer’s Association revised and proposed the new diagnostic criteria with three stages: preclinical AD, mild cognitive impairment due to AD, and probable dementia due to AD (Albert et al., 2011;
McKhann et al., 2011; Sperling et al., 2011) (Table 1.1.3). These revised criteria incorporates the idea that AD progresses over time and manifests over a continuum. Therefore, it may be possible to detect AD pathophysiology preclinically with biomarkers. The current CSF biomarker analyses involve observing the decreased level of Aβ42 peptide and elevated Tau or phospho-Tau levels in CSF. One of the major problems with this approach is that detectable Aβ accumulation may not occur years before the apparent cognitive symptoms – detection at preclinical, asymptomatic stage poses its limitations. By contrast, clinically-relevant symptoms of dementia due to AD occur during the last stage of the disease progression. Thus, biomarkers of early brain amyloidosis and biomarkers of dementia may behave differently as predictors of future cognitive decline. Brain image scans are performed by directly monitoring the progression of atrophy (via MRI) and by monitoring chemical tracers such as [C-11]-labelled Pittsburgh Compound B ([C-11]PiB) that binds to Aβ peptides (via PET). Yet, none of these test are definitive, therefore, AD diagnosis is performed on combinations of these laboratory tests, which can be time consuming and cost-inefficient. The only definitive diagnostic test is based on post-mortem brain tissue analyses. Therefore, there is an urgent need to translate insights into the molecular etiology into rational approaches for early AD diagnosis.

Apart from the APP substrate, there are many other TM protein substrates that get processed by γ-secretases (Table 1.1.4) (Hemming et al., 2008; Wakabayashi and De Strooper, 2008). These are involved in various cellular functions including, but not limited to, cell fate determination (Notch and Jagged), cell-cell adhesion (N-and E-cadherins, CD-44, Nectin-1α), regulation of ion conductance (β2 subunit of the voltage-gated sodium channel), and growth factor–dependent receptor signalling pathway (p75 NTR)(Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). The broad specificity of the γ-secretase complex poses a challenge in efforts to devise a specific therapeutic strategy. The general characteristics for γ-secretase substrate are:

1. a Type 1 TM helix,
2. a small ectodomain,
3. permissive determinants in the juxtamembrane and cytoplasmic domains, and
4. residues that can destabilize the helical configuration of the TMD around the cleavage site (Beel et al., 2008; Li et al., 2009).
Table 1.1.3 – Diagnostic criteria of Alzheimer’s disease.

<table>
<thead>
<tr>
<th>Stages of AD</th>
<th>Features</th>
</tr>
</thead>
</table>
| **Preclinical** (Sperling et al., 2011) | **Stage 1:** Asymptomatic Amyloidosis  
- High PET amyloid tracer retention  
- Low CSF Aβ1-42  
**Stage 2:** Amyloidosis + Neurodegeneration  
- Neuronal dysfunction on FDG-PET/fMRI  
- High CSF tau/p-tau  
- Cortical thinning/hippocampal atrophy on sMRI  
**Stage 3:** Amyloidosis + Neurodegeneration + Subtle Cognitive Decline  
- Evidence of subtle change from baseline level of cognition  
- Poor performance on more challenging cognitive tests  
- Does not yet meet the criteria for MCI |
| **MCI** (Albert et al., 2011) | Cognitive concern reflecting a change in cognition (i.e., historical or observed evidence of decline over time)  
- Impairment in one or more cognitive domains:  
  - Memory  
  - Executive Function  
  - Attention  
  - Language  
  - Visuospatial skills  
- Maintain independence in functional abilities  
- Rule out vascular, traumatic, medical causes of cognitive decline, where possible  
- Provide evidence of longitudinal decline in cognition, when feasible  
- Report history consistent with AD genetic factors, where relevant  
- Does not yet meet the criteria for dementia |
| **Dementia** (McKhann et al., 2011) | Meets the criteria for all-cause dementia:  
- Insidious onset - gradual cognitive decline  
- Cognitive deficits that:  
  - Interferes with independence  
  - Evidenced by clear decline in history  
  - Are not due to delirium or another psychiatric disorder  
  - Has Impairments in two or more cognitive domains:  
    - Memory  
    - Reasoning and judgement  
    - Personality or behaviour  
    - Language  
    - Visuospatial skills  
- Evidence on either amnestic (learning and recall) or nonamnestic (language, visuospatial, and executive dysfunction) presentations.  
- Not evidenced by following conditions:  
  - Cerebrovascular disease  
  - Dementia with Lewy bodies  
  - Behavioral variant frontotemporal dementia  
  - Primary progressive aphasia  
  - Another concurrent, active neurological disease, or a non-neurological medical comorbidity or use of medication that could have a substantial effect on cognition. |
Table 1.1.4 γ-Secretase substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Physiological Functions</th>
<th>Secreted Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>Cell adhesion and migration, neurite outgrowth, synaptogenesis</td>
<td>Aβ, p3</td>
</tr>
<tr>
<td>APLP-1, APLP-2</td>
<td>Cell adhesion and migration, neurite outgrowth, synaptogenesis</td>
<td>ND</td>
</tr>
<tr>
<td>Notch 1, 2, 3 and 4</td>
<td>Notch signaling receptor; multiple cell differentiation processes</td>
<td>Nβ</td>
</tr>
<tr>
<td>ErbB-4</td>
<td>Receptor tyrosine kinase, receptor of various growth factors</td>
<td>ND</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Cell adhesion</td>
<td>ND</td>
</tr>
<tr>
<td>LRP1</td>
<td>LDL receptor superfamily, endocytosis of a wide range of ligands, regulation of signaling pathways</td>
<td>ND</td>
</tr>
<tr>
<td>CD44</td>
<td>Cell adhesion, tumor growth, metastasis</td>
<td>CD44β-peptide</td>
</tr>
<tr>
<td>Nectin-1α</td>
<td>Cell adhesion, synaptic contact</td>
<td>ND</td>
</tr>
<tr>
<td>NRG-1</td>
<td>Growth and differentiation factor, ligand for ErbB receptors, nervous system development</td>
<td>ND</td>
</tr>
<tr>
<td>Delta 1</td>
<td>Notch signaling ligand, multiple cell differentiation processes</td>
<td>ND</td>
</tr>
<tr>
<td>GluR3</td>
<td>Ionotropic glutamate receptor</td>
<td>ND</td>
</tr>
<tr>
<td>p75NTR</td>
<td>Neurotrophin receptor, regulation of axonal outgrowth, cell survival</td>
<td>ND</td>
</tr>
<tr>
<td>DCC</td>
<td>Commissural axon guidance, receptor for netrin-1</td>
<td>ND</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Cell adhesion</td>
<td>ND</td>
</tr>
<tr>
<td>Jagged</td>
<td>Notch signaling ligand, multiple cell differentiation processes</td>
<td>ND</td>
</tr>
<tr>
<td>apoER2/LRP8</td>
<td>LDL receptor superfamily, maintenance of synaptic plasticity, LTP</td>
<td>ND</td>
</tr>
<tr>
<td>Syndecan-3</td>
<td>Proteoglycan, receptor/co-receptor of cytokines, growth factors and extracellular matrix components</td>
<td>ND</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Receptor tyrosine kinase, cell proliferation and differentiation</td>
<td>ND</td>
</tr>
<tr>
<td>Alcadeinα, β, γ</td>
<td>Cadherin-related membrane protein family, postsynaptic Ca2+-binding protein</td>
<td>β-Alc</td>
</tr>
<tr>
<td>Megalin/LRP2</td>
<td>LDL receptor superfamily, regulating several extracellular signaling by endocytosis</td>
<td>ND</td>
</tr>
<tr>
<td>NRADD</td>
<td>Induction of apoptosis in neuronal cells</td>
<td>ND</td>
</tr>
<tr>
<td>Pcdh</td>
<td>Cadherin-related adhesion molecules, neuronal connectivity and survival</td>
<td>ND</td>
</tr>
<tr>
<td>CD43</td>
<td>Immune synapse, T-cell proliferation and migration, cell-cell interactions in leucocytes</td>
<td>ND</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor, activation of signaling pathways</td>
<td>ND</td>
</tr>
<tr>
<td>VGSCb</td>
<td>Modification of voltage-gated sodium channel functions</td>
<td>ND</td>
</tr>
<tr>
<td>Tyr, Tyrp1,DCT/Tyrp2</td>
<td>Conversion of tyrosine to DOPA, pigment synthesis</td>
<td>ND</td>
</tr>
<tr>
<td>EphrinB1, B2</td>
<td>Ligands for receptor tyrosine kinase Eph receptors, neuritogenesis, angiogenesis</td>
<td>ND</td>
</tr>
<tr>
<td>SorLA, Sortilin, SorCS1b</td>
<td>Vps10p sorting receptors, intracellular sorting and trafficking of various cargo proteins</td>
<td>Sorβ</td>
</tr>
<tr>
<td>RPTPε</td>
<td>Receptor protein tyrosine phosphatase, cell adhesion</td>
<td>ND</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>MHC class I protein, T-cell development, immune cell responses, synaptic plasticity</td>
<td>ND</td>
</tr>
<tr>
<td>LAR</td>
<td>Receptor protein tyrosine phosphatase, formation of synapses and neuronal networks</td>
<td>ND</td>
</tr>
<tr>
<td>EphB2</td>
<td>Receptor tyrosine kinase, neuritogenesis, angiogenesis</td>
<td>ND</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Receptor tyrosine kinase, cell proliferation</td>
<td>ND</td>
</tr>
<tr>
<td>Tie1</td>
<td>Receptor tyrosine kinase, vascular development</td>
<td>ND</td>
</tr>
</tbody>
</table>

Adapted from Wakabayashi and De Strooper (2009)

*ND: not done, which means no experimental evidence available.
Recently, PS genes and their protein products have gained interests as potential therapeutic targets for the treatment of early-onset AD. However, these approaches targeting \(\gamma\)-secretase are still unsatisfactory. For example, the use of \(\gamma\)-secretase inhibitor LY-411,575 not only alters the levels of A\(\beta\)40 and A\(\beta\)42 in brain and plasma, but also affects lymphocyte development and causes profound alterations in gastrointestinal tract tissue morphology – potentially mediated by the concomitant inhibition of Notch S3 cleavage (Wong et al., 2004). The indiscriminative inhibition of \(\gamma\)-secretase may not be the ideal therapeutic solution. Therefore, the goal of to advanced \(\gamma\)-secretase inhibitor (GSI) strategies is to lower A\(\beta\) production in the brain enough prevent A\(\beta\) oligomerization and fibril formation, while preserving enough Notch signalling to avoid toxic side effects. The first reported in vivo testing of GSI involved dipeptidic compound N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and showed the reduction of A\(\beta\) peptides in brain, plasma and CSF of young APP-transgenic mice in a dose dependent manner within 3 hours of treatment (Dovey et al., 2001). Important to note is that DAPT binds only active \(\gamma\)-secretase complex with cleaved CTF, not the immature PS holoprotein. More specifically, DAPT binds to CTF at a site distinct from the catalytic site or substrate binding site, and may cause an allosteric conformational change to the complex which affects A\(\beta\) peptide production (Morohashi et al., 2006).

More recent derivatives of DAPT include the more potent inhibitors Compound E, benzodiazepine analogue LY411,575 and benzolactam LY450,139 (Best et al., 2005; Fleisher et al., 2008; Seiffert et al., 2000; Siemers et al., 2006). Although, these GSI displayed effective reduction of A\(\beta\) levels, issues of toxicity in both mice and human have been noted that require more attention. For example, long term treatment of LY411,575 caused severe gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice, which may be due to Notch-related side effects (Searfoss et al., 2003; Wong et al., 2004). In addition, LY450,139 treatment in randomized phase 2 human trials have been reported to cause various side effects including hair colour changes and transient bowel obstruction (Fleisher et al., 2008). Similarly, L685,458, a transition-state analogue of GSI that targets the catalytic aspartates and therefore binds NTF and CTF also caused side-effects related to inhibition of other substrates, which diminished interest in it as a therapeutic drug (Li et al., 2000; Shearman et al., 2000). Nevertheless, its ability to bind to PS allowed L685,458 to be an excellent experimental tracer for \(\gamma\)-secretase activity in vivo (Xiong et al., 2007). Another GSI called BMS-299897, a
sulphonamide inhibitor, has been claimed to display specific inhibition of APP processing over notch by about 15-fold (Anderson et al., 2005). In conclusion, effort to find GSIs that can be safely used in therapeutical applications are still under investigations and studies so far have uncovered a surprising complexity of an underlying biology that ties γ-secretase to multiple novel pathways.

The second strategy for targeting Aβ oligomers is to prevent the oligomerization of Aβ or to enhance its clearance from the cerebral cortex. As indicated earlier in this chapter, Aβ oligomers have been suggested as the major culprit for cellular toxicity and as a precursor for insoluble plaque formation. To eliminate Aβ oligomers, immunotherapy using an active or passive Aβ immunization has been suggested (Klyubin et al., 2005; Necula et al., 2007; Townsend et al., 2006). It has been shown that in vivo administration of Aβ monoclonal antibodies can prevent the inhibition of hippocampal long-term potentiation caused by secreted Aβ (Klyubin et al., 2005). On the other hand, other study has shown that the conditional inactivation of PS1 in APP-transgenic mice failed to rescue the contextual memory and hippocampal synaptic deficits, while amyloid accumulation is effectively prevented (Saura et al., 2005). Nevertheless, despite conflicting reports, antibody strategy displays the promising therapeutic advantages that may offer a strategy for the reversal of cognitive impairment. In addition, various Aβ oligomer-selective degradation reagents including insulin-degrading enzyme (IDE), plasmin, cathepsin B, neprilysin and an Aβ oligomer destabilizer called Alzhemed are currently under investigation for therapeutic applicability (Chow et al., 2010; Haass and Selkoe, 2007; Walsh et al., 2002; Walsh et al., 2005).

The third therapeutic strategy targeting Aβ is based on γ-secretase modulators (GSMs). GSMs alter the cleavage site within APP and, as a consequence, increase the production of less toxic shorter Aβ peptides. It has been hypothesized that the binding of GSMs to APP leads to a change in how APP is presented to the γ-secretase catalytic site. In one model, a hidden allosteric site within γ-secretase may become accessible to GSMs only after the binding of substrates. Allosteric binding of GSMs then may induce PS to undergo a subtle conformational adjustment which affects its alignment with the APP substrate and, consequently, the exact cleavage site (Uemura et al., 2010). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most studied GSMs. It has been shown that certain NSAIDs reduce the production of neurotoxic Aβ42
peptides without affecting Aβ40 levels and, in fact, may increase the production of shorter, less harmful Aβ peptides, including Aβ38 (Czirr et al., 2008; Weggen et al., 2001; Weggen et al., 2003). Still, there had been debates on whether such NSAIDs efficacy is sufficient to warrant human trials. At this time, one component, called (R)-flurbiprofen (tarenflyurbil; Flurizan), did enter phase 3 clinical trials but failed to show beneficial effects (Green et al., 2009). In addition, NSAIDs usage was shown to cause gastrointestinal and renal side-effects due to the inhibition of cyclo-oxygenases (COX1) (Gasparini et al., 2005; Tabet and Feldman, 2002). Other NSAIDs, without COX-inhibitory activity, were subsequently developed and tested. However, to date these derivatives failed to deliver promising results in clinical trials (Bergmans and De Strooper, 2010).

Other possible therapeutic strategies include modulating cholesterol homeostasis by using cholesterol-lowering drugs, and efforts to chelate Cu²⁺ and Zn²⁺metal ions, which have been shown to support Aβ aggregation (Sparks, 2011). Additionally, the anti-cancer drug Imatinib (Gleevec) has been shown to selectively decrease Aβ production by targeting γ-secretase activating protein (GSAP), without affecting Notch cleavage (He et al., 2010; Wolfe, 2006). In conclusion, although impressive progress has been made in attempts to target complex molecular machinery that affects abnormal levels of Aβ production in AD, the effectiveness of current inhibitors is limited and no safe AD intervention strategy has emerged.

Interactome studies using Mass Spectrometry

For an enzyme like γ-secretase, which does not utilize a common substrate recognition sequence, quantitative proteomics may be the most effective and unbiased method for identifying endogenous substrates and protein interactors. In recent years, several attempts have been made to study γ-secretase using proteomic analysis. First, Hemming and colleagues studied the interactome of γ-secretase in HeLa cells using a method referred to as stable isotope labelling with the amino acids in cell culture (SILAC), which involves metabolic labelling of cells with amino acids containing either heavy or light isotopic variants (Hemming et al., 2008). In this particular analysis, membrane proteins were labelled with light or heavy lysine and arginine amino acids, purified, and then analyzed using SDS-PAGE and LC-MS/MS. Several PS substrates including APP, APP homolog APLP2, CD44, human leukocyte antigen, low-density lipoprotein receptor, syndecan were identified. Another group approached the objective to
identify PS interacting proteins in HEK293 cells by using SDS-PAGE and LC-MS/MS but did not incorporate protein labelling into their workflow. In this study, Winkler and colleagues identified CD147, Niemann-Pick type C1 protein (NPC1), ATP1A1, ATP1B3, CD98, M6PR, TMP21 and three other p24 protein families: p24a (TMED2), gp25L2 (TMED9), and p24b (TMED4) as PS interacting partners (Winkler et al., 2009). Another \( \gamma \)-secretase interactome study was performed by Wakabayashi and colleagues (2009). This study attempted to examine the \( \gamma \)-secretase interactome by re-introducing calmodulin-flag tagged PS1 and PS2 into PS1/PS2 double knockout mouse embryonic fibroblast (MEF) cells, purify \( \gamma \)-secretase complex with tandem affinity purification (TAP) and analyze it with LC-MS/MS. In this study, they identified 59 proteins that co-purified with PS, including previously published interactor proteins, including Tmp21, \( \beta \)-catenin, \( \gamma \)-catenin, and Rab-11, but without quantification. Additionally, they have identified several tetraspanin proteins, which are involved in various cell adhesion, cell signalling and proteolysis (Wakabayashi et al., 2009).

To date, we are not aware of attempts that succeeded in examining the molecular environments of distinct \( \gamma \)-secretase sub-complexes, allowing to quantitatively assign interactor protein to subcomplexes. As previously indicated, PS1 and PS2 form distinct \( \gamma \)-secretase sub-complexes and their knockout models of mice have demonstrated two very dissimilar phenotypes. This may indicate distinct functions of PS1- and PS2-containing \( \gamma \)-secretase complexes. To understand the etiology of AD progression, an in-depth study of distinct \( \gamma \)-secretase complexes and their interaction partners is critically needed.
1.2 An Overview of Proteomics and Mass Spectrometry

1.2.1 Introduction to Proteomics

Proteomics, the study of proteins, has emerged as a powerful tool for studying biological processes. The word “proteomics” was first used by Dr. Peter James in 1997 as an analogy to genomics, the study of genes (James, 1997). Proteomes are highly dynamic and complex. Indeed, the major challenges of proteomics research lie with the diversity of proteins. The transcription and translation of any gene typically gives rise to multiple distinct proteins due to various biological processes including alternative splicing, sequence polymorphisms, random mutations and a large number of possible post-translational modifications. Each protein acquires a unique three-dimensional conformation with distinct physicochemical and functional properties. To describe the structure of proteins, a terminology is used that distinguished four levels of organization. Primary structure refers to the amino acid sequence of a protein. Secondary structure refers to local folds that are frequently stabilized by hydrogen bonds forming alpha helix and beta sheet structures. Tertiary structure is formed when secondary structure elements assemble into a larger fold that encompasses the entire single gene-encoded protein molecule. Finally, the quaternary structure refers to the assembly of several protein molecules, which work in concert within a protein complex. All of the aforementioned levels of structural organization can influence protein-protein interactions, intracellular localization, and function. In addition, the expression of a given protein can vary considerably depending on the type of cell that it originates from, the stage within the cellular life cycle or within growth, development and age of a cell it is expressed at, and the environmental conditions its host cell is exposed to.

1.2.2 Challenges in studying protein-protein interactions

Proteins are engaged in dynamic interactions with other proteins to complete their diverse cellular functions (Aloy and Russel, 2002; Blackstock and Rowley, 1999; Gavin et al., 2006; Neubauer and Wilm, 1999; Sobott and Robinson, 2002). The specific interaction of a protein is commonly referred to as the ‘interactome’ (Berggard et al., 2007; Charbonnier et al., 2008; Drewes and Bouwmeester, 2003). Most common biochemical technique that is employed during the identification of interacting protein is the co-purification. In particular, the combination of protein tagging and affinity chromatography of protein complexes has been proven to be a
powerful tool for interactome analysis (Burgess and Thompson, 2002; Gingras et al., 2005). However, not all proteins are suited for this type of analysis. For example, protein-protein interactions involving membrane-embedded proteins are notorious for the difficulties they pose. One challenge is in predicting how well a given protein-protein interaction involving membrane protein will tolerate exposure to a specific detergent. As such, a generic solubilization strategy cannot be devised (Hooker et al., 2007; Reisinger and Eichacker, 2008).

**Tagging technology: GS-TEV tag, cloning strategy, lentiviral transduction**

Each protein has its unique properties, which in turn, call for unique purification conditions. Additionally, there are several important considerations to be taken into account during purification procedures. First, proteins should, as much as possible, maintain near-native properties and activities during their purification. Second, effective purification conditions should limit the co-purification of nonspecific interactor proteins and other contaminants. Third, purification of protein complexes from biological sources should yield a sufficient quantity for downstream analyses such as mass spectrometry. Therefore, the overall objective is to devise an optimized purification strategy which is suited to isolate a sufficient quantity of biologically active, pure protein complexes using the fewest possible number of steps.

The recent development of several protein tagging technologies has greatly advanced this objective. For example, the tandem affinity purification (TAP) tagging method is a two-step affinity purification protocol that enables rapid purification under native conditions of proteins (Burckstummer et al., 2006; Puig et al., 2001). This method involves two preparative steps, the fusion of the TAP tag to the C- or N-terminus of a target protein, and the introduction of the expression construct into a suitable host cell. The original TAP tag had been developed for the purification of proteins from yeast (Rigaut et al., 1999). It consists of two IgG binding domains of *Staphylococcus aureus* Protein A (ProtA) and a calmodulin binding peptide (CBP) separated by a tobacco etch virus (TEV) protease cleavage site (Rigaut et al., 1999). This original TAP tag can also work for the purification of proteins from mammalian cells, but may not give rise to a satisfactory yield (Burckstummer et al., 2006). These shortcomings have provoked the development of an improved tagging technology based on Protein G (ProtG; 60 amino acids) and a streptavidin binding peptide (SBP; 38 amino acids) that can be eluted by biotin (Figure 1.2.1A, B) (Burckstummer et al., 2006; Xu et al., 2010). This improved tag with a combined molecular
weight of 18.8 kDa, hereafter referred to as the GS-TEV tag, has been shown to produce a tenfold increase in protein yield with improved specificity in human embryonic kidney 293 (HEK 293) cells compared to the original yeast tag (Burckstummer et al., 2006; Collins and Choudhary, 2008; Kocher and Superti-Furga, 2007). Once the protein of interest is fused with the TAP tag on the desired protein terminus, it can be delivered to the host cell system using viral vectors.

Lentiviral vectors, which are derived from human immunodeficiency virus (HIV) and hepatitis B virus (HBV), can mediate the efficient delivery, stable integration, and long-term expression of transgenes (Naldini et al., 1996; Tiscornia et al., 2006; Trono, 2000). The transduction process...
using lentivirus is depicted in Figure 1.2.2A. The lentiviral system we employed follows the protocol from Trono and colleagues (2000). This system requires three separate delivery vectors: envelop, package, and transfer. A protein expression construct is inserted into a transfer vector and together with envelop and package vectors, a virus is formed to invade the cell nucleus causing its genomic integration (Trono, 2000). To generate the lentiviral particle required for transduction, the three vectors are together transfected into a human embryonic kidney 293T (HEK293T) cell. HEK293T cells are subvariant of HEK293 cells that have been modified to produce the simian virus 40 (SV40) large tumor antigen, known to increase the replication of transfected vectors containing the SV40 origin of replication. This amplification of transfected vectors results in extended expression of the desired gene products within two to three days of transfection (DuBridge et al., 1987; Pear et al., 1993). Successful transfection can be tracked and evidenced using integrated green fluorescence protein (GFP) marker incorporated into the transfer vector. Thus, immunofluorescing cells can be easily identified under the fluorescence microscope and the cells stably expressing the transgene can be selected by using a fluorescence activated cell sorting (FACS) machine. Once the transfection is successful, HEK293T cells produce lentivirus expressing protein of interest (Trono, 2000). Lentivirus are collected and transduced into HEK293F cells, yet another subvariant of HEK293 cells that has been adapted to grow non-adherently in suspension culture. Stable lentiviral integrant clones expressing the protein of interest can be selected using an antibiotic resistance gene which is translated from an internal ribosome entry site (IRES).

One important advantage of the lentiviral delivery is its ability to replicate in both mitotic and non-mitotic cells such as neurons (Naldini et al., 1996). For example, a retroviral delivery system cannot transfer genes into host cells that do not engage in mitosis (Lewis and Emerman, 1994). Moreover, several biosafety features have been incorporated in the lentiviral system we employed. First, the lentiviral vector was engineered as a self-inactivating (SIN) HIV-derived vector, which causes it to lose its transcriptional capacity once transferred to target cells (Miyoshi et al., 1998; Zufferey et al., 1998). This minimizes the risk of replication-competent recombinants (RCR) originating in the host cells. Second, to minimize the number of crossovers that would be required to recreate RCR, the distribution of sequences encoding its various vector components is produced from as many independent DNA units as possible (Dull et al., 1998; Farson et al., 2001). Therefore, the overlap between vector and helper sequences, which may
A. Envelope vector pMD2G
Packaging vector pPAX2
Transfer vector pWPI

Transfection

HEK 293T

Transduction

Protein-expressing lentivirus

HEK 293F expressing protein

B. pBudCE4
4596 bp

Promotor EF1-α and PS1 inserted into pBud

pBud-EF1 Alpha.PS1
7363 bp

PS1 inserted into pWPI

Xma 1

pWPI.NEO. MCS+
11437 bp

Fse 1

C. pWPI.NEO. PS1
13378 bp

Marker
pWPI.NEO.MCS-
pWPI.NEO.PS1

20000
10000
5000
2000
1500

Ladder

EMCV IRES
hEF1

Ps1 or 2 insertion site

5′UTR

SV40 ORI

Ampr

LTR

REP

LTR3′IN

Lori

WPHE

pA
components is produced from as many independent DNA units as possible (Dull et al., 1998; Farson et al., 2001). Therefore, the overlap between vector and helper sequences, which may have the potential for homologous recombination, has been reduced to a few tens of nucleotides (Dull et al., 1998; Trono, 2000). Third, over 60% of the HIV genome is completely eliminated with its packaging system comprising only three of the nine HIV-1 genes: gag, pol and rev (Dull et al., 1998). Therefore, the possibility of the parental virus reconstituting itself in host cells is abolished.

One downside of using a lentiviral vector system is its large vector size. Cloning large insert into a pWPI lentiviral transfer vector, approximately 12 Kb in size, exacerbates the cloning difficulties. For our study, we devised a strategy that aimed to sub-clone a portion of the vector containing multiple cloning sites (MCS) into an alternative, smaller eukaryotic intermediate vector pBudCE4 (Invitrogen), which is 4.5kb in size. This effort facilitated a more efficient cloning of the 2.5 Kb insert, which was later shuttled back into the final lentiviral vector (Figure 1.2.2B). The insertion of a transgene can be confirmed by an analytical restriction enzyme digestion on an agarose gel and sequencing (Figure 1.2.2C). The use of the intermediate vector also allowed rapid analysis of cloning expression efficiency in cells using a simple transfection assay which produced results within a day.
Multistep Purification

As previously noted, the ability to purify significant amounts of protein complexes in their near-native state is the key to success in studying protein-protein interactions. Finding optimal cell solubilization conditions that will effectively homogenize the cells without disrupting the protein complexes of interest is crucial. Solubilisation of highly hydrophobic membrane-associated proteins is notoriously difficult in aqueous conditions. Therefore, detergent treatment which helps to extract proteins and mediates solubility in aqueous solutions is a prerequisite for protein purification. Detergents are amphipathic molecules consisting of a polar head group and an extended hydrophobic hydrocarbon chain. They are usually categorized by the nature of their chemical properties as non-ionic, anionic, cationic, and zwitterionic (Arnold and Linke, 2007). In general, ionic detergents (e.g. SDS) are considered relatively harsh as oppose to non-ionic detergents (e.g. DDM, digitonin) which have milder solubilization properties. Not surprisingly, zwitterionic detergents (e.g. CHAPSO and CHAPS) combine properties of both ionic and non-ionic detergents (Seddon et al., 2004). The choice of a detergent used strongly influences the outcome of the experiment, with harsh detergent treatments frequently changing the native state of proteins and, consequently, causing problems related to undesired aggregation or disruption of physiological interactions. A few considerations in selecting detergents for purification are: (1) the stability of the protein complexes, (2) whether the detergent of choice is compatible with downstream protein handling steps such as mass spectrometry or NMR, and (3) whether the detergent is dialyzable and, therefore, can be removed (Arnold and Linke, 2007; Seddon et al., 2004). For protein complexes such as γ-secretase that are difficult to maintain, a combination of several detergents may result in better protein solubilization than the use of a single detergent. Several studies had prior to this work attempted to purify the active γ-gamma secretase complex using various detergent conditions (Cacquevel et al., 2008; Fraering et al., 2004; Gu et al., 2004; Hur et al., 2008). It had been reported that 1% CHAPSO is the optimum detergent for preserving γ-secretase activity in cell-free membrane preparations. It was also known that γ-secretase activity is significantly reduced in the presence of 0.25% digitonin and completely abolished by 1% Triton X-100, 1% n-octyl glucoside, and many other detergents (Gu et al., 2004). DDM-solubilized γ-secretase complexes were reported to contain all four known γ-secretase components, and seemed to contain larger amounts of solubilized nicastrin and Pen-2 when compared with γ-secretase complexes purified in the presence of 1% CHAPSO or 1% digitonin.
(Fraering et al., 2004). Yet, higher concentrations of DDM had been shown to dissociate and inactivate \( \gamma \)-secretase in a concentration-dependent manner, and 1% DDM resulted in a substantial decrease in activity (Fraering et al., 2004). Therefore, in the pilot studies we undertook prior to this work, we have found it to be beneficial to use combinations of these two detergents at lower concentrations. For example, when purifying active \( \gamma \)-secretase complexes, the combination of both CHAPSO (0.25%) and DDM (0.05%) for solubilization was more beneficial than using either CHAPSO or DDM alone (data not shown). This combination of detergents could also relatively easily remove in a last step of the purification method and, therefore, would not interfere with subsequent analysis by mass spectrometry.

Once desired solubilization conditions are achieved, lysates could be subjected to multistep purifications using the TAP tagging strategy (Figure 1.2.3). The GS-TEV tag is designed to be employed in a two-step purification scheme (Burckstummer et al., 2006). The first step of the purification is based on the ProtG motif that can be isolated using IgG-sepharose resin. The second purification step is based on streptavidin-based purification using the streptavidin binding peptide (SBP) for binding and competitive displacement with biotin for elution. Additional affinity purification steps may exploit inherent properties of the protein complex. For example, the \( \gamma \)-secretase complex, which contains heavily N-glycosylated nicastrin, can be additionally purified by performing a lectin chromatography step with wheat germ agglutinin (WGA) serving as the lectin. The result of purification can be confirmed with Western blotting analysis and the functional activity can be confirmed with enzyme-linked immunosorbent assay (ELISA) analysis.
Figure 1.2.3 Multistep purification strategy of γ-secretase complex based on GS-TEV tag and N-glycans present on nicastrin subunit. In the initial step, the TAP-tagged protein is purified using the ProtG moiety, and then released by TEV cleavage. Next, the eluted sample is subjected to lectin chromatography using WGA to select the subpopulation of fully assembled, nicastrin-containing γ-secretase complexes. Finally, the sample is enriched using the SBP tag and eluted using an excess of biotin. Control samples can be prepared by pre-incubating the samples with biotin prior to the streptavidin purification step (N-acetylglucosamine, Nac-GlcN; Streptavidin binding peptide, SBP; Wheat Germ Agglutinin, WGA)
1.2.3 Introduction to Mass Spectrometry

In the last few decades, mass spectrometry (MS) has emerged as one of the major technologies driving proteomics and has become the most favoured method in identifying large subsets of proteins and peptides (Cravatt et al., 2007; Patterson and Aebersold, 2003; Steen and Mann, 2004). The general scheme of MS-based proteomics is as follows (Khan et al., 2011; Nesvizhskii, 2007; Perkins et al., 1999; Steen and Mann, 2004; Xie et al., 2011):

1. peptides in a sample are ionized and converted to gas phase ions,
2. the mass-to-charge ratio \(m/z\) of the precursor ion is generated and the mass of the peptide is determined implicitly,
3. selected precursor ions are isolated and subjected to a further gas phase,
4. precursor ions are fragmented,
5. fragment-ion masses are analyzed and product-ion spectra are recorded, and
6. the data are processed and analyzed using computer software.

MS is a method that measures the \(m/z\) ratio of charged peptides and their fragments to identify and analyze proteins. The two most significant features of mass spectrometry which account for the popularity of this technology are its sensitivity (being able to detect proteins as low as fmol levels) and its ability to generate vast amounts of data on large numbers of proteins in a relatively short period of time. Mass spectrometers are modular in their design and consist fundamentally of three main compartments: the ionization apparatus, the mass analyzer, and the detector (Figure 1.2.4). The ionization apparatus converts molecules into charged ions. Two most widely used soft ionization techniques are electrospray ionization (ESI) (Fenn et al., 1989) and matrix-assisted laser desorption/ionization (MALDI) (Tanaka et al., 1988). ESI is often connected to online high performance liquid chromatography (HPLC) systems that are employed to reduce the complexity of a given sample prior to its injection into the mass spectrometer. For example, reversed phase (RP) HPLC separates a peptide sample based on hydrophobicity with a non-polar stationary phase and an aqueous, moderately polar mobile phase. With these RP stationary phases, retention times are longer for more hydrophobic peptides (i.e., less polar), with more hydrophilic peptides (i.e., more polar) eluting earlier. Once samples are separated and eluted from HPLC, they are injected into a high electric potential ESI apparatus. This generates highly charged droplets, which then undergo repeated cycles of fission and shrinkage until
**Figure 1.2.4 Fundamental modular design of a mass spectrometer.** A basic mass spectrometer consists of three components: (1) the ionization apparatus, which facilitates ionization of sample peptides and converts them to gas phase ions, (2) the mass analyzer, which exploits the mass-to-charge ratio ($m/z$) of the precursor ion for separation and selects precursor ions for further fragmentation if needed, (3) the detector, which determines the mass-to-charge ratio ($m/z$). The detector is connected to a recording device, which records the product-ion spectra, and processes the data using computer software.

Analyte ions are free of solvent (Steen and Mann, 2004). In contrast, MALDI uses a chemical matrix that absorbs light within the ultraviolet light spectrum and therefore can be activated by a suitable laser (e.g., a N2 laser) (Tanaka et al., 1988). Once generated, analyte gas phase ions from both ionization techniques are steered into the mass analyzer.

As the name suggests, the mass analyzer separates ions based on their mass-to-charge ($m/z$) ratio. Popular analyzers employ time-of-flight (TOF) technology, quadrupoles or ion traps (Fenn et al., 1989; Glish and Vachet, 2003; Hillenkamp and Karas, 1990; Kocher and Superti-Furga, 2007; Patterson and Aebersold, 2003; Steen and Mann, 2004). A majority of today’s mass spectrometers combine multiple mass analyzers and, as a result, are capable of tandem mass spectrometry (MS/MS) experiments. TOF mass spectrometer is based on the time it takes ions to travel through an electric-field-free and gas-evacuated flight tube. Since ions are accelerated to the same kinetic energy ($E_{\text{kinetic}}$; $E_{\text{kinetic}} = mv^2$), the velocity ($v$; $v = \text{displacement/time}$) of a given ion depends on its mass ($m$) (Chernushevich et al., 2001; Patterson and Aebersold, 2003). More specifically, the lighter ions travel faster than the heavier ions, hence reaching the detector faster, hence by clocking ion impacts at high frequency a calibrated TOF mass analyzer can infer the
m/z ratio of a given ion. The quadrupole ion trap instrument traps ions in the space between alternating current (AC; oscillating, non-static) and direct current (DC; non-oscillating, static) electric fields (Jonscher and Yates, 1997). The ejection of ions requires application of a third electric field which can be gradually ramped to generate a selective instability (corresponding to the m/z value) of the flight path of ions within the trap and causes their ejection from a detector which record impact. During MS/MS, trapped ions can be isolated and further fragmented within the trap. Orbitrap mass analyzer is a modified ion trap with high mass accuracy, a high resolving power and a high dynamic range that uses electrostatic field, instead of radio frequency or magnet, to trap ions (Scigelova and Makarov, 2006).

The last essential component of any mass spectrometer is the detector. The detector measures either the charge induced or the current produced when an ion hits its surface. Typically, impact signals are amplified and mass spectra are recorded using software such as Analyst. Interpretation of data (protein identification and quantification) can be performed using algorithm software (e.g. Mascot, Sequest, ProteinPilot).

The most widely used proteomic method for identifying a large set of proteins in complex samples is shotgun or discovery proteomics (Domon and Aebersold, 2006; Fournier et al., 2007; Marcotte, 2007; Patterson and Aebersold, 2003). This method is compatible with most modern tandem mass spectrometers connected to online liquid chromatography (LC) systems (Lu et al., 2008; Steen and Mann, 2004; Xie et al., 2011). Once a peptide solution is separated by two-dimensional LC (2D-LC), it is subjected to MS in full scan mode, which scans the spectrum in full m/z ranges. Using data-dependent acquisition based on signal intensity, a precursor ion can be selected during the survey scan. Then the precursor is subjected to fragmentation (e.g., by collision-activated dissociation (CAD)) and its fragments recorded during a product-ion scan on the basis of their m/z ratios. The resulting MS/MS spectra, in conjunction with the precursor ion information, determine the amino acid sequence of the fragmented peptide and can lead to protein identification. The signal intensity from the survey scan can be used as a method for quantification.
Mass spectrometry sample preparation

The most critical factor for performing a successful MS analysis is the purity and quality of the protein sample. The effective digestion of proteins into peptides requires dissociation of complexes and denaturation of their protein constituents. This requires the use of harsh denaturing conditions which may, for example, be achieved in the presence of concentrated urea, an effective chaotropic agent. Subsequently, disulfide bonds are irreversibly reduced with chemicals containing sulfhydryl or phosphine groups such as dithiothreitol (DTT) or tris-2-carboxyethylphosphine hydrochloride (TCEP) and alkylated with iodoacetamide or 4-vinylpyridine (4-VP) to re-arrange into optimal structures (Gray, 1993). This chemical modification ensures higher peptide yield and sequence coverage (Hale et al., 2004). Next proteins are digested into smaller peptides, a step which most often is realized by the addition of the enzyme trypsin. Trypsin is an endoprotease that cleaves peptides at the carboxyl-terminal side of arginine and lysine residues (Olsen et al., 2004). Because these amino acids comprise approximately 10% of combined amino acids within mammalian protein, trypsinization results in peptides with a length of ~10 amino acids that terminate on a basic C-terminal residue. The predominant benefit of analyzing peptides over proteins emerges from the fact that a majority of mass spectrometers operate at their highest sensitivity and resolution in the low mass range populated by peptides (Steen and Mann, 2004). Attempts to analyze intact proteins directly by mass spectrometry (“top-down” analyses) are increasingly being pursued. Confounders in these analyses are that many proteins are unstable or not soluble in solvents suitable for MS analyses (Dubois et al., 1996). The latter aspect plays a minor role for peptide-based studies (“bottom-up” proteomics). Finally, because proteins undergo a multitude of post-translational modifications that result in many different isoforms, analyses at the protein level are prone to suffer from complexity not seen with peptide-based studies. Naturally, the flipside of this argument is that the reductionist peptide-centric approach can be inferior if the objective is to capture this molecular isoform complexity which exists at the protein level.

Once peptides are generated, they are separated by online HPLC column connected to the mass spectrometer. Because the signal intensity collected for a given analyte is proportional to its concentration, chromatographic columns with narrow inner diameters (for example, between 50 to 150 μm) are popular (Kocher and Superti-Furga, 2007).
To further reduce sample complexity, the separation by RP chromatography may be preceded by peptide fractionation by conventional strong cation exchange (SCX) chromatography or isoelectric focusing (IEF). SCX is based on charge differences between peptides, helps to remove impurities such as salts, and reduces the overall peptide complexity of the sample. During SCX, the sample is loaded into a low ionic strength mobile phase and eluted by increasing the ionic strength of the mobile phase using a salt gradient or by increasing the pH, alternative strategies to decrease the net positive charge of peptides, leading to weaker binding and shorter retention times. Between chromatography steps and in preparation for the final injection, Zip-Tip clean-up or related methods can be used to remove impurities, exchange solvents and concentrate samples. Zip-Tips are pipette tips with a bed of chromatography media embedded at their tip-end. Due to their low dead volume the method is particularly suited for the clean-up of small quantities of peptides or proteins. Another advantage of using Zip-Tips is their ease of operation and minimal risk to cross-contaminate samples. Once impurities have been removed from the sample by one of the aforementioned methods, it is ready to be subjected to MS analysis.

1.2.4 Quantitative methods: using iTRAQ labelling

The term quantitative proteomics refers to measuring the changes in the levels of abundance of proteins or peptides in different samples (Domon and Aebersold, 2010). A common approach in these studies relies on the use of isotopic labelling. The use of quantitative chemical labelling has had a long history in MS analyses of small molecules but was first introduced to protein MS by Gygi and colleagues (1999), who demonstrated the use of an isotope-coded affinity tag (ICAT) to quantify protein expressions in yeast *Saccharomyces cerevisiae* grown on ethanol or galactose as their carbon source. The ICAT technique quantifies pairs of heavy and light isotopically-tagged peptides based on the intensity of the m/z signal they give rise to in a precursor scan (Gygi et al., 1999; Shiio and Aebersold, 2006). To achieve this objective, ICAT reagents consist of three parts: an affinity biotin tag, a linker with stable heavy or light isotopes, and a chemically reactive group which will form covalent bonds with cysteines available in the target protein sequence (Steen and Mann, 2004). The ratio between the peak intensities of heavy and light isotope pairs provides the relative abundance of the peptides as a quantification measure (Figure 1.2.5A). Conspicuous shortcomings of this method are its limited reactivity toward proteins
which harbor cysteines (approximately 8% of mammalian proteins do not contain any cysteine) and the fact that it only allows the simultaneous comparison of two samples (Shiio and Aebersold, 2006; Wiese et al., 2007).

Another labelling method is called stable-isotope labelling by amino acids in cell culture (SILAC). This method relies on metabolic incorporation of isotope-labelled (e.g. deuterium, 13C, 15N) amino acids, typically lysine and arginine. The labeled amino acids are either added to the culture media of cells or the feed of animals, which inserted them metabolically into their proteome(s) (Mann, 2006; Ong et al., 2002; Ong et al., 2003a; Ong et al., 2003b; Steen and Mann, 2004). In a typical SILAC experiment control cells or animals fed with normal media (‘light’) are compared against cells or animals fed with isotopically labeled amino acids (‘heavy’) and subsequently subjected to MS analyses (Figure 1.2.5B). One notorious technical complication with this labelling method is the metabolic conversion of labelled arginine to proline. It has been shown that more than 50% of tryptic digested samples contain proline and 10-25% of the total proline is found to contain isotope labels (Park et al., 2009; Van Hoof et al., 2007). Therefore, unless corrections are made to account for this undesired side effect proline-containing peptides may affect the accurate quantitation. Also, the limitation of comparing just two conditions can constrain experimental design. Finally, SILAC also is expensive for experiments, which require comparisons of biological materials dissected from whole organisms.

Such drawbacks have spurned efforts to develop isotopic labelling methods which can overcome some of these drawbacks, including the generation of isobaric tags for relative and absolute quantitation (iTRAQ). The iTRAQ reagents are non-polymeric, with the isobaric tagging reagents consisting of a reporter group, a balance group and a peptide reactive group, which upon liberation in MS/MS analyses allow for protein identification and quantification (Figure 1.2.6A) (Unwin et al., 2010; Zieske, 2006). The peptide reactive group covalently links to primary amines and labels lysine side chains or the N-terminus of a peptide in a given tryptic digest. During the fragmentation process, the reporter group retains a charge and can subsequently be detected in the low-mass region of MS/MS spectra within the m/z window covering the mass range of 114-117 (for fourplex iTRAQ) (Figure 1.2.6B). One advantage of using the iTRAQ technique is its ability to label up to eight samples in one MS run, and because all iTRAQ reagents are isobaric (have the same mass) labeled peptides derived from identical
Figure 1.2.5 Flowcharts depicting ICAT and SILAC quantitative methods. (a) Schematic representation of ICAT (isotope-coded affinity tag) method. ICAT labels consist of three parts: an affinity biotin tag, a linker with stable isotopes, and a thiol reactive group. Two ‘heavy’ and ‘light’ isotopically labelled proteins are combined, digested and subjected to MS/MS analysis. Relative quantification is performed by comparing the peak intensities of a given ICAT-labeled peptide. (b) Schematic representation of SILAC (stable-isotope labelling by amino acids in cell culture) method. Isotopically labelled amino acids in a cell culture medium are incorporated into cells. Cell lysates grown in the presence of different isotopically labeled amino acids are combined, lysed and subjected to MS/MS analysis. As with ICAT, relative quantification relies on a comparison of precursor peak intensity levels (adapted with permission from Steen and Mann, 2004. Copyright (2004) Nature Publishing Group).
peptides accumulate to single peaks in the parent spectrum increasing the chance of fragmentation (Wiese et al., 2007). By quantifying the area under iTRAQ signature ion peaks within the MS/MS spectrum, the relative contributions of biological samples to the identification of a given peptide can be measured and quantified with accuracy levels that may reach as high as two orders of magnitude (Ow et al., 2009). One limitation of this method, however, is that since iTRAQ is applied after enzymatic digestion, the labelling efficiency is directly proportional to the completeness of peptide digestion. In addition, iTRAQ suffers like other methods from a tendency to identify abundant proteins in complex biological samples. Therefore, the accuracy and the precision of low abundance proteins are compromised (Ow et al., 2009). One solution to this problem is to reduce the sample’s complexity by preceding analysis with additional separation techniques such as SCX or IEF (Unwin et al., 2010). Once iTRAQ-labelled peptides have been analyzed by MS/MS, a number of software packages are available (including ProteinPilot and Mascot), which facilitate the computing-intense task to interpret the tandem MS spectra, identify the proteins which were present within the samples and quantify their relative abundance.

Figure 1.2.6 iTRAQ labelling method. (a) iTRAQ reagents consist of a reporter group, a balance group and a peptide reactive group. During the fragmentation, iTRAQ reagents undergo neutral loss of their balance group, but retain a charge on their reporter group that is detected in the low-mass region between m/z of 114-117. (b) Schematic representation of iTRAQ method. Proteins from four samples (four-plex iTRAQ reagents) are purified and enzymatically digested into peptides. After labelling with iTRAQ 114 to 117, the four samples are combined and subjected to MS/MS analysis. The bottom panel of the flow chart depicts a hypothetical MS/MS spectrum derived from an iTRAQ labelled peptide. While sequence ions assist in protein identification, the relative and absolute quantitation of iTRAQ reporter ion ratios assist in peptide quantification. In the hypothetical example, iTRAQ labels 115 to 117 display a 3 to 4-fold increase when compared to iTRAQ label 114. This can be interpreted to indicate that the identified peptide was present at 3- to 4-fold higher levels in the samples labelled with iTRAQ 115, 116 and 117 than in the sample labelled with iTRAQ 114.
A. 

Isoleptic Tag (Total mass = 145) 

**Reporter** (Mass = 114 thru 117) 

- Peptide Reactive Group 

**Balance** (Mass = 31 thru 28) 

B. 

Sample 1 → Sample 2 → Sample 3 → Sample 4 → 

Protein purification and enzymatic digestion 

- iTRAQ 114 
- iTRAQ 115 
- iTRAQ 116 
- iTRAQ 117 

Combine iTRAQ labelled samples 

Analyze by MS/MS 

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Chapter 2:
Rationale, Hypothesis, and Objectives
2.1 Rationale

Alzheimer’s disease (AD) is characterized pathologically by: extensive neuronal loss, the presence of intracellular neurofibrillary tangles and extracellular amyloid plaques. Amyloid plaques are predominantly composed of aggregated beta amyloid (Aβ) peptides, which are the cleavage products of the Amyloid Precursor Protein (APP). The generation of Aβ requires a series of proteolytic cleavages mediated by secretase enzymes. Among them is, γ-secretase, a unique intramembrane proteolytic enzyme that constitutes a hetero-tetrameric core complex of presenilin (PS), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2) proteins. γ-Secretase plays a pivotal role in the production of aggregation-prone, neurotoxic Aβ42, which has been recognized to be a key molecular player that is abnormally increased in AD patients (Francis et al., 2002; Goutte et al., 2002; Kimberly et al., 2003; Sato et al., 2007; Seshadri et al., 2010; Wolfe, 2007; Yu et al., 2000). PS, an aspartyl protease, is the catalytic component of the γ-secretase complex (Laudon et al., 2005; Spasic et al., 2006; Wolfe et al., 1999). The human genome contains two PS genes which code for 67% identical presenilin 1 (PS1) and presenilin 2 (PS2) proteins (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995; Tandon and Fraser, 2002). These presenilins harbor the catalytic aspartates required for regulated intramembrane proteolysis and contribute to the assembly of distinct subpopulations of γ-secretases. Currently, 185 AD-causing mutations for PS1 and 13 mutations for PS2 have been reported (summarized in the AD and FTD Mutation Database (www.molgen.ua.ac.be/admutations)). Characterizations of PS1 and PS2 knockout mice suggest that these two paralogs may fulfill distinct roles, with PS1−/− mice displaying late embryonic lethality, primarily linked to a notch signaling impairment phenotype, and PS2−/− mice being viable, fertile and exhibiting only a subtle phenotype characterized by minor liver and lung perturbations (Herreman et al., 1999). PS1 and PS2 are incorporated in molecularly distinct γ-secretase complexes in humans (Franberg et al., 2010; Sato et al., 2007). It has been shown that the relative levels of Aβ secreted from blastocyst-derived cell lines and mice differ depending on whether their generation was driven by PS1- or PS2-containing γ-secretase complexes with PS1 being predominantly responsible for the bulk of Aβ production (Lai et al., 2003; Mastrangelo et al., 2005). In contrast, PS2-containing γ-secretase exhibits lower proteolytic activity toward APP substrates than PS1-containing γ-secretase complexes (Ghidoni et al., 2007; Lai et al., 2003; Shirotani et al., 2007). It is possible that part of this dichotomy may be based on PS1- and PS2-
containing γ-secretase complexes being embedded in distinct molecular environments, with each complex having access to its own subset of interacting proteins and substrates. A careful examination of the molecular environment of distinct γ-secretase complexes in mice and cell may shed light on the proteins these enzyme complexes are surrounded by. In addition, for enzyme complexes like γ-secretase, which display broad substrate specificity, there is the hope that quantitative proteomics may also uncover a subset of their substrates. Finally, it is hoped that comparative interactome investigations of PS1-and PS2-containing γ-secretase complexes may provide insights which may translate into rationale approaches for early diagnosis and/or selective inhibition of Aβ plaque formation in AD.
2.2 Hypothesis

Our main hypothesis is that a comparative interactome investigation of PS proteins and isoforms will provide insights into the substrate specificity and cellular organization of distinct γ-secretase complexes and will reveal candidate interactors that may modulate the Aβ generating proteolytic function of γ-secretase complexes through their protein-protein interactions.

2.3 Objectives

To implement a research program which investigates our primary hypothesis the following specific aims were pursued:

Chapter 3:

1. Perform purification from transgenic mice expressing human wild-type or mutant PS1 variants and conduct comparative interactome analyses of iTRAQ-labelled γ-secretase complexes using ESI-MS/MS.
2. Design lentiviral vectors containing GS-TEV tandem affinity purification (TAP) tagged PS1 and PS2, which will display near-physiological levels of bait protein expression and establish a eukaryotic system for stable expression of PS proteins.
3. Perform multistep purifications to generate samples which contain all known components of γ-secretase complexes and are proteolytically active and conduct comparative interactome analyses of iTRAQ-labelled PS1- and PS2- containing γ-secretase complexes using ESI-MS/MS.
4. Validate a specific candidate interactor and examine the significance of the proposed biochemical interaction between the candidate interactor and the γ-secretase complex.

Chapter 4:

1. Validate interaction between PS and SPP in reciprocal IPs.
2. Perform knockdown/knockout study to examine whether PS and SPP mutually affect their level of expression or maturation.
3. Assess whether manipulating levels of SPP affects Aβ peptide generation in cellular models of AD.

4. Examine whether SPP exhibits other functions in the context of the known biology of PS, such as contributing to presenilinase activity, by performing pharmacological inhibition and functional mutagenesis studies.

Chapter 5:

1. Perform purification of endogenous SPP and co-immunoprecipitate candidate interactors.
2. Conduct comparative interactome analyses of iTRAQ-labelled control and SPP complexes using ESI-MS/MS.
3. Validate a specific candidate interactor, vigilin.
4. Examine the significance of the proposed biochemical interaction between the candidate interactor and SPP.
Chapter 3:
Comparative interactome analyses of mature γ-secretase complexes reveals distinct molecular environments of PS1 and PS2

Please note that majority of this chapter contains materials from the following articles:


Miss Amy Jeon performed DNA/vector synthesis, mass spectrometry analyses, cell cultures, purifications, biochemical validations and drafted the manuscript; Dr. Christopher Böhm performed purification optimization, γ-secretase activity assay on HEK cells. Dr. Fusheng Chen performed purification optimization and mouse brain sample preparation.
3.1 Abstract

γ-Secretase is an intramembrane cleaving protease (i-CLIP) that plays a pivotal role in the production of neurotoxic amyloid β-peptide (Aβ), the principal component of plaques observed in the brains of individuals afflicted with Alzheimer’s disease (AD). γ-Secretase consists of a heterotetrameric core complex of presenilin (PS), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2) proteins. The human genome codes for two presenilin genes, PS1 and PS2, which are 67% identical at the amino acid sequence level and harbor the catalytic aspartates. Gene knockout studies suggest that PS1 and PS2 orthologs in mice may fulfill distinct roles, with PS1−/− mice primarily exhibiting a notch signaling impairment phenotype and PS2−/− mice demonstrating a subtle liver and lung perturbation. To begin to understand the underlying causes for this dichotomy, in-depth quantitative comparisons of the molecular neighbourhood of γ-secretase complexes containing PS1- or PS2-subunits were undertaken. A similar large-scale interactome comparison was undertaken to compare the molecular environment of wild-type PS1 and its derivative carrying point mutations known to cause heritable early-onset AD. Biological source materials were mice engineered to express wild-type or mutant PS1, as well as stable integrant clones in HEK293 cells which code for PS paralogs equipped with N-terminal tandem-affinity purification (TAP) tags. The analyses corroborate the previously known composition of γ-secretase core complexes and revealed their interaction with the cellular catenin-cadherin network, as well as the molecular machinery that targets and fuses synaptic vesicles to cellular membranes, and the H+-transporting lysosomal ATPase macro-complex. Whereas members of the catenin/cadherin family of proteins were almost exclusively found associated with PS1, another member of the i-CLIP family, signal peptide peptidase (SPP), was pre-dominantly found to co-purify with PS2-containing γ-secretase complexes.
3.2 Introduction

Alzheimer disease (AD) is a complex, progressive neurodegenerative disease that is the most common cause of dementia. A defining pathological hallmark of AD is the deposition of extracellular plaques, largely consisting of the 38 to 43 amino acid amyloid β-peptide (Aβ). Aβ is generated by consecutive cleavages of the amyloid precursor protein (APP) by two proteolytic activities, β-secretase and γ-secretase. To date, all mutations leading to early-onset familial forms of AD (<10% of all AD cases) occur either in the APP cleavage substrate or in the presenilin components of the γ-secretase complex. γ-secretases are membrane-embedded multi-protein complexes consisting of at least four different proteins (presenilin, nicastrin, Pen-2 and Aph-1) proposed to be present in single copies in the mature complex (Lazarov et al., 2006; Sato et al., 2007). Presenilins constitute intramembrane proteases of ~50 kDa and are presumed to adapt a 9-transmembrane topology and to harbor the aspartate-based catalytic APP cleavage activity of the γ-secretase core complex (Laudon et al., 2005; Spasic et al., 2006; Wolfe et al., 1999b). The proteolytic activity of presenilins is directed toward Type-1 transmembrane proteins which are cleaved by them at sites embedded within their respective membrane-spanning domains (Edbauer et al., 2003; Sato et al., 2007; Wolfe, 2007). Though initially considered unique in their ability to carry out regulated intramembrane proteolysis (RIP), it has become apparent that presenilins are members of an ancient family of intramembrane cleaving proteases (i-CLIPs) that also comprise site-2 proteases (S2P), the signal peptide peptidases (SPPs) and the rhomboids (Spasic and Annaert, 2008; Wolfe et al., 1999a). Assuming a simple 1:1:1:1 stoichiometry of its four core constituents, the γ-secretase core complex would be expected to have a size of less than 200 kDa. Paradoxically, the observed size of active γ-secretase has—dependent on the methodology employed—been estimated to range from 200 kDa to more than 1 MDa, and is thereby significantly larger than its predicted size. It is currently unclear what accounts for this discrepancy. However, more than a dozen proteins have been proposed to interact and, at least temporarily, associate with this protein complex. In previous work we have, for example, shown that TMP21 is a protein which binds to the γ-secretase complex and modulates its APP cleavage activity (Chen et al., 2006). Others have reported interactions with CD147 (Chen et al., 2006; Vetrivel et al., 2008; Zhou et al., 2005), the cadherin/catenin adhesion system (Baki et al., 2001; Georgakopoulos et al., 1999; Kouchi et al., 2009; Serban et al., 2005; Stahl et al., 1999), gSAP
(He et al., 2010) and a subset of tetraspanin proteins, including CD81 and Upk1b (Wakabayashi et al., 2009).

Most investigations which explored the composition of the γ-secretase complex published to date have conceptually glossed over the fact that the human genome codes for two PS genes, PS1 on chromosome 14 and PS2 on chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995), and two Aph-1 genes, Aph-1a and Aph-1b (Serneels et al., 2005). Little is known about how these alternative gene products contribute to the assembly of distinct subpopulations of γ-secretase complexes. Previous evidences suggested that PS1 and PS2 paralogs which exhibit 67% amino acid sequence identity, carry out at least partially distinct functions (Mastrangelo et al., 2005). In support of this notion, the two PS paralogs (i) display overlapping but distinct expression profiles, with PS1 expression highest in testis and lung, and PS2 expression highest in heart, pancreas and brain (Hutton and Hardy, 1997); (ii) generate distinct knockout phenotypes, with PS1 knockout mice characterized by late embryonic lethality, disturbed somitogenesis, cranial hemorrhage, and PS2 knockout mice being viable and fertile but exhibiting mild pulmonary fibrosis and hemorrhage with age (Herreman et al., 1999); (iii) display differences in APP processing and γ-secretase activity (Franberg et al., 2010; Martoglio and Golde, 2003); and (iv) may influence distinct signaling pathways, with PDGF signalling, for example, being influenced only by PS2 (Kang et al., 2005).

More than 50 γ-secretase substrates have been identified, all of which are Type-I transmembrane proteins (Beel and Sanders, 2008; Hemming et al., 2008), including APP (Sastre et al., 2001), Notch (Struhl and Adachi, 1998), Sorla (Bohm et al., 2006), CD44 (Lammich et al., 2002), Sortilin (Nyborg et al., 2006), SorCS1b (Nyborg et al., 2006), and LRP (May et al., 2002). Whereas Notch-cleavage by γ-secretase has been shown to be critically important for neurodevelopment, the physiological significance of γ-secretase-mediated cleavages of other substrates has been less apparent. Some of the γ-secretase substrates are abundantly expressed (e.g., APP, LRP1, cadherins) while others were observed to reach relatively low levels of expression (e.g., Notch, p75). This has raised the question as to how substrates compete for access to limited quantities of active γ-secretase complexes at the cell surface. Inhibiting the γ-secretase-mediated production of Aβ peptides may hold promise as an AD intervention strategy if a method could be devised that selectively targets aberrant APP cleavages and, thus, avoids
concomitant perturbation of vital signal transduction processes that depend on the physiological cleavage of other γ-secretase substrates. Similarly, shifting the PS cleavage activity so it favors generation of shorter and less amyloidogenic Aβ peptides may be a promising therapeutic strategy. Recent work on a protein called gSAP (Wakabayashi et al., 2009) as well as studies which explore the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Choi et al., 2010; Kretner et al., 2011; Lleo et al., 2004) indicate that these objectives may become within reach once the existing molecular complexity surrounding substrate selection and cleavage site preference are better understood.

The question arises whether differences in protein-protein interactions PS1 and PS2 engage in can explain differences in their biology and serve as starting points for refining therapeutic approaches which may target their APP cleavage activity. Similarly, it may be instructive to know whether mutant PS1 is surrounded by a different set of proteins than wild-type PS1. To our knowledge, no previous study has revealed differences in the interactomes of distinct γ-secretase complexes (Hemming et al., 2008; Wakabayashi et al., 2009; Winkler et al., 2009).

We report on a quantitative comparative analysis of wild-type and L286V mutant PS1-containing γ-secretase complexes purified from mice which were engineered to express near-physiological levels of these bait proteins (Janus et al., 2000). We further report on the gentle purification of active and intact PS-containing γ-secretase complexes from human lentiviral integrant clones derived from HEK293 parental cells which express PS1 or PS2 variants equipped with an N-terminal tandem affinity purification (TAP) tag at near-physiological levels in the context of endogenous nicastrin, Aph-1 and Pen-2. Quantitative mass spectrometry was employed to distinguish specific interactors from non-specific binders. Interactome data tables confirmed a number of previously reported PS interactors, shed doubt on others and revealed predominant co-enrichment of the catenin/cadherin molecular machinery with PS1-containing complexes. In contrast, signal peptide peptidase (SPP), a member of the i-CLIP family that adapts an inverse orientation to presenilins relative to the lipid bilayer and, consequently, cleaves substrates which acquire a Type-II orientation, was primarily associated with PS2-containing complexes (Spasic and Annaert, 2008; Wolfe et al., 1999a). Subsequent biochemical validation experiments confirmed a bias of SPP for co-purifying with the PS2 paralog and established an influence of SPP levels on the cellular release of Aβ.
3.3 Materials and Methods

Lentiviral expression system
The TAP-tag cassette was amplified from pRV_NTAP (Burckstummer et al., 2006) through PCR with the forward primer TTTTTGGATCCGACCATGGGCACCCCCGCAGTCAC and backward primer TTTTTGAATTCCCGTCTGCTGCCC. Human PS1 was amplified with TTTTTCTGCAGAGAGTTACCTGCAC and TTTTTCTCGAGCTAGATAAAATTGA from pCMV_PS1. Human PS2 was amplified with primer pair TTTTTCTCGAGTCAGATGGAGCTGATGG and TTTTTGAATTCTGCTCACATTCATGGCCTCTGAC. TAP-tag, PS1 or PS2 PCR products were digested with the restriction enzymes BamHI/EcoRI and PstI/XhoI (NEB, Ipswich, MA), respectively, and inserted into the pcDNA4 eukaryotic expression vector pre-digested with the same restriction enzymes. Subsequently, TAP-PS cassettes assembled in this manner were amplified by PCR, the resulting products digested with NdeI/BamHI and transferred into the pre-cleaved cloning cassette of the lentiviral pWPI.Neo.MCS+ vector. Lentiviral particles were generated by transfecting HEK293T cells with the CalPhos transfection reagent kit (Clontech, Mountain View, CA) and harvesting the cell medium after two days of incubation. Subsequently, lentivirus particles were enriched by ultracentrifugation (Beckman SW32ti) at 120,000 x g for 2 h at 4°C, and HEK293F cells were transduced overnight with lentivirus particles. After an additional 24 h of incubation, a neomycin-based selection of successfully transduced cells was initiated by the addition of antibiotic selection marker G418 (Gibco/Invitrogen, Carlsbad, CA) to the cell medium. Following clonal selection by dilution method, individual clones were analysed by western blot analyses to confirm PS1 or PS2 expression. For an integrant clone to be selected for downstream interactome analyses it had to express the TAP-tagged PS paralog at near near-physiological levels and demonstrate a degree of PS endoproteolysis comparable to endogenous wild-type PS.

Antibodies
Mouse monoclonal anti-PS1 IgG1 antibody (NT1) directed against human residues 41-49 (amino acids RRSLGHPEP) does not cross-react with mouse PS1 and was provided by PMM. Affinity-purified polyclonal rabbit anti-PS1-NTF (A4) antibody was provided by PEF. Commercially obtained were rabbit polyclonal anti-SPP (Abcam, Cambridge, MA), anti-Nct (Sigma), anti-Aph-1 (O2C2; Affinity Bioreagents), and anti-Pen-2 (Anaspec, Fremont, CA) antibodies.
Purification procedure from transgenic mice expressing human wild-type or mutant PS1 variants

Transgenic mice expressing wild-type or L286V mutant human PS1 had been described before (Janus et al., 2000). 10 brains each of age-matched (12 week old) transgenic mice expressing wild-type PS1 or mutant PS1 were rapidly dissected, and snap frozen in liquid nitrogen. Whole brains were cut into 1 mm³ pieces with a razor blade and pieces further ground with pestle and mortar. Subsequently cells were lysed in 25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, protease inhibitor cocktail (Roche, Basel, Switzerland). Following centrifugation membrane pellets were dissolved with 1% (w/v) CHAPSO (Anatrace, Maumee, OH) and 0.05% (w/v) DDM (Anatrace). The sample was incubated on ice for 1 h and insoluble material was removed by centrifugation at 12,000 x g for 10 min. The supernatant was diluted in the CHAPSO/DDM buffer used for lysis to a total protein concentration of 0.5 mg/ml (BCA Protein Assay, Pierce/Thermo Scientific, Rockford, IL). Solubilized membrane proteins were loaded overnight at 4°C onto wheat germ agglutinin (WGA) lectin resin (Vector Laboratories, Burlingame, CA) using a 1 to15 volume ratio of resin to eluate. The WGA resin was collected in a disposable column and washed with CHAPSO/DDM lysis buffer. Complexes were eluted by the addition of four volume equivalents (relative to the WGA resin wet volume) of 0.5 M N-acetyl-D-glucosamine (Sigma, St. Louis, MO) in CHAPSO/DDM lysis buffer and the eluate split in two. One part of the eluate was incubated with NT-1 antibody resin the other part with cognate peptide-saturated NT-1 antibody resin (which served as a negative control) for 2 h at 4°C. The resin was sedimented by gravity, washed twice with CHAPSO/DDM lysis buffer, and once with lysis buffer in which CHAPSO/DDM had been replaced with 0.5% (w/v) DDM. Finally, purified complexes were eluted from the antibody resin by pH drop elution with 0.2% trifluoroacetic acid and 20% acetonitrile, pH 1.9.

Cell Culture

HEK293T cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin (Gibco/Invitrogen) and maintained in a humidified incubator with 5% CO₂. HEK293F cells were cultured in 293 Serum-Free Medium (SFM)-II (Gibco/Invitrogen) containing 5% GlutaMAX-I supplement (Gibco/Invitrogen) and 1% penicillin/streptomycin and maintained in constantly stirred 1L suspension culture flask in a humidified incubator with 5% CO₂.
Purification procedure from HEK293F cells

HEK293F cells stably expressing TAP-PS1 or TAP-PS2 were harvested and lysed in 25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, protease inhibitor cocktail (Roche, Basel, Switzerland) with 0.25% (w/v) CHAPSO (Anatrace, Maumee, OH) and 0.05% (w/v) DDM (Anatrace). The sample was incubated on ice for 1 h and insoluble material was removed by centrifugation at 12,000 x g for 10 min. The supernatant was diluted in the CHAPSO/DDM buffer used for lysis to a total protein concentration of 0.5 mg/ml (BCA Protein Assay, Pierce/Thermo Scientific, Rockford, IL). A 1:2000 volume equivalent of pre-washed IgG-resin was added to the diluted supernatant. Following overnight incubation with gentle rotation at 4°C, the resin was collected in a disposable column (Bio-Rad, Hercules, CA) and washed with a 10-fold volume (relative to the IgG-resin wet volume) of CHAPSO/DDM lysis buffer. Subsequently, a suspension of 1:1 resin to CHAPSO/DDM lysis buffer was incubated for 2 h at 4°C with 1 U/ml tobacco etch virus (TEV) protease (Invitrogen, Carlsbad, CA) and 1 mM DTT (Invitrogen TEV kit). The TEV cleavage step was repeated once and the ensuing two TEV eluates were combined and loaded overnight at 4°C onto wheat germ agglutinin (WGA) lectin resin (Vector Laboratories, Burlingame, CA) using a 1 to 15 volume ratio of resin to eluate. The WGA resin was collected in a disposable column and washed with CHAPSO/DDM lysis buffer. Complexes were eluted by the addition of four volume equivalents (relative to the WGA resin wet volume) of 0.5 M N-acetyl-D-glucosamine (Sigma, St. Louis, MO) in CHAPSO/DDM lysis buffer and the eluate split in two. One part of the eluate was incubated with streptavidin resin (Pierce) the other part with biotin-saturated streptavidin resin (which served as a negative control) for 2 h at 4°C. The resin was sedimented by gravity, washed twice with CHAPSO/DDM lysis buffer, and once with lysis buffer in which CHAPSO/DDM had been replaced with 0.5% (w/v) DDM. Finally, purified complexes were eluted from the streptavidin resin with 2 mM Biotin in the aforementioned DDM buffer.

Western blot analyses and antibodies

Samples were prepared in SDS sample buffer, separated on 4-12% Bis-Tris gels (Invitrogen), and transferred to nitrocellulose membranes. Proteins were detected by enhanced chemiluminescence (ECL) following incubations with primary antibodies and peroxidase-conjugated secondary antibodies.
Silver staining analyses
Samples were prepared in SDS sample buffer, separated on 4-12% Bis-Tris gels (Invitrogen), and silver stained using SilverXpress staining kit (Invitrogen) according to the manufacturer’s instructions.

In-gel trypsinization
Silver stained protein bands were excised into 1 mm pieces using clean razor blades. Gel pieces were destained with 50 μL destaining buffer containing 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate at room temperature until they were destained. The destaining was stopped with water and the solution was discarded. The gel pieces were washed with 5 to 10 X the volume of 25 mM ammonium bicarbonate solution, then acetonitrile (ACN)/25 mM ammonium bicarbonate (v/v 20/80) solution with gentle agitation. The solution was removed and the gel pieces were dried completely by SpeedVac concentrator (Thermo Scientific, Waltham, MA). The gel pieces were re-swelled with 25 mM ammonium bicarbonate solution containing 12.5 ng/μL TPCK-treated porcine Trypsin (Promega) on ice for 5 min. Subsequently, the gel pieces were covered with additional 25 mM ammonium bicarbonate solution without Trypsin to just enough to cover the gel pieces. The sample was incubated at 37°C for 6 h. The peptide extraction was proceed as follow: (1) Remove the solution and collect it the microcentrifuge tube. (2) Vortex the trypsinized gel pieces for 3min with 0.1% formic acid (FA), spin for 3min, and collect solution in the same tube as the previous step. (3) Vortex 3min with 0.1% FA in 30% ACN, spin for 3min, and collect solution. (4) Vortex 3min with 0.1% FA in 60% ACN, spin for 3min, and collect solution. (5) Sonicate the gel pieces for 5min with 0.1% FA in 60% ACN, spin for 3min, and collect solution. Repeat this step once more. (6) Add 5 μL of ACN, spin for 1min, and collect solution. Remove the organic solvents (e.g. ACN) with SpeedVac concentrator and concentrate the sample down to 5 μL for subsequent mass spectrometric analysis.

In vitro γ-secretase activity assay and Ab40 ELISA
Determination of γ-secretase activity by cell-free assay was as previously described (Chen et al., 2006). Briefly, samples were incubated with recombinant Flag-tagged APP-C100. Aβ40 generated by proteolysis was measured by ELISA (Invitrogen/Biosource International) according to the manufacturer’s instructions.
Protein reduction, alkylation and trypsinization

Eluates from multistep purification were concentrated down to the volume of 5 µL using a SpeedVac concentrator (Thermo Scientific). Samples were subsequently denatured in the presence of 9 M ion exchanged urea in HPLC-grade water for 10 min at room temperature, followed by reduction with 5 mM tris-(2-carboxyethyl)-phosphine (TCEP) for 30 min at 60°C and alkylation with 9 mM 4-vinylpyridine (4-VP) for 1 h at room temperature in the dark. Samples were diluted five-fold to ensure that the concentration of urea did not exceed 2 M. Tryptic digestion was initiated by the addition of 1% (wt/wt) of side chain-modified, TPCK-treated porcine trypsin and allowed to proceed at 37°C for 6 h.

iTRAQ labelling of peptides

Individual iTRAQ labeling reagents (Applied Biosystems, Foster City, CA) were reconstituted in ethanol according to the manufacturer’s recommendation, added to peptide mixtures derived from the tryptic digestion of eluates (negative control PS1: iTRAQ114; PS1: iTRAQ115; negative control PS2: iTRAQ116; PS2: iTRAQ117) and incubated at room temperature in the dark for 3 h with occasional mixing every 1 h. Equal labeling with all four reagents was verified by documenting equal intensities of 114:115:116:117 signature ion peaks within collision-induced dissociation (CID) spectra assigned to a small number of peptides observed for trypsin which had undergone autolysis. Any strong deviation from this ratio would have indicated problems with the labeling reaction, or recovery of individual samples prior to the sample mixing step.

Two-dimensional liquid chromatography

Strong cation exchange (SCX) chromatography was used to achieve peptide fractionation of the complex digest mixture. Samples digested with trypsin were adjusted to 25% acetonitrile and acidified (pH 3.0) by 20-fold dilution in 25% acetonitrile, 20 mM KH₂PO₄, pH 2.5. HPLC was carried out using the Ultimate System (Dionex, Sunnyvale, CA) equipped with a microflow calibration cartridge, a Valco injection port and a 180 nl volume UV cell. Separation was achieved on a self-packed 0.5 mm x 110 mm Luna SCX (Phenomenex, Torrance, CA) column with a steep salt gradient from 0–700 mM KCl in 25% acetonitrile, 20 mM KH₂PO₄, pH 2.5. Fractions eluted from the SCX column were desalted with ZipTipC₁₈ tips (Millipore, Billerica, MA) and subsequently subjected to nano-flow RP-HPLC using the Ultimate LC system (Dionex,
Sunnyvale, CA) equipped with a nanoflow calibration cartridge at a flow rate of 250 nL/min. Peptides were separated on a 75-µm ID self-packed column containing Proteo C12 reverse-phase matrix (Phenomenex, Torrance, CA) using a 100-min gradient from 2%–80% acetonitrile in water, with 0.1% (wt/vol) formic acid as the ion-pairing agent.

**ESI-QqTOF mass spectrometry analysis**

The column effluent was coupled directly via a fused silica capillary transfer line to a QSTAR XL hybrid quadrupole/time-of-flight tandem mass spectrometer (Applied Biosystems Sciex, Concord, ON) equipped with a MicroIonSpray source. The progress of each LC/MS run was monitored by recording the total ion current (TIC) as a function of time for ions in the m/z range 375 to 1400. At 5 s intervals through the gradient, a mass spectrum was acquired for 1 s, followed by one collision-induced dissociation (CID) acquisition of 4 s each on ions selected by preset parameters of the information-dependent acquisition method, using nitrogen as the collision gas. Singly-charged ions were excluded from CID selection. The collision energy was adjusted automatically for each CID spectrum using an empirically optimized formula which considers the charge state and m/z value of the precursor ion.

**Database searches**

Peak lists for database searching were created using Mascot Distiller (Version 1.1.2.0; MatrixScience, London, England). Searches were performed using designated MS/MS data interpretation algorithms within ProteinPilot Version 2.0; Applied Biosystems Sciex) and Mascot (Version 2.2.04; MatrixScience). Modifications considered were oxidation of methionine, phosphorylations of serine and threonine, N-terminal (pyro)Glu and alkylation with 4-vinylpyridine. Searches further considered up to one missed cleavage and charge states ranging from +2 to +4. For a protein to be listed in the data tables it had to be identified by both search algorithms. A total of four biological replicates of the TAP experiment with downstream mass spectrometry analyses were used. Because repeat experiments were not conducted under exactly identical conditions, we chose to select one of the data tables obtained for presenting these data and provide for each protein identified information on the number of times (out of four near-identical replicates) a confident identification was made. The majority of proteins were identified on the basis of Mascot scores and ProteinPilot confidence assignments which easily exceeded thresholds conventionally applied for confident identifications. The mass tolerance range
between expected and observed masses used for database searches was ±150 ppm for MS peaks, and ±0.15 Da for MS/MS fragment ions. These relatively large thresholds were used to capture more of the low intense peaks that frequently display broader distribution and thus are assigned with lower mass accuracy. Threshold levels were optimized based on LC/MS/MS datasets of tryptic digests of standard proteins. All samples were searched against SwissProt and the human IPI database (releases: June 2011) and ‘decoy’ databases in which all entries of the above databases had been inverted. iTRAQ ratios were determined with quantitation algorithms embedded in the software packages Mascot and ProteinPilot. Both software packages also contain a feature that was used to correct raw iTRAQ ratios for impurity levels of individual iTRAQ reagent lots determined by the manufacturer.
3.4 Results

Strategy for quantitative interactome mapping of wild-type and mutant PS1 in mice

The objective of a first series of experiments was to develop a method for characterizing the molecular interactions of wild-type human PS1 or a mutant PS1-(hPS1-L286V), known to cause inherited early onset AD in humans. Brains of transgenic mice which expressed the aforementioned PS1 bait proteins from the neuron-specific prion-protein promoter were used as biological source material. The analysis was to be based on an immunoprecipitation strategy followed by mass spectrometry of co-purifying bait protein interactors. It was desirable to achieve rapid purification of mature γ-secretase complexes while avoiding artificial modifications to their core protein constituents. Because the method targeted unmodified bait proteins, the latter objective had to rely on intrinsic features of the known γ-secretase core components for purification. Thus, lectin affinity chromatography based on wheat germ agglutinin (WGA) resin was employed to enrich for the subpopulation of fully-assembled γ-secretase complexes (Arawaka et al., 2002; Kimberly et al., 2002). This affinity capture step exploits the presence of N-glycans on mature nicastrin (Tomita et al., 2002; Yang et al., 2002). Following the removal of unbound protein, binding to the lectin matrix was reversed by competitive elution with an excess of N-acetyl-D-glucosamine. A second orthogonal capture step was based on the PS1 bait protein and made use of a high-affinity monoclonal antibody (NT1) which selectively recognizes a short epitope within the N-terminal domain of human PS1 (residues 41-49, amino acid sequence ‘RRSLGHPEP’) that is not present in mouse PS1. To generate a negative control for the downstream interactome analysis, half of the WGA eluate sample was side-by-side incubated with an identical NT1 immunoaffinity matrix which had been pre-saturated with the synthetic peptide antigen the NT1 antibody had been raised against (Figure 3.1A). To further distinguish specific from non-specific interactors peptides in negative control samples and specific samples were conjugated to distinct isotopic tags for relative and absolute quantitation (iTRAQ114: negative control; iTRAQ115: PS1; iTRAQ116, PS1 mutant) (Zieske, 2006). Subsequently, samples were combined, fractionated by two-dimensional liquid chromatography (1st dimension: strong cation exchange; 2nd dimension: reversed phase) and introduced by electrospray ionization (ESI) into a quadrupole time-of-flight (QqTOF) tandem mass spectrometer.
A.

## A. DDM/CHAPSO solubilization of purified membranes

- WGA agarose
- NT1 agarose
- Control
- wtPS1
- mutPS1

## B. Reduction, Alkylation, Trypsinization

## C. iTRAQ labeling

- 114
- 115
- 116

## D. Only included in subset of experiments

## E. SCX/RPHPLC

## F. ESI MS/MS

## B. Lysates NT1 Co-IP

- MW [kDa]
  - 98
  - 38
  - 28

- NCT
- PS1-FL
- PS1-NTF

## C. Composite Western blot

- MW [kDa]
- 98
- 38
- 28

- hPS1 WT
- hPS1 L286V

- Tg mouse brain
- Aβ40 generation pg/ml/5hr
- Aβ42 generation pg/ml/8hr

- Aβ40
- Aβ42
- Aβ42/Aβ40
Figure 3.1 Strategy for purification of PS1-containing γ-secretase complexes from transgenic mice expressing near-physiological levels of wild-type or mutant PS1. (a) Flow-chart depicting two-step purification strategy of γ-secretase complexes from transgenic mouse brain expressing human wild-type PS1 or mutant PS1-L286V. Following the solubilization of cellular membranes by the addition DDM/CHAPSO, the presence of N-glycans on mature nicastrin is exploited for the capture of mature γ-secretase complexes on wheat germ agglutinin (WGA) agarose. Next, bait protein complexes are eluted in the presence of an excess of N-acetylglucosamine (NAcGlcN) and loaded onto a pre-generated NT1-immunoaffinity matrix. To generate a negative control sample, half of the WGA agarose eluate fraction was instead incubated with an affinity matrix which had been pre-saturated with the NT1 antigenic peptide. (b) Pilot experiment which documented successful immunoaffinity capture of γ-secretase complexes with anti-PS1 monoclonal NT1 antibody that selectively recognizes human PS1. (c) Relative activity of purified complexes in a standard ELISA assay that monitors the production of Aβ peptides and can distinguish between Aβ40 and Aβ42 variants. Note that γ-secretase complexes containing mutant PS1-L286V exhibit a diminished overall activity toward the APP-C100 assay substrate but generate a higher ratio of Aβ42/Aβ40.

The membrane-embedded nature of the heterotetrameric γ-secretase core complex with its combined 19 transmembrane domains and the objective to restrict the analyses to mature and proteolytic active complexes posed a formidable challenge during the early stages of this project but also offered a means to validate and optimize the purification strategy. It provided two orthogonal assays based on which the physiological relevance of purified complexes could be gauged, namely (1) the choice of detergent used for its purification had to maintain the integrity and assembly of the four known core constituents, and (2) the complex had to retain proteolytic activity toward a well-established proteolytic substrate throughout its purification. Small-scale pilot experiments established that the levels of expression of wild-type PS1 and its mutant counterpart in the respective transgenic mouse models was comparable and that the epitope which is recognized by the NT1 antibody is indeed accessible in mature γ-secretase complexes under physiological conditions (Figure 3.1B). A comparative analysis of γ-secretase activities in solubilised membrane fractions, which were obtained from brains of the transgenic mice by a standard lysis and centrifugation method, established that the complexes containing human wild-type mutant PS1 were active. As expected, further characterization of the respective activities with an ELISA assay which can distinguish between Aβ40 and Aβ42 cleavage products revealed
that γ-secretase complexes containing human wild-type PS1 had a higher overall activity but led to a lower Aβ42:Aβ40 ratio than the respective complexes based on mutant PS1 (Figure 3.1C).

Peripheral association of the wild-type PS1-containing γ-secretase core complex with members of the catenin/cadherin family, proteins of the synaptic fusion machinery, proteolipid protein, and lysosomal H+ transporting ATPase

It is to be expected that presenilins engage in dynamic interactions with other proteins to exert their biological functions. A gentle and rapid purification of bait proteins under near-physiological conditions may be critical for maintaining many of these interactions. A particular challenge for the study of protein complexes involving membrane proteins poses the need to utilize detergents and, at the same time, avoid conditions which inadvertently disrupt physiologically relevant protein interactions. Naturally, it is fundamentally impossible to know which biochemical treatment will preserve a yet to be identified relevant protein-protein interaction. In the absence of this information, lysis conditions which preserve the integrity of the bait protein complex with regard to its known constituents may be the next best choice. Therefore, various detergents and detergent mixtures were tested to formulate a lysis buffer composition most compatible with obtaining stable and active γ-secretase complexes. The idea to solubilize cellular membrane pellets in CHAPSO alone, a mild zwitterionic detergent which had repeatedly been shown to preserve γ-secretase activity, was abandoned when it was observed that it led to lower yields in recovered γ-secretase complexes than a subset of mixed detergent formulations. Eventually, a mixture of 1% CHAPSO and 0.025-0.05% DDM emerged as the detergent conditions which best preserved γ-secretase activity solubilised from mouse brain membranes (Figure 3.2A).

A first series of large-scale interactome analyses focused on uncovering the molecular environment of wild-type PS1 alone and employed relatively mild conditions for pre-elution wash steps of the affinity matrix once the bait protein complexes had been captured (referred to as “Low stringency” in the accompanied data tables). Both this initial and subsequent experiments which included the double mutant variant of PS1 were based on 10 brains each obtained from age-matched (12 week old) transgenic mice. To verify the success of the purification and gauge the level with which non-specific binders to the affinity matrix may be present, 1% aliquots of the final eluates were subjected to denaturing SDS-PAGE followed by
A. 

Heat Map visualization of iTRAQ ratios
Protein family/Functional Classification

- Gamma Secretase Core Complex
- Catenin Family
- Cadherin Family
- Synaptotagmin-associated
- Synaptic vesicle glycoprotein
- Syntaxin-associated protein
- Snare core Complex
- Other Synaptic-associated Protein
- Lysosomal ATPase, H+ transporting
- Proteolipid
- Others

B. 

Composite Western blot

Silver stain

γ-Secretase activity

-DMM [\%]
-CHAPSO [\%]

MW [kDa]

105
98
78
55
45
34
17
16
7
4

NCT

PS1-NTF

C.

Spectral Counts / MW

Log$_2$ (115:114 iTRAQ Ratios)
Figure 3.2 PS1-containing γ-secretase core complexes engage in loose interactions with members of the catenin-cadherin cell adhesion machinery, the synaptic fusion apparatus, clathrin and the vATPase complexes. (a) Activity-based optimization of detergent mixture for purification of active γ-secretase complexes from mouse brain. Brains from transgenic mice expressing wild-type human PS1 were homogenized in the absence of detergent, the fraction enriched for cellular membranes obtained by centrifugation and proteins extracted by the addition of defined combinations of n-Dodecyl β-D-maltoside (DDM) or 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPSO). (b) Analysis of eluate fractions obtained after two-step purification (see Figure 3.1A) by denaturing SDS-PAGE followed by silver-staining or immunoblotting. (c) Chart depicting bait-specific enrichment of proteins within interactome data set (based on iTRAQ signature mass ion intensity ratios) against spectral counts underlying identification of individual proteins (normalized by molecular weight). Note that each signal in the graph represents a protein. Color shading is used to indicate members of protein families or to group proteins based on their known functional association. Please see Table 1 for details of candidate interactors identified and the assignment of proteins to heat map values indicated above the graph.

silver-stain analysis or western blotting with antibodies which detect nicastrin or the N-terminus of PS1 (Figure 3.2B). A comparison of band intensities seen in the PS1-specific and non-specific affinity capture eluates suggested that the two-step purification method had led to a strong enrichment of PS1 and nicastrin, indicative of the presence of mature γ-secretase complexes, and established that only trace amounts of these two proteins could be seen in the non-specific eluate fraction. Furthermore, differences in silver-stained protein profiles indicated that multiple protein bands were specifically co-enriched together with PS1. However, this analysis also confirmed the anticipation that a considerable number of proteins would bind to the affinity matrix non-specifically, emphasizing the need to incorporate isobaric peptide labels into the downstream sample work-up scheme. Thus, in this first study iTRAQ 114 and 115 reagents were used to tag proteins in the negative control eluate and the specific wild-type PS1 eluate, respectively.

Computational searches of the human International Protein Index (IPI) database at the European Bioinformatics Institute (EBI) with masses extracted from collision-induced dissociation (CID) spectra were used to generate an initial non-curated interactome dataset containing specific and non-specific interactors. Searches against a ‘decoy database’ in which all sequence entries were
inverted did not give rise to any protein identification that passed significance thresholds which had been applied (see Experimental Procedures for details). In total, this filtering approach led to the identification of more than 150 proteins (Supplementary Table 3.1). When biological replicates of the study were undertaken, and the proteins were sorted according to their known classifications and functional associations, the list shrunk to ~80 proteins which repeatedly were seen in the PS1 wild-type sample (Figure 3.2C, Table 3.1). In subsequent biological replicates a higher number of washing steps (referred to as “High stringency” condition) was applied to the affinity matrix upon capture of bait protein complexes to reduce non-specific interactors and monitor how this change in procedure may alter the list of wild-type PS1 candidate interactors.

On the basis of iTRAQ 115:114 ratios it was evident that members of the cadherin/catenin network of calcium-dependent cell adhesion molecules co-purified robustly with wild-type PS1 but gave rise to somewhat lower enrichment ratios than the known γ-secretase core complex constituents (Table 3.1). In particular, catenin alpha2, catenin beta 2 and plakophysin 4, as well as the cadherins 2 and 11 were repeatedly and strongly enriched in the PS1-specific sample. It further was evident that proteins which contribute to a molecular machinery that targets and fuses synaptic vesicles to cellular membranes were co-purifying with PS1. In addition, proteolipid protein 1 and multiple subunits belonging to V0 and V1 macro-subcomplexes of the H⁺-transporting lysosomal ATPase co-enriched together with PS1. Interestingly, the H⁺-transporting mitochondrial ATPase, known to acquire a similar molecular architecture to its lysosomal counterpart was robustly detected but, apparently, was captured non-specifically by the affinity matrix as it did not selectively co-enrich with PS1. Finally, cell adhesion molecules harboring fibronectin-type 3 or IgG-like subdomains, heat shock proteins and constituents of clathrin triskelia were modestly but inconsistently enriched in wild-type PS1 eluates.

Other proteins mostly gave rise to iTRAQ 115:114 signature mass ion ratios near ~1 which exposed them as non-specific interactors to the affinity matrix. Note that some of these non-specific interactors, including members of the Na/K ATPase, the aforementioned mitochondrial H⁺-transporting ATPase or valosin-containing protein (VCP) were observed with high spectral counts.
Table 3.1 Summary of data tables comparing the molecular environment of PS1 in transgenic mouse brains or HEK293 cells.

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Identical molecular environment of wild-type PS1 or mutant PS1- L286V

The refined methods and data obtained during the analysis of the wild-type PS1 interactome formed the basis for a next series of experiments which aimed to uncover whether the molecular environments are different for mature γ-secretase complexes comprising wild-type or mutant PS1. Differences in the characteristics with which wild-type or mutant PS1-containing γ-secretase complexes cleave an APP-related substrate (Figure 3.1C) may not manifest as differences in protein-protein interactions with non-core constituents of the complex but instead can be based on subtle differences in the molecular architecture of the catalytic cleavage centre which may escape mass spectrometry-based detection. To our knowledge, however, these alternative scenarios had not been experimentally addressed by quantitative interactome analyses. Here, repeat side-by-side interactome analyses of wild-type and mutant PS1 complexes failed to uncover differences in the proteins which co-enriched with wild-type or mutant PS1 bait proteins (Supplemental Table 3.2). More specifically, when on the basis of iTRAQ signature mass peak intensities the levels of enrichment were compared for proteins which co-purified with wild-type (iTRAQ 115:114 ratio) or mutant PS1 (116:114 ratio), no significant difference in enrichment levels (iTRAQ 116:115 ratio) was observed for any candidate interactor.

Taken together, the mouse brain-based PS1 interactome analyses described uncovered many robust and shared interactors of mature γ-secretase complexes harboring wild-type or mutant PS1 but did not reveal whether these interactions occur in a single cell-type or represent the cumulative molecular environment PS1 encounters across multiple cell-types in the brain. Also, experiments up to this point did not address whether differences exist in the interactomes of PS1 and PS2.

Stable cell lines expressing PS1 or PS2 with N-terminal mammalian TAP-tag

To compare the molecular environments of PS1 and PS2 and compare the activity of γ-secretase complexes harboring these paralogs, a cell type was needed which is known to express both proteins naturally. Here, human embryonic kidney cells (HEK293), a cell model frequently employed for γ-secretase studies, were selected for the comparison of PS1- and PS2-containing γ-secretase complexes. Though excellent antibodies for the detection or immunocapture of PS1 or PS2 are available, the benefit of working with endogenous proteins might in this application be outweighed by the risk to inadvertently introduce sample-to-sample variance through the use
of non-identical antibody capture reagents with idiosyncratic cross-reactivities. To avoid these confounders a tandem affinity purification (TAP) tag comprised of tandem IgG binding domains derived from *Staphylococcus aureus* Protein G (ProtG) and a streptavidin binding peptide (SBP) separated by a tobacco etch virus (TEV) protease cleavage site were attached to the N-terminus of PS1 or PS2 bait proteins (**Figure 3.3A**). Previous work by others documented this particular TAP-tag to afford gentle purification of transiently interacting partners under near-native conditions and to give rise to enhanced yields for the purification of mammalian protein complexes, relative to some alternative TAP-tags which primarily have found applications in yeast-based or prokaryotic interactome studies (Burckstummer et al., 2006). The N-terminal attachment site was selected on the basis of prior data which demonstrated partial loss of γ-secretase activity in the presence of modifications to the C-termini of presenilins (Bergman et al., 2004). To achieve near-physiological levels of bait protein expression, lentiviral integrant clones were generated and tested for their expression level and ability to process full-length presenilins into N- and C-terminal fragments at a level comparable to endogenous PS1 or PS2 (Trono, 2000). HEK293F cells were adapted for growth in serum-free suspension cultures allowing the relatively rapid production of suitable quantities of cells (**Figure 3.3C**).

In preparation of PS1 and PS2 complex purifications lentiviral integrant cells expressing TAP-tagged PS variants were side-by-side lysed in the presence of the aforementioned CHAPSO/DDM detergent mixtures. Interestingly, it was observed that for the HEK293 cell paradigm the solubilization of mature γ-secretase complexes required a fourfold lower concentration of 0.25% CHAPSO to preserve maximum γ-secretase activity (not shown), as opposed to the 1% CHAPSO concentration which provided highest activity levels when cellular membranes derived from mouse brains were solubilized (**Figure 3.2A**). Next, soluble membrane proteins were passed through IgG-Sepharose and released from the resin by TEV cleavage, leaving the bulkier N-terminal portion of the TAP tag behind. Next, lectin affinity chromatography based on wheat germ agglutinin (WGA) resin was again employed to enrich for the subpopulation of fully assembled, nicastrin-containing γ-secretase complexes. Subsequently, recovered bait protein complexes were passed through streptavidin agarose resin capitalizing on the presence of a 38-amino acid streptavidin binding peptide (SBP) within the modular TAP-tag (Burckstummer et al., 2006) (**Figure 3.3B**).
A. Preincubated with Biotin

B. Cell lysates
   PS1  PS2
   IgG agarose
   WGA agarose
   Streptavidin agarose
   Preincubated with Biotin
   Biotin

PS1 control  PS1
PS2 control  PS2

C. HEK293F
   HEK293T
   Fluorescence
   Brightfield
Figure 3.3 Design elements of TAP-PS constructs and three-step protocol for purification of PS paralogs from HEK293 cells. (a) Schematic representation of the ProtG-TEV-SBP TAP-tag which consists of two IgG binding domains of *Staphylococcus aureus* Protein G (ProtG) and a streptavidin binding peptide (SBP) separated by a TEV protease cleavage site, fused to the N-terminus of PS1 or PS2. (b) Flow-chart depicting key steps of purification strategy. In the initial step, the TAP-tagged PS1 or PS2 bait proteins are captured on IgG resin by means of their tandem ProtG moieties. Following the release of bait protein-containing complexes by TEV cleavage, the presence of N-glycans on mature nicastrin is exploited for capture of mature γ-secretase complexes on wheat germ agglutinin agarose. Next, bait protein complexes are eluted in the presence of an excess of N-acetylglucosamine (NAcGlcN), followed by recapture on streptavidin resins by means of the streptavidin binding peptide (SBP) embedded in the TAP tag. Finally, purified complexes are obtained following their competitive displacement from the streptavidin resin in the presence of an excess of biotin. (c) Immunofluorescence analysis of n-terminally tagged PS1 integrant clones generated in both HEK293F suspension cells and HEK293T adherent cells.

Biochemical comparison of PS1- and PS2-containing γ-secretase complexes

The identity of the four strongest protein bands observed following silver staining was determined by in-gel trypsinization and tandem mass spectrometry (Figure 3.4B). As expected, this analysis confirmed PS-containing γ-secretase core complexes to be comprised of nicastrin, PS, Aph-1 and Pen-2 (Figure 3.4A, B). Some of the unspecific bands in the first purification step were identified as IgG gamma chain C region, tubulin, actin and, TEV protease. These unspecific bands were removed after subsequent purification steps. The side-by-side comparison of denatured complex components further revealed that the relative abundance and migration of proteins which constitute the γ-secretase core are shared between PS1- and PS2-containing γ-secretase complexes. The only γ-secretase core subunits that migrated with different apparent MW were the PS paralogs themselves, an observation which had been anticipated based on differences in the MWs of PS1 and PS2 (Figure 3.4C). To compare molecular weights of native PS1- and PS2-containing γ-secretase complexes and assess their integrity and heterogeneity, Blue Native Gel (BNG) analyses and Western blotting were conducted (Figure 3.4D). These analyses revealed that both PS1- and PS2-containing γ-secretase complexes migrated at a single MW of approximately 350 kDa in the presence of mixed micelles composed of CHAPSO and DDM. Consistent with the notion that both γ-secretase preparations largely contained intact
Figure 3.4 Purification of TAP-tagged γ-secretase complexes. (a) Western blot analyses of informative fractions collected during key steps of multi-step purification procedure. The successful incorporation of the heterologously expressed TAP-PS proteins into mature γ-secretase complexes can be estimated by the relative intensities of signals attributed to the TAP-PS-FL precursors which migrated with an apparent MW of 58-62kDa and the faster migrating TAP-PS-NTF cleavage products seen at 46-50kDa. (b) Denaturing SDS-PAGE analysis of three representative eluate fractions collected during the multi-step purification of PS1-containing γ-secretase complexes followed by silver staining. Unspecific bands are eliminated through each purification steps: 1, IgG gamma chain C region; 2, Tubulin; 3, Actin; 4, TEV protease. Final elute displays clear enrichment of four strongest protein bands: 5, Nicastrin; 6 and 8, PS NTF and CTF, 7, Aph-1; 9, Pen-2. (c) Denaturing SDS-PAGE analysis of final streptavidin agarose eluates followed by Coomassie staining. To facilitate assessment of relative amounts of γ-secretase core components present two different volumes of each eluate fraction were subjected to analysis. A densitometric comparison of signal intensities suggested that γ-secretase core subunits were represented at equal stoichiometries in both complexes. Note the expected differences in migration of the N-terminal fragments (NTF) of PS2 and PS1 and the absence of unprocessed full-length TAP-PS signals in these fractions, indicative of the selective purification of fully mature γ-secretase complexes. (d) Non-denaturing Blue Native Gel analysis documenting migration of purified PS1- and PS2-containing γ-secretase complexes in single bands of similar apparent MW. Western blot analyses indicated that non-PS core components were also exclusively present in the PS-containing protein bands consistent with homogeneity and mature post-translational modifications within purified complexes. (e) PS1-containing γ-secretase complexes exhibit stronger responsiveness to established γ-secretase inhibitors in an in vitro ELISA assay that monitors the release of Aβ40 from a recombinant APP-C100 substrate. PS paralog-specific γ-secretase complexes are active and are inhibited by inhibitors L685,458 and Compound E in a concentration-dependent manner.

mature complexes, BNG analyses did not reveal the presence of molecular species which migrate with lower apparent MWs. Furthermore, a standard in vitro γ-secretase activity assay based on a recombinant APP-C100-Flag substrate documented that PS1- and PS2-containing γ-secretase complexes were not only active, but also were responsive to inhibition by small-molecule pharmacological inhibitors that are routinely used in the field and have repeatedly been shown to suppress γ-secretase proteolytic activities (Figure 3.4E). More specifically, when adjusted for total amounts of presenilins present, purified γ-secretase complexes containing PS1 exhibited approximately 30% higher absolute cleavage activity toward the APP-C100-FLAG substrate than the respective complexes containing PS2 (not shown). The cleavage activity of both types
of complexes toward the APP-C100-FLAG substrate could be inhibited in a concentration-dependent manner in the presence of both L685,458, a transition state analogue inhibitor (Shearman et al., 2000), or Compound E, a non-transition state analogue inhibitor (Seiffert et al., 2000). Also, when activity charts were normalized and plotted based on relative activity levels of naive purified complexes, PS2-containing complexes required higher inhibitor concentrations to achieve comparable levels of inhibition.

Paralog-dependent protein interactions of PS1- or PS2-containing γ-secretase complexes

To generate a more comprehensive comparative interactome data set of PS1- and PS2-containing γ-secretase complexes, streptavidin eluate fractions were, in subsequent experiments, not subjected to gel-based analyses but directly trypsinized in solution. Negative control samples which were intended to facilitate the distinction of specific and non-specific interactors to the affinity matrix were in these studies generated by passing 50% of the protein material over a streptavidin resin which had been pre-saturated with biotin (Figure 3.3B). As for the cell interactome studies of PS1, peptides in negative control samples and specific samples were conjugated to distinct isotopic tags (iTRAQ114, PS1 negative control; iTRAQ115: PS1; iTRAQ, PS2 negative control; iTRAQ117, PS2) and processed in an identical manner to the samples described above. In total, this approach led to the identification of 39 proteins, which repeatedly passed significance thresholds in consecutive biological repetitions (Table 3.2).

The inspection of iTRAQ signature mass peaks visible in the low mass region within individual CID spectra revealed that iTRAQ intensity ratios observed were largely consistent across CID spectra which supported the identification of a single protein. Based on distinct iTRAQ signature mass peak intensity patterns, identified proteins could be grouped into four candidate categories (Figure 3.5): (I) PS1/PS2 Specific Binder Category: proteins assigned to this category displayed iTRAQ signature mass peak distributions consistent with the interpretation that they were found in both PS1- and PS2-specific samples but not in the non-specific control samples. The known γ-secretase core constituents nicastrin, Pen-2, Aph-1A and Aph-1B were assigned to this category, as well as Tmp21, CD63 and a few other proteins (Figure 3.6C, D). (II) PS1 Specific Binder Category: proteins in this group were characterized by an iTRAQ signature mass peak pattern in which only the intensity of the iTRAQ 115 signature peak was elevated. PS1 itself (Figure 3.6A), beta-catenin, delta-catenin, cadherin-2, plakophilin-4 and a few other proteins were observed to
## Table 3.2 Summary data table depicting quantitative comparison of TAP-PS1 versus TAP-PS2 interactomes in HEK293 cells.

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Figure 3.5 Sample processing of TAP-purified complexes and experimental setup for quantitative PS1 and PS2 interactome comparisons. (a) Flow-chart depicting steps in preparation of quantitative mass spectrometry analyses, including assignments of iTRAQ conjugation reagents to samples compared. Once labeled samples were combined and subjected to offline strong cation exchange (SCX) and online reverse-phase (RP) liquid chromatography coupled to electrospray (ESI) tandem mass spectrometry (MS/MS). (b) Cartoon depicting iTRAQ signature mass peak intensity patterns (observed in the low mass region of collision-induced dissociation spectra) according to which proteins can be grouped into four categories: (i) PS1/PS2 specific binder category; (ii) PS1 specific binder category; (iii) PS2 specific binder category; (iv) Non-specific binder category.
A. Presenilin 1 (ENSP00000326366), MS/MS of m/z 741.00
QVVEQDEEDEELTLK (AA 61-76) + iTRAQ114 (N-term)

B. Presenilin 2 (ENSP00000355747), MS/MS of m/z 544.57
QGPEDGENTAQWR (aa 36-48) + iTRAQ114 (N-term)
C. Nicastrin (ENSP00000357042), MS/MS of m/z 592.39
SGAGVPAVILR (aa 404-414) + iTRAQ114 (N-term)

D. Aph1A (ENSP00000358105), MS/MS of m/z 732.35
ADEGLASLSEDGR (aa 97-109) + iTRAQ114 (N-term)
E. Delta Catenin (p120)(ENSP00000352907), MS/MS of m/z 599.65
ALSAIADLLTNEHEER (aa 711-725) + iTRAQ114 (N-term)

F. Signal Peptide Peptidase (ENSP00000365296), MS/MS of m/z 733.36
NASDMPETITSR (aa 62-73) + iTRAQ114 (N-term)
Figure 3.6 Representative mass spectra demonstrating γ-secretase complexes. CID spectra assigned to tryptic peptide. (a) ‘QVVEQDEEDEEELTLK’ of PS1. (b) ‘QGEPEGENTAQWR’ of PS2. (c) ‘ADGLASLSEDGR’ of Aph-1A. (d) ‘SGAGVPAVILR’ of nicastrin. (e) ‘ALSAIADLLTNEHER’ of delta catenin documenting selective enrichment of this protein in PS1-specific eluate fractions labelled with iTRAQ115 reagent. (f) ‘NASDMPETITSR’ of SPP documenting selective enrichment of this protein in PS2-specific eluate fractions labelled with iTRAQ117 reagent. Selective enrichments of Aph-1A and nicastrin parent proteins are present in both PS1- and PS2-specific eluate fractions labelled with iTRAQ115 and iTRAQ117 reagents, respectively. Insets: high resolution graphs depicting isotopic envelopes of precursor ions and iTRAQ signature mass peak regions within CID spectra. The distribution of iTRAQ signature ion mass peaks not only documents a selective enrichment of PS paralogs as per the design of the experiment but also argues against the existence of γ-secretase complexes with mixed PS1 and PS2 content.

co-enrich in this manner (Figure 3.6E). (III) PS2 Specific Binder Category: analogous to the previous category except that CID spectra which underlie the identification of proteins in this group only showed a relative intensity elevation of the iTRAQ 117 signature peak. Only PS2 itself (Figure 3.6B), SPP and SCAMP3 could be assigned to this category (Figure 3.6F). (IV) Non-specific Binder Category: a small subset of proteins appeared to have been non-specifically captured by the TAP procedure or was introduced during sample handling, intentionally or inadvertently. Proteins in this group were recognizable by an iTRAQ signature mass peak pattern displaying similar intensities for all iTRAQ signature masses. As expected from the silver and Coomassie staining analyses of SBP eluate fractions (Figure 3.4B) very few proteins were in this manner interpreted to be non-specifically carried through the TAP purification procedure. As such, this category was primarily based on CID spectra which could be assigned to TEV protease, autolysis of trypsin, streptavidin or keratins.

The presence of all known constituents of the γ-secretase core complex in the interactome data set, including the small protein Pen-2, which was detected on the basis of 3 CID spectra, served as a positive control in this analysis. Additional proteins identified as candidate PS1- and PS2-interactors that had previously been shown to co-purify with γ-secretase complexes were members of the catenin/cadherin cell adhesion system, TMP21, monocarboxylate transporter 1
(MCT1) and proteolipid protein. Altogether, eighteen proteins co-purified with both PS paralogs, ten proteins selectively co-enriched with PS1 and only two proteins showed preferential association with PS2.

**Preferential association of SPP with PS2-containing γ-secretase complexes**

A detailed analysis of the distribution of iTRAQ signature mass peaks observed in SPP-specific CID spectra suggested that this candidate interactor was co-purifying predominantly with PS2, i.e., whereas the ratio of 117:116 iTRAQ signature mass peaks indicating the relative enrichment of SPP in PS2-specific versus negative control samples averaged a value of 103.8, the corresponding ratio of 115:114 iTRAQ signature mass peaks for PS1 averaged a value of 1.5 (Table 3.2). To validate the preferential co-enrichment of SPP with PS2-containing complexes, we next analyzed purified PS1- and PS2-containing γ-secretase complexes by Western blotting using an SPP-directed antibody for its detection. When levels of PS1- and PS2-containing mature γ-secretase complexes were normalized based on band intensities of their shared Pen-2 and nicastrin subunits, the amount of SPP which was co-enriched with PS2-complexes strongly exceeded SPP levels detected in PS1-complex samples (Figure 3.7A), consistent with the preferential enrichment of SPP with PS2 that iTRAQ mass peak patterns within CID spectra assigned to SPP had indicated (Figure 3.6F). To assess whether the co-purification of SPP merely represented an artefact of the heterologous expression of the bait proteins in lentivirally transduced HEK293 integrant clones, reciprocal co-immunoprecipitation (co-IP) experiments were next conducted with naive HEK293 cells that express exclusively endogenous PS paralogs. Western blot analyses of SPP-specific and negative control co-IPs based on a nonspecific immunoglobulin established that SPP-capture leads to the specific co-enrichment of endoproteolytically processed N-terminal fragments of PS2 (Figure 3.7B). Thus, taken together, these experiments validated the interaction between SPP and PS2.
Figure 3.7 Validation of SPP-PS2 interaction. (A) Validation of preferential co-enrichment of SPP together with PS2-containing γ-secretase complexes. SBP eluate fractions from multistep purifications of PS1- and PS2-containing γ-secretase complexes were subjected to western blot analyses. Two different volumes of each eluate fraction were analyzed side-by-side in order to identify a loading quantity that would give rise to near-identical signals derived from the presence of Pen-2 and Nct in the samples (the two proteins were selected because no paralogs or isoforms are known for them which could skew the intended comparison or PS1- or PS2-containing γ-secretase complexes). Please note the stronger SPP-derived signal in preparations of PS2- versus PS1-containing γ-secretase complexes in samples that contained near-identical amounts of Pen-2 or Nct. (B) Reciprocal co-IP analysis of endogenous SPP in wild-type HEK293 confirms co-enrichment of PS2. Signals labeled with an asterisk most likely represent a band derived from the light-chain of the polyclonal antibody employed for the immunoprecipitation of SPP.
3.5 Discussion

Data collected in this study (i) confirmed the hetero-tetrameric composition of mature \( \gamma \)-secretase core complexes; (ii) documented that \( \gamma \)-secretase core complexes engage in interactions with other protein networks, including the cellular catenin-cadherin network, the molecular machinery that targets and fuses synaptic vesicles to cellular membranes, and the \( \text{H}^+ \)-transporting lysosomal ATPase macro-complex; (iii) validated several previously proposed \( \gamma \)-secretase interactors (e.g., catenins and proteolipid protein) and identified novel candidate interactors (e.g., synaptic proteins, including VAMP); (iv) provided examples of proteins which can engage in robust interactions with \( \gamma \)-secretase complexes in individual cell types but may escape detection when whole brain interactome studies are conducted (e.g., CD63, Tmp21, SCAMPs, heat shock protein 70); (v) revealed mature \( \gamma \)-secretase complexes containing PS1 or mutant PS1 to be indistinguishable in their protein composition; (vi) firmly established a predominant association of the catenin-cadherin network with PS1-containing \( \gamma \)-secretase complexes; and (vi) uncovered a selective enrichment of SPP with PS2-containing \( \gamma \)-secretase complexes, a surprising development which appears to exert an effect on cellular A\( \beta \) secretion.

Specific versus non-specific interactors

The scale of interactome analyses presented in this study was comparable to two previous reports (Wakabayashi et al., 2009; Winkler et al., 2009). However, to our knowledge, this study was the first to identify by mass spectrometry all known constituents of the \( \gamma \)-secretase core complex, including Pen-2, a small membrane protein of 12 kDa, and Aph-1b, a protein which exhibits 55% sequence identity to the more commonly observed Aph-1a paralog. The identification of both proteins in this work based on multiple CID spectra which could be assigned to non-overlapping peptides may have been facilitated by the inclusion of the WGA lectin affinity capture step in the sample work-up procedures (Figure 3.1 and 3.3). The latter step restricted the interactome analyses to fully-assembled \( \gamma \)-secretase complexes which carry mature N-linked glycans attached to the nicastrin core constituent. This interpretation is further supported by the conspicuous absence of a subset of previously reported candidate interactors in this dataset. This subset of proteins, which includes calnexin, protein disulfide isomerase (PDI), calreticulin and BIP, is known to predominantly localize to endoplasmic reticulum (ER) or Golgi compartments, and as such may engage in contacts with immature \( \gamma \)-secretase constituents during their passage through
the secretory pathway but is less likely to bind to fully-assembled complexes in post-Golgi compartments.

To our knowledge, the study constituted the first investigation of the \( \gamma \)-secretase interactome which employed isotopic labeling for distinguishing specific from non-specific interactors. This conceptual improvement was critical for direct comparisons of interactomes. Thus, although one of the large-scale \( \gamma \)-secretase interactome studies which preceded this work could draw qualitative conclusions about PS1 and PS2 interactomes being similar, the consecutive analysis of PS1 and PS2 interactomes precluded a confident assessment of differences in the interactomes of the PS paralogs (Wakabayashi et al., 2009). In contrast, the sample-specific iTRAQ labels which were employed in this work enabled concomitant analyses of control and bait-specific affinity capture eluates, thereby avoiding notorious run-to-run variance confounders in mass spectrometry analyses, and allowing relative comparisons. A notable exception to this observation posed a subset of CID spectra (for example, those assigned to the peptide with the amino acid sequences \textquoteleft SVLVGK\textquoteright or \textquoteleft MLVETAQER\textquoteright) which could not be unambiguously assigned to PS1 or PS2 due to the 67% percent sequence identity amongst these baits and contrasted in their iTRAQ distribution additional CID spectra observed for these proteins which could be assigned to paralog-specific sequence segments. Nevertheless, these comparisons not only helped to flag several proteins (e.g., Na/K ATPase subunits and valosin-containing protein), which had previously been proposed to represent \textit{bona fide} candidate interactors (Wakabayashi et al., 2009), as probably belonging to the non-specific binder category but also led to the identification of catenins and SPP as candidate interactors, which did not associate with all mature \( \gamma \)-secretase complexes at equal levels but exhibited a surprising degree of binding bias toward PS1- and PS2-containing \( \gamma \)-secretase complexes, respectively.

**Distinct molecular environments of presenilin paralogs**

To minimize disruptions to the authentic molecular environment of \( \gamma \)-secretase complexes, for the comparison of PS1 and PS2 interactomes only the PS bait proteins were equipped with affinity tags and their expression was adjusted to near-physiological levels by selecting appropriate lentiviral integrant clones. This strategy set our study conceptually apart from the majority of previous reports which were either based on cell models that concomitantly overexpressed multiple affinity-tagged \( \gamma \)-secretase core constituents or targeted endogenous
complexes using conventional immunoprecipitation protocols. One of the aforementioned PS interactome studies employed a similar TAP approach based on an earlier version of a tandem affinity tag which had been optimized for the purification of protein complexes from yeast cells (Wakabayashi et al., 2009). However, whereas the latter study purified γ-secretase from mouse embryonic fibroblasts which had been derived from a presenilin double-knockout mouse, studies in this report were based on HEK293 cells, the most widely used cell model for studying the biology of γ-secretase complexes.

Data presented in this work validated the observation made in previous reports that a majority of presenilin candidate interactors associate with both PS1- and PS2-containing γ-secretase complexes but also document that a small number of proteins exhibit preferential co-enrichment with one or the other type of γ-secretase complex. In the latter category belong members of the catenin/cadherin family (catenin delta-1, catenin beta-1, plakophilin-4 and cadherin-2) which exhibited a strong bias for co-enrichment with PS1 in this work. This conclusion was unequivocally supported by the presence of more than three dozen CID spectra which could be assigned to peptides derived from these proteins and displayed strong signals for the PS1-specific iTRAQ115 signature mass peak and only weak signals—barely above background levels observed in negative control samples—for the PS2-specific iTRAQ117 signature mass peak (Figure 3.6E, Supplemental Table 3.3). Cadherins are membrane-spanning proteins involved in calcium-dependent cell adhesion and in the transduction of signals into the cell, a biology which is at least partly mediated by their interaction with catenins (Kouchi et al., 2009). MS results presented in this study extend previous data that showed the direct interaction of PS1 with a subset of catenins (p120 delta catenin) and an enzyme-substrate relationship with a subset of cadherins (for example, N- and E-cadherins) (Georgakopoulos et al., 1999; Kouchi et al., 2009; Parisiadou et al., 2004; Serban et al., 2005). It has been proposed that a large cytoplasmic loop domain within the C-terminal endoproteolytic fragment of PS1 (amino acid residues 330 to 360) is required for the direct association of PS1 with delta-catenin (Kouchi et al., 2009). Our data are consistent with this interpretation and document that PS2 exhibits a much reduced propensity to associate with members of the cadherin/catenin cell adhesion system. It will be of interest to investigate whether the divergent evolution of the PS1 loop domain which can be observed in multiple alignments of presenilin orthologs is a direct consequence of this functional specialization of PS1 (Figure 3.8).
Previous reports established that APP intramembrane cleavages are \textit{in vivo} foremost carried out by $\gamma$-secretase complexes containing PS1 (Ghidoni et al., 2007; Lai et al., 2003). In support of this conclusion, PS1 knockout cells displayed nearly complete abolishment of APP-directed $\gamma$-secretase activity, in contrast to PS2 knockout cells which displayed only minor reductions in this activity (Martoglio and Golde, 2003). These observations do not appear to merely reflect differences in the levels of expression (Huynh et al., 1997) although PS1 is known to be higher expressed during development (Lee et al., 1996). In contrast to biochemical data which preceded this work (Shirotani et al., 2007), highly purified PS1- or PS2-containing complexes were observed to process the APP substrate at similar rates \textit{in vitro}. Differences in the ability to turn over the APP substrate only emerged in this study when the response to treatments with two well-characterized $\gamma$-secretase inhibitors was compared and inhibitor dose response curves were recorded. It was evident that PS2-containing $\gamma$-secretase complexes were less sensitive to either of the two inhibitors tested, i.e., for achieving similar levels of inhibition of APP substrate processing higher concentrations of inhibitors had to be administered to purified PS2-containing $\gamma$-secretase complexes. The basis for these differences in inhibitor responsiveness is not known at this time.

In conclusion, we have revealed that mature PS1- and PS2-containing $\gamma$-secretase complexes form distinct molecular environments. The functional significances of each interaction between $\gamma$-secretase proteins and their candidate interactor proteins require further investigation to understand the potential role in A$\beta$ generation.

\begin{figure}
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\caption{Sequence alignment of PS1 and PS2 loop region. Amino acid sequences of PS1 (265-407) and PS2 (271-387) are aligned. Sequences in bold depict the low consensus.}
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Chapter 4:

Biochemical validation of Signal Peptide Peptidase, a PS2 interacting protein

Please note that some of this chapter contains materials from the following article:


Miss Amy Jeon performed 90% of the experiments and drafted the chapter; Dr. Christopher Böhm performed SPP knockdown and γ-secretase activity assay.
4.1 Abstract

Signal Peptide Peptidase (SPP) is a member of intramembrane cleaving aspartyl proteases (iCLIPs) family, which cleaves Type II transmembrane proteins. In our previous interactome analyses, SPP has been reported as a protein which binds to presenilin (PS)-containing $\gamma$-secretase complexes, with preferential binding to PS2-containing complexes. To better understand the relationship between SPP and PS, we characterized the biochemical properties of SPP and compared them to PS paralogs in various paradigms. First, the interaction between SPP and PS2 was confirmed by Western blotting analyses and co-immunoprecipitation (co-IP) of SPP and PS proteins. Having established the authenticity of the interaction, an shRNA-based knockdown of SPP was performed to begin the examination of the possible physiological relevance between the SPP-PS interaction. The Western blot analyses uncovered no significant expression changes in PS1, PS2 and nicastrin, and only a moderate decrease in Aph-1 and Pen-2 expressions in response to the cellular SPP knockdown. Interestingly though, there was a significant reduction in secreted A$\beta$40 peptide in cells in which SPP expression was diminished compared to wild-type cells. This finding, in conjunction with previous results, indicated a possible role of SPP in the $\gamma$-secretase-dependent processing of APP. From these observations, the intriguing possibility that SPP may contribute to an activity, commonly referred to as presenilinase, which cleaves holo PS protein into an active form, has emerged. To explore this possibility, three different types of inhibitors that inhibit (1) both $\gamma$-secretase and SPP (L685,458), (2) only SPP (Z-LL)$_2$-Ketone), and (3) only $\gamma$-secretase (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)), were administered in vivo to human embryonic kidney (HEK) 293 cells and in vitro to mouse embryonic fibroblast (MEF) and HeLa cells. The results indicated an effective inhibition of PS endoproteolytic cleavage in the presence of L685,458, but not in the presence of (Z-LL)$_2$-Ketone or DAPT. Moreover, single point mutation studies of the conserved aspartyl residue on both SPP-D257A and PS2-D263A in various cell types did not show clear evidence of SPP contributing to presenilinase activity.
4.2 Introduction

SPP, a member of the family of intramembrane cleaving aspartyl proteases (iCLIPs) cleaves Type II transmembrane (TM) proteins to release membrane stubs left behind after their precursors have been shed of their ectodomains or are secreted (Nyborg et al., 2004a; Spasic and Annaert, 2008; Weihofen et al., 2002; Wolfe, 2010). In addition, SPP is known as presenilin-like homologue (PSH), on the basis of a low level sequence homology of SPP and presenilins. More specifically, SPP shares identical active site motifs YD at TM4 and PAL and GxGD at TM5 with PS, which raised the spectre that PS and SPP may employ common catalytic mechanisms (Wang et al., 2004; Xia and Wolfe, 2003) (Table 4.1). However, the topology of SPP is inverted relative to PS resulting in SPP cleaving Type II TM substrates as opposed to Type I TM \( \gamma \)-secretase substrates (Figure 4.1) (Krawitz et al., 2005; Martoglio and Golde, 2003; Weihofen et al., 2002). Also, in contrast to \( \gamma \)-secretase, SPP does neither appear to require other co-factors, or undergo endoproteolytic cleavage (Sato et al., 2006; Weihofen et al., 2002; Wolfe, 2006).

Added complexity emerges from the fact that SPP is one of five genes that code for a small family human SPP paralogs comprising: SPP, SPPL2a, 2b, 2c and SPPL3 (Ponting et al., 2002). These five proteins may share non-redundant functions since they display different phenotypes in knockdown studies and reside in different locations throughout cells. For example, a SPP knockdown in zebrafish results in neuronal cell death, whereas the respective knockdown of SPPL2b led to an observation of an enlarged caudal vein without any detectible cell death (Krawitz et al., 2005). Whereas SPP and SPPL3 are predominantly localized in the ER, SPPL2b

Table 4.1 PS and SPP homolog comparison table (adapted with permission from Martoglio and Golde, 2003. Copyright (2003) Oxford University Press).

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Figure 4.1 Comparison of members of the iCLIP family. Individual proteins within this family comprise two conserved catalytic aspartate residues embedded within YD and GxGD sequence motifs. PS and SPP display an inverted orientation to each other, which gives rise to these proteins mediating Type I and Type II TM cleavages, respectively.

is localized in endosomes/lysosomes (Krawitz et al., 2005). Not much is known about the subcompartmental localization of these proteins except for a report that located a subset of SPP in lipid rafts (Nyborg et al., 2006).

Under mild lysis conditions, mature SPP with its N-linked glycans is primarily detected at an apparent MW of 95 kDa, consistent with it forming a dimer in vivo (Martoglio and Golde, 2003; Nyborg et al., 2004b). In 2008, a high resolution atomic model of Escherichia coli SPP was presented based on X-ray crystallographic data (Kim et al., 2008). SPP was shown to form a tetrameric structure, which was shaped like a bowl with the predicted membrane-associated surface opening at its base of approximately 96Å in diameter. An inner ridge inside the bowl restricted the opening to approximately 40Å in diameter. A concave surface was seen to be formed by the base opening and the ridge creating a substrate binding pocket for four catalytic active sites within the tetramer structure (Figure 4.2A). More recently, the 3D crystal structure of human SPP was solved at a resolution of 22Å (Miyashita et al., 2011). In this work, active SPP was again observed to form a slender, bullet-shaped homotetramer with dimensions of 85 x
Figure 4.2 3D structures of bacterial and human SPPs. (a) A section through the *E. coli* SPP tetramer revealed a bowl-shaped structure with the base of ~96 Å in diameter, an inside ridge of approximately ~40Å, and a top opening of ~22Å. A concave surface indicated by the double sided arrow creates the substrate binding pocket. The depth of the tetramer is ~50Å from base to axial opening (adapted with permission from Kim *et al*. 2008. Copyright (2008) Elsevier). (b) 3D image of human SPP in different angles revealed the bullet-shaped tetramer structure with large interior chamber. The height of the tetramer is ~130Å (adapted from Miyashita *et al*. 2011).
85 × 130Å and a spacious chamber inside the molecule. The authors suggested that a tetramer is an essential and minimal form of functional SPP, that its N-terminal region works as a structural scaffold for the tetramer assembly and plays a modulatory function for intramembrane cleavage (Figure 4.2B) (Miyashita et al., 2011).

SPP shows broad substrate specificity for Type II TM proteins; SPP substrates include signal peptide-derived human lymphocyte antigen (HLA)-E-binding epitope during immune surveillance, hepatitis C virus (HCV), preprolactin, eosinophil cationic protein (ECP), p120/inhibin-binding protein IgSF1, and preprocalcitonin (Golde et al., 2009; Lemberg et al., 2001; McLauchlan et al., 2002; Targett-Adams et al., 2008). Interestingly, it has been reported that SPP may also cleave PS1 holo-protein between amino acids 432 and 445 in the middle of its most C-terminal TM domain (Moliaka et al., 2004). Although less is known about the substrate specificity of SPP-like (SPPL) proteins, SPPL2a and 2b have been shown to cleave tumour necrosis factor α, Fas ligand (FasL), and dementia-associated protein Bri2 (Fluhrer et al., 2006; Kirkin et al., 2007; Martin et al., 2009; Martin et al., 2008).

The definitive biological function of SPP is largely unknown. However, there are a few possible functions of SPP. First, SPP may be involved in the regulation of immunological surveillance via HLA-E-binding epitope cleavage (Lemberg et al., 2001). Second, SPP may dislocate membrane proteins from the ER during virus infection (Loureiro et al., 2006). Human cytomegalovirus (HCMV)-encoded immunoevasin US2 induces the dislocation of class I major histocompatibility complex (MHC) heavy chains from ER membranes and target them for proteasomal degradation during viral infection. It was shown that the interaction of SPP with US2 was essential for US2-mediated ER dislocation of class I HMC heavy-chain molecule (Loureiro et al., 2006). Third, SPP may be involved in normal development. SPP-deficient D. melanogaster (Casso et al., 2005), C. elegans (Grigorenko et al., 2004) and zebrafish (Krawitz et al., 2005) displayed severe developmental phenotypes which resulted in lethality. Forth, in a protease-independent manner, SPP may contribute to the stabilization of misfolded proteins (Schroder and Saftig, 2010; Schrul et al., 2010). For example, misfolded opsin accumulated in the presence of catalytically active SPP without the concomitant accumulation of cleaved opsin fragments. The purpose of an involvement of SPP in this biology is unclear.
PS endoproteolytic cleavage – Presenilinase activity

Full-length PS has to undergo endoproteolytic cleavage at around residues 292 and 298 within a hydrophobic domain of the loop connecting TMDs 6 and 7 in order to mature to its active heterodimer comprising NTF and CTF (Figure 4.3) (Dillen and Annaert, 2006; Podlisny et al., 1997; Xia, 2008; Xia and Wolfe, 2003). This process is commonly referred to as presenilinase activity. During the proteolysis, each fragment reveals catalytic aspartates that are critical for the activity of the fully assembled $\gamma$-secretase complex. Importantly, point mutagenesis of either of the two aspartate residues is enough to abolish Aβ generation (Nyabi et al., 2003; Wolfe et al., 1999; Xia, 2008; Yu et al., 2000).

The general consensus in the scientific community is that PS cleavage occurs by autoproteolysis (Edbauer et al., 2003; Kimberly et al., 2000; Steiner et al., 1999a; Wolfe et al., 1999). One piece of evidence for this model comes from a PS1 point mutation study where mutagenesis of

Figure 4.3 Detailed representation of PS1. The conserved aspartate residues D257 and D385 are shown in yellow. The endoproteolytic cleavage site is within the hydrophobic domain between TM domains 6 and 7, shown next to ‘Presenilinase’ (adapted with permission from Dillen and Annaert, 2006. Copyright (2006) Elsevier).
methionine 292 to aspartate sufficiently abolished endoproteolytic activity (Steiner et al., 1999b). However, there are a few paradoxical questions that cannot be explained through autoproteolysis model. First, pharmacological evidence has indicated that γ-secretase is distinct from presenilinase. It has been shown that potent γ-secretase inhibitors dipeptidic compound N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and compound E, which can reduce Aβ generation with an IC₅₀ of 20 nM and 0.3 nM, displayed no, or very little, effect on presenilinase activity even at concentrations of 200 μM and 400 μM (Campbell et al., 2003; Dovey et al., 2001; Seifert et al., 2000). Second, biochemical evidence has shown that catalytically inactive mutant PS still undergoes partial processing into NTF and CTF by a presenilinase-like activity (Nyabi et al., 2003). Third, a structural conundrum emerged from the fact that the hydrophobic domain of PS, which serves as the presenilinase substrate, is expected to acquire a Type II orientation, rather than the Type I topology γ-secretase is known to cleave (Figure 4.4A, B, C). Therefore, it has been speculated that PS endoproteolytic cleavage may involve other proteins, which may contribute to the presenilinase activity.

In our interactome study (Chapter 3), SPP was identified as a PS2-interacting partner. The close association between these two aspartyl proteases PS2 and SPP which cleave Type I and Type II TM proteins, respectively, raised the tantalizing possibility that these cleavage enzymes may act in concert with each other and that in fact SPP may contribute to presenilinase activity. SPP exhibits similar pharmacological inhibition profile to presenilinase. Both presenilinase and SPP can be inhibited with aspartyl protease inhibitor pepstatin A and not affected by DAPT (Campbell et al., 2003; Dev et al., 2006). In addition, since SPP cleaves substrates with Type II topology, SPP involvement may assist in explanation for the structural paradox. Therefore, if it is indeed the case that SPP is contributing to presenilinase activity, it may provide a means to address the paradoxical questions.

Multiple strands of investigation were pursued to understand the biochemical relationship between PS and SPP. Co-IP experiments validated the interaction of SPP and PS2. The shRNA-based knockdown of SPP was pursued to address whether mere alterations of levels of SPP affect the expression and maturation of γ-secretase core components. The effect of the γ-secretase and SPP inhibitor L685,458, the γ-secretase-specific inhibitor DAPT, and the SPP-specific inhibitor (Z-LL)₂ Ketone on presenilinase activity was monitored by observing the
generation of PS NTF fragments in various *in vivo* and *in vitro* paradigms. In addition, mutant PS2-D263A and SPP-D257A, generated by single point mutations of conserved aspartate residues within PS2 and SPP, facilitated the dissection of the relative contribution of PS2 and SPP to presenilinase activity.

Figure 4.4 Presenilinase paradox and possible SPP function. (a) Presenilinase cleavage occurs in the hydrophobic domain in loop region between TM domains 6 and 7. It has been suggested that this presenilinase cleavage process is autoproteolysis. However, a few paradoxical questions arise: (b) First, the cleavage occurs less than 20 amino acids away from TM domain 6, which is too short for the hydrophobic domain to adjust itself in the Type I orientation. If the hydrophobic domain is not in Type I orientation, it cannot be processed via γ-secretase cleavage. (c) Second, if the hydrophobic domain is inserted into the membrane in a Type II orientation due to the length constraint in the previous scenario, the loop requires another hydrophobic domain for it to remove itself from the membrane and correctly insert into CTF.
4.3 Materials and Methods

shRNA knockdown experiments
HEK293 cells stably expressing Swedish APP (HEKswAPP) were transfected with the SPP shRNA vector acquired from Open Biosystems (Thermo Fisher scientific/Open Biosystems, Huntsville, AL). After 48 hr incubation, the selection of cells stably expressing SPP shRNA was initiated by adding 1 μg/ml puromycin (Gibco). Individual clones were selected for their decrease in SPP protein levels.

Western blot analyses and antibodies
Samples were prepared in SDS sample buffer, separated on 4-12% Bis-Tris gels (Invitrogen), and transferred to membranes. Proteins were detected by enhanced chemiluminescence. Antibodies used were: rabbit polyclonal anti-SPP (Abcam, Cambridge, MA), Nct (Sigma), PS1 A4, Aph-1 O2C2, Pen-2 (Anaspec, Fremont, CA). Densitometric analyses were performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Aβ40 ELISA and cell-free assays
Aβ40 levels were measured by ELISA assays using conditioned medium collected from HEK293 cells either with or without stably expressed the SPP shRNA. The enzyme-linked immunoabsorbent assay, ELISA, was performed according to the manufacturer’s instructions (Biosource International/Invitrogen). Determination of γ-secretase activity by cell-free assay was performed as previously described (Chen et al., 2006). Briefly, membrane fractions were isolated from HEK293 cells and incubated with recombinant FLAG-tagged APP-C100. Aβ40 peptide generated by γ-secretase proteolysis was measured by ELISA.

PS2 loop and N-terminus swapping experiments
The expression of SPP in the presence or absence of PS2 was analyzed by lentiviral transduction in PS double knockout mouse embryonic fibroblast (PS1−/−/PS2−/− MEF) cells with the following constructs: (1) PS2, (2) PS2 with cytosolic loop swapped with PS1 loop, and (3) PS2 with N-terminus swapped with the PS1 N-terminus (constructs were gifts from Dr. Shinsuke Matsuzaki, University of Toronto). Cells expressing each construct were lysed in DDM/CHAPSO lysis buffer (25mM HEPES pH7.4, 150mM NaCl, 2mM EDTA, protease inhibitor cocktail (Roche,
Basel, Switzerland), 0.25% (w/v) CHAPSO (Anatrace, Maumee, OH), and 0.05% (w/v) DDM (Anatrace)) and analyzed using Western blotting analysis.

**SPP and γ-Secretase Inhibitors in Cells**

Inhibition of SPP and PS endoproteolytic activity in live cells was assayed by treating HEK293 cells with L685,458 (Calbiochem), (Z-LL)2 Ketone (Calbiochem), and N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Calbiochem). To find the optimal duration, three inhibitors were treated for 48, 72, and 96 hours. In addition, to find the optimal concentrations, three inhibitor treatments were performed using various concentrations (1-10 μM for L-685, 458; 10-200 μM for (Z-LL)2 Ketone; 10-100 μM for DAPT) for 48 hours. After incubation, cell pellets were homogenized and lysed in DDM/CHAPSO lysis buffer. Samples were analysed by Western blotting analyses.

**Subcellular Fractionation**

The method was modified from the subcellular fractionation protocol from Xia and colleagues (Xia et al., 1998). Mouse embryonic fibroblast (MEF) and HeLa cells were detached from dishes and briefly washed with 20 mM EDTA in ice-cold PBS. All following steps were performed on ice. Cells were pelleted and resuspended in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, and protease inhibitor mix (5ug/ml leupeptin, 5ug/ml aprotinin, 0.25mM phenyl methane sulfanyl fluoride, PMSF). The use of commercially available protease inhibitor cocktail was avoided due to the presence of pepstatin A, an aspartyl protease inhibitor. Cells were then homogenized by 10 strokes of 700 rpm speed Potter-Elvehjem homogenizer (RW D2M.N homogenizer; Ika-labortech-nik, Germany). Homogenates were passaged three to five times through a 27-gauge needle. The collected sample was centrifugated at 1500 g for 10 min at 4°C and the supernatants were collected for further processing.

**In vitro Inhibition and de novo PS NTF/CTF generation**

This section of de novo PS fragment generation was modified from Campbell and colleagues (Campbell et al., 2002). To determine de novo PS fragment generation in vitro, samples generated by subcellular fractionation were divided into two aliquots. One aliquot was incubated at 4°C for 0 and 2 hours for determination of basal PS fragment levels. The other aliquot was incubated at 37°C for 2 hours for determination of de novo fragment generation. PS fragment
levels in both aliquots were probed by Western blotting analysis. In addition, to observe the effect of pharmacological inhibitions of SPP and γ-secretase *in vitro*, samples generated by the subcellular fractionation were incubated with three inhibitors used in cell study. For all of the inhibitors, samples were incubated with various concentrations of inhibitors on ice for 30 min, then at 37°C for 2 hours. The concentrations used for (Z-LL)$_2$ Ketone were 80 to 120 μM, L-685,458 were 8 to 12 μM, and DAPT were 40 to 50 μM. After incubation, *in vitro* processing of the samples was stopped by adding SDS sample buffer and the samples were subjected to western blotting analysis.

**PS and SPP single point mutation**

The conserved catalytic aspartic acid residues of PS2 (D263) and SPP (D257) were single point mutated into alanine residues through PCR amplification with the forward primer CTGGGCGCCATCTCTGTATGCTCCTCGTGGCTGTGCTGTGCTCCC and reverse primer GGGACACAGCACAGCCACGAGAGCATACACAGAGATGGCGCCCA for PS2 and the forward primer CTGGGCGGACTCTTCATCTACGCTGTCTTCTGGGTATTTGGCACC and reverse primer GGTCGCCAAATACCCAGACAGACGCTGTAGATGAAGAGTCCGCCCAC for SPP. For transduction into cells, SPP knocked down, HEKswAPP cells (generated in SPP ShRNA knockdown experiment; HEKswAPP SPP kdown), MEF with both PS1 and PS2 knocked out (MEF$^{-/-}$), and N2A cells were used. Each cell line was transfected using Lipofectamine 2000 (Invitrogen) with according to following DNAs: (1) PS2 control, (2) PS2 mutant, (3) SPP control, (4) SPP mutant, (5) PS2 control plus SPP control, (6) PS2 control plus SPP mutant, (7) PS2 mutant plus SPP control, and (8) PS2 mutant plus SPP mutant. After 48 hours, cells were collected, lysed using DDM/CHAPSO lysis buffer. Lysates were analysed by western blotting analyses using rabbit polyclonal anti-SPP and PS2 DT2.

**Electroporation Transfection Method**

The MEF Nucleofector Kit 1 was purchased from Amaza Biosystems (Lonza/Amaza Biosystems, Walkersville, MD). MEF$^{-/-}$ cells were transfected with DNAs in the same conditions as used in previous Lipofectamine transfection using MEF Nucleofector Kit 1 on Nucleofector II Device (Lonza/Amaza Biosystems) according to the manufacturer’s protocol. After 48 hr incubation, cells were collected, lysed using DDM/CHAPSO lysis buffer and subjected to Western blotting analysis using rabbit polyclonal anti-SPP and PS1 A4.
4.4 Results

Significance of SPP-PS2 interaction for \( \gamma \)-secretase mediated A\( \beta \) release

The authenticity of the preferential interaction between PS2 and SPP was confirmed in experiments presented in the previous chapter. Next, we examined whether SPP and PS influence the expression or post-translational modifications of each other. To this end SPP levels were suppressed by shRNA-based knockdown in HEK293 cells stably expressing the Alzheimer’s disease Swedish variant of APP. Next, equal levels of total protein from cell extracts were analyzed by western blotting and it was determined by densitometry that levels of SPP expression were silenced in shRNA treated cells to less than 40% signal intensities observed in naive cells (Figure 4.5A, B). When the same fractions were probed with antibodies specific for \( \gamma \)-secretase core constituents no significant changes in PS1, PS2 or nicastrin expression levels were observed. However, the SPP knockdown caused a considerable decrease in the expression levels of Aph-1 (35.5%) and Pen-2 (32.91%) that approximately matched the level of signal reduction seen for SPP itself (Figure 4.5A, B).

Finally, we examined whether SPP is involved in the production/release of cellular A\( \beta \). The experiment was based on a sandwich ELISA and relative \( \gamma \)-secretase activities in cellular extracts were compared \emph{in vitro} by monitoring A\( \beta \)40 release following intramembrane endoproteolysis of the APP-C100 substrate. A comparison of HEK293 cells which had been transfected with SPP-specific or mock siRNAs revealed a significant reduction in secreted A\( \beta \)40 peptide levels in cells which exhibited diminished SPP levels (Figure 4.5C). Taken together, these experiments support a model whereby SPP may play a physiological role in the cleavage or release of cellular A\( \beta \). In light of the interactome data we presented it is plausible that this influence of SPP on secreted A\( \beta \) levels may predominantly be mediated by its preferential interaction with PS2.

Presence/absence of PS does not affect SPP expression

The previous paragraph described experiments which indicated that PS expression was not altered by a decrease in SPP expression. We next conducted reciprocal analyses to investigate whether SPP levels are altered in cellular extracts derived from mouse embryonic fibroblasts (MEF) cells which express: (1) no PS (PS1/PS2 double knockout cells), (2) PS2 re-introduced by transfection into PS1/PS2 double knockout cells, (3) PS2 cytosolic loop swapped with PS1 loop
Figure 4.5 Knockdown of SPP causes a reduction in Aβ production. (a) Multi-panel western blot data documenting shRNA-mediated knockdown of SPP in HEK293 cells stably expressing the Swedish variant of APP. Whereas levels of PS1, PS2 and nicastrin were not altered in cells which expressed reduced levels of SPP, levels of Aph-1 and Pen-2 were moderately but significantly decreased when SPP expression levels were diminished. (b) SPP, Aph-1 and Pen-2 display similar levels of signal reductions. (c) Significant reduction of γ-secretase activity in cellular extracts obtained from HEK293 cells that had been subjected to shRNA mediated SPP knockdown. Measurements of γ-secretase activity were based on APP-C100 substrate assay which determines the quantities of secreted Aβ40 by ELISA.
transfected into PS1/PS2 double knockout cells, (4) PS2 N-terminus swapped with PS1 N-terminus re-introduced by transfection into PS1/PS2 double knockout cells. The domain swapping component of these experiments constituted an attempt to begin to understand the nature of the preferential association of SPP with PS2. However, the results of these studies revealed no differences in SPP protein expression levels when either both PS were deficient or PS2 was re-inserted into a PS-deficient background, corroborating the view that SPP and PS do not influence the expression of each other (Figure 4.6). In addition, it can be concluded from the experiments that the swapping of structural domains which have evolved most divergently between PS1 and PS2 does not affect SPP expression. For example, exchanging the most variable regions, PS2 cytosolic loop and N-terminus to PS1 counterparts, did not influence SPP expression. Thus the differential SPP-PS interactions we observed with PS1- or PS2-containing γ-secretase complexes did not translate into differences in the expression of SPP.

Does SPP represent the elusive presenilinase?

In light of the interaction between SPP and PS paralogs, we wondered whether SPP may contribute to the endoproteolytic activity, which is known to generate NTF and CTF fragments from full-length PS. Although the precise mechanism and enzyme responsible for this cleavage is not known, the activity is commonly referred to as the presenilinase activity. The predominant view in the field has been that the immature PS full-length protein is somehow capable to activate itself through auto-endoproteolysis. To examine the effect on PS maturation when SPP is pharmacologically inhibited, HEK293 cells were treated with three different types of inhibitors, namely a compound refered to as L685,458, (Z-LL)2 Ketone and DAPT for durations of 48, 72, or 96 hrs (Figure 4.7A). (Z-LL)2 Ketone was the only SPP-specific inhibitor in this experiment and would be expected to only affect presenilinase cleavage of the full-length PS if this process was indeed dependent on SPP (Narayanan et al., 2007). However, among the three inhibitors tested only L685,458, which inhibits both γ-secretase and SPP, was seen to inhibit the presenilinase activity evidenced by the accumulation of full-length PS (Figure 4.7B). As expected, levels of PS2 full-length and PS2 NTF display a reciprocal relationship in this study that is reflective of their substrate-product relationship. The inhibition can be seen to lose its effectiveness after 48 hrs. DAPT, a potent γ-secretase inhibitor that is known to inhibit γ-secretase-dependent Aβ peptide production but not presenilinase cleavage or pro-lactin

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Figure 4.6 Expression levels of SPP in mouse embryonic fibroblasts (MEFs) are not affected by the presence or absence of PS paralogs. (a) Western blot documenting equal expression of SPP. (b) PS2 N-terminus antibody DT2 is able to display PS2 expressions in PS1/2 double knockout cells transduced with PS2 or PS2 cytosolic loop swapped with PS1 loop. (c) PS1 N-terminus antibody A4 display expression in cell transduced with PS2 NTF swapped with PS1 NTF, which indicates the successful swapping of PS2 N-terminus with PS1 counterpart.
Figure 4.7 SPP-specific inhibitor does not affect presenilinase activity *in vivo*. (A) Structural formulas of L685,458, DAPT, and (Z-LL)₂ Ketone. (B) Cells were treated for 48, 72 and 96 hrs with inhibitors as indicated. Among the three inhibitors, only administration of L685,458 to the medium led to a detectable inhibition of presenilinase cleavage evidenced by the accumulation of uncleaved full-length PS. However, the efficacy of L685,458 decreased with time. SPP expression levels were not altered in the presence of inhibitors. (C) In a repeat experiment inhibitors were offered at various concentrations for duration of 48 hrs. At higher concentration of L685,458 more full-length PS substrate was seen to accumulate. Both DAPT and ((Z-LL)₂ Ketone showed no effect on presenilinase even at the highest concentrations tested. (FL, full length; NTF, N-terminal fragment; (Z-LL)₂, (Z-LL)₂ Ketone).
processing by SPP, does not appear to inhibit presenilinase activity in this experimental paradigm. A shortcoming of the experimental approach taken was the possibility that the inhibitors available for this analysis may have different ability to pass the cellular membrane, possibly leading to low effective concentrations in the secretory pathway (the cellular compartment known to host presenilinase activity). To begin to address this shortcoming, HEK293 cells were in a repeat experiment treated with 10 to 20-fold increased inhibitor concentration for 48 hrs (Figure 4.7C). However, similar to the aforementioned result, (Z-LL)2 Ketone and DAPT failed again to document any inhibitory effect on presenilinase activity, whereas L685,458 continued to show the expected concentration-dependent increase in full length PS accumulation. One interpretation of these data is that the inhibition of SPP may indeed have no influence on PS endoproteolysis. However, despite the increased levels of inhibitors applied in this repeat experiment, the absence of a suitable in vivo control which could be used to monitor the efficacy of SPP inhibition, inhibitor solubility problems may still be the main factor limiting our ability to fully interpret these data. Taken together, the pharmacological inhibition of SPP in cells did not allow us to conclusively evaluate a possible SPP contribution to presenilinase activity.

In vitro inhibition of SPP did not alter the de novo PS NTF/CTF generation

Although the SPP inhibitor was unable to effectively inhibit presenilinase activity when offered in the growth medium to HEK293 cells, we realized that this result may merely indicate a very low penetration of this (Z-LL)2 Ketone into cells, an observation also reported by others (Weihofen et al., 2003). Consequently, there was a possibility that inhibiting SPP in vitro following mild solubilization of cellular membranes might provide a more conclusive answer. To this end, we next solubilized MEF and HeLa cells with a homogenization buffer that contained multiple conventional protease inhibitors but lacked an inhibitor of aspartyl proteases. It has been shown previously that presenilinase is an aspartyl protease that can be inhibited by pepstatin A or other inhibitors directed against this subclass of cellular proteases (Campbell et al., 2003). Next, the three aforementioned inhibitors L685,458, (Z-LL)2 Ketone, and DAPT, were added to cell lysates on ice for 30 min to allow the inhibitors to soak into the membrane preparation, and then incubated at 37°C for 2 hours to observe a possible inhibitory effect on presenilinase activity (Figure 4.8A). This experimental design was empirically derived from observations which indicated that presenilinase enzymatic activity surges within 10 min of incubation at 37°C, but is
not reproducibly observed when the 30 min incubation on ice is omitted, possibly because the inhibitors lack the appropriate time to associate with their target sites. After the incubation, samples were subjected to western blotting analysis and \textit{de novo} NTF/CTF generation in the absence or presence of inhibitors was monitored. As expected, L685,458 treated lysates displayed an accumulation of uncleaved full-length PS substrate to a level that was comparable to the 4°C negative control sample we had included in the analysis in both MEF and HeLa cell lines (\textbf{Figure 4.8B, C}). Moreover, similar to the experiments we had undertaken in cells, (Z-LL)$_2$ Ketone and DAPT did not interfere with presenilinase cleavage \textit{in vitro}. Therefore, it may seem that SPP is not part of the presenilinase complex. However, there are still a few possible scenarios which make the outcome of this experiment less than conclusive. Most importantly, because the pharmacological profile of SPP-like proteases is not known (Nyborg et al., 2006), their presence in the sample could undermine interpretation if their activity is not inhibited by the
Figure 4.8 Pharmacological inhibition of presenilinase in vitro. (a) Schematic diagram of strategy. Cells are collected and homogenized using a Potter-Elvehjem homogenizer. Sample is further passaged through a 27-gauge needle and subjected to centrifugation. Supernatant is collected and divided into six aliquots: control collected at 0h incubation, 4°C control at 2h incubation, 37°C control at 2h incubation, and three inhibitor treatment samples at 2h incubation. Samples were analyzed by Western blotting analyses. (b) Western blot displaying the PS1 expression in MEF cells. Incubation at 37°C can be seen to allow presenilinase activity to proceed evidenced by a decrease in levels of PS1 FL substrate when compared to the negative control at 4°C. Only L685,458 treatment inhibited presenilinase activity. (c) Western blot displaying PS1 expression in HeLa cell. The result shows similar outcome to the previous MEF cell experiment documenting that only L685,458 treatment inhibited presenilinase activity (Cont, Control; L685, L685,458; ZLL, (Z-LL)2 Ketone; FL, full length; NTF, N-terminal fragment).

SPP inhibitor (Z-LL)2 Ketone but is able to compensate for the reduced presenilinase activity SPP might contribute to. Second, because no SPP substrate assay was available for this study and (Z-LL)2 Ketone is known to be a less potent inhibitor of SPP than on L685,458, the experiment may not have been able to capture the window of inhibitory activity required to observe an effect of (Z-LL)2 Ketone on SPP.

PS and SPP point mutation
Both PS and SPP harbor two conserved aspartyl residues that play a pivotal function for their proteolytic activities. It has, for example, previously been documented that the PS2 endoproteolytic activity vanishes when either one of the conserved aspartate residues is mutated
Figure 4.9 SPP and PS2 single point mutation. (a) Amino acid sequences of SPP and PS. Both proteins show conserved aspartic acids shown in bold font and underlined. Point mutations were generated to replace the first conserved aspartic acid residue within both SPP and PS2 with an alanine. (b) DNA sequencing results confirming successful mutagenesis of SPP and PS2. Single amino acid changes are shown in the red rectangles.
(Xia, 2008). Similarly, mutation of one of the conserved aspartyl residue in SPP decreases intramembrane proteolytic cleavages mediated by SPP (Fluhrer et al., 2008; Fluhrer and Haass, 2007). Therefore, mutagenesis of one of the two catalytic aspartates should be an alternative strategy to explore if SPP contributes to presenilinase activity. Introduction of the analogous mutation in PS should enable a comparative analysis of the relative contribution of the catalytic aspartates to presenilinase activity in cells transfected with combinations of wild-type or mutated PS and SPP. Perturbation of presenilinase activity can in this experimental paradigm again be monitored by the accumulation of full-length PS2 protein. Thus, single point mutations were introduced to PS2 and SPP to replace the aspartate residue within their conserved YD motifs to alanine (giving rise to mutant PS2 D263A and SPP D257A;GAT → GCT) (Figure 4.9A, B). These constructs were transiently transfected into HEKswAPP SPP knockdown, MEF PS1/PS2 double knockout and N2A cell lines in various combinations: (1) wild-type control, (2) PS2, (3) PS2 mutant, (4) SPP, (5) SPP mutant, (6) PS2 and SPP, (7) PS2 and SPP mutant, (8) PS2 mutant and SPP, and (9) PS2 mutant and SPP mutant (Figure 4.10A). It was observed that the PS2 mutation diminished PS endoproteolysis, documented by the absence of PS2 NTF in lanes 3 of both the MEF and N2A cell analyses. The consequence of introducing the mutation into PS2 is less clear within HEKswAPP cells, possibly because of a more robust endogenous PS2 expression in these cells (compare lanes 1 in the three different cell type panels). In contrast, the introduction of the point mutation into SPP caused more of this mutant to migrate at the level of monomer than seen in side-by-side analyzed samples from cells expressing wild-type SPP (see lanes 5 of the MEF and N2A cell type panels). However, it did not alter the levels of intensity at which SPP dimers are observed. The side-by-side analysis of cells which were co-transfected with PS2 and SPP or SPP mutant did not reveal an effect of the mutant on the levels at which PS2 NTF signals can be seen, arguing that the catalytic aspartate within the YD motif of SPP does not contribute to presenilinase activity (compare lanes 6 and 7). To ensure that the outcome is not a result of merely having achieved relatively low levels of transfection when using Lipofectamine, an alternative electroporation-based transfection method was tested on MEF cells which are notoriously refractory to conventional transfection (Figure 4.10B). The electroporation result corroborated the previous conclusion that SPP proteolytic activity did not contribute to PS2 endoproteolysis in MEF PS1/PS2 double knockout cells. Therefore, the result validated that SPP does not contribute to presenilinase activity.
Figure 4.10 Co-expression of heterologous PS and SPP wild-type and aspartate mutants in a range of host cells indicates that presenilinase activity does not depend on SPP. (a) Transient transfection of constructs into HEKswAPP SPP knockdown, MEF PS1/PS2 double knockout and N2A cell lines. The expression of the PS2-D263A mutant led to the accumulation of full-length PS2-D263A signals and a decrease of PS2-D263A NTF signals. On the other hand, expression of SPP-D257A caused a slight increase in SPP monomer expression relative to cells transfected with wild-type SPP. A side-by-side comparison of cellular extracts from cells which were co-transfected with PS2 and SPP or SPP mutant did not reveal changes to the levels of intensity at
which PS2 NTF signals were observed. (b) Electroporation-based transfection of constructs into MEF cell. This method improved the transfection efficacy, which can be evidenced by the absence of PS2 NTF in PS2 mutant. However, the observations from this experiment corroborated the conclusion that heterologously expressed SPP did not contribute at a significant level to PS2 endoproteolysis.
4.5 Discussion

Experiments conducted in this chapter explored the functional significance of an interaction between PS2 and SPP association that was uncovered by our previous PS directed comparative interactome investigation. This observation was initially based on multiple CID spectra which allowed an unequivocal identification of SPP and, importantly, displayed consistent iTRAQ signature mass distributions which constituted strong evidence for its association with PS2. Consistent with previous reports (Nyborg et al., 2006; Wahrle et al., 2002), our biochemical analyses revealed co-fractionation of SPP and presenilins in sucrose gradients but also suggested that SPP does not exert an influence on the cellular localization of presenilins detectable by this fractionation technique (not shown). However, when SPP levels were experimentally reduced by the use of shRNAs, cellular extracts of HEK293 cells exhibited lower γ-secretase activity in a conventional assay that measures the release of Aβ40 peptide. Further experiments is needed to establish whether this observation is the result of SPP affecting the maturation or localization of γ-secretase or its APP substrate, or of SPP influencing the γ-secretase-mediated intramembrane proteolysis step more directly. Noteworthy in this context is a report from a few years ago in which authors described that SPP can clip a C-terminal peptide from PS1 by cleaving its C-terminal domain at a site near the PAL motif (Moliaka et al., 2004). Because the study was based on a paradigm in which both proteins were overexpressed and the physiological relevance of this observation was not apparent, the finding was merely discussed as a curious and unexpected precedent of a multi-spanning transmembrane domain protein serving as a substrate of another i-CLIP family member. In this study, the possibility of SPP contributing to PS endoproteolytic cleavage, referred to as presenilinase activity, was thoroughly examined using γ-secretase and SPP inhibitors in vitro and in vivo, and manipulating PS and SPP catalytic aspartates in various relevant human and mouse cell lines. Taken together, results from these experiments argued against a significant contribution of SPP to presenilinase activity.

Throughout the work we undertook to explore the physiological significance of the PS2-SPP interaction, we were aware of the existence of multiple additional SPP-like (SPPL) proteins, which could exhibit degrees of functional redundancy with SPP (Krawitz et al., 2005). It is currently unknown to which extent SPPL can replace SPP in SPP deficient cells. It also is unclear to which extent the inhibitors we employed can functionally impair these SPP paralogs.
Therefore, although our result using inhibitors and point mutation failed to indicate a significant SPP contribution to presenilinase activity, we are cognizant that our work does no strictly rule out the possibility that members of the SPP/SPPL may contribute to the endoproteolysis of PS proteins.

**Alternative SPP function in relation to PS**

SPP is one of only a handful of proteins which together with presenilins form the mammalian i-CLIP family. Relative to presenilins, SPP is expected to adapt an inverted membrane topology. Thus, whereas presenilins are known to carry out intramembrane cleavages on substrate proteins which acquire a Type 1 transmembrane topology, SPP is expected to cleave Type II substrate proteins. Much speculation surrounds the broader significance of presenilin-mediated cleavages of Type I transmembrane proteins. Whereas for a subset of its substrates, a physiological role of γ-secretase cleavage products could be identified (for example, for the notch intracellular domain), for other substrates this has not been straightforward. The broad spectrum of γ-secretase substrates has invoked the analogy of γ-secretase as a “secretosome” or “proteasome of the membrane”. According to this model γ-secretase may patrol cellular membranes to rid them of the cumulative burden posed by Type I transmembrane stubs left behind from ongoing cellular sheddase activities (Kopan and Ilagan, 2004; Schenk, 2000; Yu et al., 2001). The surprising association of PS2 and SPP revealed in this work adds fuel to this concept. Just as the proteasome is equipped with multiple proteolytic activities to be able to deal with a diverse range of substrates, the cell may have devised an analogous machine for the removal of transmembrane stubs by pairing i-CLIPs which exhibit proteolytic activity against transmembrane domains embedded in the lipid bilayer by a range of topologies.

A functional benefit which may arise from the close proximity of SPP and γ-secretase may represent the possibility that these proteins work together and contribute to cellular efforts to prevent the “clogging” of membranes from by-products of cellular protein secretions – quality control of membrane proteins. Importantly, by conjoining proteolytic activities directed against both Type I and Type II TM domains, the cell may have mastered the trick to digest Type III multi-spanning transmembrane proteins, which can be viewed an assembly of transmembrane domains with alternating Type I and Type II orientation. To this end, it would of future interest
to work toward a cell-free assay and explore whether a synergistic combination of SPP and $\gamma$-secretase would be able to digest Type III TM proteins, such as rhodopsin or Sec71p.

In conclusion, SPP does not contribute to presenilinase activity, but it may function as part of a high-molecular weight complex that interacts with different proteins, including PS2, to facilitate the “unclogging” of the membrane traffic and participate in cellular quality control systems aimed at membrane proteins.
Chapter 5:

Vigilin interacts with Signal Peptide Peptidase

Please note that this chapter contains materials from the following article:


Miss Amy Jeon performed mass spectrometry analysis and contributed in drafting the manuscript; Mr. Stephen Lu performed purification and biochemical validations.
5.1 Abstract

Signal peptide peptidase (SPP), a member of the presenilin-like intra-membrane cleaving aspartyl protease family, migrates on Blue Native (BN) gels as 90 kDa, 200 kDa and 450 kDa species. SPP has recently been implicated in other non-proteolytic functions such as retro-translocation of MHC Class I molecules and binding of misfolded proteins in the endoplasmic reticulum (ER). These high molecular weight SPP complexes might contain additional proteins that regulate the proteolytic activity of SPP or support its non-catalytic functions. In this study, an unbiased iTRAQ-labeling mass spectrometry approach was used to identify SPP-interacting proteins. We found that vigilin, a ubiquitous multi-KH domain containing cytoplasmic protein involved in RNA binding and protein translation control, selectively enriched with SPP. We have identified and validated vigilin as a novel interacting partner of SPP that could play an important role in the non-proteolytic functions of SPP. This data adds further weight to the idea that intramembrane-cleaving aspartyl proteases, such as presenilin and SPPs, could have other functions besides the proteolysis of short membrane stubs.
5.2 Introduction

Signal peptide peptidase (SPP) is a member of the intramembrane cleaving aspartyl protease family that also includes the presenilins and SPP-like proteases (Weihofen et al., 2002). Members of this family share characteristic YD, GxGD and PAL motifs (Fluhrer et al., 2009). However, the presenilins cleave Type I transmembrane proteins (McCarthy et al., 2009), while the SPP-like proteases cleave Type II transmembrane proteins (Weihofen et al., 2002). Amongst the SPP-like proteases, the ER-resident SPP is thought to cleave the membrane-bound stubs of some secreted proteins following proteolysis of the signal peptide by the signal peptidase (Weihofen et al., 2002; Weihofen et al., 2000), and in doing so presumably releases the stubs from the ER membrane. SPP may also have additional activities in protein control in the ER. For example, SPP is required for the dislocation or retro-translocation of MHC Class I molecules in the presence of the human cytomegalovirus (hCMV) US2 protein (Loureiro et al., 2006). Others have reported that both catalytically inactive and wild-type SPP may bind and stabilize misfolded membrane proteins (Crawshaw et al., 2004; Schrul et al., 2010).

Preliminary biochemical and biophysical studies by this and other groups (Schrul et al., 2010) have revealed that the SPP-like proteases in general, and SPP in particular, exist in cell lysates as higher molecular mass species than would be predicted from the calculated molecular weight of their respective monomeric polypeptides (Figure 5.1). One potential explanation for this observation is that the SPP proteins might exist as dimers (Nyborg et al., 2006; Nyborg et al., 2004) or higher order assemblies. However, this observation also raises the possibility that there may be ancillary proteins which, although not required for SPP proteolytic activity, may nevertheless modulate its function. We therefore set out to determine whether the high molecular weight SPP complexes might contain other proteins, and if so, whether these other proteins modulated the SPP protease activity function of the SPPs. Using an unbiased mass spectrometry (MS) based approach, vigilin was identified as a candidate SPP interacting protein. Reciprocal co-immunoprecipitations confirmed the interaction between SPP and vigilin. Additionally, SPP and vigilin co-localized in restricted cellular domains, co-fractionated biochemically and co-migrated as a single band on BN gels.
Figure 5.1 SPP exists in higher molecular weight complexes. *Top panel*, HEK293 lysate solubilized in 0.5% DDM and resolved on 16% Bis-Tris Blue Native-PAGE gels, revealing SPP-containing complexes at 450 kDa, 200 kDa and 100 kDa. *Lower panel*, lysates resolved on the BN-PAGE were then resolved on the second dimension/SDS-PAGE. SDS-stable SPP dimer can be found in all three high molecular weight bands suggesting that the 100 kDa band observed in the first dimension is a SDS-stable SPP dimer.
5.3 Materials and Methods

Antibodies and Resins
The following antibodies were used in this study: rabbit anti-SPP C-terminus polyclonal antibody (SPP-CT, Abcam, Cambridge, UK), rabbit anti-SPP N-terminus polyclonal antibody (SPP-NTF, Abcam), rabbit anti-vigilin polyclonal antibody (Abcam), mouse anti-FLAG monoclonal antibody (Clone M2, Sigma-Aldrich, Dorset, UK), rabbit anti-GM130 polyclonal antibody (Abcam), rabbit anti-calnexin polyclonal antibody (Abcam) and mouse anti-myc monoclonal antibody (Invitrogen). The HA-agarose and FLAG-agarose affinity gels were obtained from Sigma-Aldrich and Protein G-sepharose was from GE Healthcare (Little Chalfont, UK).

Molecular Cloning
Vigilin cDNA (ORFeome clone number: 100011448) was amplified by PCR using the forward (sequence: 5’-GGCGCCATGAGTTCCGTTGCAGTTTTG-3’) and reverse (sequence: 5’-GGCCGGTTACTACTTTGTCATCGTCATCCTTTGTAAGTCTCGTGGGCGCCAAGGGAG-3’) primers and inserted into the TA-cloning site of the pcDNA3.3 mammalian expression vector (Invitrogen, Paisley, UK) with a single FLAG tag (N-DYKDDDDK-C) introduced directly to the C-terminus of the vigilin cDNA.

Cell culture and transfection
Human Embryonic Kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Following sequence verification, the pcDNA3.3-vigilin-FLAG plasmid was transfected into HEK293 cells with FuGene 6 (Roche Applied Science, Burgess Hill, UK). Stable transfectants were clonally selected with 1 mg/ml Geneticin G418.

Membrane Preparation and Affinity Purification of Endogenous SPP
Antibodies were cross-linked to Protein G sepharose with 0.025 M Borax pH 9.45 in the presence of 20 mM dimethylpimedilate (Sigma-Aldrich) and residual uncross-linked antibody was removed with 100 mM glycine HCl pH 3.0. For the preparation of microsomal membranes, the cell pellet was resuspended and homogenized in sucrose lysis buffer (25 mM HEPES pH 7.4, 4 mM EDTA, 0.25 M sucrose, complete protease inhibitor cocktail (Roche)). Following centrifugation at 2,000 × g for 10 min, the supernatant was further centrifuged at 100,000 × g (Ti45 rotor, Beckman Coulter, High Wycombe, UK) for 60 min to isolate...
microsomal membranes. Membrane lysates were solubilized with 0.5% (wt/vol) n-dodecyl-β-D-maltoside (DDM, Anatrace) in HEPES buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 4 mM EDTA, complete protease inhibitor cocktail). Lysates were pre-cleared with pre-immune serum cross-linked to protein G sepharose overnight, and SPP was then purified with SPP-CT antibody cross-linked to protein G sepharose. Next, the beads were subjected to at least 5 washes with HEPES buffer containing 0.02% (wt/vol) DDM and a single high-salt wash step in HEPES buffer with 300 mM NaCl. SPP and its interacting proteins were eluted by pH drop with Tris-HCl pH 3.0. To generate a negative control, anti-SPP antibody was pre-saturated with 100 µg/ml of cognate peptide (sequence: N-TESKEGTEASASKGLEKEK-C) and washes with 100 µg/ml of the aforementioned peptide was also included.

**Protein reduction, alkylation and trypsinization**
Protein-containing fractions were denatured in the presence of 9 M urea, followed by reduction with 5 mM tris-(2-carboxyethyl)-phosphine for 30 min at 60°C and alkylation with 9 mM 4-vinylpyridine for 1 h at room temperature in the dark. Samples were diluted five-fold to ensure that the concentration of urea did not exceed 2 M. Tryptic digestion was initiated by the addition of 1% (wt/wt) of side chain-modified, TPCK-treated porcine trypsin and allowed to proceed at 37°C for 6 h.

**iTRAQ labeling and mass spectrometry**
The steps for the labeling of peptides with iTRAQ reagents, two-dimensional separation of peptide mixtures by offline strong-cation exchange (SCX) and online reversed phase (RP) liquid chromatography and subsequent analysis by electrospray tandem mass spectrometry have been described previous chapter. Samples derived from control and SPP-specific purifications were iTRAQ-labeled with iTRAQ-116 and iTRAQ-117 reagents, respectively.

**Database searches**
Collision induced dissociation (CID) spectra were analyzed using ProteinPilot (Version 2.0, Applied Biosystems, MDS Sciex). The lists of candidate interacting proteins were subjected to the following filters: (i) all identifications of proteins had to be based on at least two CID spectra which passed the 95% confidence score returned by the ProteinPilot software; (ii) assignments to non-iTRAQ-labeled peptides or CID spectra with individual confidence scores of less than 90% were not included in the calculation of enrichment ratios based on
iTRAQ signature mass signal intensities. Raw iTRAQ ratios were corrected for impurity levels of individual reagent lots determined by the manufacturer. The mass tolerance range between expected and observed masses used for database searches was ±150 ppm for MS peaks, and ±0.15 Da for MS/MS fragment ions. All samples were searched against the International Protein Index (IPI) database disseminated through EMBL/EBI.

Co-immunoprecipitations and Western Blotting
Homogenized HEK293 cells were solubilized in ice-cold HEPES buffer with 0.5% DDM for 60 min and centrifuged at 100,000 × g for 30 min to remove insoluble material. Co-immunoprecipitations were carried out with 500-1000 µg of solubilized total lysate utilizing antibodies against SPP-CT and FLAG-tag, with pre-immune rabbit serum and antibody against the HA-tag serving as the control for the SPP and FLAG IPs, respectively. After pre-clearing, lysates were incubated overnight at 4°C with antibody and Protein G Sepharose or pre-conjugated affinity resins. Resin was then washed five times with 0.02% DDM in HEPES buffer. The precipitated proteins were eluted with 1 × sample buffer (LDS, Invitrogen). For immunoblotting, the samples were resolved on 12% Bis-Tris NuPAGE gels (Invitrogen), transferred onto polyvinylidene fluoride membranes (PVDF, Millipore, Watford, UK) and probed with appropriate primary and secondary antibodies.
5.4 Results

Isolation of endogenous SPP complexes

SPP and its potential interacting partners were isolated from native HEK293 cells using a single-step affinity purification protocol (for a schematic of the protocol see Figure 5.2A). Specifically, microsomal membrane isolates were solubilized in 0.5% N-Dodecyl-β-D-maltoside (DDM) and subjected to anti-SPP affinity purification using anti-SPP-CT antibody, which recognizes the C-terminal amino acids 358-377 of SPP. In order to generate the negative control with which to identify and exclude non-specific binders, the capture antibody was pre-blocked with cognate peptide in the control purification, and the cognate peptide was further included during the subsequent affinity matrix washing step (Figure 5.2B).

A.

**Protocol for Endogenous SPP and Control Purifications**

| Membrane preparation and solubilization (0.5% DDM) |  |
| Pre-clear with serum-resin | Control: Incubate with 100μg/ml cognate peptide |
| Purify with antibody-resin | Control: Additional wash with 100μg/ml cognate peptide |
| Washes (0.02% DDM) |  |
| Wash (300mM NaCl) |  |
| pH drop elution | SPP: iTRAQ-117 |
| Trypsination, iTRAQ-labelling and pooling of samples | Control: iTRAQ-116 |
| Strong Cation Exchange Fractionation |  |
| Online reversed phase separation |  |
| Quantitative electrospray MS/MS |  |
Figure 5.2 Purification of SPP containing complexes. (a) Schematic showing the protocol for the purification and iTRAQ-based mass spectrometry analysis of the SPP interacting proteins. (b) The blocking of the capture anti-SPP antibody with 100 µg/ml of cognate peptide and the additional wash with cognate peptide, as detailed in purification protocol, prevented SPP protein from being immuno-purified.

The purified proteins were resolved by SDS-PAGE and visualized by Western blotting. In eluates obtained following SPP-specific capture, SPP-immunoreactive bands were observed at 42 kDa for monomeric SPP (predicted to be 47 kDa) and at 70 kDa, interpreted to be SDS-stable dimers. The discrepancy between the actual molecular weight and the observed molecular weight on SDS-PAGE is a common observation, in particular for membrane proteins, and in part reflects the effect of bound detergent (Rath et al., 2009). However, in the control purification, no SPP signal was observed, demonstrating that the cognate peptide was able to block the isolation of SPP. Parenthetically, this method was used because it was much more effective at reducing SPP binding in the control purification than siRNA knockdown of SPP (data not shown). The identity of the co-purified proteins and their level of enrichment in the cognate-peptide-blocked control equipment were then determined by iTRAQ-labelling coupled with mass spectrometry (Figure 5.3A).
iTRAQ-based analysis of the interactome of SPP

Inspection of the iTRAQ data revealed a robust enrichment of SPP (HM13) (for a list of proteins identified and their iTRAQ 117:116 ratios, see Table 1), while non-specific binders such as the frequently encountered protein HSP70, had iTRAQ 117:116 ratios for the signature mass peaks of less than 1.5 (Figure 5.3A). However, selective co-enrichment (117:116 ratios ≥ 1.5) of the ubiquitous 140 kDa RNA-binding protein vigilin was observed together with SPP (Figure 5.3B). Thirteen unique peptides were identified from vigilin in total.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession Number</th>
<th>Symbol</th>
<th>Unique Peptide</th>
<th>Coverage (%)</th>
<th>iTRAQ Ratio (117:116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal peptide peptidase</td>
<td>IPI:PI00152441.3</td>
<td>SPP</td>
<td>6</td>
<td>22.0</td>
<td>331.3</td>
</tr>
<tr>
<td>Vigilin</td>
<td>IPI:PI00022228.1</td>
<td>VIGLN</td>
<td>13</td>
<td>14.6</td>
<td>4.3</td>
</tr>
<tr>
<td>10 kDa heat shock protein, mitochondrial</td>
<td>IPI:PI00220362.4</td>
<td>HSPE1</td>
<td>9</td>
<td>72.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Microfibrillar-associated protein 1</td>
<td>IPI:PI00022790.1</td>
<td>MFAP1</td>
<td>5</td>
<td>20.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Heat shock protein 60</td>
<td>IPI:PI00472102.3</td>
<td>HSP60</td>
<td>23</td>
<td>63.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Calnexin precursor</td>
<td>IPI:PI00020984.1</td>
<td>CNX</td>
<td>9</td>
<td>15.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Alpha-enolase</td>
<td>IPI:PI00465248.4</td>
<td>ENO1</td>
<td>14</td>
<td>37.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>IPI:PI00219018.6</td>
<td>GAPDH</td>
<td>8</td>
<td>33.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Protein C14orf166</td>
<td>IPI:PI00006980.1</td>
<td>C14orf166</td>
<td>7</td>
<td>39.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Stress-70 protein, mitochondrial precursor</td>
<td>IPI:PI00007765.5</td>
<td>HSPA9</td>
<td>43</td>
<td>71.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4 gamma 1</td>
<td>IPI:PI00552639.2</td>
<td>EIF4G1</td>
<td>8</td>
<td>13.6</td>
<td>1.0</td>
</tr>
<tr>
<td>ATP synthase O subunit, mitochondrial precursor</td>
<td>IPI:PI00007611.1</td>
<td>ATP5O</td>
<td>10</td>
<td>59.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Actin, cytoplasmic 1</td>
<td>IPI:PI00021439.1</td>
<td>ACTB</td>
<td>9</td>
<td>47.5</td>
<td>1.0</td>
</tr>
<tr>
<td>130 kDa leucine-rich protein</td>
<td>IPI:PI00329745.4</td>
<td>LPPRC</td>
<td>11</td>
<td>12.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>
A. HSP70 (IPI00029744), MS/MS of m/z 753.42
AQFEGIVTDLIR (aa 349-360) + iTRAQ114 (N-term)

B. Vigilin (IPI00022228), MS/MS of m/z 565.33
EQLAQAVAR (aa 271-283) + iTRAQ114 (N-term)
Figure 5.3 Representative CID spectra documenting specific co-enrichment of vigilin with SPP and non-specific co-purification of HSP70. Parallel co-purification with presaturated or naive anti-SPP antibody was followed by iTRAQ-based quantitation and ESI-MS/MS analyses. iTRAQ labeling reactions were setup with the iTRAQ116 label as the negative control, and iTRAQ117 label as peptides derived from the SPP-specific purification. (a) CID spectrum assigned to tryptic peptide AQFEGIVTDLIR which contributed to the identification of HSP70. iTRAQ signature mass peak ratios documented that this peptide was contributed equally by negative control and specific IP eluates. (b) Representative CID spectrum assigned to vigilin-derived peptide EQLAQAVAR which exhibited a strongly skewed iTRAQ ratio, demonstrating that this peptide was primarily contributed by the specific sample. Graphs on the side depict expanded views of the isotopic envelope of the respective precursor masses and the iTRAQ signature mass peaks.

Vigilin physically interacts with SPP

To validate the iTRAQ results, reciprocal co-immunoprecipitation experiments from HEK293 cells were undertaken. As expected, endogenous vigilin was captured when using endogenous SPP as the bait protein (Figure 5.4A). The reciprocal co-immunoprecipitation experiments using anti-vigilin antibody was inconclusive because the SPP bands migrated very closely to the denatured IgG, making it difficult to differentiate SPP bands from non-specific IgG bands. In order to detect vigilin interacting with SPP after vigilin immunoprecipitation, a HEK293 cell line that stably expressed FLAG-tagged vigilin was generated. Expression of FLAG-tagged vigilin in this cell line allowed the use of mouse anti-FLAG antibody, instead of the rabbit anti-vigilin antibody. Similar to the endogenous SPP co-immunoprecipitation experiments, the capture with anti-SPP antibody was able to specifically co-immunoprecipitate vigilin-FLAG and SPP while no bands were observed from control co-immunoprecipitations with pre-immune rabbit serum (Figure 5.4B). Co-immunoprecipitation experiments using anti-FLAG antibody (Figure 5.4C) captured vigilin-FLAG as well as its interacting partner SPP. Interestingly, the FLAG IP captured predominantly dimers and trimers of SPP but not monomers.
Figure 5.4 SPP interacts with vigilin. (a) Co-immunoprecipitation with anti-SPP-CT antibody pulled down endogenous vigilin. The blot was probed with anti-vigilin antibody. HEK293 cell lysate was used to align the band. (b) In a HEK293 cell line stably expressing FLAG-tagged vigilin, co-immunoprecipitation with SPP-CT antibody pulls-down vigilin-FLAG. The blot was probed with anti-FLAG antibody. (c) Co-immunoprecipitation with anti-FLAG antibody pulled-down SDS-stable SPP dimers and trimers in the FLAG-tagged vigilin expressing HEK293 cell line. The blot was probed with anti-SPP-CT antibody. Data shown are representative blots from three independent experiments.
5.5 Discussion

Signal peptide peptidase (SPP), which exists as a component of several different high molecular weight membrane-bound protein complexes, has important proteolytic and non-proteolytic functions (Crawshaw et al., 2004; Loureiro et al., 2006; Schrul et al., 2010; Weihofen et al., 2002). The latter functions, which are not well-characterized, may relate to protein quality control. The biochemical and cellular data presented here suggest that the SPP-vigilin interaction is genuine and highly selective, although it is also clear that only a minor proportion of both proteins are involved in this interaction. Despite this seemingly minor interaction, vigilin molecules interact with SPP with high affinity on the basis that despite a high concentration of DDM in the solubilization step, the interaction between SPP and vigilin was still preserved. This interaction is also highly selective for SPP in high molecular weight complexes, both in immunoprecipitation and BN-PAGE experiments (not shown).

A possible function of vigilin is to modulate the activity of SPP. Indeed, one obvious hypothesis is that vigilin might affect the proteolytic activity of SPP in a manner analogous to the selective modulating effect of TMP21 (Chen et al., 2006) and gSAP (He et al., 2010) on presenilin complex–mediated γ-secretase activity. However, we found that vigilin does not alter the protease activity of SPP, suggesting that the SPP-vigilin interaction might be involved in the non-proteolytic functions of SPP (not shown). It is also possible that SPP may modulate the biological activity of vigilin, although this could not be tested as no vigilin-specific biochemical assay exists.

An alternative effect of the SPP-vigilin interaction is that vigilin could modulate the putative functions of SPP in protein quality control in the ER. Testing this hypothesis will require much further work, and is beyond the focus of the current study. However, vigilin has previously been shown to bind RNA, presumably via its KH domains (Kruse et al., 1996; Kugler et al., 1996; Musco et al., 1996), and can be localized with free and membrane-bound ribosomes (Batlle et al., 2011; Kruse et al., 2003; Vollbrandt et al., 2004). The binding of mRNA by vigilin may stabilize the mRNA molecules and could be required for the localization of mRNA to actively translating ribosomes (Cunningham et al., 2000; Dodson and Shapiro, 1997; Goolsby and Shapiro, 2003; Kruse et al., 2003). This observation, together with SPP’s putative function in binding misfolded protein, suggests that the vigilin-
SPP complex could potentially be involved in the translation and quality control of membrane proteins. For instance, the vigilin-SPP interaction could ensure that SPP is near to the site of newly synthesized polypeptides destined for insertion into the ER membrane. If so, this might allow the vigilin-SPP complex to be involved in the regulation of translation and the binding/removal of newly-made misfolded membrane proteins. The latter might be achieved by a combination of: 1) dislocation/translocation back across the ER membrane (as shown for SPP’s role in MHC Class I molecules (Loureiro et al., 2006)); and 2) proteolytic cleavage of fragments in the membrane itself.

In conclusion, using an unbiased iTRAQ mass spectrometry-based approach, we have identified the multi-KH domain protein vigilin as a potential interacting protein of SPP. We have confirmed the interaction between SPP and vigilin using a series of experiments. Vigilin does not appear to modulate the protease activity of SPP but instead vigilin-SPP complex could have a role in membrane protein quality control in the ER.
Chapter 6:
Conclusions and Future Directions
6.1 Preamble: Hypothesis Revisited

The γ-secretase complex plays a pivotal role in the production of neurotoxic Aβ peptide in Alzheimer’s disease progression. Presenilin (PS), the catalytic subunits of γ-secretases exists in humans as two paralogs, PS1 and PS2. Despite their structural similarities, they participate in the formation of distinct γ-secretase complexes. In addition, as evidenced in mouse knockout studies, the absence of PS1 or PS2 leads to two different biological phenotypes – PS1 knockout results in neonatal death, whereas PS2 knockout results in viable, fertile mice with minor respiratory complications. Therefore, in order to understand the differences in PS1 and PS2 and their roles in γ-secretase function, further understanding of the distinct γ-secretase complexes is necessary. The formulation of the hypothesis presented in Chapter 2 is, thus, primarily a response to the lack of understanding of underlying factors that contribute to the substrate specificity and cellular organization of distinct γ-secretase complexes both in animal and cell models and to examine the revealed candidate interactor’s role in modulating Aβ generation. The research presented throughout this thesis identifies the presence of a distinct subset of candidate interactors for PS1- or PS2-containing γ-secretase complexes that may play important proteolytic and non-proteolytic roles in normal biology and in AD. We have followed a rational, step-wise approach: 1) the purification of wild-type and mutant PS1-containing γ-secretase complexes in mice; 2) quantitative peptide labelling using iTRAQ technology; 3) liquid chromatographic techniques and ESI-MS/MS to identify candidate interactors; 4) the generation of TAP-tagged PS variants and lentiviral transduction to generate eukaryotic cells stably expressing PS1 or PS2; 5) multi-step purification of PS1- and PS2-containing complexes; 6) quantitative peptide labelling using iTRAQ technology; 6) liquid chromatographic techniques and ESI-MS/MS to identify candidate interactors; 7) comparison of animal and cell γ-secretase interactomes; and 8) biochemical validation of interactions (Chapter 3). One of the candidate interactors identified was signal peptide peptidase (SPP), which interacted preferentially with PS2-containing γ-secretase complexes. The work presented in Chapter 4 represents a more in-depth biochemical validation of SPP in a number of relevant cell lines. Here, we explore and suggest possible proteolytic and non-proteolytic roles of the SPP interaction with γ-secretase complexes. Furthermore, SPP interactome was investigated in Chapter 5, and vigilin was identified as a novel interactor, which suggested another non-proteolytic function of SPP. Overall, we uncovered insights into the biology of γ-secretase complexes and their interactor proteins and
confirmed the key hypothesis of this work, namely that specific cellular functions are the result of distinct $\gamma$-secretase complexes interact with distinct subsets of interactors (Figure 6.1).

**Figure 6.1 Diagram of the interrelationship between $\gamma$-secretase proteins (PS1, PS2, Nicastrin, Aph-1, Pen-2) and SPP.** The above diagram summarizes the organization of this thesis. PS1-containing and PS2-containing $\gamma$-secretase complexes displayed distinct protein-protein interactions (Chapter 3). We have identified SPP as a PS2-predominant interactor, which appears to modulate its proteolytic function, but also engages in interactions with other proteins such as vigilin (Chapter 4 & 5). We assessed whether SPP contributes to the maturation of $\gamma$-secretase complex as a part of presenilinase machinery (Chapter 4). While the functional significance of the SPP-PS interaction is only beginning to emerge through this work, it is likely that these two aspartyl proteases work in concert in the quality control and molecular homeostasis of cellular membranes.
6.2 Conclusion and Future Direction

Two distinct subsets of interactors for PS1- and PS2-containing γ-secretase complexes

Prior to the commencement of the work presented in Chapter 3, the presence of distinct subsets of interactor proteins for two human PS paralogs that form distinct γ-secretase complexes was speculated, but undefined. Our approach to use an optimized and mild TAP multistep procedure and quantitative iTRAQ labelling enabled a comparative analysis of two human PS paralogs and was intended to minimize the inadvertent disruption of protein-protein interactions. Using this approach, we were able to confirm several previously known γ-secretase interactors (e.g., TMP21, PLP2) and identified novel candidate interactors (e.g., SCAMPs 1 and 3, and synaptic glycoprotein-2) which bind equally well to γ-secretase complexes comprising PS1 or PS2. Second, we documented that γ-secretase core complexes engage in interactions with other protein networks, including the predominant association of the catenin-cadherin molecular cell adhesion network with PS1-containing γ-secretase complexes, thereby extending observations which preceded this work (Kouchi et al., 2009). Third, we uncovered a novel predominant-association between PS2-containing γ-secretase complexes and SPP, a Type II TM domain cleaving aspartyl protease, which shares identical active site motifs YD, PAL and GxGD with presenilins (Wang et al., 2004; Xia and Wolfe, 2003).

Comparative interactome analyses of animal vs. cell models of studying Alzheimer’s disease

The interactome analyses presented in Chapter 3 was the first extensive, large-scale quantitative mass spectrometry, which identified all known γ-secretase core proteins, including Pen-2 and Aph-1b. Especially, we were able to identify four out of eight available Pen-2 tryptic peptides, which is to our knowledge the most number of Pen-2 peptide ever shown in γ-secretase mass spectrometry analyses (Figure 6.2 and Supplementary table 3.3).

A comparison of the two wild-type PS1 whole brain interactome data tables collected following low and high stringency pre-elution washing of affinity capture matrices versus the HEK293 PS1 interactome further allows some interesting conclusion to be drawn. Not surprisingly, both, increasing the stringency of washing steps during the final affinity capture steps or the move to a defined cell system, led to a reduction in the number of candidate interactors of mature γ-secretase complexes containing wild-type PS1. However, whereas the increase in stringency
Figure 6.2 Tryptic digestion sites of Pen-2. The arrows display a possible Pen-2 tryptic digestion sites. In Chapter 3, we have identified four tryptic peptides (GYVWR, VSNEEK, MNLER, WGALGDYLSF) that are shown in bold.

primarily was accompanied by a mere reduction in the number of candidate interactors which were revealed and, as such, was largely quantitative in nature, the move to the HEK293 cell system caused a qualitative shift in the candidate proteins identified. While a thorough understanding of the reasons behind interactome differences in whole brain versus HEK293 cells will require follow-up experiments beyond the scope of this study, it is probable that differences in the level of differentiation and cellular specialization are responsible for some of these differences. For example, the absence of members of the synaptic fusion apparatus in the wild-type PS1 interactome dataset derived from HEK293 cells may not be surprising, given that these cells are not known to develop synaptic structures. The reproducible enrichment of multiple proteins (e.g., SCAMPs, VAMP3) known to contribute to vesicle fusion processes at the plasma membrane in the PS1 interactome of these cells, however, suggests that even in HEK293 cells the overall propensity of mature PS1-containing γ-secretase complexes to interact with or reside in close spatial proximity with proteins belonging to the vesicle fusion network is maintained. The notion that mature γ-secretase complexes may in brain and HEK293 cells engage in interactions with related protein complexes is even more evident when considering that PS1 was shown to interact in this work with proteolipid protein 1 in brain but proteolipid protein 2 in HEK293 cells, and whereas Cadherin 2 is only modestly enriched in the PS1 interactome from brain (in contrast to a robust enrichment of Cadherin 11), a very strong co-enrichment of this candidate interactor with PS1 was observed with HEK293 cells as the biological starting material.
Novel PS2-SPP interaction

The PS2-SPP interaction is of particular interest since the physiological role of PS2-containing γ-secretase complexes is not well understood. Previous reports established that APP intramembrane cleavages are foremost carried out by PS1-containing γ-secretase complexes (Ghidoni et al., 2007; Lai et al., 2003; Martoglio and Golde, 2003; Zhang et al., 2000). And a PS2 knockout in mouse brain did not alter the cleavage of two of the most important γ-secretase substrates, APP and Notch, that are altered in PS1 knockout mice (Franberg et al., 2010). The identification of SPP as a PS2-specific interacting partner opened multiple avenues for further research and constitutes a first step toward assigning functional significance to PS2-containing γ-secretase complexes. For the first step towards biochemical validation of this interaction, it was necessary to demonstrate conclusively that the SPP-PS2 interaction is authentic. In Chapter 3, we used TAP-tagged PS to purify the active γ-secretase complex and its interacting partners, including SPP. One possible way of confirming the interaction was to use an SPP antibody in a reciprocal co-immunoprecipitation assay with PS2 as the bait protein. Our result validated the co-immunoprecipitation of SPP and PS2, and indicated that its existence does not depend on heterologous expression of PS2. Additionally, the observation that SPP co-fractionated with PS2 in lipid raft enriched fractions was consistent with a physiological interaction between the two proteins. It will be of interest to map the interface between PS2 and SPP to shed light on the cause for the preferential binding of SPP to PS2, as opposed to PS1. It is reasonable to predict that the differences in substrate binding specificity between PS1 and PS2 are affected by other proteins which may either engage in contacts with PS near their active sites or alter substrate recognition allosterically. Therefore, it will be interesting to evaluate which of the two principal modes of PS2 binding SPP adapts and whether the formation of the PS2-SPP interface limits access of PS2 substrates to the catalytic centre.

Possible SPP contribution to presenilinase activity

SPP and presenilins are part of the same iCLIPs family of proteases that share identical active site motifs YD, PAL and GxGD with inverted topology resulting in Type II and Type I TM cleavages, respectively (Krawitz et al., 2005; Martoglio and Golde, 2003; Wang et al., 2004; Weihofen et al., 2002; Xia and Wolfe, 2003). The close interaction between these two proteases raised the exciting possibility of SPP contributing to PS endoproteolysis during γ-secretase complex formation, known as presenilinase activity. Full-length PS must undergo
endoproteolytic cleavage into active NTF and CTF (Dillen and Annaert, 2006; Podlisny et al., 1997; Xia, 2008; Xia and Wolfe, 2003). Since both fragments contain catalytic aspartic acid residues that are critical for endoproteolysis, it has been previously thought that PS cleavage could occur by autoproteolysis (Kimberly et al., 2000; Wolfe et al., 1999; Xia, 2008). However, some aspects of the data available to date are not easily explained by this model, a circumstance sometimes referred to as the ‘presenilinase paradox’. A number of biochemical properties of SPP made it an attractive alternative presenilinase candidate which could reconcile some of the key contradictions which constitute the presenilinase paradox.

In Chapter 4, we examined this possibility of SPP’s contribution to presenilinase activity from various perspectives. First, we examined the effect of PS2 expression change on SPP and found out that there was no significant change in SPP expression. Second, we examined the effect of SPP knockdown on PS2 expression and again observed that there were no significant changes. However, a decrease in SPP expression resulted in a decrease in other γ-secretase core proteins, namely Aph-1 and Pen-2. Additionally, it also caused a reduction in secreted Aβ peptides. Consequently, SPP may play a physiological role in the γ-secretase-dependent cleavage or release of cellular Aβ. Third, the effect of γ-secretase and SPP inhibitors was evaluated in human and mouse cell lines and in vitro. We used L685,458, DAPT and (Z-LL)₂-ketone inhibitors in these studies. L685,458 is a transition-state analogue mimic of the catalytic site of aspartyl proteases, which had been shown to inhibit both γ-secretase and presenilinase activities (Esler et al., 2000; Shearman et al., 2000; Wrigley et al., 2004). DAPT is a peptidic inhibitor, which binds to PS CTF at a site distinct from the catalytic site or the presumed substrate binding site and inhibits γ-secretase but not presenilinase activity. Therefore, it was of interest to analyze the effect of (Z-LL)₂-ketone, a SPP-specific inhibitor, and compare its inhibitory profile toward presenilinase with the corresponding profile of the other γ-secretase inhibitors. If SPP was contributing to presenilinase activity, addition of (Z-LL)₂-ketone would interfere with PS endoproteolysis in a similar manner as L685,458. However, it was observed that (Z-LL)₂-ketone had no effect on PS endoproteolysis. Regrettably, the data remain somewhat inconclusive and on their own are insufficient to dismiss the hypothesis, in part because we lack an assay to determine whether the membrane-permeability of the inhibitor was sufficient to reach the inhibitory concentration for effectively blocking endogenous SPP. Fourth, proteolytically inactive point mutants of both PS2 and SPP were created and analyzed. Previous reports had
established that when either one of the two conserved aspartyl residues within PS or SPP are mutated, proteolytic activities of these intramembrane proteases are abolished (Fluhrer et al., 2008; Fluhrer and Haass, 2007; Xia, 2008). If SPP was contributing to presenilinase activity, impairment of its catalytic centre by mutagenesis would be expected to interfere with PS endoproteolysis. Results from our experiments indicated that the generation of a functional SPP knockout by the introduction of a aspartate-to-alanine mutation in the catalytic centre did not affect presenilinase cleavage. One issue to note is that SPP-like (SPPL) protein variants exist, which may have functional redundancy with SPP (Krawitz et al., 2005). To our knowledge, it is unknown to which extent SPPL can replaces SPP function when expression levels of SPP are decreased or eliminated by knockdown or knock-out technologies. Consequently, although our results using inhibitors and point mutants did not indicate a significant SPP contribution to presenilinase activity, these data cannot categorically reject the notion that SPP or SPPL may contribute to endoproteolysis of PS proteins. Evidence for or against SPP involvement in presenilinase activity may derive from future studies which target multiple SPP and SPPL variants together. This approach may not only provide additional insights into the specificity of the interaction between SPP and PS but may also help to determine the role SPP plays at a mechanistic level for an intricate Aβ release biology. It is unlikely that it will be technically feasible to eliminate all SPP and SPPL molecules concomitantly from a cell. Therefore, a combination of knockdown/knockout genetic approaches and pharmacological methods for the selective inhibition of individual members or a subset of these proteins might be needed to elucidate their true involvement in the maturation or endoproteolysis of PS paralogs. Some insights into the level of redundancy with which SPP and SPPL molecules can process overlapping substrates might initially be derived from investigations which probe the ability of individual members of this family to cleave a well-known Type II TM substrate protein such as prolactin. These studies would be easier to conduct as this assay can be easily set up in vitro and may provide a first step toward cell-free characterizations of the SPP/SPPL cleaving activities. It is envisioned that the ultimate validation of a possible involvement of SPP/SPPL molecules in the endoproteolytic processing of PS would similarly require a cell-free assay system that is based on highly purified and defined components only.

SPP interactome suggests non-proteolytic role

In Chapter 5, the SPP interactome was investigated and vigilin was identified as a novel
candidate interactor. Through a series of validation experiments, the authenticity of the SPP-vigilin interaction was confirmed and biochemical data suggested that this interaction may play a role for the non-proteolytic functions of SPP. In addition, this interaction was confirmed to be specific and not to extend to other SPPL protein or PS1 (not shown). One possible significance of the SPP-vigilin interaction may relate to protein translation processes. Vigilin has been shown to not only function in cholesterol mobilization (Fidge, 1999) but also to contribute to mRNA stabilization during protein translation (Cunningham et al., 2000; Dodson and Shapiro, 1997; Goolsby and Shapiro, 2003; Kruse et al., 2003), whereas SPP has been shown to bind to misfolded proteins. On the basis of this prior knowledge, one may hypothesize that the vigilin-SPP interaction may recruit PS2 to newly-synthesized polypeptides to aid in the binding/removal of misfolded proteins. Further work is required to explore this hypothesis.

Concluding Remark

In conclusion, we have taken a fresh look at a protein-protein interaction network that plays a central role in Alzheimer’s disease. The study provided evidence of PS1- and PS2-containing γ-secretase core proteins existing within distinct high-molecular weight complexes whose components may contribute to an emerging network of complex regulatory processes or facilitate the task to direct presenilins toward specific substrates.

A cell needs to straddle two seemingly conflicting needs: it needs to be able to (1) closely control intramembrane cleavages of a subset of transmembrane stubs for which carboxy-terminal fragments have acquired important signaling functions, and (2) counteract broadly the accumulation of a diverse range of membrane stubs before their cumulative burden threatens to choke its membranes. It is plausible that a divergent evolution of mammalian PS paralogs may have served the purpose to address this logistical challenge. Consistent with this view, data from this work suggest that PS1 and PS2 paralogs may divide up the task of handling a broad range of membrane stubs at least in part by associating with different molecular environments. Novel interactions between SPP – PS and SPP – vigilin have generated multiple interesting and testable hypotheses. Further studies will be needed to fully understand the intricate regulation that must underlie a biology that has to strike the right balance between the need for bulk membrane stub removal and specific and time-sensitive substrate recognition.
Appendix 1:

Time-Controlled Transcardiac Perfusion Crosslinking for in vivo Interactome Studies

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Summary

The time-controlled transcardiac perfusion crosslinking (tcTPC) method differs from conventional perfusion fixation in that the crosslinking reagent is administered throughout the circulatory system for only a relatively short period of time, thereby allowing limited crosslinking to occur. Bait protein complexes are isolated by affinity capture (AC) under stringent conditions and are recovered from the AC matrix by acidic elution. Affinity purified proteins are reduced, alkylated and digested with a specific endoproteinase, such as trypsin. Subsequently, peptides are isotopically labeled, separated by reversed-phase chromatography and analyzed by quantitative tandem mass spectrometry (MS/MS). The proteins crosslinked to the bait protein during tcTPC are identified by database searches with conventional protein identification software. The tcTPC strategy offers unique advantages over alternative approaches for studying a subset of protein complexes which require a particular environment for their structural integrity, such as membrane protein complexes that are notorious for their tendency to dissociate upon detergent solubilization. The sensitivity and utility of this method is influenced by the spatial distribution of chemical groups within the bait protein complexes that can engage in productive crosslinks.
1. Introduction

Individual proteins do not act in isolation but engage in complex and dynamic interactions with other proteins to fulfill their diverse cellular roles (Aloy and Russel, 2002; Blackstock and Rowley, 1999; Gavin et al., 2006; Neubauer and Wilm, 1999; Sobott and Robinson, 2002). Numerous methods have been devised to provide insights into the specific interactions a protein engages in, commonly referred to as the interactome of a given protein (Berggard et al., 2007; Charbonnier et al., 2008; Drewes and Bouwmeester, 2003). Increasingly, interactions of a protein of interest, hereafter referred to as the bait protein, are probed biochemically based on the identification of proteins that co-purify with the bait protein during its isolation. In particular, the combination of protein tagging and affinity chromatography of protein complexes has proven to be powerful (Burgess and Thompson, 2002; Gingras et al., 2005). A subset of proteins, however, seem refractory to this kind of analysis. Notorious amongst these are membrane proteins, partly because no generic solubilization strategy can be devised for them and it cannot be predicted how well a given membrane protein complex will tolerate the exposure to a specific detergent (Hooker et al., 2007; Reisinger and Eichacker, 2008). Alternative approaches are also needed if the objective is to characterize the molecular environment of a protein of interest, including those proteins which reside in spatial proximity but do not engage in bona fide interactions.

The time-controlled transcardiac perfusion crosslinking (tcTPC) method stabilizes existing next neighbor relationships amongst proteins by formaldehyde crosslinking prior to the disruption of tissue integrity (Figure 1) (Schmitt-Ulms et al., 2004). Mild formaldehyde crosslinking has been employed extensively for the study of nucleosomal protein interactions (Fragoso and Hager, 1997; Jackson, 1999; Orlando et al., 1997; Wells and Farnham, 2002). Some features that make formaldehyde crosslinking attractive are: (i) the water solubility of the reagent; (ii) the absence of reagent-induced rearrangements of the proteins; and (iii) the crosslink bonds are short (2–3 Å), endure harsh treatments and are reversible (Jackson, 1999). The tcTPC procedure is followed by a rapid tissue dissection and an optimized sample work-up scheme that includes some modifications to conventional protocols for the co-affinity capture of proteins. The covalent stabilization of protein interactions translates into the ability to treat affinity captured protein complexes with stringent salt and detergent washing steps. Compared with
Appendix I Figure 1. Schematic outline of tcTPC method. (a) The formaldehyde crosslinking solution is pumped through the circulatory system of the mouse in a time-controlled manner. (b) Protein complexes are purified by stringent affinity capture, (c) then reduced, alkylated, digested and conjugated to iTRAQ reagents. (d) Samples are combined and subjected to reversed phase liquid chromatography and (e) quantitative tandem mass spectrometry, which is followed by (f) computationally-aided protein identification.
alternative protocols that do not make use of crosslinking or employ \textit{in vitro} crosslinking, the move to \textit{in vivo} crosslinking further reduces the risk of being misled in situations where non-physiological interactors bind directly to the bait protein or to the affinity-purified protein complex only when present in an extract but do not physiologically interact with the bait protein when cellular integrity is maintained.

Nevertheless, unspecific interactors can be present in interactome samples as a result of (i) aggregated proteins in the sample that co-sediment with the affinity matrix, (ii) proteins that bind directly to the affinity matrix, (iii) proteins which under physiological conditions are found in a different cellular compartment than the bait protein, but have an intrinsic propensity to bind to the bait protein when present in an extract, (iv) abundant cellular proteins that populate affinity purification eluate fractions when samples are subject to less than the most stringent washing conditions, (v) proteins that originate from the AC matrices themselves e.g. if crude antibody preparations were coupled to chemically activated matrix beads, and finally (vi) proteins such as trypsin, human skin and hair proteins introduced into the sample during handling procedures. With this many possible sources of unspecific proteins, rather than aiming to eliminate all contaminants, a feasible objective is to minimize their occurrence and more importantly, to know their identities. To this end, we strategically embedded an isotopic labeling step at the peptide level, which affords the quantitative and comparative analysis of the sample of interest and a negative control sample. The following method represents the most recent version of a protocol we have gradually improved over the years.
2. Materials

The tcTPC method was developed in North America and, thus, reagents and equipment items listed in the following paragraphs were primarily sourced in this region. Due to the generic nature of most materials required, we expect that equivalent substitutes for all items can be purchased from alternative suppliers and manufacturers.

2.1. Biological Source Material
1. The tcTPC method was developed utilizing mice. However, the method can also be applied to rats, hamsters and other small rodents and should be adaptable to any species that is equipped with a circulatory system for the rapid delivery of formaldehyde to the tissue(s) of interest.
2. Negative controls: Excellent bait-specific and negative control samples which differ in the presence or absence of a specific bait protein can be obtained from the crossbreeding of animals which are heterozygote for the bait protein of interest. Alternative approaches to generating negative controls are available should no knockout animals be available (see Section 3.1).

2.2. Time-Controlled Transcardiac Perfusion Crosslinking
1. Deep Anaesthesia Cocktail: 10:1 mix (w/w) of Ketamine (Bioniche Life Sciences, Belleville, ON) and Xylazine (Bayer, Leverkusen, Germany).
2. Purging Buffer: Phosphate-buffered saline (PBS) supplemented 1 Unit/mL of Heparin (commercially available as a 1000 Units/mL stock solution e.g. from American Pharmaceutical Parners, Schaumburg, IL).
3. tcTPC Solution: Freshly made solution of 2–4% formaldehyde (Bioshop Canada, Burlington, ON) in Purging Buffer (see Note 1).
2.3. Generation of Soluble Extract
1. Rod homogenizer (PowerGen 120; Thermo Fisher Scientific, Waltham, MA).
2. Homogenization Buffer: 150 mM NaCl, 50 mM NH₄Cl, 100 mM Tris/HCl (pH 8.0), 1× Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) (see Note 2).
3. Extraction Buffer: 150 mM NaCl, 1% deoxycholate, 1% NP-40, 20 mM Tris/HCl, pH 8.0 (see Note 3).

2.4. Generation of Affinity Matrix
1. AC matrix: Affi-Gel 10 agarose (Bio-Rad, Hercules, CA), Protein A or G agarose (Sigma-Aldrich, St. Louis, MO) or alternative magnetic bead matrix.
2. Affinity-purified bait-specific antibody.
4. High Salt Wash Buffer: 1 M NaCl, 20 mM Tris/HCl, pH 8.0.
5. Pre-Elution Buffer: 10 mM HEPES, pH 8.0 (see Note 4).
6. Acidic Elution Buffer: 15% acetonitrile (ACN), 0.2% trifluoroacetic acid (TFA), pH 1.9.
7. Buffer A: 1% NP-40, 150 mM NaCl, 25 mM HEPES, pH 7.5.

2.5. Affinity-Purification of Bait Protein Complexes
1. High Salt Buffer A: 1% NP-40, 500 mM NaCl, 25 mM HEPES, pH 7.5.
3. 0.65 mL Safe Seal Microcentrifuge Tubes (PGC Scientific, Frederick, MD).
4. Storage Buffer: 0.05% NaN₃ in PBS.

All solutions listed in this section are made fresh immediately prior to use.
1. Denaturation Buffer: 9 M urea in water (see Note 5).
2. pH Adjustment Buffer: 1 M HEPES, pH 8.0.
3. Reducing Agent: 75 mM tris (2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) stock solution in water.
4. Alkylation Agent: 150 mM 4-vinylpyridine (Sigma-Aldrich) stock solution in water.
5. Trypsin Solution: Dissolve side chain–modified, L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)–treated porcine trypsin in water to a concentration of 0.4 μg/μL (Promega, Fitchburg, WI).

6. iTRAQ Reagent: Dissolve individual isobaric tags for relative and absolute quantitation (iTRAQ) labeling reagents (Applied Biosystems, Foster City, CA) in 70 μL ethanol, vortex for 1 min and subject to brief spin to collect liquid at bottom of tube.

2.7. Tandem Mass Spectrometry

1. ZipTipC18 tips (Millipore, Billerica, MA).
3. Methods

3.1. Design of Negative Control

Whenever bait knockout animals are available we recommend the use of an approach we refer to as the bait exclusion strategy (Figure 2A). In this strategy, side-by-side affinity purifications are carried out from starting materials which differ in the presence or absence of the bait protein. Because all steps during the AC procedure employ the same reagents, the bait exclusion method makes it easy to attribute differences in the interactome lists of sample and control to the expression versus ‘knock-out’ of the protein of interest. While the conceptual simplicity of this approach is compelling, the experimenter needs to be cognizant that a protein may nonetheless end up in the bait protein-specific eluate as a result of its ability to bind nonspecifically to the bait or its physiological interactors. In instances where no knockout animals are available, a derivative strategy can be used, which employs the side-by-side AC from identical wild-type extracts but employs for the negative control an AC matrix which has been pre-saturated with a small ligand that competes with the bait protein-containing complexes for binding. In most instances, this small ligand will represent the peptide-antigen, which was originally used to raise the bait-specific antibody. The success of this approach depends on the relative affinity of bait protein and competitive ligand pair for binding to the affinity matrix. Analogous design concepts can also be employed if the AC matrix is not based on a bait-specific antibody but capitalizes on the existence of a small-molecule ligand (inhibitor, cofactor, etc.) known to strongly and selectively bind to the bait protein. We advise against the generation of negative control data based on the conjugation of unrelated antibodies, for example generic immunoglobulins (IgGs) not known to recognize the bait protein, due to the poor discriminative power this strategy offers for the detection of crossreactivities of the bait-specific capture antibody. Regardless of the approach used, we recommend incorporation of an isotopic labeling step at this time (Ong and Mann, 2005) but are cognizant that further improvements to label-free quantitative mass spectrometry (MS) methods may soon eliminate the need for this extra labeling step (Zhu et al.). Quantitative data are indispensable for interactome studies aimed towards abundant cellular proteins where, without quantitation, it may be impossible to distinguish bona fide interactors from unspecific contaminants. Whereas for cell culture-based work metabolic isotopic labeling strategies are attractive (Zhang and Neubert, 2009), methods which conjugate
isotopic tags ‘post harvest’ to peptides are more cost-effective and straight-forward for animal based methods such as tcTPC (Figure 2B). Several alternative reagents are available for this purpose and should be equally applicable (Chen et al., 2007). Most of these approaches reduce the MS analysis time (by enabling pooling of samples and controls) and reduce the risk to misinterpret performance fluctuations and run-to-run variances in the analyses of complex samples to reflect sample-to-sample differences (Rudnick et al., 2010).
Appendix I Figure 2. Cartoon depicting Bait Exclusion strategy for the identification of unspecific interactors. (a) The Bait Exclusion strategy employs side-by-side affinity capture from starting materials which differ in the presence or absence of the bait protein. As a result, bait-specific interactors represent a subset of proteins exclusively found in eluate fractions derived from the bait-containing biological source material. (b) Expected iTRAQ reporter ion signature profiles assuming peptides in the control and bait-specific eluates were labeled with iTRAQ114 and iTRAQ115 reagents, respectively.
3.2. Time-Controlled Transcardiac Perfusion Crosslinking

1. Deep anesthesia of rodents: Determine body weight of rodents and inject Deep Anesthesia Cocktail intraperitoneally to achieve a concentration of 100 mg Ketamine/10 mg Xylazine per kg of body weight.

2. Mounting of rodent and surgical procedures: Place deeply anaesthetized animal (unresponsive to paw pinch test) on gridiron (e.g. lid of animal cage or similar grid) mounted on top of a plastic or aluminum reservoir. Stretch out limbs with hemostats and make incisions from caudal of sternum up along the sides of rib cage without injuring underlying organs using artery scissors. Secure open chest and skin with hemostats. Attach 20-gauge perfusion needle (30 mm length) with barrel tip and Luer lock hub to end of peristaltic pump tubing. Feed tubing through peristaltic pump and immerse opposite end in Purging Solution.

3. tcTPC: Grab tip of the heart with curved serrated forceps or hemostat and make 2-3 mm incision into the left ventricle (recognizable by its lighter colored appearance). Insert perfusion needle into the left ventricle (Figure 3). Clamp the needle in place with a hemostat and secure stable position of tools. Make another incision into the right atrium to enable the purging of blood and excess crosslinking reagent from the circulatory system during the perfusion step. Perfuse animal with PBS at 10 mL/min for 2 min to purge the circulatory system of blood. The success of this step can be observed by the decoloring of the liver and the two vessels that flank the midline of the animal’s ribcage (Figure 3). Switch perfusion solution to tcTPC crosslink solution and continue pumping at 10 mL/min for an additional 6 min. Conjugation chemistry of formaldehyde crosslinking is shown in Figure 4 (see Notes 6 and 7).

4. Rapid dissection of brain: Remove head with sharp scalpel or large scissors. Open skin by sagittal cut from neck to nose. Remove residual muscular tissue from neck of animal. Use angled dissector scissors to cut through bone along sagittal midline of skull. Force skull open by spreading movement with back of dissector scissors. Remove brain with round-ended spatula (see Note 8). Immerse dissected tissue in tcTPC Solution (for example in Falcon tube) for up to 9 min (this includes the time for dissection).
Appendix I Figure 3. Still-image of mouse captured during a critical step of tcTPC procedure. The image depicts a mouse with its chest cavity opened and mounted on grid-covered container, with perfusion canula inserted into the left ventricle of heart.

Appendix I Figure 4. Conjugation chemistry of formaldehyde crosslinking involving amino groups present within proteins. The reaction proceeds in two steps: Initially, formaldehyde mounts a nucleophilic attack toward the amino group provided by a peptide to form a Schiff-base conjugate. Subsequently, the reaction of this intermediate with a nearby primary amine is paralleled by the concomitant elimination of water and the formation of a covalent bond.
3.3. Generation of Soluble Extract

All steps in this section are to be carried out at 4°C.

1. Homogenization of tissue: Pool appropriate amount of tcTPC-treated rodent tissue (see Note 9) and homogenize with rod homogenizer (e.g. 5 x 30 s strokes) at maximum power setting using a ratio of 10:1 (w/w) of Homogenization Buffer over tissue. Prevent extracts from warming by keeping samples on ice between and following the homogenization steps.

2. Solubilization of bait protein complexes: Add Extraction Buffer (same volume as Homogenization Buffer used in previous step) to extract membrane proteins and incubate samples for 30 min with gentle agitation.

3. Removal of insoluble material: Initially, remove cellular debris from the samples by low-speed centrifugation (1000 g, 5 min). Transfer supernatants to ultracentrifugation tubes and remove all insoluble protein material by high-speed centrifugation (100,000 g, 1 h).

3.4. Generation of Affinity Matrix

The method will work with a wide range of AC matrices, including chemically activated or Protein A/G-derivative matrices. Similarly, both conventional crosslinked agarose and more recent products based on improved bead-based technologies can be employed (Yingyongnarongkul et al., 2003). The bait-specific capture antibody has to be covalently crosslinked to the matrix to avoid its inadvertent release during the protein elution step. The antibody conjugation step should follow vendor instructions for the specific matrix employed. Most commercial matrices allow the covalent conjugation of 1 to 5 mg of purified antibody per mL of matrix. A typical interactome investigation requires the matrix equivalent of 100 μg conjugated antibody per sample. The following instructions should be followed regardless of choice of AC matrix used. For steps which require the collection of AC matrix, minimize physical compression by employing gravity sedimentation, by applying minimal centrifugal force (determined by pilot sedimentation tests) or by employing magnetic bead materials.

1. Determine amount of antibody conjugated by comparing protein concentration in purified antibody solution before and after antibody capture step, for example, with the use of BCA assay by following manufacturer instructions.
2. Prior to its use, wash the bait-specific AC matrix with at least 10 volumes each of 1 x PBS, High Salt Wash Buffer, Extraction Buffer, Pre-Elution Buffer, Acidic-Elution Buffer (see Notes 10 and 11).

3. Finish preparative steps for AC matrix by three consecutive washes with Buffer A.

### 3.5. Affinity-Purification of Bait Protein Complexes

Throughout the next sample handling procedures collect and repeatedly set aside aliquots from all samples to aid with troubleshooting if necessary. Prior to their use, rinse all tubes and reservoirs which will come in contact with samples with water and ACN to remove unwanted polymer residue and minimize dust load. We describe an implementation of this method based on Handee Spin Cup Columns (HSCCs). Similar commercial products from other manufacturers can be used instead.

1. Add tissue extract obtained from high-speed centrifugation step to AC matrix and incubate for 2 to 24 h with gentle agitation on turning wheel (see Note 12).

2. Following the capture of bait-containing protein complexes, collect AC matrix and save supernatant for future use.

3. Subject AC matrix to consecutive washes by briefly swirling, and subsequently sedimenting, the AC matrix in 500-fold excess (v/v) of Buffer A, High Salt Buffer A, Buffer A and Pre-Elution Buffer.

4. Transfer AC matrix to HSCC reservoir (with bottom plug removed) and allow the buffer to pass through the settling AC matrix leaving only enough buffer behind to just submerge the matrix (see Note 13).

5. Acidic detachment of protein complexes from AC matrix: Plug bottom of HSCC and add 300 μL of Acidic Elution Buffer. Close the screw-cap lid and gently invert HSCC to suspend beads in Elution buffer. Incubate for 3 min (Figure 5).

6. To collect the eluate, position HSCC above 0.65 mL Safe Seal Microcentrifuge Tube, remove plug from bottom of HSCC and unscrew lid. When liquid levels in the HSCC have dropped to the front of the AC matrix, pipet an additional 100 μL of Acidic Elution Buffer into the HSCC without disrupting the settled AC matrix. Continue elution until 400 μL of eluate have accumulated in the capture tube (which should coincide with Acidic Elution Buffer reaching the AC matrix front).
Appendix I Figure 5. Cartoon depicting steps for the elution of bait protein complexes from the AC matrix. (a) Add 300 µL of Elution Buffer to HSCC. (b) Tilt column gently to resuspend beads. (c) Use syringe to adjust back pressure for an elution speed of 1 drop/5s and collect eluate in pre-rinsed 0.65 mL microcentrifuge tube. (d) Add 100 µL of Elution Buffer along the tube wall without disturbing the settled AC matrix. (e) Store HSCC for future use in Storage Buffer. Proceed with speed vacuum concentration.

7. Storage of matrix: Unplug bottom of HSCC and wash AC matrix with Storage Buffer. When pH of flow-through has returned to neutral, close HSCC at both ends and store for future use at 4°C.

Many alternative directions can be pursued for the downstream handling of eluate fractions. The following section describes one implementation of a robust method for the denaturation, reduction, alkylation, trypsinization and iTRAQ labeling steps.
1. Reduce volume of eluate fractions in a speed vacuum concentrator to 10 µL. Add 150 µL of water and continue speed vacuum concentration to a volume of 5 µL. Repeat the preceding step once more.
2. Denaturation: Add 10 µL of Denaturation Buffer and incubate for 10 min at room temperature.
3. Test the pH by spotting a small volume (e.g. 0.1 μL) on pH paper. If pH is below 8.0, add 1 μL of pH Adjustment Buffer, gently vortex for a few seconds and collect liquid at bottom of tube by centrifugation. Repeat preceding step until a pH of 8.0 has been reached.

4. Reduction and alkylation: Add 1 μL of Reducing Agent stock solution and incubate for 30 min at 60°C. Add 1 μL of Alkylation Agent stock solution and incubate for 1 h at room temperature in the dark. Dilute samples with water to a combined volume of 50 μL (see Note 14).

5. Trypsinization: Add 5 μL (2 μg) of Trypsin Solution to samples and incubate at 37°C for 6 h (see Note 15).

6. iTRAQ labeling: Add 70 μL of iTRAQ Reagent to digested samples (control: iTRAQ 114, sample 1: iTRAQ 115, sample 2: iTRAQ 116, etc.), vortex for 30 s and collect solution at bottom of tube by brief centrifugation. Incubate the mixtures at room temperature in the dark for 3 h with occasional brief agitation.

3.7. Tandem mass spectrometry

From here on the objective is to obtain an in-depth inventory of peptides present in the combined iTRAQ labeled samples. Many alternative strategies are available and the details of steps will as much depend on personal preferences as on available equipment. Thus, rather than providing a detailed step-by-step protocol we will restrict instructions to generic steps and point out possible obstacles. Before combining iTRAQ labeled samples and embarking on the final steps of sample preparation, it is advisable to subject a small aliquot of each sample to analytical tandem mass spectrometry (MS/MS) analysis (see Note 16).

1. Combine 5% (v/v) of individual iTRAQ labeled samples into a fresh 0.65 mL Microcentrifuge Tube and concentrate its volume by speed vacuum concentration.

2. Carry out ZipTipC18 (or equivalent) clean-up as described by manufacturer and subject eluate to MS/MS analysis (see Note 17).

3. Verify that iTRAQ labeling proceeded with equal efficiency by inspecting iTRAQ signature mass peak region within MS/MS of spectra which can be assigned to peptides common to sample and control (e.g. trypsin auto-proteolysis peptides) (Figure 2B).

4. Once data on the integrity, quantity and complexity of the sample have been obtained, informed decisions can be made regarding the most suitable downstream analysis method.
5. If either the quantity or complexity of the peptide mixture is relatively low, reversed-phase separation followed by tandem analysis of the sample is recommended.

6. For samples of high complexity, it is advisable to precede the reversed-phase separation with an orthogonal fractionation either by strong cation exchange fractionation, high pH reversed-phase or isoelectric focusing.

7. Perform MS/MS analysis on an instrument suitable for the detection of low-mass iTRAQ reporter ions (e.g. a quadrupole/time-of-flight (QqTOF), a time-of-flight/time-of-flight (TOF/TOF), or an orbitrap mass spectrometer).

8. Conduct iTRAQ ratio analyses using one of several alternative software packages designed to facilitate this step (e.g. ProteinPilot (Applied Biosystems), Mascot (Matrix Science), SpectrumMill (Agilent), Warp LC (Bruker), Peaks (Bioinformatics Solutions Inc) ProteinProspector (University of California, San Francisco), and others) (see Note 18).
4. Notes

1. Clarification on the terminology of formaldehyde and paraformaldehyde: In aqueous solution, formaldehyde slowly polymerizes to paraformaldehyde, which is poorly soluble and cannot be used as a fixative. Aqueous solutions of 4-10% formaldehyde are often referred to as formalin. Since methanol slows the spontaneous polymerization of formaldehyde, it is often added by chemical suppliers at a concentration of 10-15% (v/v). Methanol-free formaldehyde can be obtained by hydrolysis of paraformaldehyde in the presence of heat. Methanol-free formaldehyde solutions are also commercially available and are most frequently obtained in gas sealed ampoules. Upon air exposure, these containers should only be used for up to one week.

2. The combination of Homogenization and Extraction buffer presented here works well for diverse bait proteins. However, its composition may need to be adjusted to accommodate biochemical idiosyncrasies of a given bait protein.

3. Deoxycholate will precipitate at a pH < 8.0.

4. All subsequent procedures until the iTRAQ conjugation step avoid the use of reagents containing primary amines as their presence would interfere with the isobaric labeling chemistry. Therefore, we move to HEPES instead of Tris at this step in the procedure.

5. In aqueous solution small amounts of urea gradually decompose to ammonium and cyanate which reacts with various amino acid side chains to form their respective carbamyl derivatives. To minimize these unwanted modifications we advise preparing the 9 M urea stock immediately before its use and deplete cyanate ions by incubation with a mixed bed ion exchange resin (prior to the addition of HEPES buffer).

6. Reliable placing and securing of perfusion canula: Indicators of a successful perfusion are signs of convulsions, absence of blood in tail and limbs and overall hardening of animal. Too deep insertions of the perfusion canula may lead to the rapid filling of lungs with perfusion liquid.

7. Purging, perfusion, dissection and post-fix crosslinking steps take a combined 17 min. To increase the throughput of tcTPC to 10 animals per h, individual perfusions can be initiated every 6 min in a workflow that requires the parallel operation of two peristaltic pumps. A team of three people is required to achieve this rate of perfusion. A convenient arrangement
for subdividing tasks is to have one person carry out the intraperitoneal injections, preparative surgery and mounting of the animals, a second person handle the perfusions and a third person be in charge of dissection and the postfix step.

8. The tcTPC method can also be applied for the study of protein interactions occurring outside the brain. For instructions describing the rapid dissection of alternative tissues please consult the respective primary literature.

9. The amount of tissue required may need to be adjusted following a small-scale pilot investigation. Parameters that will influence the outcome are the expression-level of the bait protein, the availability of functional groups that support crosslinking and the percentage of formaldehyde used. We have collected brain interactome datasets for individual bait proteins with starting material that ranged from 1 to 100 tcTPC-treated mouse brains.

10. The washing step with Acidic Elution Buffer may need to be omitted if the antibody employed does not recover from exposure to low pH even when this step is performed rapidly.

11. To avoid physical shearing of AC matrix during transfer steps cut the end of pipet tips to generate a sufficiently wide opening.

12. The avidity and affinity of the antibody employed as well as the stability of protein complexes containing the bait protein are likely to play an important role for the capture yield. For uncrosslinked material shorter incubation times appear to be beneficial for the capture of weak interactors (Cristea et al., 2005); this is less of a factor for this method which uses covalently crosslinked biological material.

13. During this and subsequent handling steps of the HSCC the liquid flow can be manually adjusted by pressing air into the reservoir with the help of an empty syringe. To this end, the syringe can be mounted to the column with the help of the Luer lock hub present on alternative HSCC lids included with this kit.

14. The dilution with water is necessary to ensure that the concentration of urea does not exceed 1.5 \( M \) during the trypsinization step.

15. (Optional) Following trypsinization, spike a known amount of a synthetic peptide (for example, 1 pmol (Glu1)-Fibrinopeptide B (GluFib) (Sigma-Aldrich, St. Louis, MO)) to samples. Equal iTRAQ labeling can then be confirmed by verifying equal intensities of 114:115:116:117 iTRAQ signature mass peaks upon fragmentation of the GluFib parent ion.
16. This is to verify that AC, digestion and iTRAQ labeling steps were successful, to assess the complexity and quantity of the peptide mixture and to confirm that the sample is not inadvertently dominated by common contaminants such as polymers or keratins.

17. The sample needs to be acidified and depleted of organic solvent prior to ZipTip clean-up or reversed-phase separation steps. If the complexity of the sample is to be reduced prior to reversed-phase capture, fractionation by strong cation exchange is recommended as it does not require the prior removal of ethanol.

18. The relative quantitation works well for the filtering of candidate interactors in a qualitative sense (as long as relative iTRAQ ratios exceed a practical discriminatory threshold of ~1.5 fold) but tends to underestimate abundance ratios in instances when one of the samples contains much higher levels of a given protein. Biological and/or technical repetitions of the experiment are frequently accompanied by changes in the relative ratios of peptides but are not expected to alter the qualitative assignment of iTRAQ ratios. Inconsistent iTRAQ ratios for different peptides derived from the same protein may indicate that this protein exists in multiple isoforms which differ in their propensity to be retained during the AC step.

Acknowledgments

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Appendix II:
Proteome-wide Identification of Mycobacterial Pupylation Targets

Please note that this chapter contains materials from the following article:

Appendix II Figure 1. Specific enrichment of pupylated peptide in polyhistidine affinity captured eluate. (A, B) Annotated CID spectra that contributed to the identification of (A) MSMEG_2352 and (B) MSMEG_4326 as a pupylation targets. The CID spectrum of (A) was derived by fragmentation of a quadruple-charged precursor ion observed at m/z [766.91]+4H]+ and was matched to the branched peptide GVGSAENFK(QGG)IVEELADSLGGAVGASR carrying iTRAQ labels at the N-termini of both its main and branched chain. Please note the absence of a detectable iTRAQ114 signature mass peak (inset), indicating that this peptide was exclusively contributed by the pupylated iTRAQ 115-labeled sample. Also note the double charged nature of branched peptide fragments due to the retention of an additional charge by the primary amine present within the N-terminal glycine of the GGQ pupylation stub. The CID spectrum of (B) was derived by fragmentation of a triple charged precursor ion observed at m/z [693.37]+3H]+ and was matched to the branched peptide YGVK(QGG)IPDEDLAGLR, carrying iTRAQ labels at the N-termini of both its main and branched chain.
**Appendix II Table 1. Protein targets from *M. smegmatis* with one or more confirmed PUP modifications.** Protein targets with pupylated sequences that have been identified by both methods, ESI QqTOF mass spectrometry analysis and 2D gel analysis, are highlighted in gray. Column annotation: Global MS, peptide index from ESI-QqTOF analysis; 2D gel, peptide index from 2D gel electrophoresis; App. MW (calculated), apparent MW versus calculated MW (all targets identified by 2D gel electrophoresis show an apparent MW that exceeds the calculated MW of the same target); Gene locus (*M. smegmatis*); Detected pupylation peptides, PUP GGE sites are highlighted by superscript; Pupylated lysines, sequence residue numbers; Gene locus (*M. tuberculosis*) for *M. smegmatis* genes with identified paralogs in *M. tuberculosis*; Conservation of lysine pupylation site, based on *M. smegmatis/M. tuberculosis* sequence alignments of targets genes (data not shown); Functional annotation (*M. tuberculosis*); Functional categorization (*M. tuberculosis*), category identifiers have been taken from Camus et al, 2002. 0, virulence, detoxification, adaptation; 1, lipid metabolism; 2, information pathways; 3, cell wall and cell processes; 7, intermediary metabolism and respiration; 8, proteins of unknown function; 9, regulatory proteins; 10, conserved hypothetic proteins. Target gene clusters, pair or multiple pupylation targets that are encoded from genes with neighboring loci, generally found in joint operons, are indicated I–VI.

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**Notes:**
- **Target No.** refers to the target number.
- **2D Gel spots** is the spot number on the 2D gel.
- **Gene locus** (M. smegmatis) and **Gene locus** (M. tuberculosis) refer to the gene loci in the Mycobacterium smegmatis and M. tuberculosis genomes, respectively.
- **Detected Pupylation Sequences** indicates the sequences detected for pupylation.
- **Pupylated lysines** refer to the lysine residues that are putylylated.
- **Gene locus** (M. tuberculosis) indicates the gene loci in M. tuberculosis.
- **Pupylation site** refers to the putylylation site.
- **Functional Annotation** provides information about the functional aspects of the proteins.
- **Gene cluster** categorizes the target proteins into clusters.
Appendix III:
A first-generation integrated cell interrogation platform (ICIP)
1. Introduction

A first generation integrated cell interrogation platform (ICIP) in multiplexing western blot analysis format is introduced to assist in efficient validation of interactome data. Analyzing candidate interactor proteins for global disease conditions such as AD and other neurodegenerative diseases, is a time-consuming and cumbersome task for a large interactome data set. Most laboratories investigating neurodegenerative diseases routinely measure the production of Aβ, monitor tau phosphorylation, determine the levels of synaptic marker proteins or query signs of oxidative or other types of cellular stress, and others. Currently, these tests are applied in a non-standardized and somewhat arbitrary manner – making it both inefficient and costly. These shortcomings contribute to uncertainties regarding the commonalities and differences of disease etiologies, both in human disease but also in animal models. They further have contributed to fragmented literatures, in which, observations of individual researchers can often not be cross-referenced with findings by others, and the validation of novel findings is difficult and time-consuming. Because proteins work in concert as a part of intricate networks and signalling cascades, an argument can be made that valuable information of the status, metabolism and health of a cell can be obtained by restricting the analysis to a subset of proteins which are most relevant within a given disease context. ICIP assay is devised as a strategy, which can interrogate expression levels and post-translational modifications of many relevant proteins concomitantly in a disease-state using multiplexing Western blot analysis.
2. Materials and Methods

To generate a standardized ready-to-use repository of samples, various types and conditions of cell lines (HEK293, MEF, PC12, SH-SY5Y), mouse brains, and human brains were collected. First, brains were cut into pieces with 1 mm cross-section using razor blades or homogenized using a mortar and pestle under liquid nitrogen. The wet-weights of samples are measured and incubated for 5 min in 10X sample wet-weight amount of the 90°C pre-heated extraction buffers (2% SDS, 100 mM DTT, 62.5 mM Tris-HCl, pH 7.5). Transfer 1-1.5 ml of extracts to 2 ml straight-sided tubes preloaded with 1 ml of 0.5 mm (cells) or 2.5 mm (brain tissue) glass beads. Add extra volume if needed to minimize the amount of residual air in the tube. Solubilize samples through 3 x 1 min Beadbeater-8 (BioSpec Products, Bartlesville, OK) ‘Homogenization’ disruption procedure. Between bead beating steps, incubate the samples at 90°C for 1 min. At the end of the procedure, incubate the samples at 90°C for 5 min. Transfer supernatants to ultracentrifuge test tubes. Centrifuge samples for 20 minutes at maximum speed of over 12,000 x g. Collect supernatant and aliquot 500 ul into 1.5 ml tubes. Add 500 ul of dilution buffer (2% SDS, 100 mM DTT, 30% glycerol, 62.5 mM Tris-HCL, pH 6.5, 0.001% Bromophenol Blue). Sample is ready to use for western blot analyses or to store at -80°C.

To generate platform that will describe the broader protein status, antibodies were collected, categorized, and optimized according to their relevance. Category 1 is antibodies for proteins that are directly implicated in neurodegenerative disease etiologies: Aβ (6E10; Covance), tau (BD Biosciences), presenilin (A4), TDP-43 (ProteinTech Group, Chicago, IL), alpha-synuclein (Covance), nicastrin (Sigma Aldrich). Category 2 is antibodies for proteins that are involved in activities of signaling pathways strongly implicated in diseases: p120 catenin (BD Bio), N-cadherin (BD Bio), p35of CDK5 (Cell signaling, Danvers, MA), PHF-Tau (Thermo Pierce Biotechnology, Rockford, IL). Category 3 is antibodies for proteins that are involved in endocytosis and autophagy: EEA1 (BD Bio), Rabaptin5 (BD Bio), CD63 (Santa Cruz Biotechnology), TSG101 (Sigma Aldrich), Rab11 (BD Bio), Anti-LC3B (Cell Signaling). Category 4 is antibodies for proteins that are indicators of various cell status: Drebrin (MBL International, Watertown, MA), Munc18 (BD Bio), proteosome 19S S5a (Abcam), MnSOD (Assay Designs, Ann Arbor, MI), Caspase 3 (Cell Signaling), Histone H4 acet. at K5 (Millipore).
3. Results

Validation strategy: Integrated cell interrogation platform (ICIP)
ICIP assay consists of two components: a generation of standardized, ready-to-use samples and multiplexing western blot analyses designed for the parallel screening of samples with a panel of over 20 well-characterized antibodies (Appendix III Figure 1A, B).

For neurodegenerative diseases it would be desirable to minimally monitor proteins which (1) are directly implicated in disease etiologies (abeta, tau, presenilin, TDP-43, alpha-synuclein, PrP, progranulin, DJ-1, SOD-1 etc.); (2) can report on the activity of signaling pathways strongly implicated in disease (Wnt/catenin-cadherin, CDK5, GSK3, MARK, etc.); (3) can indicate status for endocytosis and autophagy (EEA1, Rabaptin5, CD63, TSG101, Rab11, LC3B; (4) serve as cell status reporters providing information on the integrity of synapses, cellular stress, proteasomal activity, or report on the health and status of mitochondria, lysosome (drebrin, munc18, proteosome 19S S5a, MnOD, caspase 3, histone H4 acet. at K5); or (4) are coded by genes recently linked to neurodegenerative disease (Picalm, Clusterin, FUS, etc.).

The pilot ICIP assay incorporates the above design concepts in a multiplex western blot format. In its current implementation it provides information about expression levels and post-translational modifications of 24 proteins, with individual western blots optimized for the concomitant detection of six proteins. Appendix III Figure 1C displays the example of category 1 multiplex western blot analyses. Proteins that are directly implicated in neurodegenerative diseases are blotted on one western blot concomitantly against various mouse models of AD. The advantage of ICIP assay is that the procedure requires 24 hours from start to finish, consumes 50 ug of cell or tissue extract per six proteins detected and is largely based on antibodies with documented cross-reactivity toward human and mouse proteins. It utilizes a semi-automated workflow, including: bead beating steps for tissue homogenization, an automated western blotting procedure, premixed stocks of antibodies, standardised ready-to-use samples. This strategy is optimized with a view to avoid inadvertent cross-interference.
**A.**

**ICIP Western Blotting Analysis**

- Harvest and add 90°C preheated extraction buffer
- Add glass beads
- Transfer tubes to bead beater
- Collect supernatants
- Centrifuge the sample and collect the supernatant
- Heat samples in water bath at 90°C

**Category X antibodies**

**B.**

**Mouse Brain**

- Mouse Brain gel
- Proteins: Drebrin, Munc18, S5a, MnSOD
Appendix III Figure 1. Integrated cell interrogation platform (ICIP). (a) Generation of standardized, ready-to-use sample. Cell/tissue are harvested, subjected to bead beading extraction, and performed western blot analysis (b) Optimization of multiplexing western blot analyses for the parallel screening of samples. For example, drebrin (115 kDa), Munc 18 (64 kDa), proteosome 19S S5a (40 - 55 kDa), MnSOD (25kDa) are concentration-optimized and blotted concomitantly in one western blot analysis. (c) An example of ICIP assay. Proteins that are implicated in AD are western blotted against various mouse brain conditions. The transgenic CRND8 (TgCRDN8) is a mouse model of AD-like amyloid pathogenesis that expresses a double-mutant form of human APP 695. The antibodies used in the multiplex analysis only recognize the human form of APP, hence only CRND8 mouse display expression of APP.
4. Conclusion

The first generation of ICIP assay has promisingly displayed its advantages and future prospective as an efficient way to gain global cellular information of newly identified candidate interactors. The use of this platform will greatly benefit the initial prioritization process of the more critical candidates from a large list of interactome data set. This relatively short 1-2 day process will decrease the time and increase the validation efficiency. Therefore, a more comprehensive version of this assay may be applicable towards study in all global disease conditions.
REFERENCES


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