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Functional Role of Each Component in Gamma Secretase Complex

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To the Graduate Council:

I am submitting herewith a dissertation written by Chen Hu entitled "Functional Role of Each Component in Gamma Secretase Complex." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Functional Role of Each Component in Gamma Secretase Complex

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DEDICATION

I dedicate my work to:

My family

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ABSTRACT

Amyloid hypothesis is widely accepted as the centerpiece of Alzheimer's disease (AD) pathogenesis. It is believed that the accumulation of amyloid beta (Aβ) is the major deterministic factor of AD and the most important causative factor is the ratio of Aβ42/Aβ40. Gamma(γ)-secretase defines the length of Aβ and is composed of at least four subunits: presenilins (PS1 or PS2), nicastrin (NCT), anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2). They have been reported to have different roles in γ-secretase. For example, PS were believed as the catalytic components in γ-secretase; NCT was recognized as a substrate receptor; Pen-2 was regarded as necessary for the endoproteolysis of PS which necessary for the activity of PS; and Aph-1 was known as important for stabilization of the other γ-secretase components. However, these notions having been challenged by new and controversial findings, which make the functions of these components remain elusive. Therefore, the goal of my research projects is to address these controversial issues by systematically investigate the function of these components in γ-secretase activity and in apoptosis.

Our results demonstrate that 1) Aph-1 is dispensable for γ-secretase catalyzed processing of both Notch and amyloid beta precursor protein (APP); 2) NCT is crucial for APP processing, but is not absolutely required for Notch processing; 3) Pen-2 is necessary for the processing of both Notch and APP processing;

4) Pen-2 is the most important component for recruiting substrates; 5) Knockout of Aph-1 sensitizes cells to apoptosis; 6), PS1 accounts for the majority of the γ-secretase activity the $PS1C_{299}$ (from amino acid 299 to the end amino acid 467) is the most active form of PS1 C. These new findings not only significantly contribute to our knowledge of the biochemistry of γ-secretase and its catalyzed Notch and APP processing, but also provide valuable information for the development of therapeutic strategy of prevention and treatment of AD.

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LIST OF ABBREVIATIONS

aa, amino acid

AD, Alzheimer's disease

AICD, APP intracellular C terminal domain

Akt, Protein kinase B

Aph-1, anterior pharynx-defective 1

APOE, Apolipoprotein E

APP, Amyloid precursor protein

APPsw , Swedish mutant APP

Aβ, Amyloid beta

BACE, Beta Site APP-Cleaving Enzyme

Bak, Bcl-2 homologous antagonist/killer

Bcl2, B-cell lymphoma 2

cdk5/p35, cyclin-dependent kinase 5/p35

CHOP, C/EBP homologous protein

CompE, compound E

CTFα, APP C terminal fragment after α–secretase cleavage

CTFβ, APP C terminal fragment after β–secretase cleavage

DAPM, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine methyl ester

E-cadherin, epithelium cadherin

ER, endoplasmic reticulum

ERK, Extracellular signal-regulated kinases

FAD, Familial Alzheimer's disease

GSI , γ-secretase inhibitors

GSK3β, glycogen synthase kinase 3 beta

GSM, γ-secretase modulators

JNK, c-Jun N-terminal kinase

MAP, Mitogen-activated protein

MEF, mouse embryonic fibroblast

N-cadherin, neural cadherin

NCT, nicastrin

NF-κB, nuclear factor kappa-light-chain-enhancer of activated **B** cells

NICD, Notch intracellular domain

Notch molecule

NotchΔE, plasmid expressing the extracellular region truncated and myc-tagged

NTFs, intracellular neurofibrillary tangles

p53, Tumor protein **p53**

PARP, poly ADP ribose polymerase

Pen-2, presenilin enhancer 2

PKC, Protein kinase C

PS1, Presenilin1

PS1C, presenilin C terminals

$PS1C_{293}$, PS1 C terminal from aa 293 to aa 467

$PS1C_{296}$, PS1 C terminal from aa 296 to aa 467

$PS1C_{299}$, PS1 C terminal from aa 299 to aa 467

$PS1C_{334}$, PS1 C terminal from aa 334 to aa 467

$PS1C_{346}$, PS1 C terminal from aa 346 to aa 467

$PS1_{D257,385A}$, PS1 mutant, two point mutant of aspartic acid 257 and 385 to Alanine

$PS1_{D257A}$, PS1 mutant, single point mutant of aspartic acid 257 to Alanine

$PS1_{D385A}$, PS1 mutant, single point mutant of aspartic acid 385 to Alanine

PS1N, presenilin N terminal, from aa1 to aa 292

PS2, Presenilin 2

stat3, Signal transducer and activator of transcription 3

INTRODUCTION

Alzheimers' Disease

Dementia is a common brain disease, especially among aged people, from which not only patients but also their families deeply suffer. Among all kinds of dementia, about 50% to 70% are Alzheimer's disease (AD). It has been more than 100 years since the description of the first AD case by the Germen psychiatrist Alois Alzheimer. However, there is still no cure available for treatment of this devastating disease. AD is a neurodegenerative disease which is clinically characterized by memory loss, inability of carrying conversation, impaired judgement, difficulty of recognizing people and eventually death (Alzheimer's 2013). As a progressive disease, it takes several years to develop which is emotionally, physiologically and financially consuming.

Although age is recognized as the major risk factor of AD, AD is not a normal part of aging and more than 5 percent of AD patients have earlier onset (younger than 60). AD ranks as the sixth death cause in United States (Sherry et.al 2013), and even worse, the death caused by AD increase by 68% between 2000 and 2010, while the death caused by other listed disease decreased (Arialdi et al 2002 and Sherry et.al 2013). With the globally population ageing, AD will become a severe threaten with increasing shadows to people and our society.

Molecular Mechanism of AD

Amyloid β (Aβ) hypothesis

AD is pathologically characterized by the presence of extracellular plaques enriched in amyloid-beta (Aβ) peptides and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein (Selkoe 1998). Mounting evidence suggests that the abnormal accumulation of Aβ is not only a hallmark of AD, but is a primary causative factor of AD, and this theory is known as amyloid hypothesis (Hardy and Selkoe 2002). Based on this hypothesis, it is believed that the accumulation of Aβ is the direct cause of AD, although, the amyloid hypothesis has and still does confront lots of doubts and rejections (Herrup 2015). Another hallmark of AD is intracellular neurofibrillary tangles (NTFs), which are composed of phosphorylated tau protein. Together with extracellular plaque, NTFs were found in AD patient's brain. Researchers have found that both NTFs and plaques could result in neuron loss which contributes dramatically to AD. However, compare to Aβ aggregation, NTFs is not the predominant one. Since the tangle formation could be influenced by both Aβ and APP, but the plaque formation is barely affected by tau overexpression, Aβ is suggested to be the primary and deterministic factor of AD (Lewis, Dickson et al. 2001). Preclinical studies also support amyloid cascade hypothesis since Aβ immunotherapies could help in preventing AD (Lemere and Masliah 2010). Therefore, amyloid cascade hypothesis is still the most widely accepted one in AD physiology.

APP processing and Aβ production

Aβ is produced from the amyloid precursor protein (APP). APP is a type I single transmembrane protein which could be processed in two different pathways: amyloidogenic pathway and non-amyloidogenic pathway. In these pathways, APP is first cleaved by β-secretase or α-secretase and produce C terminal fragments, CTFβ or CTFα, respectively. The CTFβ and CTFα will be subsequently processed by γ-secretase into Aβ and p3, respectively and, concomitantly, a common APP intracellular C terminal domain (AICD) (Figure I) (Xu 2009). In fact, the first disease causative gene identified is the APP gene. The other two are Presenilin1 (PS1) and Presenilin 2 (PS2) genes. PS1 and PS2 are two proteins sharing high homology with each other. Mutations in these two presenilin genes account for the majority of familial Alzheimer's Disease (FAD) cases (De Strooper 2007). Interestingly, the mutations in all the disease causative genes, *APP*, *PS1* and *PS2,* lead to one common consequence: the increased production of Aβ. Based on the accumulation of knowledge and evidences, amyloid cascade hypothesis was first summarized at the 1992 by Dr. Hardy and Dr. Higgins (Hardy and Higgins 1992). They suggest that the accumulation of Aβ is the direct cause of AD.

BACE

As mentioned above, Aβ is produced from APP through sequential cleavage of β–secretase and γ–secretase. β–secretase, also known as BACE (Beta Site

APP-Cleaving Enzyme) (Vassar, Bennett et al. 1999) has two homologues: BACE1 and BACE2, among which BACE1 has the major activity at processing APP and producing CTFβ which could be further processed into Aβ (Farzan, Schnitzler et al. 2000, Basi, Frigon et al. 2003). BACE1 is confirmed of required for production of Aβ through knock out experiments in mice (Cai, Golde et al. 1993). The knockout of BACE in mice also cause dramatic neonatal lethality (Dominguez, Tournoy et al. 2005), which suggests that BACE is significantly necessary for some other functions in vivo. This critical role of BACE in the development leads to the difficulties of targeting it for the treatment of AD.

γ–secretase components

Further researches discovered that the ratio of Aβ42 versus Aβ40 (Aβ42/Aβ40) is critical for AD development (Hardy and Selkoe 2002, Kumar-Singh, Theuns et al. 2006). The γ–secretase controls the length of Aβ. Therefore, γ–secretase has come under the spotlight of AD research and for the understanding of mechanism of Aβ production and AD treatment. γ secretase is composed of at least four components: presenilins (PS1 or PS2), nicastrin (NCT), anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2)

Among those, the nine transmembrane protein presenilin (PS1 or PS2 isoforms) is thought to be the catalytic subunit, since the mutation of the two aspartyl residues results in the loss of γ–secretase activity (Wolfe, Xia et al. 1999, Wolfe 2002). NCT is suggested to be substrate receptor (Shah, Lee et al. 2005). A more recent research suggests NCT acts as a molecular gatekeeper for

substrate binding and catalysis through actively excludes larger substrates with steric hindrance (Bolduc, Montagna et al. 2016). Aph-1 might be required for the stabilization of other γ–secretase components (Francis, McGrath et al. 2002, Lee, Shah et al. 2002, Steiner, Winkler et al. 2002). Pen-2 was believed to be required for the endoproteolysis of presenilin which is a necessary step in γ–secretase maturation (Luo, Wang et al. 2003, Takasugi 2003). However, recent researches have raised questions to this notion, for example Pen-2 is only partially required for endoproteolysis of presenilin as demonstrated by recent studies (Mao, Cui et al. 2012, Holmes, Paturi et al. 2014) and NCT is not absolutely required for the processing of Notch which is one of γ–secretase substrate (Zhao, Liu et al. 2010). These controversial results casted shadows over the current view regarding the functional role of each component in γ–secretase complex. In addition, the catalytic functions of PS1 and PS2 have been well defined, however, the mechanisms remain elusive. Therefore, we set out to address these issues by the following proposed study.

γ secretase substrates

As discussed above, based on Amyloid hypothesis, one way for the treating AD is to inhibit γ–secretase activity in order to reduce the production of Aβ. However, more than 90 type I transmembrane proteins have been identified as γ–secretase substrates (Haapasalo and Kovacs 2011), including APP (De Strooper, Saftig et al. 1998), Notch (De Strooper, Annaert et al. 1999), E-cadherin, N-cadherin,

ErbB4 and so on. Specifically, γ–secretase catalyzed Notch processing plays an important role in controlling cellular homeostasis and cell fate determination. Thus, it becomes difficult to simply inhibit γ–secretase activity as a therapeutic strategy for treating AD. Notch pathway requires the processing of γ–secretase to produce a Notch intracellular domain (NICD) in order to regulate the transcription of important genes for cell development, proliferation, differentiation and so on (Kopan and Ilagan 2009, Andersson, Sandberg et al. 2011). Thus, the inhibition of γ–secretase will result in other severe consequences through failure of Notch signaling regulation. Therefore, it becomes important to find a way to selectively inhibit γ–secretase catalyzed APP processing without affecting the Notch processing, which is one of the goals of my research project.

Apoptosis in AD

The importance of apoptosis in AD has been discussed in 1998 by Marcia Barinaga (Barinaga 1998), in which they speculate that apoptosis could play a role in neuron death found in AD. Since then, scientists found more evidence to support this idea. The anti-apoptotic protein level Bcl2 was found lower in AD brain, the active forms of caspase 3 was detected and so does the fragmentation of DNA (Shimohama 2000). In vitro experiments have also demonstrated that expression of Aβ could induce the activation of caspases (Ivins, Thornton et al. 1999). Lots of signaling pathway, like JNK (Troy, Rabacchi et al. 2001), GSK3β (Lucas, Hernandez et al. 2001), even PS1 (Soriano, Kang et al. 2001) itself have been reported of involving in the apoptosis of AD (Bamberger and Landreth

2002). Besides PS1, some other γ–secretase components, like Aph-1 and Pen-2 have been found contribute to apoptosis as protective roles through p53 pathway due to the fact that they could help in keeping the integrity of γ–secretase complex (Dunys, Kawarai et al. 2007). Since we have different γ–secretase components knock out cells, alongside with investigations about their roles in γ–secretase activity, we also studies their role in apoptosis. Our result demonstrates that the knock out Aph-1 lead to higher tendency of apoptosis in mouse embryonic fibroblast (MEF) cells when triggered by MG132 through Akt-GSK3β pathway. Combined with the results of γ secretase activity: Aph-1 is not definitely required for the processing of both Notch and APP, but more importantly for the stability of γ–secretase and cell survival (Hu, Zeng et al. 2015). Therefore, the targeting of Aph-1 in treating AD is completely unreasonable theoretically.

Therapy

Several anti-Aβ monoclonal antibodies like Bapineuzumab and crenezumab were tested in clinical trials. They have been demonstrated to have no significant effect on cognition but only decrease some level of aggregated Aβ or soluble Aβ (Doody, Thomas et al. 2014, Salloway, Sperling et al. 2014). Some β-secretase inhibitors are under test at the clinical trial phase 2 or 3. They have been found to be able to reduce 80% production of Aβ, but the effects on cognition and prevention of AD need to be further determined (Ayutyanont, Langbaum et al.

2014) (Bateman 2015). Lots of γ–secretase inhibitors (GSI) and γ–secretase modulators (GSM) are at phase 2 or 3 stage of clinical trials, but most of them are either not efficient or have significant side effects and toxicity since including Notch, plenty of γ–secretase substrates and their pathways are influenced (Cummings 2010, Samson 2010, Schor 2011). When comparing with GSI, the toxicity of GSM is much less since they only regulate the length of Aβ rather than the activity of γ–secretase. Since the longer forms of Aβ have been demonstrated to be easier at aggregation and forming plaque (Xu 2009), if proper GSM which could reduce the production of longer Aβ, that would be helpful in treat AD. Alternatively, we might be able to switch to targeting γ–secretase components, for example NCT, since we have found that the knockout of NCT could selectively inhibits APP processing, but has less effect on Notch processing (Hu, Zeng et al. 2015). Hope our research on the structure and function of γ secretase could provide further supports on the development of AD treatment.

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Appendix

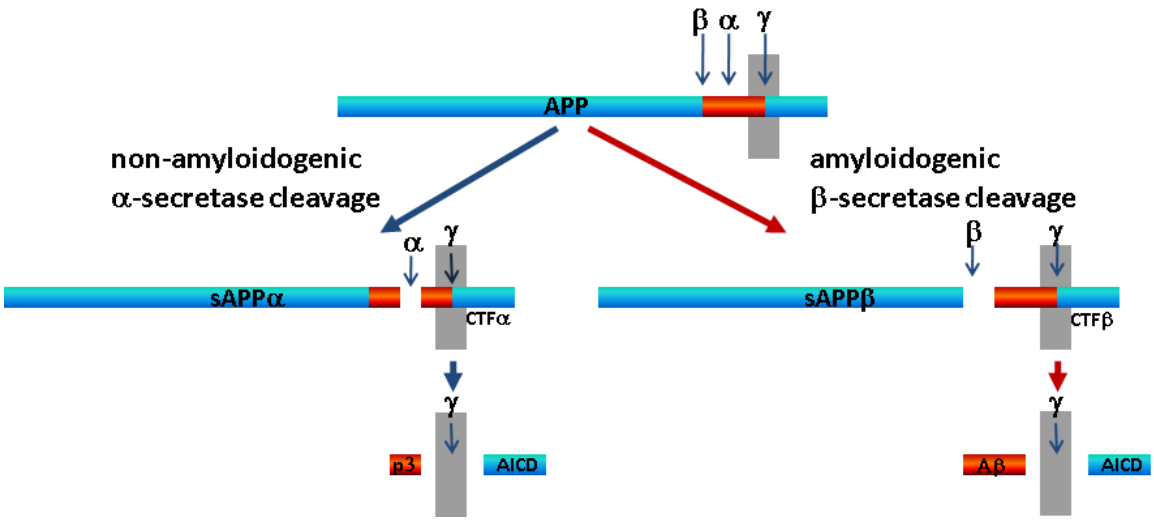

Figure I (Xu 2009)

CHAPTER I

NICASTRIN IS REQUIRED FOR APP BUT NOT NOTCH PROCESSING, WHILE APH-1 IS DISPENSABLE FOR PROCESSING OF BOTH APP AND NOTCH

This chapter is a slightly revised version of a paper by the same name published in the Journal of Neurochemistry in 2015 by Chen Hu, et al:

Hu, C., L. Zeng, T. Li, M. A. Meyer, M. Z. Cui and X. Xu (2015). "Nicastrin is required for APP but not Notch processing, while Aph-1 is dispensable for processing of both APP and Notch." J Neurochem.

My use of “we” in this chapter refers to my co-authors and myself. I contributed to all the data and most writing of this paper.

Abstract

The γ-secretase complex is composed of at least four components: presenilin (PS1 or PS2), nicastrin (NCT), anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (pen-2). In this study, using knockout cell lines, our data demonstrated that knockout of NCT, as well as knockout of Pen-2, completely blocked γ-secretase-catalyzed processing of CTFα and CTFβ, the C-terminal fragments of β-amyloid precursor protein (APP) produced by α-secretase and β-secretase cleavages, respectively. Interestingly, in Aph-1-knockout cells CTFα and CTFβ were still processed by γ-secretase, indicating Aph-1 is dispensable for APP processing. Furthermore, our results indicate that Aph-1 as well as NCT is not absolutely required for Notch processing, suggesting that NCT is differentially required for APP and Notch processing. In addition, our data revealed that components of the γ-secretase complex are also important for proteasome- and lysosome-dependent degradation of APP and that endogenous APP is mostly degraded by lysosome while exogenous APP is mainly degraded by proteasome.

Introduction

One of the hallmarks of Alzheimer's disease (AD) is the abnormal production and accumulation of β-amyloid peptide (Aβ) in the brain. According to the amyloid hypothesis, the ratio of the long Aβ species, Aβ42, versus the short Aβ40 (Aβ42/Aβ40) has been considered to play a critical role in AD (Hardy and

Selkoe 2002). An increased Aβ42/Aβ40 ratio appears to correlate with early-onset familial AD cases caused by presenilin mutations (Kumar-Singh, Theuns et al. 2006). Aβ is derived from the amyloid precursor protein (APP) by successive action of the β- and γ-secretases. APP can be processed via two pathways, the non-amyloidogenic pathway or the amyloidogenic pathway. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase to release a soluble N-terminal ectodomain and a membrane anchored C-terminal fragment (CTFα); in the amyloidogenic pathway, APP is first cleaved by β-secretase to remove the N-terminal fragment and generate a membrane-anchored C-terminal fragment of APP (CTFβ). Both CTFα and CTFβ are then subsequently cleaved within the transmembrane domain by γ-secretase to produce a common APP intracellular domain (AICD) and lead to the generation of a p3 fragment from CTFα and the full-length Aβ from CTFβ (Xu 2009). Since the γ-secretase-catalyzed cleavage determines the C-termini of Aβ species and the ratio of Aβ42/Aβ40, dissecting the biological and biochemical nature of γ–secretase is important for understanding the mechanism of Aβ formation. Thus far at least four polypeptides have been identified as necessary components for γ-secretase activity (Dries and Yu 2008, Zhang, Li et al. 2014). These four components are presenilins (PS1 or PS2), nicastrin (NCT), anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2). Mutation of the two conserved aspartyl residues in PS1 and PS2 results in the loss of γ-secretase activity (Wolfe 1999), and affinity labeling experiments have demonstrate that γ-secretase inhibitors

bind directly to PS1 (Esler, Kimberly et al. 2000, Li, Xu et al. 2000); therefore, the nine transmembrane protein presenilin (PS1 or PS2 isoforms) is thought to function as the catalytic subunit of γ-secretase (Wolfe 2002). The identification of a substrate-binding domain in NCT strongly suggests that NCT functions as the substrate receptor (Shah, Lee et al. 2005). Using siRNA technology, studies suggested that the seven transmembrane protein Aph-1 is required for stabilization of the PS1 endoproteolysis products PS1N and PS1C (Francis, McGrath et al. 2002, Lee, Shah et al. 2002, Steiner, Winkler et al. 2002) and that the two transmembrane protein Pen-2 is required for endoproteolysis of PS1 (Luo, Wang et al. 2003, Takasugi, Tomita et al. 2003). However, recent studies have shown that Pen-2 is dispensable for endoproteolysis of PS1 (Mao, Cui et al. 2012, Holmes, Paturi et al. 2014). One study also showed that NCT is not absolutely required for γ-secretase activity (Zhao, Liu et al. 2010). To further determine the role of each component of the γ-secretase complex in γ-secretase activity, we used knockout cell lines to examine the effect of deletion of each component on the processing of CTFα and CTFβ. Our data demonstrated that knockout of Pen-2, as well as NCT, almost completely blocked the processing of both CTFα and CTFβ. However, knockout of Aph-1 had no significant effect on the processing of CTFα and CTFβ, indicating Aph-1 is dispensable for APP processing. Furthermore, our results revealed that NCT is differentially required for γ-secretase-catalyzed processing of APP and Notch. In addition, our data

suggest that the components essential for γ-secretase-dependent APP processing are also important for APP degradation.

Materials and Methods

Cell culture

Mouse embryonic fibroblast (MEF) cells established from PS1/PS2-double knockout (PS1/2$^{-/-}$) cells (Herreman, Serneels et al. 2000), PS1-knockout (PS1$^{-/-}$) cells (De Strooper, Saftig et al. 1998), PS2-knockout (PS2$^{-/-}$) cells (Herreman, Hartmann et al. 1999), Pen-2-Knockout (Pen-2$^{-/-}$) cells (Bammens, Chavez-Gutierrez et al. 2011), and wild-type mouse embryonic fibroblasts were all kindly provided by Dr. Bart De Strooper (Center for Human Genetics, Belgium). Nicastrin-knockout (NCT$^{-/-}$) cells (Li, Ma et al. 2003) and Aph-1abc-triple-deficient (Aph-1$^{-/-}$, deficient in all three Aph-1a, Aph-1b, and Aph-1c isoforms) cells (Chiang, Fortna et al. 2012) were kindly provided by Dr. Tong Li (John Hopkins University). The wt-7 cells (N2a cells stably expressing wild-type presenilin 1 [PS1wt] along with Swedish mutant APP [APPsw]) were kindly provided by Drs. Sangram S. Sisodia and Seong- Hun Kim (University of Chicago). All cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine (Lonza, Walkersville, MA, USA), 100 units/mL penicillin (Lonza), and 100 µg/mL streptomycin (Lonza).

Inhibitors and reagents

Proteasome inhibitor MG132 was purchased from Peptides International (Louisville, KY, USA). Gamma-secretase inhibitors compound E and L685, 458 and proteasome inhibitor lactacystin were purchased from EMD Millipore (Billerica, MA, USA). Lysosome inhibitors chloroquine, leupeptin, and NH_4Cl were purchased from Sigma (St. Louis, MO, USA). The general caspase inhibitor, benzyloxycarbonyl-Val- Ala-Asp-fluoromethylketone (Z-VAD-fmk) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Complete protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN, USA). Lipofectamine LTX with plus reagent was purchased from Invitrogen (Carlsbad, CA, USA).

Antibodies

Anti-PS1C, anti-NICD (#4147, which specifically recognizes the processed Notch), anti-caspase3, and anti-caspase-6 were purchased from Cell Signaling (Danvers, MA). Anti-NCT was from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies anti-Aph-1aL and anti-PEN-2N were from Covance (Princeton, NJ, USA). Anti-Aph-1bc was from NOVUS (Littleton, CO, USA). Polycolonal antibody C15 was raised against the last 15 amino acids at the very C terminal of APP (Zhao, Mao et al. 2004). Anti-myc antibody, C-Myc (9E10), was purchased from Santa Cruz (Dallas, TX, USA). Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was from EMD Millipore.

Plasmids

Plasmid expressing the truncated ectodomain and myc-tagged Notch molecule (NotchΔE) containing the murine Notch-1 leader peptide (1-23 amino acids) (Kopan, Schroeter et al. 1996) was kindly provided by Dr. Raphael Kopan (Washington University) and Dr. Masayasu Okochi (Osaka University, Japan). The plasmid APPsw, which expresses a C-terminal myc-tagged Swedish mutant APP (APPsw) (Thinakaran, Teplow et al. 1996), was kindly provided by Dr. Gopal Thinakaran (University of Chicago).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out as described previously (Hao, Tan et al. 2010). Total RNA was isolated from MEF cells mentioned above using an RNeasy mini-prep kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2 μg total RNA using the ThermoScipt RT-PCR kit (Invitrogen). The cDNA products were amplified using GeneAmp PCR core reagents (Applied Biosystems, Foster City, CA, USA) and a Stratagene Mx3000P thermocycler (Agilent, Santa Clara, CA, USA) with the following program: 5 min at 95°C followed by 28 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s followed by a final extension for 7 min at 72°C. The primers used were as follows: Aph-1a, forward 5′-ACGGAAGATCACCCAT-3′ and reverse 5′-TGTCAGAAGGTGACTCCCA-3′; Aph-1b,c, forward 5′-CCTGACGCATCTGGTGGTG-3′ and reverse 5′-GTTCCAAGATACAGGGG-3′; and NCT, forward 5′-TCTTCTCACACATGCACGCC-3′ and reverse 5′-

CATGGGATCTGTGTGCATCC-3′. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously (Tan, Mao et al. 2008, Zhao, Liu et al. 2010). MEF cells were cultured for 24 h. Conditioned media (CM) were supplemented with an inhibitor cocktail (Millipore) containing AEBSF (4-[2-aminoethyl] benzenesulfonyl fluoride hydrochloride) at a final concentration of 1 mM. The CMs were analyzed with a mouse $A\beta_{40}$-specific ELISA kit (Invitrogen), according to the manufacturer's instructions.

Cell-free assay

In vitro AICD (APP intracellular domain) generation was determined by cell-free assay using the protocol reported by Tesco et al (Tesco, Ginestroni et al. 2005). MEF cells were grown at a density of 150,000 cells/cm^2 for 24 h. Cells were scraped in 1 ml buffer A (50 mM HEPES, 150 mM NaCl, 5 mM 1,10-phenanthroline monohydrate [PNT], pH=7.4) and homogenized by passing them through 25-gauge 5/8 needles 10 times. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C. The membrane fraction obtained was washed once with buffer A and centrifuged at 10,000 × *g* for 5 min at 4℃. Total protein was measured in the membrane fraction, and protein aliquots were incubated with 50 µl buffer B (50 mM HEPES, 150 mM NaCl, 5 mM PNT, cocktail protease inhibitor, chloroquine (10 µM), pH=7.0) for 2 h at 37°C in the presence or

absence of L685, 458 to induce the production of AICD. After incubation, samples were centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were collected and analyzed by Western blot using anti-APP-CTF antibody, C15.

SDS-PAGE and Western blotting

For analysis of endogenous APP processing, 10 h after splitting, cells were incubated overnight in the presence or absence of the following inhibitors compound E (5nM), L685, 458 (0.5 μM), lactacystin (10 μM), MG132 (5uM), chloroquine (10 μM), leupeptin (5 μg/ml), and NH_4Cl (1mM). For analysis of the exogenous APP and Notch processing, the cells, 24 h after splitting, were transfected with plasmids expressing APPsw or NotchΔE with lipofectamine LTX. Ten hours after transfection, inhibitors were added and the cells were further incubated overnight. Cell lysis and Western blot analysis were carried out as described previously (Zhao, Mao et al. 2004). Briefly, cells were lysed with sonication for 20 s on ice in Western blot lysis buffer (50 mM Tris–HCl, pH 6.8, 8 M urea, 5% mercaptoethanol, 2% SDS, and protease inhibitor mixture). After addition of 4 × SDS sample buffer and boiling at 100°C for 7 min, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS PAGE, 16% for APP CTFs; 14% for PS1 C terminals, caspases, and GAPDH; 10% for Notch and C-Notch; 6% for APP). The membranes were probed with appropriate antibodies as described in figure legends.

Statistical analysis

Data are expressed as mean ± SEM and assessed for significance by Student's *t* test. When P > 0.05, differences were considered not significant.

All methods used are approved by University of Tennessee (Registration #309-13).

Results

Aph-1 is dispensable for γ-secretase-catalyzed processing of CTFα

To determine the role of the components of the γ-secretase complex in APP processing activity, we examined the effects of deletion of each component of the complex on the processing of CTFα. As shown in Figure 1. 1a, as expected, in the absence of inhibitor, almost no CTFα was detectable in wild type (wt) cells (lane 9). However, when the cells were treated with transition state γ-secretase inhibitor L-685,458, a significant amount of unprocessed CTFα was accumulated (lane 10). As reported previously (Herreman, Serneels et al. 2000), a dramatic accumulation of unprocessed CTFα was observed in the PS1 and PS2 double knockout (PS1/2$^{-/-}$) cells (compare lanes 13 and 14) regardless of the presence or absence of γ-secretase inhibitor. Similarly, significant accumulation of CTFα was also observed in nicastrin-knockout (NCT$^{-/-}$) cells (lanes 3 and 4) and Pen-2-knockout (Pen-2$^{-/-}$) cells (lanes 5 and 6) regardless of the presence or absence of γ-secretase inhibitor.

However, in contrast to knockout of NCT or Pen-2, a significant decrease in the level of CTFα was detected in the Aph-1-knockout (Aph-1$^{-/-}$) cells in which all three murine Aph-1 alleles–termed Aph-1a, Aph-1b, and Aph-1c–were knocked out (Figure 1. 1a, lane 1). More interestingly, the decrease in the level of CTFα was completely blocked by γ-secretase inhibitor (lane 2). In addition, we also observed that knockout of PS2 had almost no effect on the turnover of CTFα (lane 11) and this decrease in CTFα in PS2-knockout (PS2$^{-/-}$) cells was completely inhibited by γ-secretase inhibitor (lane 12). This result indicates that knockout of PS2 did not cause significant reduction in γ-secretase activity. However, a significant amount of CTFα was detected in the PS1-knockout (PS1$^{-/-}$) cells in the absence of inhibitor (lane 7), indicating a substantial reduction in γ-secretase activity.

Previous studies have reported that CTFs of APP undergo degradation by a proteasome-dependent mechanism distinct from γ-secretase (Skovronsky, Pijak et al. 2000, Nunan, Shearman et al. 2001, Nunan, Williamson et al. 2003). To determine whether the decrease of CTFα detected in the Aph-1-knockout cells is indeed due to γ-secretase, we examined the effect of proteasome inhibitor on the turnover of CTFα. As shown in Figure 1. 1b, treatment of cells with proteasome inhibitor MG132 caused a slight increase in the level of CTFα in Aph-1^{-1-} cells (compare lane 2 with lane 1). A similar result was also observed in PS1-knockout cells (compare lane 8 with lane 7) and wt cells (compare lane 14 with lane 13). However, the extent of the increase in CTFα caused by MG132 is much less

than that caused by γ-secretase inhibitor (compare Figure 1. 1b with 1a). These results indicate that, similar to wt cells, the turnover of CTFα in the Aph-1$^{-/-}$ cells is mainly catalyzed by γ-secretase activity. In addition, MG132 showed no significant effect on the level of CTFα in NCT$^{-/-}$ cells (compare lanes 4 with lane 3), Pen-2$^{-/-}$ cells (compare lane 6 with lane 5), nor PS1/2$^{-/-}$ cells (compare lane 12 with lane 11). It was noted that no CTFβ was detected in these experiments, suggesting a possibility that the mouse endogenous APP was mostly processed via the α-secretase pathway and that the low level of CTFβ was undetectable under our experimental conditions.

If the turnover of CTFα in Aph-1$^{-/-}$ cells were catalyzed by γ-secretase activity rather than by random degradation, the AICD produced by γ-secretase activity would be detectable. However, AICD was not detected in the experiments shown in Figure 1. 1a and b, possibly due to rapid degradation of this peptide in living cells [Cupers, 2001 #7150]. Thus, we performed a cell-free assay using the procedure described previously (Tesco, Ginestroni et al. 2005). As shown in Figure 1. 1c, in the absence of γ-secretase inhibitor, a significant amount of AICD was readily detected in membrane prepared from wt (lane 5), Aph-1$^{-/-}$ (lane 7), and PS2$^{-/-}$ (lane 9) cells, and the generation of AICD in these cells was strongly inhibited by γ-secretase inhibitor L-685,458 (lanes 6, 8, and 10). Similarly, in wt-7 cells, both AICD-myc and AICDendo, produced from exogenous APP with a myc-tag and endogenous APP, respectively, were detected at very high levels (lane 15) and inhibited by L-685,458 (lane 16). However, this AICD was not detected in

NCT$^{-/-}$, Pen-2$^{-/-}$, and PS1/2$^{-/-}$ cells regardless of the presence or absence of γ-secretase inhibitor (lanes 1 to 4, and lanes 13 and 14). These results strongly indicate that the turnover of CTFα in Aph$^{-/-}$ cells is catalyzed by γ-secretase activity. AICD was hardly detected in PS1$^{-/-}$ cells (lanes 11 and 12), suggesting that PS1 accounts for the majority of the γ-secretase activity. To further ascertain whether APP is indeed processed by γ-secretase in Aph-1$^{-/-}$ cells, we performed an ELISA to determine the formation of Aβ in these cells. As shown in Figure 1. 1d, a large amount of $A\beta_{40}$ was detected in the media of wt and PS2$^{-/-}$ cells. Interestingly, a significant amount of $A\beta_{40}$ (> 50% of that detected in wt cells) was also detected in Aph-1$^{-/-}$ cells when PS1/2$^{-/-}$ cells were used as a negative control. This result provided further strong support to the notion that APP is indeed processed by γ-secretase activity in Aph-1$^{-/-}$ cells. On the other hand, only a low, but still significant, level of $A\beta_{40}$ (< 20% of that detected in wt cells) was detected in PS1$^{-/-}$ cells, and a very low level of $A\beta_{40}$ (< 8% of that detected in wt cells) was also detected in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells.

Aph-1c protein is undetectable in Aph-1abc-triple deficient cells under the experimental conditions

Since the Aph-1$^{-/-}$ cells were created by knockdown of Aph-1c in Aph-1a/b double knockout cells using shRNA technology (Chiang, Fortna et al. 2012), one concern is whether the γ-secretase activity detected in Aph-1$^{-/-}$ cells results from incomplete knockdown of Aph-1c. To address this issue, we performed a RT-PCR assay to determine the mRNA level of Aph-1c using primers corresponding

to the coding regions of Aph-1c. As controls, similar RT-PCR was also performed for Aph-1a and NCT. As shown in Figure 1. 1e, as expected, neither NCT mRNA nor Aph-1a mRNA was detected in NCT$^{-/-}$ and Aph-1$^{-/-}$ cells, respectively. However, as shown in the second panel of Figure 1. 1e, a fine PCR band was detected in Aph-1$^{-/-}$ cells, indicating the presence of a trace amount of residual or partially cleaved Aph-1c mRNA in Aph-1$^{-/-}$ cells. Thus, we further determined the protein levels of Aph-1c and other components in these knockout cells used. As shown in Figure 1. 1f, Western blot analysis using specific antibodies confirmed the absence of PS1, PS2, NCT, and Pen-2 as well as Aph-1 (Aph-1a, Aph-1b, and Aph-1c) proteins in the corresponding knockout cells. Specifically, the fact that antibody specific to Aph-1b/c did not detect any signal in Aph-1$^{-/-}$ cells suggests that the Aph-1c gene was efficiently silenced by shRNA technology.

Components of the γ-secretase complex might also play a role in regulating APP CTF degradation by proteasome and lysosome.

It was noted from the above experiments that treatment with proteasome inhibitor MG132 caused an increase in the level of CTFα in wt, Aph-1$^{-/-}$, and PS1$^{-/-}$ cells. However, MG132 showed no effect on the level of CTFα in NCT$^{-/-}$, Pen-2$^{-/-}$, and PS1/2$^{-/-}$ cells. These results suggest that knockout of different components might have different effects on the proteasome-dependent turnover of CTFα. APP and its processing products have also been reported to be subjected to lysosome degradation (Eisele, Baumann et al. 2007, Vingtdeux, Hamdane et al. 2007). Thus, next, we examined the effects of other proteasome and lysosome inhibitors

on the turnover of CTFα in these knockout cells. As shown in lanes 2 and 3 of the top four panels of Figure 1. 2a, as expected, both of the γ-secretase inhibitors, compound E (compE) and L-685,458, caused accumulation of unprocessed CTFα in wt, PS1$^{-/-}$, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells. When the cells were treated with proteasome inhibitors MG132, strong accumulation of CTFα resulted in wt, PS1$^{-/-}$, and Aph-1$^{-/-}$ cells (lane 5), but lactacystin in comparison, caused a lesser accumulation of CTFα in PS1$^{-/-}$ and Aph-1$^{-/-}$ cells (lane 4), and CTFα was hardly detectable in wt cells (lane 4). Neither MG132 nor lactacystin had a detectable effect on the CTFα level in PS2$^{-/-}$ cells (Panel 3, lanes 4 and 5). When the cells were treated with the lysosome inhibitors chloroquine, leupeptin, and NH_4Cl, significant accumulation of CTFα was observed in wt, PS1$^{-/-}$, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells. In addition, it was noted that in the presence of lysosome inhibitors, specifically, chloroquine and leupeptin, the APP intracellular c-terminal domain (AICD) produced by γ-cleavage of CTFα become detectable in wt cells, PS2$^{-/-}$ cells, and to a lesser extent in Aph-1$^{-/-}$ cells. These results suggest that lysosome is the major site for CTFα degradation. In addition, the detection of AICD in the presence of lysosome inhibitors indicates that these lysosome inhibitors have no effect on γ-secretase catalyzed processing of CTFα. As shown in the bottom three panels of Figure 1. 2a, the proteasome inhibitors lactacystin and MG132 had no effect on the level of CTFα in PS1/2$^{-/-}$, NCT$^{-/-}$, and Pen-2$^{-/-}$ cells. Lysosome inhibitors caused a slight increase in the level of CTFα in these cells.

These results indicate that CTFα was not significantly degraded by either proteasome or lysosome activity in these cells.

As mentioned above, possibly because mouse endogenous APP was mostly processed via the α-secretase pathway, the level of endogenous CTFβ was too low to be detected under our experimental conditions. To determine the effects of knockout of each γ-secretase component on the processing of CTFβ, we transiently transfected these cells with a plasmid expressing myc-tagged human Swedish mutant APP (APPsw) in the presence or absence of different inhibitors. As shown in Figure 1. 2b, recombinant APP was detected in all transfected cells. As shown in the top panel, in the wild type-cells, endogenous CTFα (CTFα[endo]) as well as CTFα-myc and CTFβ-myc produced from exogenous myc-tagged APPsw, were accumulated in the presence of the γ-secretase inhibitors compound E (lane 4) and L-685,458 (lane 5). Similarly, γ-secretase inhibitors caused accumulation of unprocessed CTFα-myc, and CTFα(endo) was also clearly detected in Aph-$1^{-/-}$, $PS2^{-/-}$, and $PS1^{-/-}$ cells. These results indicate that γ-secretase inhibitors had similar effects on both exogenous and endogenous APP in these cells, excepting that CTFβ-myc was hardly detected in these cells. In wt cells, the accumulation of CTFα(endo), CTFβ-myc, and CTFα-myc was also detected when cells were treated with the lysosome inhibitors chloroquine and leupeptin, and to a lesser extent with NH_4Cl (lanes 8–10). However, mainly CTFα-myc and CTFβ-myc, but almost no CTFα(endo), were accumulated in the presence of proteasome inhibitors lactacystin (lane 6) and

MG132 (lane 7). In PS1$^{-/-}$, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells, both CTFα(endo) and CTFα-myc were detected at various levels in the presence of these proteasome and lysosome inhibitors. However, almost no CTFβ-myc was detected in these cells, with the exception of MG132-treated Aph-1$^{-/-}$ cells (fourth panel, lane 7). A small amount of CTFα-myc was detected in PS1$^{-/-}$ cells in the absence of any inhibitors (second panel, lane 3), indicating a low γ-secretase activity in these cells in comparison with that in PS2$^{-/-}$ cells.

It was interestingly noted that in PS1$^{-/-}$, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells, treatment with proteasome inhibitors lactacystin and MG132 mainly caused accumulation of CTFα-myc (Figure 1. 2b, lanes 6 and 7), whereas lysosome inhibitors mostly caused accumulation of CTFα(endo) (lanes 8–10). These data revealed an interesting finding that exogenous APP was primarily degraded by proteasome, and the endogenous APP was mostly degraded by lysosome. This notion was further supported by the fact that exogenous full-length APP (both mature and immature forms) was detected at high levels in the presence of proteasome inhibitors in all cells (lanes 6 and 7). In contrast to the PS1$^{-/-}$, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells, neither proteasome nor lysosome inhibitors had a significant effect on the levels of CTFα(endo) and CTFα-myc in PS1/2$^{-/-}$ cells (fifth panel), NCT$^{-/-}$ cells (sixth panel), nor Pen-2$^{-/-}$ cells (seventh panel), indicating that APP CTFs were not significantly degraded by these organelles in these cells. A small amount of CTFβ-myc was also detected in these cells, specifically in cells treated with MG132 and lysosome inhibitors (lanes 7–10). The above results clearly indicate

that the effects of proteasome and lysosome on the turnover of full-length APP and APP CTFs vary in different knockout cells.

γ-secretase-catalyzed CTFα processing in Aph-1$^{-/-}$ cells is independent of proteasome and lysosome activity

Data presented in Figure 1. 2a show that AICD was detected in Aph-1$^{-/-}$ cells as well as in wt and PS2$^{-/-}$ cells in the presence of lysosome inhibitors, indicating that γ-secretase activity was not affected by these lysosome inhibitors. In other words, γ-secretase-catalyzed processing of CTFα is independent of lysosome activity in these cells. To further determine whether the γ-secretase inhibitors compound E and L-685,458 caused accumulation of CTFα in Aph-1$^{-/-}$ cells was not due to inhibition of proteasome or lysosome activity, we performed the following experiments. As shown in Figure 1. 3a, the amount of CTFα accumulated in cells treated with both compound E and MG132 (lane 7) was roughly the sum of the CTFα detected in cells treated with compound E (lane 2) and MG132 (lane 4), separately. A similar result was also observed when L-685,458 was used in combination with MG132 compared with L-685,458 and MG132 alone (compare lane 9 with lanes 3 and 4). During the course of the experiments, it was noted that treatment with MG132 could induce the activation of caspase, which has been implied in the turnover of APP CTFs (Weidemann, Paliga et al. 1999). This raised the question as to whether inhibition of caspase activation would lead to further accumulation of CTFα in cells treated with MG132. Indeed, a greater amount of CTFα was observed in MG132-treated cells

in the presence of pan caspase inhibitor Z-VAD (compare lane 6 with lane 4). When these cells were further treated with compound E, an even greater amount of CTFα was accumulated (compare lane 8 with lane 6). A similar result was also observed when L-685,458 was added with MG132 and Z-VAD (compare lane 10 with lane 6). These results indicate that γ-secretase inhibitor and proteasome inhibitor have an additive effect on the accumulation of unprocessed CTFα through different mechanisms. Furthermore, it was noted that in addition to regular PS1C produced by normal endoproteolytic processing of PS1, a short C-terminal fragment of PS1, CaspPS1C, which was produced by caspase activity (Zeng, Hu et al. 2015), was detected in cells treated with MG132 (lanes 4, 7, and 9), and the formation of CaspPS1C was completely inhibited by the addition of pan caspase inhibitor Z-VAD (lanes 6, 8, and 10).

Next, we examined the additive effect of γ-secretase inhibitors and lysosome inhibitors on the accumulation of unprocessed CTFα. As shown in Figure 1. 3b, the amount of CTFα accumulated in the cells treated with both compound E and chloroquine (lane 6) was roughly the sum of the CTFα detected in cells treated with compound E (lane 2) and chloroquine (lane 4), separately. A similar result was also observed when cells were treated with L-685,458 and chloroquine (compare lane 7 with lanes 3 and 4). Likewise, leupeptin exhibited a similar additive effect on CTFα accumulation when used in combination with compound E (compare lane 8 with lanes 2 and 5) and L685, 458 (compare lane 9 with lanes 3 and 5). These data indicate that γ-secretase inhibitor-caused accumulation of

CTFα in Aph-1$^{-/-}$ cells is not due to inhibition of proteasome or lysosome, i. e., γ-secretase-catalyzed CTFα processing in Aph-1$^{-/-}$ cells is independent of proteasome and lysosome activity.

Aph-1, as well as nicastrin is, dispensable for γ-secretase-catalyzed processing of Notch

Data presented above demonstrate that Aph-1 is not absolutely required for γ-secretase-catalyzed APP CTF processing, while NCT and Pen-2 are crucially essential for this process. In addition to APP, Notch is another well-characterized substrate of γ-secretase. We next examined the effect of knockout of different components of the γ-secretase complex on the processing of Notch. To do so, cells were transfected with a plasmid expressing NotchΔE, the ectodomain-truncated and myc-tagged Notch containing the murine Notch-1 leader peptide (1-23 amino acids) (Kopan, Schroeter et al. 1996) in the presence or absence of γ-secretase inhibitor L-685,458. As shown in Figure 1. 4a, recombinant NotchΔE was detected with anti-myc antibody at various levels in wild-type and knockout cells, possibly due to different transfection efficiency. As shown in the middle panel, NICD, which is produced by γ-secretase from NotchΔE, was detected in wild-type cells (lane 13), PS2$^{-/-}$ cells (lane 9), and PS1$^{-/-}$ cells (lane 7), Aph-1$^{-/-}$ cells (lane 1), and NCT$^{-/-}$ cells (lane 3), and the formation of this NICD was strongly inhibited by the addition of L-685,458 (lanes 2, 4, 8, 10, and 14). However, this NICD was not detected in PS1/2$^{-/-}$ cells (lane 11) nor Pen-2$^{-/-}$ cells

(lane 5). These results revealed an interesting finding that, under our experimental conditions, NCT is crucially essential for γ-secretase-catalyzed APP CTFs processing, but is not absolutely required for γ-secretase-catalyzed Notch processing.

Generation of NICD from NotchΔE is not affected by proteasome and lysosome inhibitors

Data presented in Figure 1. 2 demonstrate that CTFα was also degraded by proteasome and lysosome in a γ-secretase-independent mechanism. Next, we determined whether Notch is also subjected to proteasome and/or lysosome degradation and whether proteasome and lysosome inhibitors have any effect on NICD formation. As shown in the middle panel of Figure 1. 4b, γ-secretase inhibitors compound E (lane 4) and, specifically, L-685,458 (lane 5) strongly inhibited the formation of NICD from NotchΔE. However, the level of NICD in proteasome inhibitors-treated cells was slightly increased (lanes 6 and 7), likely due to the protection of NICD from degradation, while the lysosome inhibitors showed no effect on the generation of NICD (lanes 8–10). In addition, the level of unprocessed NotchΔE was also slightly increased in proteasome inhibitor-treated cells (top panel, lane 6 and 7), suggesting that, though to lesser extent, NotchΔE also underwent proteasome degradation. Taking together, these data suggest that both NotchΔE and NICD undergo proteasome degradation, but the proteasome and lysosome inhibitors have no effect on γ-secretase-catalyzed processing of Notch.

Recovery of PS1C does not necessarily restore the γ-secretase activity toward APP in NCT$^{-/-}$ cells.

Previous study revealed that Pen-2, Aph-1, and NCT are not necessary for endoproteolytic processing of PS1, but are required for stabilization of the PS1 endoproteolytic processing products PS1N and PS1C (Mao, Cui et al. 2012). Thus, it is speculated that the loss of γ-secretase activity toward CTFα and CTFβ might have resulted from the instability of endoproteolytic products of PS1 in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells. As shown in the top panel of Figure 1. 5, in the absence of MG132, PS1C was detected in wt cells (lane 9), in PS2$^{-/-}$ cells (lane 5), and, to a lesser but significant extent, in Aph-1$^{-/-}$ cells (lane 1). A very low level of PS1C was detected in NCT$^{-/-}$ cells at (lane 3), and only a trace amount of PS1C was detected in Pen-2 cells (lane 7). As expected, no PS1C was detected in PS1$^{-/-}$ cells (lane 11). This result confirmed again that Pen-2 is crucial for stabilizing PS1C. This result also revealed that Aph-1 is less important for stabilizing the endoproteolytic products of PS1.

When the cells were treated with MG132, a significant decrease in the level of PS1C and a concomitant significant increase in the level of CaspPS1C produced by caspase activity were detected in wt cells (lane 10), PS2$^{-/-}$ cells (lane 6), and Aph-1$^{-/-}$ cells (lane 2). In the presence of MG132, CaspPS1C was also detected in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells (lanes 4 and 8). However, in contrast to wt, PS2$^{-/-}$, and Aph-1$^{-/-}$ cells, the increase in CaspPS1C was not associated with a decrease, but rather an increase in the regular PS1C in NCT$^{-/-}$ cells (compare lane 4 with lane

3) and Pen-2$^{-/-}$ cells (compare lane 8 with lane 7). Interestingly, as shown in the bottom panel, the high levels of unprocessed CTFα(endo), CTFα-myc, and CTFβ in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells were not affected by the addition of MG132. These results indicate that recovery of PS1C does not necessarily restore γ-secretase activity toward CTFα and CTFβ. In other words, Pen-2 and, specifically, NCT, as essential components of γ-secretase, must play a direct role in γ-secretase activity in addition to their roles in stabilizing PS1 proteolytic products. In this regard, NCT has been proposed to function as a substrate receptor (Shah, Lee et al. 2005).

Discussion

Previous studies using reconstitution and knockdown approaches have suggested that the four proteins, presenilin (PS1 or PS2), NCT, Aph-1, and Pen-2, are necessary and sufficient for γ-secretase activity (Edbauer, Winkler et al. 2003, Kimberly, LaVoie et al. 2003, Takasugi, Tomita et al. 2003). However, this view was challenged by a recent study showing that Notch was processed in a γ-secretase-dependent manner in NCT-deficient cells, suggesting that NCT is not absolutely required for γ-secretase activity (Zhao, Liu et al. 2010). In the current study, by taking advantage of the availability of all cell lines deficient in one of the four components of the γ-secretase complex, we performed a series of experiments to attempt to address this controversial issue. Using these cells, our results demonstrated that knockout of PS2 had almost no effect on APP CTFs

processing and that, in contrast, knockout of PS1 strongly inhibited APP CTF processing as determined by the turnover of CTFα, as well as the formation of AICD and $A\beta_{40}$. These observations confirmed that PS1 accounts for the majority of γ-secretase activity that catalyzes the processing of APP CTFs. In addition, our results revealed several interesting findings. First, our data demonstrate that, in contrast to $NCT^{-/-}$ and $Pen\text{-}2^{-/-}$ cells, in which no significant CTFα turnover and only a small amount of $A\beta_{40}$ was detected, similar to wt cells, a low level of CTFα and significant amount of AICD were detected in $Aph\text{-}1^{-/-}$ cells. Also, the turnover of CTFα and the formation of AICD were strongly inhibited by γ-secretase inhibitor, suggesting that the turnover of CTFα and the formation of AICD in $Aph\text{-}1^{-/-}$ cells, as well as in wt cells, were catalyzed by γ-secretase. In addition, another γ-secretase substrate, Notch, was also processed in a γ-secretase-dependent manner in $Aph\text{-}1^{-/-}$ cells. Furthermore, based on the levels of $A\beta_{40}$ determined by ELISA, it is assumed that over 50% of γ-secretase activity was retained in $Aph\text{-}1^{-/-}$ cells. Although it cannot be ruled out that the trace amount of the residual Aph-1c, which was not detectable at the protein level under our experimental conditions, may contribute to a small portion of the γ-secretase activity in $Aph\text{-}1^{-/-}$ cells, all these observations strongly suggest that Aph-1 is not absolutely required for γ-secretase activity. In addition, albeit at a very low level, the detection of $A\beta_{40}$ by ELISA in $NCT^{-/-}$ and $Pen\text{-}2^{-/-}$ cells suggests that deletion of one of these two components does not completely abolish γ-secretase activity. Thus, it is very likely that Aph-1, NCT, and Pen-2 are all required for achieving

maximal γ-secretase activity; however, Aph-1 is less crucial than NCT and Pen-2 for the enzymatic activity in this γ-secretase complex.

It is proposed that γ-secretase harbors both endopeptidase-like and carboxypeptidase-like activities, catalyzing a series of sequential cleavages of APP and leading to the generation of Aβ peptide. In this model, APP is first cleaved at the ε-cleavage site by endopeptidase-like activity to release the APP intracellular c-terminal domain, AICD, and generate the membrane-bound, long $Aβ_{49}$ peptide, which is further sequentially chopped down roughly every three residues by carboxypeptidase-like activity to produce the secreted $Aβ_{40}$ and $Aβ_{42}$ and other minor, shorter Aβ species (Xu 2009). Previous studies suggest that Aph-1 might function as a scaffold involved in γ-secretase complex assembly and maturation (LaVoie, Fraering et al. 2003, Luo, Wang et al. 2003) and in the binding of substrate (Chen, Guo et al. 2010, Mao, Cui et al. 2012). In determining the specific roles of different isoforms of Aph-1 in γ-secretase-catalyzed APP processing, recent studies further suggest that Aph-1 mainly affects the carboxypeptidase-like activity that catalyzes the sequential cleavages following the initial cleavage at the ε-site and determines the C-termini of Aβ species; specifically, γ-secretase complexes containing the Aph-1b isoform favor the generation of longer Aβ peptides (Serneels, Dejaegere et al. 2005, Serneels, Van Biervliet et al. 2009, Acx, Chávez-Gutiérrez et al. 2014). This notion might provide justification for our finding that Aph-1 is dispensable for the endopeptidase-like activity of γ-secretase that catalyzes the initial cleavage of

CTFs at the ε-site, which is a decisive step in γ-secretase-catalyzed APP processing (Xu 2009).

The second important finding of the current study is the differential requirement for NCT in γ-secretase-catalyzed processing of APP and Notch. To elucidate the specific function of NCT, a well-designed study revealed that the extracellular domain of NCT is essential for recognition of the substrate of γ-secretase, suggesting that NCT functions as a receptor of substrate (Shah, Lee et al. 2005). However, a recent study showing that cells deficient in NCT were capable of processing Notch and, to a lesser extent, APP in a γ-secretase-dependent manner raised a question as to whether NCT is absolutely required for γ-secretase activity (Zhao, Liu et al. 2010). Using the same $NCT^{-/-}$ cells and the same truncated Notch-expressing plasmid as used in Zhao et al's study, our results revealed a similar finding that Notch was processed by γ-secretase activity in the absence of NCT. In addition, our results revealed that Aph-1 was also not absolutely required for Notch processing. However, in contrast to the previous study, our data demonstrate that knockout of NCT completely abolished γ-secretase-catalyzed processing of CTFα and CTFβ produced from both endogenous and recombinant APP.

These controversial observations might have resulted from the use of different experimental systems. Specifically, in the previous study, a transiently-expressed truncated APP (C99), an artificial CTFβ, was used as a γ-secretase substrate to determine the effect of knockout of NCT on the formation of AICD

from C99. In contrast, in the current study, we examined the processing of CTFα and CTFβ produced either from endogenous APP or recombinant full-length APP. After synthesis, full-length APP undergoes multiple post-translational modifications including N- and O-glycosylation, phosphorylation, and tyrosine sulphation, and these modifications not only affect the trafficking but also the processing of APP along the secretory pathway as well as the endocytotic pathway (Jiang, Li et al. 2014). It is not known whether the overexpressed C99 also undergoes similar post-translational modification and is processed at the same subcellular locations as full-length APP. Whether possible differences in post-translational modification and trafficking may account for the discrepancy between results of the current study and that reported by Zhao et al awaits further investigation. Nevertheless, the data presented in this study strongly suggest that NCT is crucially essential for γ-secretase-catalyzed processing of CTFα and CTFβ produced from full-length APP, but that NCT is not absolutely required for Notch processing. Supporting our finding, a recent study reported that mutations in NCT differentially affect Aβ production and Notch processing (Pamrén, Wanngren et al. 2011). Thus, this differential requirement for NCT in γ-secretase-catalyzed processing of APP and Notch suggests NCT as a therapeutic target for developing a strategy to restrict Aβ formation in AD without impairing Notch signaling.

The third notable finding of the current study is that components of the γ-secretase complex essential for γ-secretase-catalyzed APP processing are also

important for proteasome- and lysosome-dependent degradation of APP derivatives. Previous studies have reported that, in addition to γ-secretase-catalyzed processing, APP and CTFs of APP are also subjected to proteasome and lysosome degradation (Skovronsky, Pijak et al. 2000, Nunan, Shearman et al. 2001, Vingtdeux, Hamdane et al. 2007, Watanabe, Hikichi et al. 2012, Wang, Sang et al. 2015). In the current study, as shown in Figure 1. 1 and 1. 2, our data demonstrate that proteasome inhibitor MG132 and, specifically, lysosome inhibitors chloroquine, leupeptin, and NH_4Cl caused marked accumulation of unprocessed APP CTFs in wild-type cells. A similar effect of these inhibitors on the accumulation of APP CTFs was also observed in $PS1^{-/-}$, $PS2^{-/-}$, and $Aph\text{-}1^{-/-}$ cells, which all expressed the γ-secretase activity that catalyzes the processing of APP CTFs. However, the effects of these inhibitors on the accumulation of the APP CTFs was less significant in $PS1/2^{-/-}$, $NCT^{-/-}$, and $Pen\text{-}2^{-/-}$ cells, in which no γ-secretase-catalyzed APP processing was observed. These findings strongly indicate that presenilin (PS1 or PS2), NCT, and Pen-2, which are essential for γ-secretase-catalyzed APP processing, are also important for proteasome- and lysosome-dependent degradation of APP CTFs. One possibility is that γ-secretase activity is involved in the proteasome- and lysosome-dependent degradation of APP CTFs. However, this is very unlikely in light of the fact that γ-secretase inhibitors and the proteasome and lysosome inhibitors exhibited additive effects on the accumulation of APP CTFs. Recent studies reported that presenilin is necessary for efficient protein degradation by lysosome in a γ-

secretase-independent manner (Lee, Yu et al. 2010, Neely, Green et al. 2011, Coen, Flannagan et al. 2012, Zhang, Garbett et al. 2012). In this regard, it is noteworthy that our results suggest that lysosome plays a major role in degradation of APP CTFs. Therefore, the inefficient degradation of APP CTFs in PS1/2$^{-/-}$ cells is likely due to impaired lysosome function caused by deficiency of presenilin. Since NCT and Pen-2 are essential for stabilizing presenilin (Mao, Cui et al. 2012), the ineffective lysosomal degradation of APP CTFs in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells might have resulted from the instability of presenilin in these cells. It is also noted that the level of PS1C in Aph-1$^{-/-}$ cells is much higher than that in NCT$^{-/-}$ and Pen-2$^{-/-}$ cells, and this might account for the fact that lysosomal degradation of APP CTFs was observed in Aph-1$^{-/-}$ cells. However, it cannot be ruled out that NCT and Pen-2 may be directly involved in PS1-regulated lysosome function rather than simply stabilizing PS1C. In addition, our results strongly suggest that endogenous and exogenous APPs undergo degradation by different mechanisms, i.e., endogenous APP mainly undergoes lysosome-dependent degradation, whereas, exogenously expressed APP is primarily degraded by proteasome.

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Appendix

Figure 1. 1 Aph-1 is dispensable for γ-secretase catalyzed APP processing. Cells were cultured in the presence and absence of γ-secretase inhibitor L-685,458 (a) or MG132 (b) overnight, lysed, and subjected to 16% SDS-PAGE and Western blot analysis using antibody C15 that was raised against the very c-terminal 15 residues of APP. The membranes were also reprobed with anti-GAPDH to indicate even loading of the samples (bottom panels). All data presented in this study are representative of at least three independent experiments. (c) Cell-free assay for *in vitro* generation of AICD. AICDendo: AICD produced from endogenous APP; AICDmyc: AICD produced from myc-tagged exogenous APPSw in a wt-7 stable cell line, which was used as a positive control. (d) Effect of knockout of different components of γ-secretase on Aβ formation. Aliquots of CM samples of knockout cells were subjected to ELISA to detect $A\beta_{40}$. A significant amount of $A\beta_{40}$ was detected in Aph-$1^{-/-}$ cells, as well as in wt cells. Low amount $A\beta_{40}$ was also detected in PS$1^{-/-}$ cells, and even lower $A\beta_{40}$ was detected in NCT$^{-/-}$ and Pen-$2^{-/-}$ cells. $N = 3$, $^{*}p < 0.01$; $^{**}p < 0.001$. (e) Western blot analysis of protein levels of γ-secretase components in knockout cells. (f) RT-PCR analysis of NCT and Aph-1 genes in corresponding knockout cells. Note: Since Aph-1c is the duplicate of Aph-1b in mice, the antibody against Aph-1b also detects Aph-1c, and the RT-PCR primers used are also common to both Aph-1b and Aph-1c.

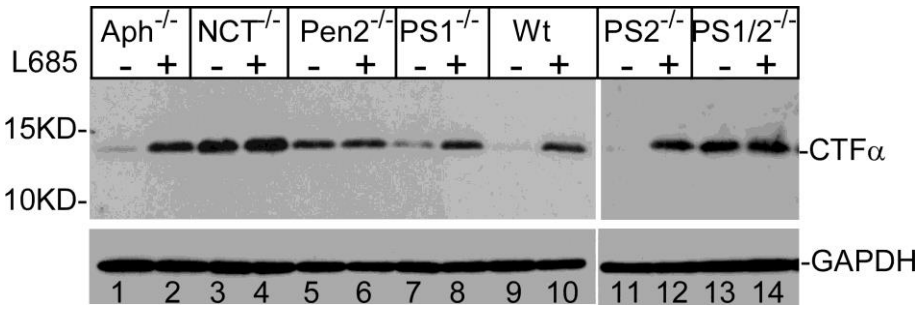
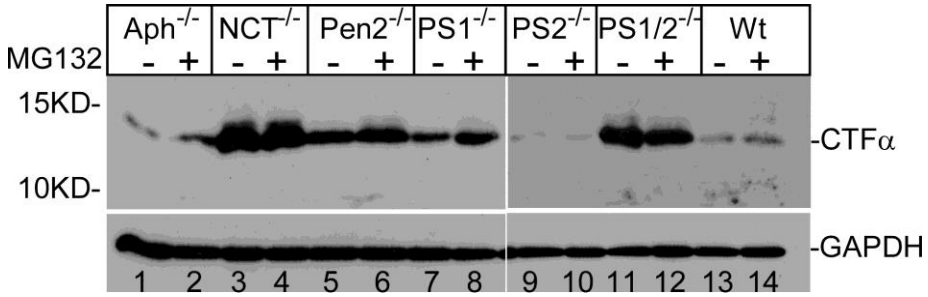


Figure 1.1 a



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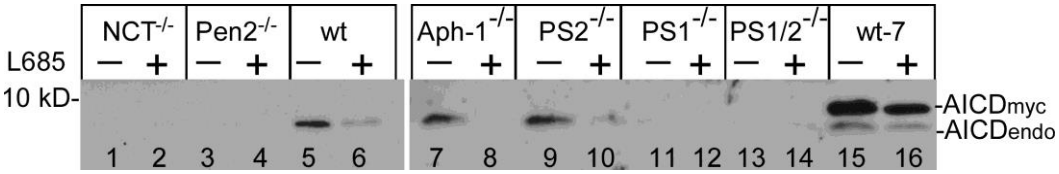

Figure. 1. 1 c

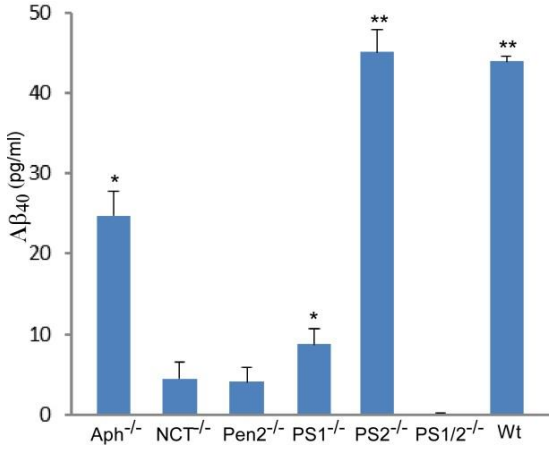

Figure 1. 1d

Figure 1.1 continued

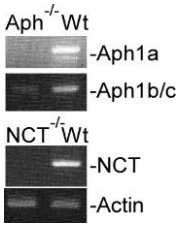

Figure 1. 1e

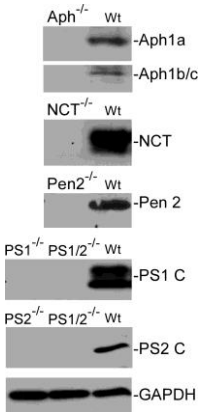

Figure 1. 1f

Figure 1.1 continued

Figure 1.2 Components of the γ-secretase complex also play a role in regulating APP degradation by proteasome and lysosome. (a) Effects of γ-secretase, proteasome, and lysosome inhibitors on the accumulation of unprocessed endogenous CTFα. The first lane is the vehicle-treated control. The last lane is the sample prepared from wt-7 cells treated with γ-secretase inhibitor compound E (compE) used as standards of CTFβ-myc and CTFα-myc. (b) In lanes 3–10, cells were transfected with human APPsw expression plasmid. In lane 2, cells were transfected with unrelated protein LacZ. In lane 1, cells were mock transfected with an empty vector. Lane 11 is the sample prepared from wt-7 cells treated with compound E used as standards of CTFβ-myc and CTFα-myc. All APP CTFs were detected using C15. (c) Quantitative analysis of the formation and turnover of APP-CTFs. Results are expressed as the mean (± SD) of three independent Western blot results shown in Figure 1. 2b.

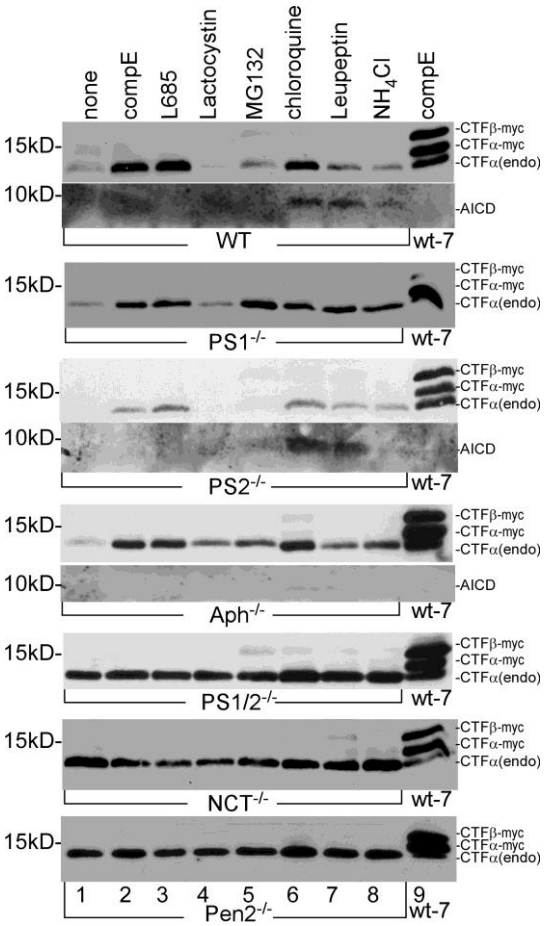

Figure 1. 2a

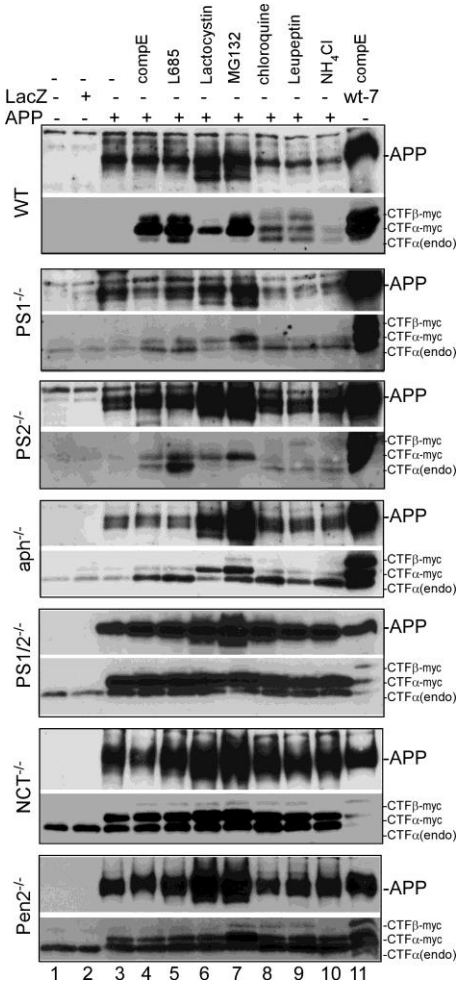

Figure 1. 2b

Figure 1.2 continued

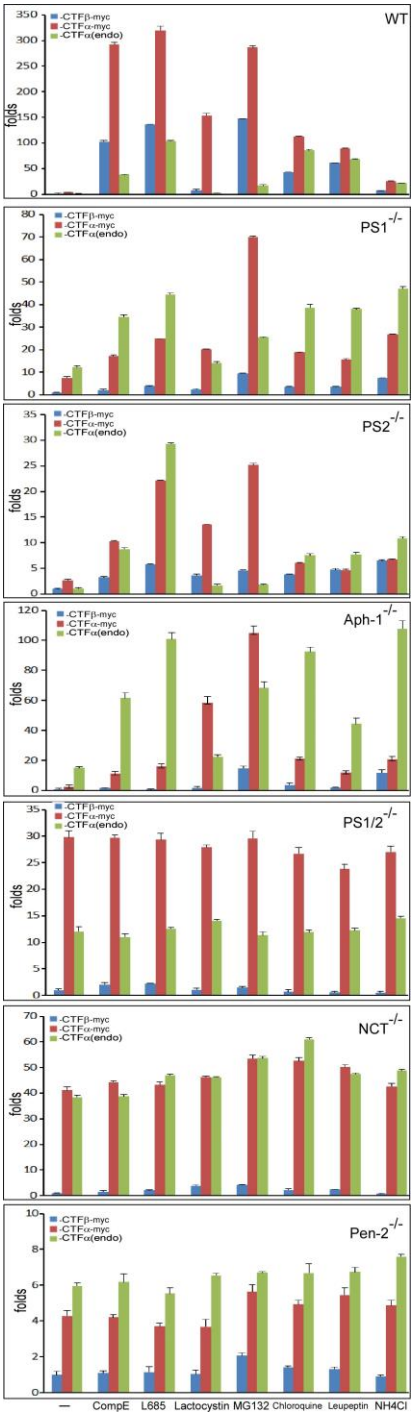

Figure 1. 2 c

Figure 1.2 continued

Figure 1.3 γ-secretase, proteasome, and lysosome inhibitors have an additive effect on CTFα accumulation in Aph-$1^{-/-}$ cells. (a) Cells in lanes 2–10 were cultured in the presence of γ-secretase inhibitors and proteasome and caspase inhibitors either individually or in combination. Top panel, immunoblot probed with C15; second panel, immunoblot probed with anti-caspase-3 to detect the formation of the active form of caspase-3; third panel, immunoblot probed with anti-caspase-6 to determine the reduction of pro-caspase-6 due to activation; fourth panel, immunoblot probed with anti-PS1C, which reacts with both regular PS1C and the caspase-produced CaspPS1C (#5643 from Cell Signaling); the immunoblot in the fourth panel was also reprobed with anti-GAPDH to indicate relative loading of samples (bottom panel). Lane 11 is the sample from wt-7 cells cultured in the presence of compound E. (b) Cells in lanes 2–10 were cultured in the presence of γ-secretase inhibitors and lysosome inhibitors either individually or in combination. Top panel, immunoblot probed with C15; bottom panel, this immunoblot was reprobed with anti-GAPDH. Lane 11 is the sample from wt-7 cells cultured in the presence of compound E.

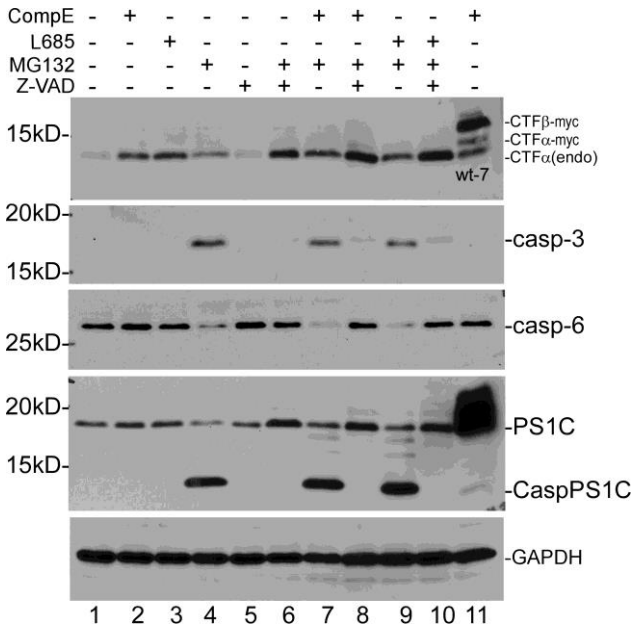

Figure 1. 3 a

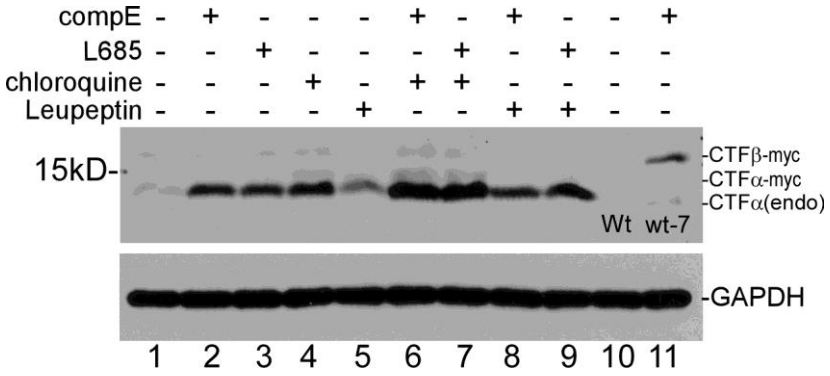

Figure 1. 3 b

Figure 1.3 continued

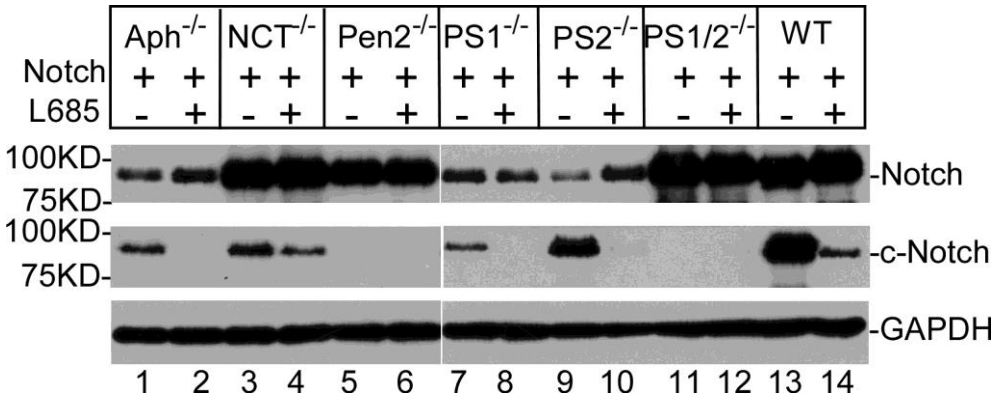

Figure 1. 4 a

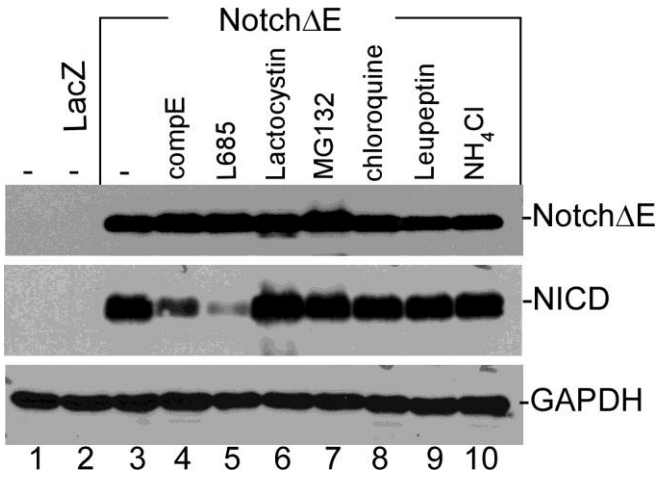

Figure 1. 4 b

Figure 1.4 Aph-1 and nicastrin are not essential for γ-secretase catalyzed processing of Notch. (a) Aph-$1^{-/-}$ cells were transfected with a plasmid expressing N-terminal truncated Notch with a C-terminal myc tag. Top panel, immunoblot probed with anti-myc to detect the unprocessed recombinant Notch. Middle panel, immunoblot probed with antibody, which specifically recognizes the N-terminus of NICD generated by γ-secretase processing. Bottom panel, immunoblot in the middle panel reprobed with anti-GAPDH. (b) Proteasome and lysosome have no significant effect on Notch metabolism. Top panel, immunoblot probed with anti-myc to determine the levels of NotchΔE in the presence of different inhibitors; middle panel, immunoblot probed with anti-NICD; bottom panel, immunoblot in middle panel was reprobed with anti-GAPDH.

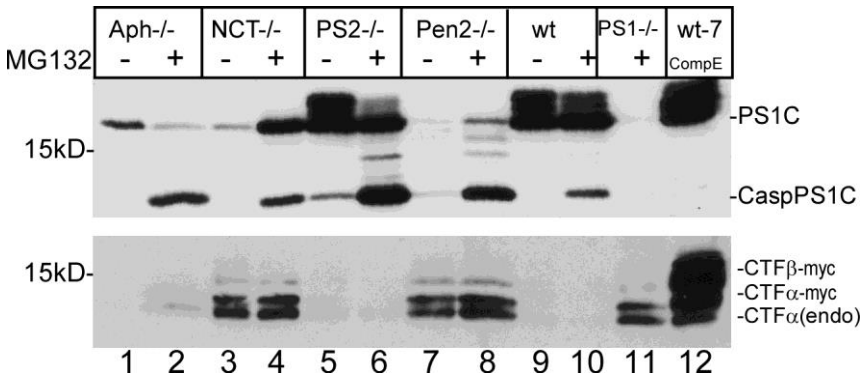

Figure 1. 5

Figure 1.5 Recovery of PS1C does not necessarily restore the γ-secretase activity toward APP in $NCT^{-/-}$ cells. Knockout cells were cultured in the presence or absence of MG132. Up panel, immunoblot probed with anti-PS1C that recognizes both regular and caspase produced PS1C; bottom panel, immunoblot probed with C15. Lane 12 is the sample prepared from wt-7 cells treated with compound E used as standards of CTFβ-myc and CTFα-myc.

CHAPTER II

THE ROLES OF GAMMA SECRETASE COMPONENTS IN APOPTOSIS AND THE FUNCITONS OF VARIED LENGTH OF PS1C

Abstract

In the previous chapter, we investigated the γ-secretase activity in various knock out cells. In this chapter, we found that the apoptosis in these knockout cells varied too. Aph-$1^{-/-}$ cells show a higher susceptibility to apoptosis, induced by MG132 via downregulation of Akt-mediated anti-apoptotic pathway. We also found several forms of PS1C, but the function of them remain unclear. In this chapter, we also used different methods to investigate the function of these forms in γ-secretase activity. The results of transfecting varied forms of PS1C back to PS1/$2^{-/-}$ double knockout cells shown that all the PS1C forms could process Notch when transfecting together with PS1N. However, only the shorter form of $PS1C_{299}$ shown significant effects on restoring γ–secretase activity toward APP processing. The phosphorylation of PS1C does not show significant effect on γ–secretase catalyzed CTFα processing under our experimental conditions.

Introduction

MG132, which is known as a proteasome inhibitor, could also induce apoptosis and autophagy at higher concentration through the upregulation of ER stress in various cells (Park, Jun do et al. 2011, Bao, Gu et al. 2016). Treatments of MAP kinase (JNK, p-38, MEK and so on) inhibitors were reported to be able to reduce apoptosis induced by MG132 (You and Park 2011), suggesting that these kinases contribute to MG132-induced apoptosis. Numbers of transcriptional

factors like NF-κB, p53 and stat3 were reported to be involved in the autophagy induced by proteasome inhibitors (Saji, Higashi et al. 2011, Pietrocola, Izzo et al. 2013). The addition of MG132 could induce the activation of a series of apoptosis related protein: like caspase 3, 7, 9, Bak, PARP and so on (Park, Jun do et al. 2011). Unlike kinases mentioned above, which promote apoptosis upon activation, the activation of Akt was shown to suppress apoptosis (Qin, Wang et al. 2010, Kahana, Finniss et al. 2011). Like ERK and PKC, Akt is regarded as a suppressive kinase in cell death induction (Scanga, Ruel et al. 2000, Franke, Hornik et al. 2003). Activated Akt (p-Akt) can phosphorylate and inactivate GSK3 (Ruvolo, Qiu et al. 2015). Therefore, the level of phosphorylated GSK3β could be used as an indicator for the activation of Akt.

In this study, we found that the same concentration of MG132 could induce varied level of apoptosis in different knock out cells. Among these cells, Aph-$1^{-/-}$ cells have a higher tendency of apoptosis based on the levels of PARP cleavage and activation of caspase 3 and caspase 7. The phosphorylation levels of kinases which mediate apoptosis, like JNK, p-38, c-Jun (Ferraris, Isoniemi et al. 2012) and transcriptional factor CHOP (Teske, Fusakio et al. 2013) were found increased in Aph-$1^{-/-}$, NCT$^{-/-}$, Pen-$2^{-/-}$, PS$1^{-/-}$, PS$2^{-/-}$ and WT cells. But unlike the other knock out cells, the level of the active form of the apoptosis suppressive kinase p-Akt is reduced in Aph-$1^{-/-}$ cells. The downregulation of p-Akt in the absence of Aph-1 might account for the higher tendency of apoptosis in Aph-$1^{-/-}$ cells. Based on the previous results, p-GSK3 located downstream of p-Akt is

phosphorylated by p-Akt. Therefore, the reduction of p-GSK3 is consistent with the reduction of p-Akt and further confirmed that the downregulation of p-Akt in the absence of Aph-1 upon treatment with MG132.

PS1 was first reported of going through endoproteolysis at 1996 and the cleavage sites range from 260aa to 320aa (Thinakaran 1996). Later report identified the more specific processing sites located on aa292. Since the mutation at aa 292 could almost abolish the endoproteolysis of PS1 (Steiner, Romig et al. 1999). Further investigation revealed that like γ-secretase substrate APP, the endoproteolyis of PS1 also follows a three-amino acid spacing manner and occurs sequentially at multiple sites at aa 293, aa 296 and aa 299 (Xu 2009, Fukumori, Fluhrer et al. 2010).

An apoptotic fragment of PS1 and PS2 were first reported of cleavage results by caspase 3. And the cleavage sites in PS1 was reported within 343aa to 346aa (Kim 1997). Two years later, besides the above one, another cleavage site was detected at 329 aa which is processed by the group of caspase 8, 6 and 11 (Craen 1999). In addition to the normal PS1C and caspase cleaved PS1Cs, phosphorylated PS1Cs were also reported. For example, the one phosphorylated by GSK3β at Ser^{353}, Ser^{357} or Ser^{397}, Ser^{401} (Kirschenbaum, Hsu et al. 2001, Twomey and McCarthy 2006), the one phosphorylated by cyclin-dependent kinase 5/p35 (cdk5/p35) on Thr^{354}(Lau, Howlett et al. 2002), a PKC phosphorylated one on Ser^{346}(Fluhrer, Friedlein et al. 2004) and so on. A recent paper have check almost all the phosphorylated PS1 and conclude that these

phosphorylation of PS1 do not modulate the activity of γ-secretase and therefore should not be considered as targets of AD treatment (Matz, Halamoda-Kenzaoui et al. 2015).

Based on our previous results and the papers mentioned about all the PS1 fragments above, including PS1N (1-292aa), PS1C ($PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$, $PS1C_{346}$ and phosphorylated PS1), we suspect that they might have different effects on γ-secretase activities. Therefore, we designed several experiments to determine their function. First, we used phosphatase to remove the phosphorylation of PS1C and check the activity of PS1C at process CTFs and found that the phosphorylation of PS1C does not contribute to gamma secretase activity. Then we also constructed different forms of PS1Cs, the $PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$ and $PS1C_{346}$ and a PS1N (1-292aa). We transfected these PS1Cs in combination with PS1N back to $PS1/2^{-/-}$ cell together with NotchΔE or APPsw. We found that the transfection with PS1C (no matter which form) alone or PS1N alone could not process NotchΔE or APP. While, when both PS1N and PS1C (no matter which PS1C form) were transfected into cells together, processing of NotchΔE was readily detectable. However, the ability of these PS1Cs to process APP was different. Our results demonstrated that the $PS1C_{299}$ is the most effective form in processing APP in comparison with other PS1C of different length. This result suggests that $PS1C_{299}$ might contribute more to APP processing.

Materials and Methods

Cell culture

MEF cells we used in this part were the same from the last chapter: including Aph-$1^{-/-}$, NCT$^{-/-}$, Pen-$2^{-/-}$, PS$1^{-/-}$, PS$2^{-/-}$, PS$1/2^{-/-}$, wild type (wt) and wt-7 cells. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MA, USA) which containing 10% fetal bovine serum, 2 mM L-glutamine (Lonza), 100 units/mL penicillin (Lonza), and 100 μg/mL streptomycin (Lonza).

Inhibitors and reagents

We used proteasome inhibitor MG132 and gamma-secretase inhibitor Compound E in this chapter and like which mentioned in last chapter: MG132 was purchased from Peptides International (Louisville, KY, USA), Compound E and DAPM were purchased from EMD Millipore (Billerica, MA, USA). Complete protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN, USA). Lipofectamine LTX and plus reagent was purchased from Invitrogen (Carlsbad, CA, USA). Phosphatase was purchased form Sigma-Aldrich (St. Louis, MO, USA).

Antibodies

Antibodies which were anti-PS1C_{5643}, PS1C_{3622}, c-Notch (#4147, which specifically recognizes the processed Notch), PARP, caspase3, caspase-7, p-cJun, p-JNK, JNK, p-p38, p38, CHOP(C/EBP-homologous protein), p-Akt, Akt,

and p-GSK3 (Ser-9) were purchased from Cell Signaling (Danvers, MA). Polycolonal antibody C15 was raised against the last 15 amino acids at the very C terminal of APP (Zhao, Mao et al. 2004). Polycolonal antibody 6E10 was purchased from Covance (Princeton, NJ, USA). Anti-myc antibody, C-Myc (9E10), was purchased from Santa Cruz (Dallas, TX, USA). Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was from EMD Millipore (Billerica, MA).

Plasmids

We used NotchΔE and APPsw plasmid which as mentioned in last chapter: plasmid expressing the extracellular region truncated and myc-tagged Notch molecule (NotchΔE) containing the murine Notch-1 leader peptide (1-23 aa) (Kopan, Schroeter et al. 1996) was kindly provided by Raphael Kopan (Washington University) and Dr. Masayasu Okochi (Osaka University, Japan). The plasmid APPsw, which expresses a C-terminal myc-tagged Swedish mutant APP (APPsw) (Thinakaran, Teplow et al. 1996), was kindly provided by Dr Gopal Thinakaran (University of Chicago). The plasmids which expressing the PS1N terminal (1-292aa), PS1C terminals: $PS1C_{293}$ (293-467aa), $PS1C_{296}$ (296-467aa), $PS1C_{299}$ (299-467aa), $PS1C_{334}$(334-467aa), $PS1C_{346}$ (346-467aa) were constructed in our lab and sequenced to confirm.

SDS-PAGE and Western blotting

For analysis of apoptosis and autophagy related proteins, 10 h after splitting, cells were incubated overnight in the presence or absence of MG132 at different concentrations (check the legends for concentration details). For the analysis of the activity of gamma secretase, gamma secretase inhibitor compound E (5nM) was applied the same like above. For analysis of the exogenous APP and Notch processing through different PS1C and PS1N terminals, the cells, 24 h after splitting, were transfected with plasmids expressing APPsw or NotchΔE with lipofectamine LTX. Twelve hours after the transfection of APPsw or NotchΔE, PS1N and PS1C terminals were transfected and the cells were further incubated for about 24 hours. For the detection of the function of phosphorylated PS1C, cells were grown for 24 hours and then collected with dephosphorylation buffer (suggested by sigma) and incubated in 37℃ for 2 hours with inhibitors and phosphatase (check figures and figure legend for details). Cell lysis and Western blot analysis were carried out as described previously (Zhao, Mao et al. 2004), which is consistent to which described briefly in last chapter. The membranes were probed with appropriate antibodies as described in Figure legends.

Results

Presenilin 1 C terminal levels varied in different knock out cells with the addition of MG132.

As a proteasome inhibitor, MG132 could block the degradation of PS1 C terminals from degrading through proteasome pathway. Therefore, the

accumulation of PS1 C supposed to be observed with the presence of MG132 in the knock out cells. Increased levels of PS1 C were observed in NCT knock out (Figure 2.1 upper panel, compare lane 4 with lane 3) and Pen-2 knock out cells (lane6 with lane5), even the volume differed. In PS2 knock out cells and WT cells, the level of regular PS1C seemed unchanged with the detection of one antibody (5643 from cell signaling, upper panel, compare lane 10 with lane 9, lane 12 with lane 11). But the levels of PS1 C were increased with the detection of another antibody (3622 from cell signaling, the lower panel), which is due to the fact that different epitopes were recognized by these two antibodies. Antibody 3622 could recognize the PS1 C from aa293 to aa313, based on our experiment, it could only recognize the longer form of PS1 C, which is from the amino acid 293 (aa) to aa467 ($PS1C_{293}$ in short), but could not recognize the other forms, like the peptide start from aa 296 to aa467 ($PS1C_{296}$ in short), aa299 to aa467 (299 in short) or the even shorter forms which are processed by caspases (picture2.1, upper panel). Based on the results detected by antibody 3622, the level of longer form: $PS1C_{293}$ was marginally expressed in different knock out cells and WT cell (lane1, 3, 5, 9 and 11, figure2.1 lower panel). The majority of PS1C was either processed into short form: $PS1C_{296}$ and $PS1C_{299}$ or go through random degradation through proteasome pathway. As shown in figure 2.1, with the addition of proteasome inhibitor MG132, PS1 C's level was dramatically increased when detected with 3622 in all knock out cells besides Aph-1 knock out cells and WT cells. When combined with the results detected by 5643

antibody which could detect all the PS1 C terminals, including the $PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$ and the caspase cleaved PS1Cs, we can see the level of total PS1 C was much lower in Aph-1 knock out cells, especially when comparing with NCT knock out cells (compare lane 2 with lane 4, figure2.1 upper panel). On the other hand, the caspase cleaved PS1 C was much higher in Aph-1 knock cells (compare lane 2 with lane 4, picture2.1 upper panel). Therefore, we speculate that the majority of PS1C were processed through caspase activity in Aph-1 knock out cells. Cells have higher apoptosis rate when induced by MG132 in Aph-1 knock out cells.

Subsequently, we conducted the following experiments to check the caspase activity in all knock out cells and WT cells with the presence of MG132.

Cells have a higher tendency of apoptosis with MG132 addition in the absence of Aph-1.

Based on the results in Figure 2.2, comparing lane 2 with lane 4, 6, 8, 10 and 12, the level of active form of caspase 3, caspase 7 and the cleavage of PARP were much higher in Aph-1 knock out cells than which induced in NCT knock out cells, Pen-2 knock out cells, PS1 knock out cells, PS2 knock out cells and WT cells by MG132. Therefore, we can conclude that the some concentration of MG132 addition trigger more apoptosis in the absence of Aph-1. We also noticed that knock out of NCT and PS2 could also result in a higher tendency of apoptosis but was much less compared to which caused by the deletion of Aph-1.

Apoptosis suppressive protein is down regulated in Aph-1 knock out cells.

Since MG132 is a proteasome inhibitor and an autophagy inducer and thus could induce apoptosis through the upregulation of ER stress with the accumulation of redundant proteins. Since the previous experiment has already demonstrated that caspase activity is much stronger in the absence of Aph-1, we examined the kinases involved in apoptosis. Based on the results in Figure 2.3, pro-apoptotic kinases p-c Jun, p-JNK, p-p38 were upregulated in a similar level in all the cells. However, the level of p-Akt (anti-apoptotic kinase) was highly differed in different cells with the addition of MG132. Compare lane 3 with lane 4, lane 5 with lane 6, MG132 induced higher expression of p-Akt in NCT knock out cells and Pen-2 knock out cells which could result in reduction of apoptosis. For PS1 knock out cells and WT cells, the level of p-Akt remained similar even with the addition of MG132. However, in PS2 knock out cells the p-Akt level was a litter bit lower. The dramatic reduction of p-Akt was found in Aph-1 knock out cell. The significant reduction of apoptosis suppressive protein: p-Akt, in Aph-1 knock out cells could be a reasonable cause that could explain the higher induction of apoptosis in Aph-1 knock out cells. The p-GSK3 which is phosphorylated through p-Akt followed the same pattern of p-Akt (Figure 2.3 the panel detected with p-GSK3). The reduction of p-GSK3 level was much obvious in Aph-1 knock out cells than the other cells (compare lane 2 with lane1, lane 4 with lane 3, lane 6 with lane 5, lane 8 with lane7, lane 10 with lane 9 and lane 12 with lane11).

The difference of apoptosis induced with MG132 become significant at the concentration of 5uM.

The concentration of MG132 we used in previous experiments is 5uM since it is a recommended concentration as a proteasome inhibitor. But how it affects apoptosis and the activation of Akt are not known, so different concentration of MG132 were tested in Aph-1 knock out cell, NCT knock out cell and WT cell. The reason we choose Aph-1 knock out cell and NCT knock out cell is that the difference of PS1C detected in these two cells were most dramatic, which indicate that the apoptosis in these two cells differ most from each other. Based on the results of Figure 2.4, we found that the induction of caspase activity and apoptosis induced kinase like p-c Jun, p-p 38, p-JNK follow a dose dependent manner. We also found the increase of caspase activity in Aph-1 knock out cells was faster which further support the idea that the absence of Aph-1 lead to higher preference of apoptosis. The reduction of p-Akt and p-GSK 3 also followed a dose dependent manner in Aph-1 knock cells and became very significant at the concentration of 5uM which was used in the previous experiments. We have also checked the effect of the addition of higher concentration of MG132. The results demonstrated that 5uM is suitable to show the difference since the increased level of MG132 (10uM) did not cause more reduction of p-Akt and p-GSK3 in Aph-1 knock out cells (Figure 2.5, compare lane 4 with lane 3). Therefore, in this project, 5uM is a suitable concentration for both proteasome degradation protection and apoptosis induction.

The only transfection of PS1C terminal fragments or PS1N fragments could not process NotchΔE.

To illustrate the potential functions of truncated PS1C terminals and N terminals at restoring gamma secretase activity, we constructed varied PS1 C terminals with the length from amino acid 293, 296, 299, 334, 346 to the very end of C terminal (name as $PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$ and $PS1C_{346}$) and PS1 N terminal starting from amino acid 1 to 292 (short as PS1N). As shown in the upper panel of Figure 2.6, with the detection of antibody 5643, PS1wt, $PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$ and $PS1C_{346}$ and PS1N were successfully transfected into the PS1/2 double knock out cells. The results shown by antibody 3622 confirmed that the band detected with 3622 was the longer form of PS1C which was $PS1C_{293}$ (lane 5), since the other forms of PS1 C could not be detected by antibody 3622 (compare lane 5 with lane 6, 7, 8 and 9). The third panel was probed with α-myc antibody which shown similar transfection level of NotchΔE in the cells transfected with varied PS1C terminals PS1N terminal. Even with the equal transfection of NotchΔE, the processing of it differed dramatically due to the transfection of varied PS1C and PS1N. In the panel detected with c-Notch which could only recognize the processed C terminal of NotchΔE, we found NotchΔE was only processed with the transfection of PS1wt rather than any other PS1 C terminals or PS1 N terminal (compare lane 4 with lane 5, 6, 7, 8, 9 and 10). This result demonstrated that the only transfection of

PS1C or PS1N could not restore the activity of gamma secretase at processing NotchΔE.

NotchΔE could be processed by the double transfection of PS1C terminal fragments (all the truncated PS1 Cs) and PS1N fragments.

Since the single transfection of PS1C or PS1N could not process NotchΔE, we suspect the processing of NotchΔE might require the integrity of PS1. So we transfect different PS1C terminals separately but together with PS1N terminals, results (Figure 2.7) shown that the transfection of PS1 Cs and PS1N were successful (upper two panels). The NotchΔE expression in all the transfected cells were equal (the third panel detected with α-myc antibody). The results shown by c-Notch immunoblot was interesting, all the PS1C transfected with PS1N terminals could process NotchΔE and the c-Notch levels were similar to which processed by PS1 wt plasmid (compare lane 5, 6, 7, 8 and 9 with lane 4 in panel 4). This result demonstrated that the PS1Cs transfection together with PS1N could restore the activity of γ-secretase at processing NotchΔE, and the restoration is not obviously affected by the length of PS1 C. No matter how the PS1C starts from 293, 296, 299, 334 or 346, they all could process NotchΔE with PS1N.

APP produced CTF α could be processed by the double transfection of PS1C terminal fragments (all the truncated PS1 Cs) and PS1N fragments differently.

Another important substrate of gamma secretase is CTFs. Our results above have already demonstrated the processing of NotchΔE does not differ with the transfection of varied length of PS1 C. The next thing is to determine the pattern of the processing of CTFs by varied PS1C terminals. Since our CTFs and PS1 levels in all the knock out cells suggest that the length of PS1 C might contribute to the ability of gamma secretase at the processing of CTFs. The upper two panels in Figure 2.8 demonstrated the success of the transfection of PS1 C and PS1 N terminals in PS1/2 double knock cells. The third panels shown the transfection of APP in all the lanes were similar. But immunoblot with C15 indicated that the levels of CTFs are different. There were three bands; the upper one was exogenous CTFβ, but the level was so low to show clear difference; the middle one was exogenous CTFα. Without the transfection of APP, these two exogenous bands could not be detected, as shown in lane 1, the only detected band is endogenous CTFα. We found that the transfection of PS1wt significantly reduced the level of CTFs (all the three bands were reduced, compare lane 4 with lane 2 and 3). The interesting thing was the transfection of $PS1C_{299}$ together with PS1N seemed processed more CTFs than the other PS1C fragments. It is suggesting that the short form of PS1 C ($PS1C_{299}$) has higher activity at processing CTFs.

Phosphorylated PS1C could not process CTFα.

In the first panel of figure 2.9, the upper band is a phosphorylated PS1C, which could be dephosphorylated by phosphatase (compare lane 2 with the other lanes). First, in the absence of DAPM, even though PS1C was dephosphorylated (compare lane 1 with lane 2 in the first panel), CTFα was not protected or processed more (shown in the second panel) which suggest that the phosphorylated PS1C was not active at processing CTFα. However, another possibility was that in the condition of dephophorylated buffer γ-secretase was not active at all. Therefore, as a γ-secretase inhibitor, DAPM (3,5-DIFLUOROPHENYLACETYL-ALA-PHG-OME) was added to test its activity. Since DAPM might be removed during sample collection by multiple wash, it was added during wash period too. Compare lane 3 and lane 2 in the second panel, the processing of CTFα was blocked, which confirmed that during the 2 hours incubation period of samples in dephosphorylation buffer with phosphatase at 37℃, the activity of γ-secretase was not disturbed. Putting all together, the dephosphorylation of PS1C did not block or promote the processing of CTFα through γ-secretase.

Discussion

Neuron loss is a major problem in AD. Therefore, apoptosis become a big issue in the searching AD treatment and therapy. It has been reported that mutant PS1 and overexpression of PS1 could induce apoptosis (Weihl, Ghadge et al. 1999,

Zeng, Hu et al. 2015). And one report mentioned that the overexpression of Aph-1a, b and pen-2 could induce an anti-apoptotic result through downregulating p-53 controlled activity of caspase 3 and the knock out of these components will result in the reverse response which is increase of caspase activity and apoptosis with the induction of staurosporine (STS). They conclude that the anti-apoptotic response is independent of γ-secretase activity but require the integrity of it (Dunys, Kawarai et al. 2007). Their theory and results are very supportive to our research here. The difference is that the apoptotic inducer we used is proteasome inhibitor MG132. We have found in previous study that the knock out of different components of γ-secretase result in varied viability of induction by MG132 (Hu, Zeng et al. 2015). But the exact roles of the γ-secretase component in apoptosis induced by MG132 and the pathways involved in MEF cells remain unclear. Therefore, we designed this study and found that in consistent to Dr. Dunys's study, indicating the knock out of Aph-1 lowering the resistance of MEF cells to apoptosis. But the knock out of Pen-2 does not increase the level of apoptotic related activities, such as the active form of caspase 3, 7, PARP and so on, which is probably because we use different inducers and the pathways involved are totally different.

For instance, in our case, the activation of p53 is not affected by MG132 (not shown). However, the level of apoptosis suppression kinase p-Akt is dramatically reduced in the Aph-1 knock out cells. And the kinase GSK3 which is phosphorylated by p-Akt decreased correspondently, further supporting the idea

that the increased apoptosis with the absence of Aph-1 is due to the lowering to p-Akt with the induction of MG132 in MEF cells(Scanga, Ruel et al. 2000, Franke, Hornik et al. 2003, Ruvolo, Qiu et al. 2015).

It still remains unknown how Aph-1 regulate p-Akt. The proper hypothesis could be p-Akt is a substrate of Aph-1 or the kinase that phosphorylated p-Akt is regulated by Aph-1 somehow. These directions need to be further pursued. Hope we will come to a more specific conclusion in the future.

The other part of this study is to compare the activity of PS1C fragments in γ-secretase through the processing of Notch and CTFs. The PS1N and PS1C fragments containing plasmids were constructed based on previous reports(Kim 1997, Craen 1999) (Steiner, Romig et al. 1999) (Fukumori, Fluhrer et al. 2010). The phorsphorylated PS1 were reported of no γ-secretase activity which is consistent with our results (Matz, Halamoda-Kenzaoui et al. 2015). The dephosphorylation of PS1C does not reduce the activity of γ-secretase.

For the processing of Notch by other PS1C fragments, we found that both PS1N and PS1C are required. It is probably due to the necessary of the intact of two aspartic site: D257 and D385 which are located on PS1N and PS1C separately (Capell, Steiner et al. 2000). Since the PS1C fragments we created in this study all include aa385 and PS1N include aa257. This hypothesis is supported by a previous report (Kim, Ki et al. 2005) and require further investigation.

However, the activity of PS1C ($PS1C_{293,}$ $PS1C_{296,}$ $PS1C_{299,}$ $PS1C_{334,}$ $PS1C_{346}$) fragment at processing Notch with the accompany of PS1N do not varied dramatically, Only $PS1C_{293}$ shown less activity when comparing to the others. However, at processing CTFs, the activity of PS1Cs varied obviously. The $PS1C_{299}$ has stronger strength than the others. Since it has been reported that the endoproteolysis and production of PS1C is also stepwise and the final produced PS1C is $PS1C_{299}$ in cells, we may conclude that the shedding of the first several amino acid is required for the processing of CTFs (Fukumori, Fluhrer et al. 2010). Apparently, in our study, the caspase produced PS1C fragments $PS1C_{334}$ and $PS1C_{346}$ do not have activity at processing CTFs. Based on above results, the processing of Notch and CTFs require different length of PS1C. The processing of CTFs is more complicated and need further investigation.

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Appendix

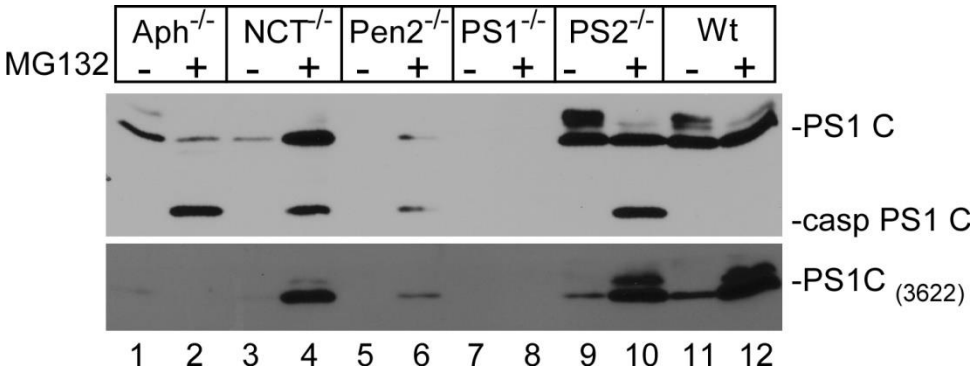

Figure 2.1

Figure 2.1 Presenilin 1 C terminal levels varied in different knock out cells with the addition of MG132. Knockout cells were cultured in the presence or absence of MG132. Up panel, immunoblot probed with anti PS1C 5643 antibody that recognizes both regular and caspase produced PS1C; bottom panel, immunoblot probed with with anti PS1C 3622 that recognizes only longer form of PS1C starts from amino acid 293.

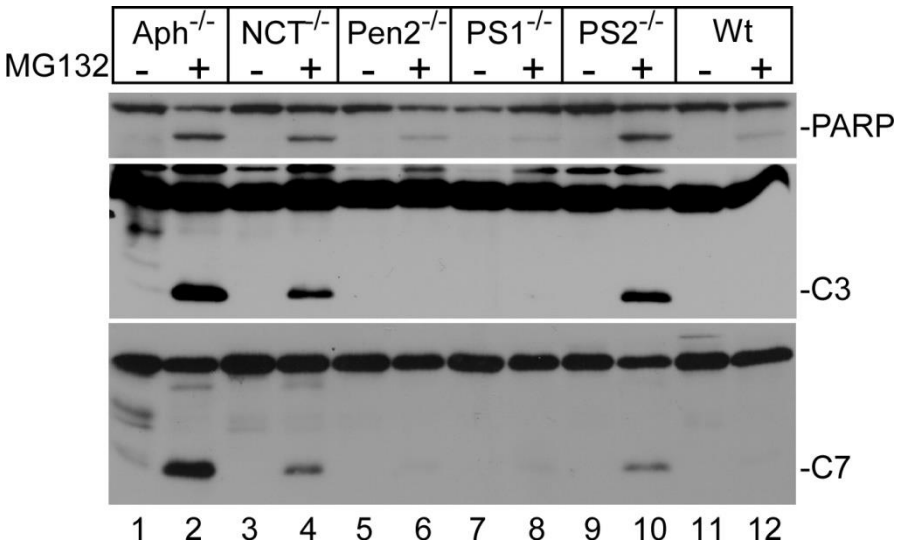

Figure 2.2

Figure 2.2 Cells have a higher tendency of apoptosis with MG132 addition in the absence of Aph1.Knockout cells were cultured in the presence or absence of MG132. Up panel, immunoblot probed with anti PARP; middle panel, immunoblot probed with caspase 3 which could recognize the full length and active form of caspase 3; bottom panel, immunoblot probed with caspase 7 which could recognize the full length and active form of caspase 7.

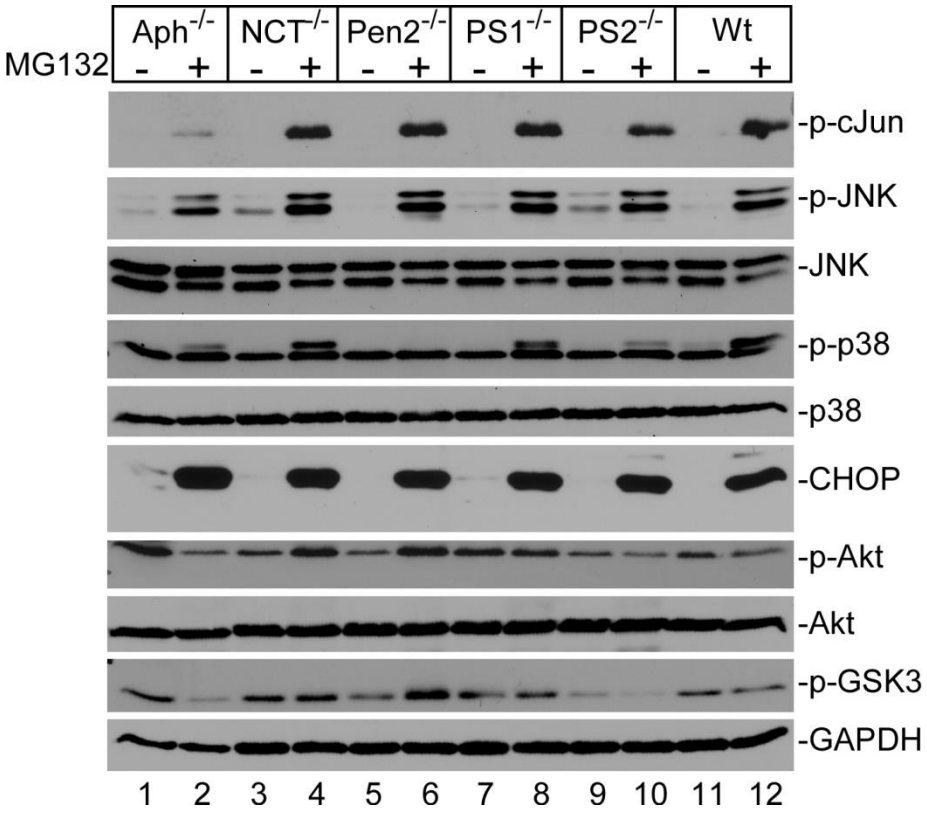

Figure 2.3

Figure 2.3 Apoptosis suppressive protein is down regulated in Aph1 knock out cells.Knockout cells were cultured in the presence or absence of MG132. And immunoblot probed with anti p-c Jun, p-JNK, JNK, p-p38, p38, CHOP, p-Akt, Akt, p-GSK3 separately. Then re-probe the p-p38 probed membrane with GAPDH for loading control.

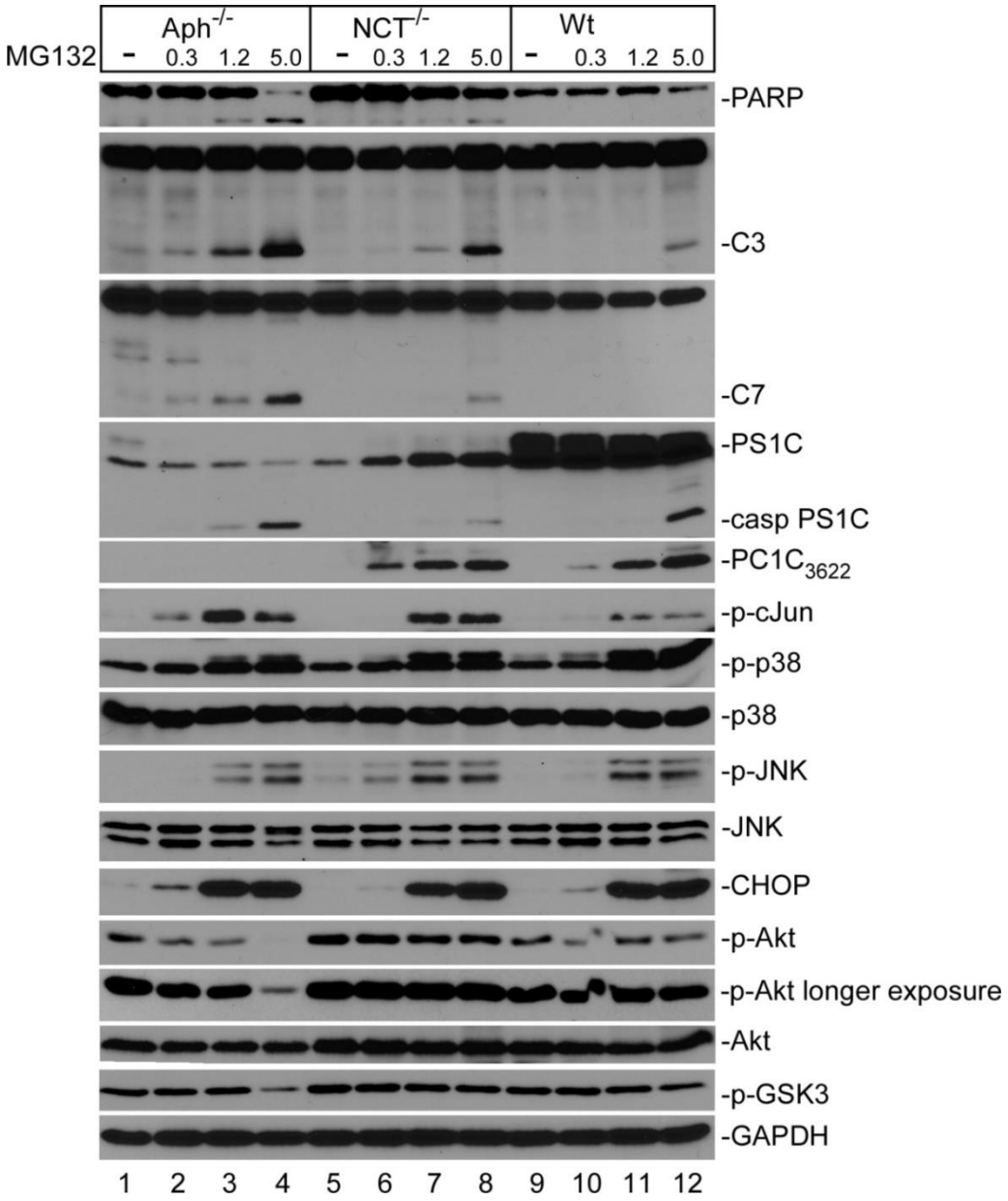

Figure 2.4

Figure 2.4 The difference of apoptosis induced with MG132 become significant at the concentration of 5uM. Knockout cells were cultured in the presence or absence of MG132. And immunoblot probed with anti PARP, caspase 3, caspase 7, PS1 C 5643, PS1C 3622, p-c Jun, p-p38, p38, p-JNK, JNK, CHOP, p-Akt, Akt, p-GSK3 separately. Then re-probe the p-p38 probed membrane with GAPDH for loading control.

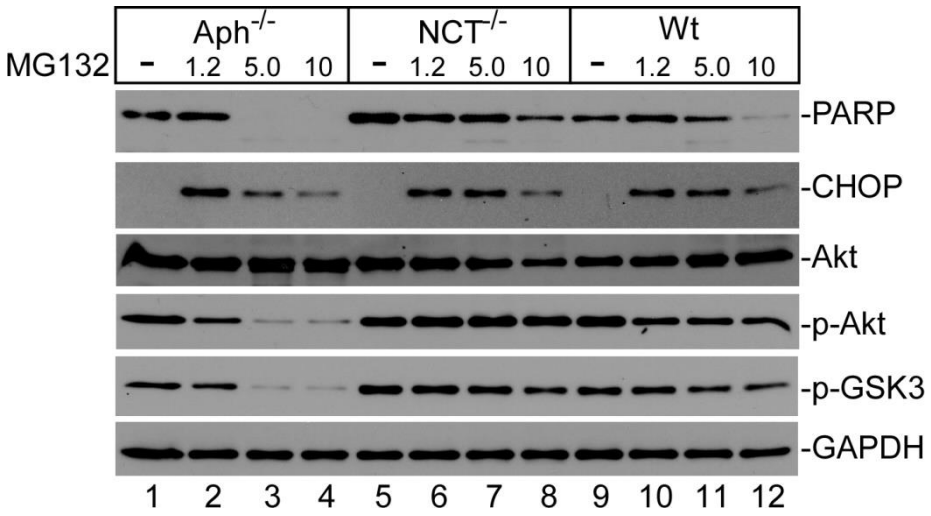

Figure 2.5

Figure 2.5 The difference of apoptosis induced with MG132 become significant at the concentration of 5uM. Knockout cells were cultured in the presence or absence of MG132. And immunoblot probed with anti PARP, CHOP, p-Akt, Akt, p-GSK3 separately. Then re-probe the CHOP probed membrane with GAPDH for loading control.

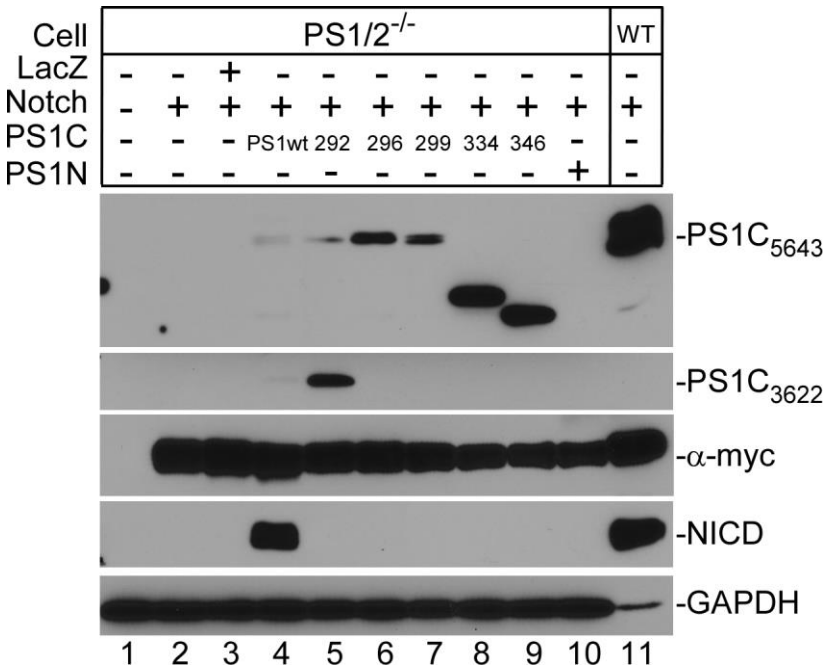

Figure 2.6

Figure 2.6 The only transfection of PS1C terminal fragments or PS1N fragments could not process NotchΔE. PS1/2 double knockout cells were transfected with different PS1 C terminals or PS1N terminals together with NotchΔE. And immunoblot probed with anti PS1C5643, PS1C3622, α-myc, c-Notch and GAPDH separately.

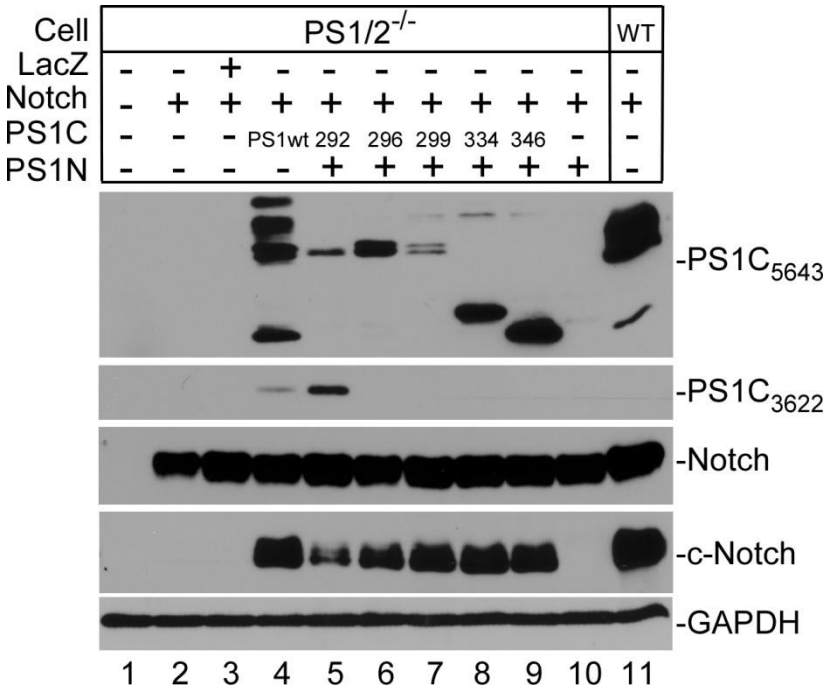

Figure 2.7

Figure 2.7 NotchΔE could be processed by the double transfection of PS1C terminal fragments (all the truncated PS1 Cs) and PS1N fragments. PS1/2 double knockout cells were transfected with different PS1 C terminals and PS1N terminals together with NotchΔE. And immunoblot probed with anti PS1C5643, PS1C3622, α-myc, c-Notch and GAPDH separately.

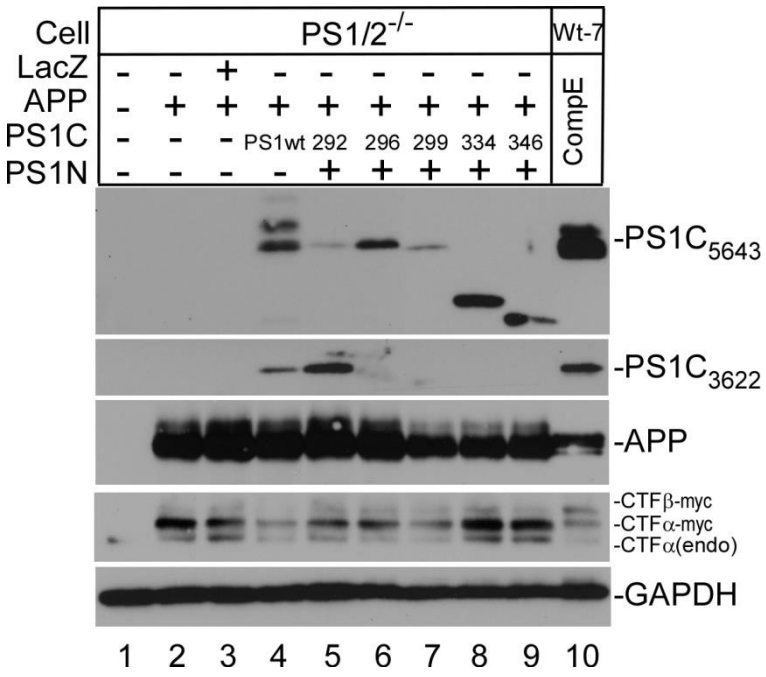

Figure 2.8

Figure 2.8 APP produced CTF α could be processed by the double transfection of PS1C terminal fragments (all the truncated PS1 Cs) and PS1N fragments differently. PS1/2 double knockout cells were transfected with different PS1 C terminals and PS1N terminals together with APP. And immunoblot probed with anti PS1C5643, PS1C3622, 6E10, C15 and GAPDH separately.

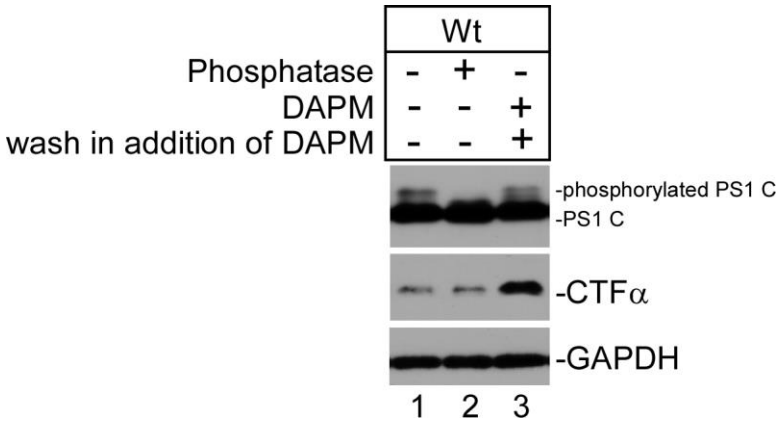

Figure 2.9

Figure 2.9 Phosphorylated PS1C could not process CTFα. WT cells were treated in the presence or absence of DAPM (100nM). Phosphatase was added in lane 2. And immunoblot probed with anti PS1C5643, C15 and GAPDH separately

CHAPTER III

PEN-2 IS REQUIRED FOR NOTCH PROCESSING AS A SUBSTRATE RECEPTOR

Abstract

Pen-2 is necessary for PS1 endoproteolysis and the stability of the heterodimer of PS1N and PS1C. Therefore, we suspect that the inability of processing Notch in Pen-$2^{-/-}$ cell is due to the instability of PS1. In order to test this possibility, PS1N and $PS1C_{299}$ alone with Notch were transfected into Pen-$2^{-/-}$ cell. However, despite the presence of significant amount of PS1N and PS1C, no γ secretase activity was detected in these Pen-$2^{-/-}$ cells. This result suggests that Pen-2 is functionally required for γ-secretase activity. Furthermore, Pen-2 and PS are the two minimal components required for Notch processing since the knockdown of NCT in Aph-1 knock out cell did not affect the processing of Notch. Furthermore, with immunoprecipitation experiments, our results demonstrate that Pen-2 might functionally required for recruit substrate Notch and assist in delivering Notch to PS for processing. As shown in chapter II, our data have demonstrated that all the PS1Cs ($PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$ and $PS1C_{346}$) examined showed no difference in catalyzing Notch processing. But $PS1C_{299}$ has a higher activity for CTFs processing. Thus, $PS1C_{299}$ was chosen as a PS1C representative to carry out the following study. Our study supports the hypothesis that Pen-2 is more than a structural component of the γ-secretase complex and may contribute to the catalytic mechanism of the enzyme (Bammens, Chávez-Gutiérrez et al. 2011).

Introduction

Presenilin enhancer 2 (Pen-2) is a 12kD, hairpin like membrane protein (De Strooper 2003). Pen-2 was found to interact closely with PS and to be required for γ-secretase activity for processing CTFβ and Notch (Francis, McGrath et al. 2002). Knockdown of Pen-2 resulted in the accumulation of holoprotein PS1 and reduction of the endproteolytic product of PS1, PS1N and PS1C. Therefore, Pen-2 was thought to be necessary for the endoproteolysis of PS (Takasugi, Tomita et al. 2003). The endoproteolysis of PS is required for the activation of PS1 functioning as the catalytic component of γ-secretase (Kopan and Goate 2000).

Pen-2 was also reported to play a role in stabilization of PS1N and PS1C heterodimer following endoproteolysis of PS1 (Prokop, Shirotani et al. 2004). Using siRNA approach, it was shown that knockdown of pen-2 also resulted in impaired NCT maturation and proteasomal degradation of other γ-secretase components, suggesting that pen-2 is also important for maintaining the integrity of γ-secretase complex (steiner, winkler et al. 2002, mao, cui et al. 2012). The important role of Pen-2 in γ-secretase activity was further confirmed by a recent study that reported that genetic knockout of Pen-2 resulted in embryo lethality and embryo absorbing at embryonic day 9.5, likely due to impaired Notch signaling (Bammens, Chavez-Gutierrez et al. 2011).

In 2012, our group found that Pen-2 might be dispensable for endoproteolysis of PS1 (Mao, Cui et al. 2012). It was found that significant level of PS1C could be recovered by addition of proteasome inhibitor MG132 in Pen-2 knockdown cells,

strongly indicating that PS1 was processed in the absence of Pen-2. This result suggests that Pen-2 is not absolutely required for PS1 endoproteolytic processing, but rather more important for the stabilization of PS1 by preventing it from proteasome degradation (Mao, Cui et al. 2012). This finding was further supported by a very recent study reporting that PS1 is processed in Pen-2 knockout cells isolated form Pen-2 knockout mice (Holmes, Paturi et al. 2014) These observations raised questions about whether Pen-2 participate in γ–secretase activity through the regulation the formation of PS1N and PS1C heterodimer or Pen-2 is directly involved in γ-secretase catalytic mechanism per se remain illusive. To elucidate the exact role of Pen-2 in γ–secretase activity, we conducted several experiments in this chapter. Our data clearly demonstrate that Pen-2 is not required for the PS1N and PS1C heterodimer formation, bur is very likely required for substrate recruitment of γ-secretase.

Materials and Methods

Cell culture

MEF cells we used in this part were: Aph-1$^{-/-}$, Pen-2$^{-/-}$, PS1/2$^{-/-}$, wild type (wt) and wt-7 cells. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MA, USA) which containing 10% fetal bovine serum, 2 mM L-glutamine (Lonza, Walkersville, MD), 100 units/mL penicillin (Lonza), and 100 µg/mL streptomycin (Lonza).

Inhibitors and reagents

Compound E, a γ–secretase inhibitor was purchased from EMD Millipore (Billerica, MA, USA). Complete protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). Lipofectamine LTX and plus reagent was purchased from Invitrogen (Carlsbad, CA).

Antibodies

Antibodies which were anti-$PS1C_{5643}$ and c-Notch (#4147, which specifically recognizes the processed Notch) were purchased from Cell Signaling (Danvers, MA). Polycolonal NCT antibody N1660 was purchased from Sigma-Aldrich (St.Louis, MO). Polyclonal antibody anti-PEN-2N was from Covance (Emeryville, CA). Anti-myc antibody, C-Myc (9E10), was purchased from Santa Cruz (Dallas, TX, USA). Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was from EMD Millipore (Billerica, MA). Anti-PS1N was raised against a peptide corresponding to residues 27–50 of human PS1 (Zhao, Cui et al. 2005).

Plasmids

We used NotchΔE plasmid which as mentioned in last chapter: plasmid expressing the extracellular region truncated and myc-tagged Notch molecule (NotchΔE) containing the murine Notch-1 leader peptide (1-23 aa) (Kopan, Schroeter et al. 1996) was kindly provided by Dr. Raphael Kopan (Washington University) and Dr. Masayasu Okochi (Osaka University, Japan). The plasmids which expressing the PS1N terminal (1-292aa), PS1C terminals: $PS1C_{293}$ (293-

467aa), $PS1C_{296}$ (296-467aa), $PS1C_{299}$ (299-467aa), $PS1C_{334}$ (334-467aa), $PS1C_{346}$ (346-467aa) were constructed in our lab and sequenced. PS1 mutants applied: $PS1_{D257A}$, $PS1_{D385A}$, $PS1_{D257,385A}$ were constructed as previously mentioned (Xu, Shi et al. 2002).

siRNA treatment

Both siRNAs and delivery reagent were purchased from Life Technologies (Carlsbad, CA), and treatment of cells with siRNAs was carried out according to the manufacturer's instruction.

Immunoprecipitation (IP)

Protein A sepharose was purchased from Pharmacia Biotech (Piscataway, NJ). After transfection for 24 hours, cells were collected with 1% Chapso buffer (20mM Tris pH 8.0, 150mM NaCl, 5mM EDTA and cocktail). Chapso was phurchased from Amresco (Solon, OH). The collected samples were then immunoprecipitated with varied antibodies (check figure for details) overnight at 4 ℃ in the presence of Protein A conjugated beads. After immunoprecipitation, samples were washed and analyzed through western blotting.

SDS-PAGE and Western blotting

For the analysis of the activity of gamma secretase, gamma secretase inhibitor compE (5nM) was applied. For analysis of the exogenous APP and Notch processing through different PS1C and PS1N terminals, the cells, 24 h after splitting, were transfected with plasmids expressing NotchΔE with lipofectamine

LTX. Twelve hours after the transfection of NotchΔE, PS1N and PS1C terminals were transfected and the cells were further incubated for about 24 hours. Cell lysis and Western blot analysis were carried out as described previously (Zhao, Mao et al. 2004), which is consistent to which described briefly in last chapter. The membranes were probed with appropriate antibodies as described in figure legends.

Results

Two aspartyl acids on PS1 were essential for the processing of Notch

The two conserved aspartic acid residues at positions 257 and 385 in PS1 have been identified as the catalytic residues in γ-secretase by a pioneer study (Wolfe 1999), and the hypothesis that these residues form the active site for the γ-secretase enzyme complex has been well supported (Steiner, Duff et al. 1999, Kimberly, Xia et al. 2000). Our results presented in the previous chapter demonstrated that γ-secretase catalyzed processing of Notch was achieved by transfection of PS1 and PS2 double knockout ($PS1/2^{-/-}$) cells with PS1N in combination with PS1C of different length. It is noted that all the PS1Cs [$PS1C_{293}$(aa293-aa467), $PS1C_{296}$(aa296-aa467), $PS1C_{299}$(aa299-aa467), $PS1C_{334}$(a334-aa467), and $PS1C_{346}$(aa346-aa467)] examined bear the aspartyl residue 385 (D385), indicating that as long as the PS1C contains the D385 residue, regardless of difference in length, it is capable of to forming a functional heterodimer with PS1N (aa1 to aa292), which bears the aspartyl residue 257

(D257), to process Notch. To test this speculation that these two aspartyl residues essential for PS1 function under our experimental conditions, I transfected PS1 plasmid and three mutant PS1: $PS1_{D385A}$, $PS1_{D257A}$, and $_{PS1D257A/D385A}$ along with NotchΔE back into $PS1/2^{-/-}$ cell. As shown in Figure 3.1, as detected with α-myc, NotchΔE, which is expressed with a myc-tag at its C-terminal, was found expressed equally in all the transfectants, except in cells transfected with NotchΔE alone (lane 2). However, NICD, the C-terminal fragment produced form NotchΔE by γ-secretase activity, was only detected in cells expressing PS1wt (lane 4), but not in cells expressing any of these mutant PS1 mutant (lanes 5-7). As shown in the third panel, significant amounts of PS1, either PS1wt (lane 4) or mutant PS1 (lanes 5-7), were detected with antibody 5643, which is specific to C-terminal of PS1. Specifically, mutant $PS1_{D385A}$ and $PS1_{D257A/D385A}$ were expressed at relatively high levels. Thus, the presence or absence of NICD in cells expressing PS1wt or mutant PS1 is unlikely due to inefficient expression levels of these PS1 variants. This result confirmed that the two aspartates are indeed essential for γ-secretase catalyzed Notch processing.

Pen-2 is directly required for Notch processing through γ-secretase.

As shown in figure 2.7, co-expression of PS1N and PS1C fragments, containing these two aspartates separately, was able to process Notch in $PS1/2^{-/-}$ cells. Pen-2 has been regarded as a necessary factor for endoproteolysis of PS1 required for maturation and activation of γ-secretase complex by previous studies

(Luo, Wang et al. 2003, Takasugi 2003). This notion raises a question as to whether Pen-2 is merely required for endoproteolysis and stabilization of PS1. If that is the case, co-expression of PS1N and PS1C together may reconstitute γ-secretase activity in the absence of Pen-2. To test this possibility, I transfected Pen-$2^{-/-}$ cells with PS1wt alone with NotchΔE or PS1N and PS1C alone with NotchΔE. As shown in figure 3.2, all PS1Cs, except $PS1C_{293}$, were expressed at substantial levels. Specifically, a significant amount of PS1C (lane 4) was detected in PS1wt-transfected cells and this result further confirmed out previous finding that Pen-2 is not absolutely required for PS1 endoproteolytic processing (Mao, Cui et al. 2012). Interestingly, despite the presence of significant amount of PS1C and PS1N, no Notch processing product NICD was detected in PS1wt or PS1N/PS1C transfected cells. This result indicates that in addition of enhancing PS1 endoproteolytic processing and stabilization of PS1, Pen-2 is directly required for the catalytic activity of γ secretase.

Knockdown of NCT in Aph-$1^{-/-}$ cells does not affect the Notch processing.

Data presented in chapter 1 demonstrated that knockout of Aph-1 or NCT separately does not affect the processing of Notch. However, it is not known whether as long as either one of Aph-1 or NCT presences will be sufficient to support the γ-secretase activity or both Aph-1 and NCT are not required for γ-secretase catalyzed Notch processing. To address this issue, using the siRNA approach, I determined the effect of knockdown of NCT in Aph-$1^{-/-}$ cells on the processing of Notch. As shown in figure 3.3, knockdown of NCT was achieved by

all three siRNAs used (lanes 3-5, third panel). In addition, transient expression of NotchΔE was detected at fair equal levels in all the transfected cells. Surprisingly, NICD was detected in all the cells (lanes 1-5). This result strongly suggests that both Aph-1 and NCT are dispensable for Notch processing. In another word, Pen-2 and PS1 are sufficient for the activity of γ secretase to process Notch.

The association of PS1N and PS1C was not disturbed by the deletion of Pen-2.

It is well established that PS1 is the catalytic component (De Strooper, Saftig et al. 1998); however the role of Pen-2 in γ-secretase remains elusive. The data presented above strongly suggest that Pen-2 is directly involved in γ-secretase activity. This finding prompts us to further investigate the mechanism by which Pen-2 is involved in γ-secretase activity. One possibility is that Pen-2 might be required for enhancing and stabilizing the PS1N/PS1C heterodimer formation. To test this possibility. I examined the effect of knockout of Pen-2 on the formation of PS1N/PS1C heterodimer. To do so, I transfected Pen-$2^{-/-}$ cells and PS1/$2^{-/-}$ cells with both PS1N and PS1C and performed co-immunoprecipitation on these cell lysates. As shown in figure 3.4, we found that PS1N could pull down varied PS1C in PS1/$2^{-/-}$ (bottom panel). This result confirmed that in PS1N and PS1C are capable of forming dimer in the absence of Pen-2. The data presented in figure 3.4 also demonstrated that PS1N is not only capable of forming complex with PS1Cs produced by normal endoproteolytic processing, such as $PS1C_{293}$, $PS1C_{296}$, and $PS1C_{299}$ (lanes 4, 5, and 6), but also capable of forming complex

with the PS1Cs produced by caspase activity, such as $PS1C_{334}$ and $PS1C_{346}$ (lanes 7 and 8). Since overexpression of PS1wt could induce apoptosis (Zeng, Hu et al. 2015), thus, the apoptotic $PS1C_{346}$ was detected in cells transfected with PS1wt (lane 3). It should be noted that, because they run at the similar migration rate, the regular $PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$ could not be distinguished from IgG light chain. These data clearly indicate that Pen-2 is not required for the PS1N/PS1C heterodimer formation.

Pen-2 is required for Notch binding to PS1.

Presenilin is the catalytic component in γ-secretase complex. The endoproteolysis of PS and the association of PS1N and PS1C were believed to be the key in γ-secretase activity. However, the deletion of Pen-2 did not affect the association of PS1N and PS1C, suggesting that Pen-2 might participate in the γ-secretase activity directly rather than indirectly through regulation of the formation and stability of the complex of PS1N and PS1C. Thus, the other possibility is that Pen-2 might play an important role in recruiting Notch. In order to test our hypothesis, I performed the following experiments.

First, all the cells were transfected with NotchΔE alone with either empty vector or plasmids expressing PS1 variants. In WT cells, certain level of Notch was co-immunoprecipitated with Pen-2 by anti-Pen-2 antibody (lane 10, upper top panel). However, PS1C antibody hardly pull down any Notch (lane 11). This is likely due to the activity of γ-secretase that processes NotchΔE into NICD, which is no longer associated with PS1. Therefore, I cultured WT cells in the

presence of γ-secretase inhibitor Compoud E, which could partially block the processing of Notch. Indeed, Notch was pulled down by PS1C antibody in WT cells in the presence of compound E (lane 14, top panel). However, it was also noted that the level of Notch pulled down by Pen-2 antibody was more than that pulled down by PS1C antibody (compare lane 13 with 14), suggesting that the association between Pen-2 and Notch may be stronger than PS1 with Notch.

To further determine the affinity of Pen-2 and PS1 for Notch, I performed the co-immunoprecipitation in Pen-2-knockout cells and PS1/2-double knockout cells. As a result, certain amount of NotchΔE was co-immunoprecipitated with Pen-2 in PS1/2$^{-/-}$ cells (lane 1, top panel). However, anti-PS1C antibody did not bring down any Notch in Pen-2 knockout cells (data not shown). Because PS1 is very unstable in Pen-2$^{-/-}$ cells, thus, this could be due to the low level of PS1 in Pen-2$^{-/-}$ cells. Therefore, I performed co-immunoprecipitation in Pen-2$^{-/-}$ cells transfected with PS1wt and it was found that only a negligible amount of NotchΔE was immunoprecipitated by anti-PS1C antibody in Pen-2$^{-/-}$ cells transfected with both PS1wt and NotchΔE, (lane 17, top panel). Again, this may be because of inefficient endoproteolytic processing of PS1 in the absence of Pen-2. Thus, I transfected Pen-2$^{-/-}$ cells with PS1N and PS1C, which have been shown to form active γ-secretase complex and process Notch in PS1/2$^{-/-}$ cells (figure 2.7). As a result, despite the fact that significant level of PS1C was detected in these cells (lane 5, third panel), no NotchΔE was co-immunoprecipitated with PS1C (lane 5, top panel). These results revealed an

interesting finding that Pen-2 could interact with Notch in the absence of presenilin, however, presenilin could not interact with Notch in the absence of Pen-2, suggesting that presenilin interacts with Notch is mediated or enhanced by Pen-2. It was also noted that in the presence of overexpressed PS1, either PS1wt or PS1C, not only anti-PS1C antibody but also anti-Pen-2 antibody brought down more NotchΔE than in cells without overexpression of PS1 (compare lanes 8 and 20 with lanes 11 and 14 for anti-PS1C antibody and compare lanes 7 and 19 with lanes 10 and 13 for anti-Pen-2 antibody). These results indicate that PS1 also enhances the interaction between Pen-2 and Notch. In addition, this set of experiments further confirmed that Notch is not processed in the absence of Pen-2 even in the presence of overexpressed PS1 (lanes 6 and 18, second panel). It should be pointed out that the binds detected by NICD-specific antibody in anti-Pen-2 immunoprecipitated samples (lanes 1, 4, 7, 10, 13, 16, and 19, second panel) are non-specific signals. It was also noted that in WT cells, anti-myc antibody could bring down PS1C (lanes 12 and 15, third panel), but not Pen-2 (lane 12 and 15, bottom panel). Also, in WT cells, anti-PS1C antibody could bring down Pen-2 (lanes 11 and 14, bottom panel), but not in PS1/2$^{-/-}$ cells expressing PS1N and PS1C (lanes 8 and 20, bottom panel).

Discussion

Among the four components of γ-secretase complex, Pen-2 is the relatively less characterized member. Studies have suggested that Pen-2 plays a role in

presenilin endoproteolysis and stabilization and nicastrin maturation (Mao, Cui et al. 2012). Using knockout mouse model, a study hypothesized that Pen-2 is more than a structural component of the γ-secretase complex and may contribute to the catalytic mechanism of the enzyme (Bammens, Chávez-Gutiérrez et al. 2011). In the study presented in this chapter, we used specific knockout cells in combination with siRNA technology to determine the role of Pen-2 in γ-secretase and the mechanism by which Pen-2 contribute to γ-secretase activity. The most interesting finding is that knockdown of NCT in Aph-1$^{-/-}$ cells had no significant effect on Notch processing. In chapter I, our data showed that knockout of either one of NCT and Aph-1 individually did not impair the enzymatic function of γ-secretase to process Notch. This finding suggests a possibility that the presence of both of NCT and Aph-1 simultaneously is not required for Notch processing. In other words, either NCT or Aph-1 along with Pen-2 and PS1 will form a functional complex to process Notch. However, data presented in this chapter surprisingly demonstrated that the knockdown of NCT in Aph-1$^{-/-}$ cells had no significant effect on Notch processing. This finding strongly suggests that Pen-2 and PS1 are the minimal required and sufficient to catalyze Notch processing. To further investigate the mechanism by which Pen-2 plays a directly role in Notch processing, our data revealed that Notch could not be co-immunoprecipitated with Pen-2 in PS1/2$^{-/-}$ double knockout cells, however, Notch was co-immunoprecipitated with PS1 in Pen-2$^{-/-}$ cells. This result indicates that Notch interacts with Pen-2 in the absence of PS1, but PS1 can't interact with Notch in

the absence of Pen-2. This finding strongly suggests a possibility that interaction between PS1 and Notch is mediated by Pen-2, i.e., Pen-2 functions as a receptor in recruiting substrate to γ-secretase complex. These novel findings revealed a important function of Pen-2 and will greatly contribute to our understanding of the molecular mechanism of γ-secretase activity.

In addition, the data presented in this chapter lead to several interesting findings.

First, using the PS1/2$^{-/-}$ double knockout cells, our data clearly demonstrated that introduction of the PS1$_{D257A}$ and PS1$_{D2385A}$ point mutations in PS1 result in an inactive γ-secretase for processing Notch and this finding confirmed again that these two aspartyl residues are essential for γ-secretase activity as reported by previous study (Wolfe, Xia et al. 1999).

Second, our data demonstrated that all the PS1 C-terminal fragments produced during endoproteolysis and by caspase activity are functional in Notch processing when expressed along with PS1N. it has been reported that during maturation, PS1 undergoes endoproteolytic processing and the produced PS1N and PS1C fragments form heterodimer. Study also reported that, similar to APP, endoproteolysis of PS1 also follows a stepwise sequential cleavage and result in the formation of PS1Cs of different length of which PS1C$_{299}$ is the major species (Fukumori, Fluhrer et al. 2010). Our data showed that co-expression of the PS1Cs with different length corresponding to the PS1Cs produced during endoproteolysis and by caspase activity along with PS1N resulted in Notch

processing. These results indicate that all these PS1Cs are functional in constituting active γ-secretase.

Third, Pen-2 is not required for PS1N and PS1C heterodimer formation. It has been concerned that the heterodimer of PS1N and PS1C might been disturbed in the absence of Pen-2 (reference is missing). However, the immunoprecipitation results demonstrated that the association of PS1N and PS1C were not affect by the deletion of Pen-2. Our finding is also supported by a previous study showing that Pen-2 did not enhance the level of heterodimer of PS1 (Shiraishi, Sai et al. 2004). Combined together, it is clearly indicated that Pen-2 is directly involved in γ secretase activity rather than indirectly by enhancing PS1N/PS1C heterodimer formation.

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Appendix

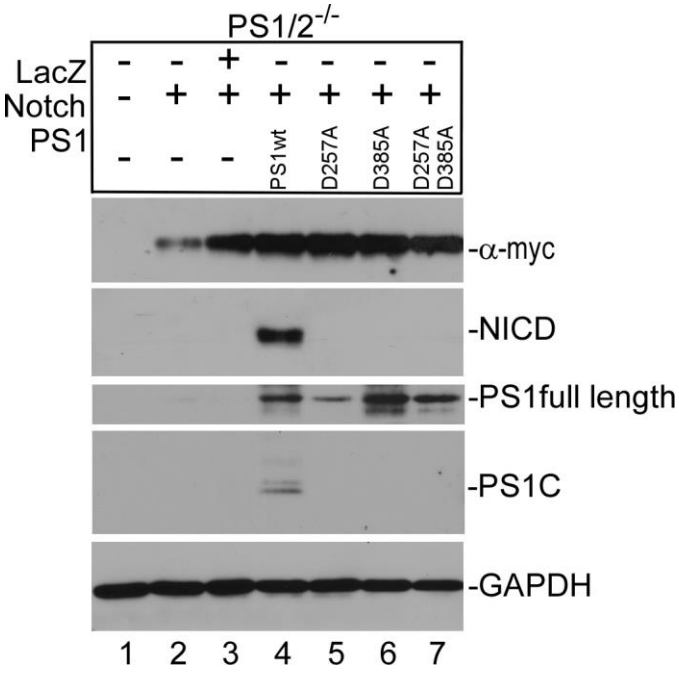

Figure 3.1

Figure 3.1 Two aspartyl acids on PS1 were essential for the processing of Notch. Notch was overexpressed in PS1/2$^{-/-}$ cell with the transfection of NotchΔE and detected with α-myc in the first panel. The processing of Notch was detected in the second panel with antibody C-Notch. Cells were also transfected with PS1 WT and PS1 mutants: PS1$_{D257A}$, PS1$_{D385A}$ and PS1$_{D257,385A}$, the expression of PS1 and PS1 mutants were reflected with antibody PS1C 5643. GAPDH was applied as loading control as shown in the bottom panel.

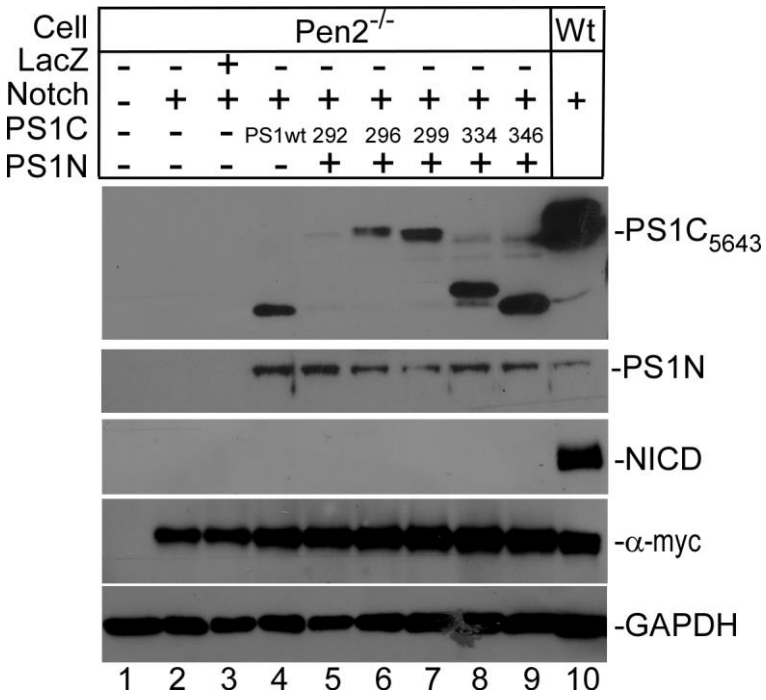

Figure 3.2

Figure 3.2 Pen-2 is directly required for Notch processing through γ-secretase. NotchΔE could not be processed by the double transfection of PS1C terminal fragments (all the truncated PS1 Cs) and PS1N fragments in Pen-$2^{-/-}$ cell. Pen-2 knockout cells was transfected with different PS1 C terminals and PS1N terminals together with NotchΔE. And immunoblot probed with anti PS1C antibody 5643, α-myc, c-Notch and GAPDH separately.

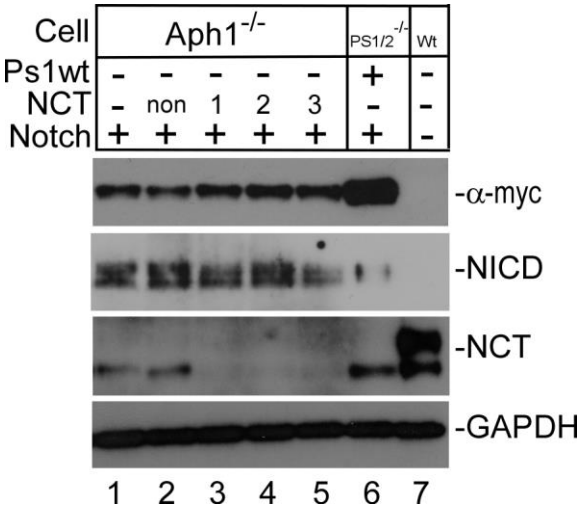

Figure 3.3

Figure 3.3 Knockdown of NCT in Aph-1$^{-/-}$ cell does not affect the Notch processing. NotchΔE was overexpressed in Aph-1$^{-/-}$ cell and detected with α-myc (first panel). PS1/2$^{-/-}$ and wt cell were used as control. Three NCT siRNA reagents (1,2 and 3) were applied and the knockdown results were detected in the third panel with antibody NCT. The processing of Notch was detected in the second panel with antibody C-Notch. GAPDH was applied as loading control as shown in the bottom panel.

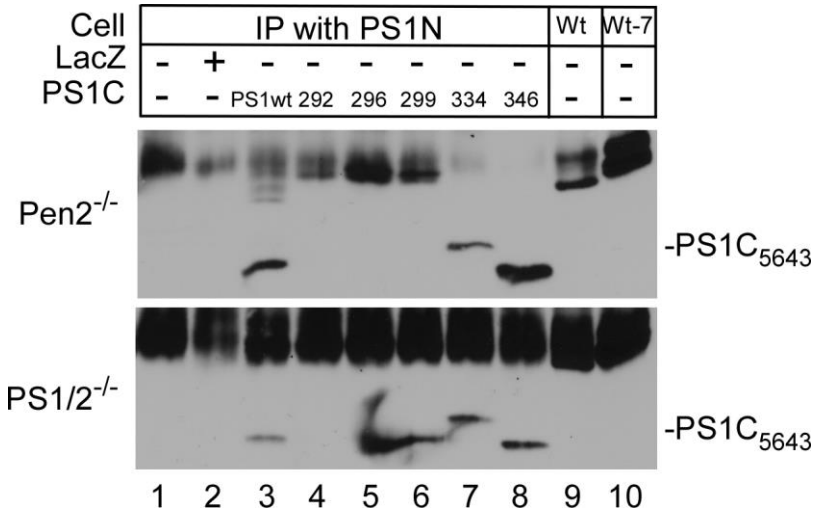

Figure 3.4

Figure 3.4 The association of PS1N and PS1C was not disturbed by the deletion of Pen-2. PS1N and varied PS1Cs ($PS1C_{293}$, $PS1C_{296}$, $PS1C_{299}$, $PS1C_{334}$, $PS1C_{346}$) were transfected into Pen-$2^{-/-}$ and PS1/$2^{-/-}$ cell. WT cell and Wt-7 cell were used as control. Samples were immunoprecipitated with antibody PS1N and the association of PS1N and PS1C were indicated with the detection of PS1 by the antibody PS1C 5643 (shown in the two panels).

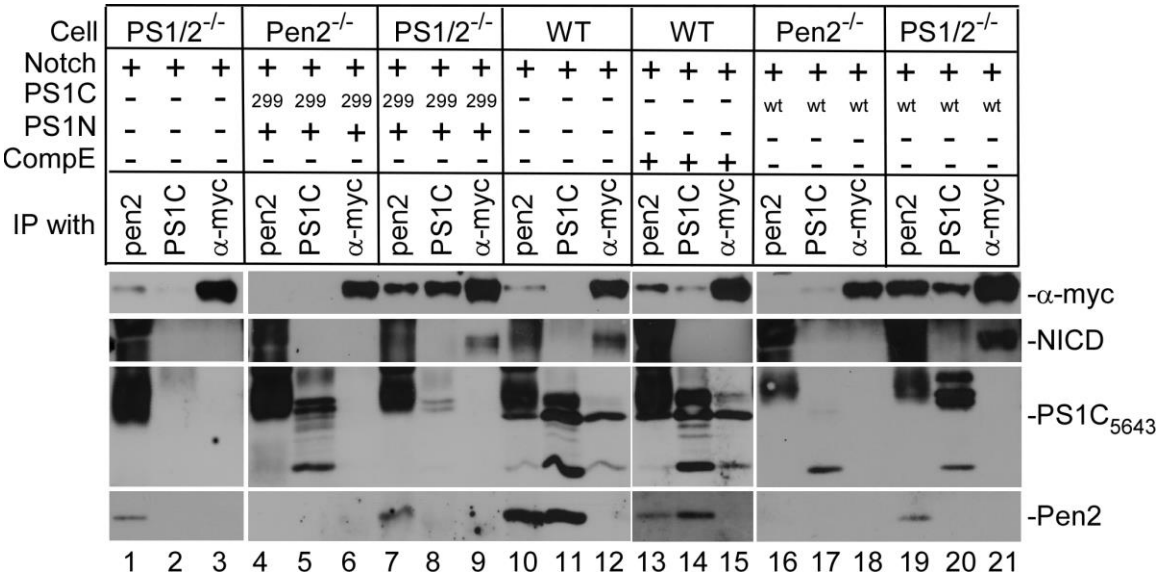

Figure 3.5

Figure 3.5 Pen-2 is required for Notch binding to PS1.Notch ΔE were transfected into Pen-$2^{-/-}$, PS1/$2^{-/-}$ and WT cell and the expression were detected in the first row with α-myc antibody. PS1WT plasmids and $PS1C_{299}$, PS1N were transfected into Pen-$2^{-/-}$ and PS1/$2^{-/-}$ cell and detected with antibody 5643 in the third row. The processing of Notch was detected with antibody C-Notch in the second row. The level of Pen-2 was detected in bottom row with anti-Pen-2 antibody. All the samples were immunoprecipitated with varied antibodies: Pen-2, PS1C 5643 and α-myc as indicated in the figure. As γ secretase inhibitor, CompE (5nM) was applied in WT cell.

CONCLUSION

Since γ-secretase is the key enzyme in Aβ production, it has attracted particular attention in Alzheimer's disease research. However, despite of numerous studies that have focused on the structure and function of γ-secretase complex, there are still a lot of questions remaining to be answered regarding the biological function and specific role of each component in the γ-secretase complex. My study is mainly focused on the role of each γ–secretase components in its activity and stability of the complex.

In conclusion, the first finding of my research is that Aph-1 is dispensable for γ secretase activity of processing both APP and Notch.

Second, our data revealed a very interesting finding that NCT is required for processing of APP, while it is not necessary for the processing of Notch. Even though the molecular mechanism of this finding needs to be further investigated, it opens a new avenue for searching for target of blocking production of Aβ without affecting the signal transduction of NICD.

Third and most importantly, our data revealed that Pen-2 and PS1 are the minimal required and essential components for constituting active γ–secretase of processing Notch. As a mechanism, our data uncovered a novel function of Pen-2 in binding and recruiting substrate Notch to γ–secretase.

In addition, our data also demonstrated that knockout of Aph-1 sensitizes cells to apoptotic stimuli.

VITA

Chen Hu was born in June 1989 in China. She graduated from Jilin University with her bachelor degree at June 2011. After that she was accepted by the Comparative and Experimental Medicine program by University of Tennessee and began her tremendous journey at pursuing her Ph.D. degree.