ROLE OF UBIQUITIN-SPECIFIC PROTEASE 25 IN THE PATHOGENESIS OF ALZHEIMER’S DISEASE IN DOWN SYNDROME

by

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B.Sc., Memorial University, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES (Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

August 2018

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Role of USP25 in the Pathogenesis of Alzheimer’s Disease in Down Syndrome

submitted by Beibei Song in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in Neuroscience

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Abstract

Down syndrome (DS) is the most common genetic cause of intellectual disabilities. Trisomy 21, an extra copy of human chromosome 21, causes the majority of DS cases. After middle age, individuals with DS inevitably develop Alzheimer’s disease, the most common form of neurodegenerative diseases characterized by extracellular amyloid plaque deposition, intracellular neuritic fibrillary tangles and neuronal loss. The extracellular amyloid plaques are made of amyloid β (Aβ) proteins derived from β- and γ- cleavage of amyloid precursor protein (APP). The abnormal accumulation of Aβ proteins plays an essential role in AD pathogenesis.

Ubiquitin-specific protease 25 (USP25) is a deubiquitinating enzyme that locates in the DS critical region of human chromosome 21. It is overexpressed in DS patients and has been shown involved in a variety of cellular processes, including immunity, myogenesis and protein degradation. However, the potential role of USP25 in neurodegenerative diseases has not been examined yet.

This thesis entails an examination of the role of USP25 in the pathogenesis of Alzheimer’s disease in Down Syndrome. First, we investigated the transcriptional regulation of human USP25 gene. We identified a functional SP1 binding site within its 5’ promoter region. We found that Sp1 signaling up-regulated USP25 transcription. Then we showed that USP25 affected APP processing by slowing down the degradation of APP and BACE1. It also altered the intracellular trafficking of BACE1 and promoted C-terminal fragment (CTF) production, indicating its role in amyloidogenic pathway in AD pathogenesis. In the third chapter, we examined the effects of USP25 on neuronal survival and proliferation. We found that USP25 overexpression facilitated oxidative stress-induced cell death and caspase-3 activation through inhibiting NF-κB activation. It upregulation also affects cell cycle regulation both during embryonic neurogenesis and adulthood cortical development.

In summary, this study investigated the effect of USP25 in the development of AD in DS. It demonstrated for the first time that USP25 overexpression contributes to the development of AD pathology by regulating APP processing, affecting neurogenesis. Our findings indicated that USP25 may serve as a potential pharmacological target for treating AD specifically in DS.
Lay summary

Down syndrome (DS) is the most common genetic cause of intellectual disabilities. Trisomy 21, an extra copy of human chromosome 21, causes the majority of DS cases. After middle age, individuals with DS inevitably develop Alzheimer’s disease (AD), the most common form of neurodegenerative diseases. We aimed to understand the underlying mechanism of AD in DS by studying genes located in human chromosome 21. We found that Ubiquitin-specific protease 25 (USP25), a gene located on human chromosome 21 and overexpressed in DS, contributes to development of AD in DS. It regulates two key proteins involved in the formation of amyloid plaques, a key neurological feature in AD. Its overexpression also promoted neurodegeneration and suppressed neurogenesis. This work provides the foundation for the development of potential intervention for treating AD specifically in DS by targeting USP25 gene.
Preface

After completing my Bachelor of science degree, I joined Dr. Weihong Song’s research team to study the molecular mechanism that underlies Alzheimer’s disease. Dr. Song introduced me to a project that focused specifically on AD in Down Syndrome. There are three parts to the project. Chapter 2 covers the first part of the project, focusing on the transcriptional regulation of human USP25 gene. I followed up a project initiated by Odysseus Zis, a former master student in the laboratory, in which he cloned a series of deletion plasmids of the 5’ flanking region of the human USP25 gene and performed the 5’ PCR to determine the transcriptional start site (TSS). We performed the luciferase assay to measure the promoter activity. I performed the gel shifting assay to show the functional binding of SP1 to USP25 promoter region and semi-quantitative PCR and western blotting to demonstrate the up-regulation of USP25 by SP1 signaling.

Chapter 3 covers the second part of the project. We investigated the effect of USP25 on the protein degradation of amyloid precursor protein (APP) and beta-site APP cleaving enzyme 1 (BACE1), proteins that are essential for C-terminal fragment production. Dr Fang Cai designed the USP25-overexpressing plasmids and USP25-transgenic mice. For the current study, I designed and carried out most of the experiments. All animal studies were approved by the University of British Columbia Animal Care Committee.

In chapter 4, we showed the effect of USP25 on cell survival and proliferation, both in vitro and in vivo. I designed and carried out most of the experiments with the exception of the Morris Water Maze, which was performed by Dr Fang Cai. Dr Jason Snyder’s lab advised me on designing animal behavioral and immunohistochemistry experiments. Dr Qin Xu helped me process the animal tissue and lysis. All the behavioral procedures on adult mice were approved by the University of British Columbia Animal Care Committee (protocol number: A14-0191).

As a collaborator on the project, I am a co-first author on two papers that we plan to submit soon. One covers the majority of the content in chapter two and the other covers the majority of the content in chapter 4. I wrote the manuscripts and revised them with the help of my supervisor Dr Weihong Song and colleagues in the lab.
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<tbody>
<tr>
<td>5’UTR</td>
<td>5’-untranslated region</td>
</tr>
<tr>
<td>5’RACE</td>
<td>Rapid amplification of 5’ complementary DNA ends</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACTA1</td>
<td>Actin alpha-1</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<td>APP</td>
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<td>Aβ</td>
<td>β-amyloid</td>
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<td>Anterior pharynx factor-1</td>
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<td>bp</td>
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<tr>
<td>BrdU</td>
<td>5-Bromo-2-DeoxyUridine</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CA3</td>
<td>Cornu Ammonis 3</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CdkS</td>
<td>Cyclin-dependent kinase</td>
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<td>CHX</td>
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<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>DG</td>
<td>Dentate gyrus</td>
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<td>CNS</td>
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<td>DUB</td>
<td>De-ubiquitinating enzyme</td>
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<td>DSCAM</td>
<td>Down syndrome cell adhesion molecule</td>
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<td>DSCR</td>
<td>Down Syndrome Critical Region</td>
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<td>DYRK1A</td>
<td>Dual specificity tyrosine-phosphorylation-regulated kinase 1A</td>
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<td>Dithiothreitol</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>endoplasmic reticulum-associated degradation</td>
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<td>ES</td>
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<td>fMRI</td>
<td>functional Magnetic Resonance Imaging</td>
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<td>NF-κB inhibitor, α</td>
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<td>interleukin</td>
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<td>mtDNA</td>
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<tr>
<td>MTM</td>
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<td>neurofibrillary tangles</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>Notch intracellular domain</td>
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<td>Receptor interacting protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxidative species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SAD</td>
<td>sporadic Alzheimer's disease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SODs</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>TSA21</td>
<td>Trisomy 21</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
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<tr>
<td>UCH</td>
<td>ubiquitin carboxyl-terminal hydrolase</td>
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<tr>
<td>UCHL1</td>
<td>ubiquitin carboxyl-terminal hydrolase L1</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin-specific protease</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage-gated sodium channels (VGSC)</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to express my gratitude to people who have helped me over the five years of working towards my Ph.D. in Neuroscience. First of all, I would like to thank Dr Weihong Song, my supervisor, for your guidance and support. Thank you for taking me as a graduate student and providing me with the opportunity and resources to work on a project that I am passionate about. Your support helped me throughout the hard time when a lot of my experiments were not working. I appreciate that you gave me the freedom to explore different aspects of the project. I would also like to acknowledge the financial support during my doctoral training from CIHR and a four-year doctoral fellowship from UBC.

I would also like to thank my supervisory committee members, Drs. Ann Marie Craig, Jason Snyder, and Yutian Wang. They provided valuable constructive advice on my PhD project and helped me improve throughout the years. I would also like to thank Dr Honglin Luo, who was the external examiner of my comprehensive examination, for advising me both during and after the exam.

To my current and past colleagues in Song lab, thank you for your support for the last five years. In particular, I would like to thank Drs Fiona Zhang, Juelu Wang, Qin Xu and Yun Zhang for helping me through the tough time and taught me experimental skills with every patience. I would not make it through without you. Special thanks to Juelu for taking care of me since I joined the lab. She encouraged me to see the bright side of things when I was frustrated. It was my pleasure to work with all of you.

To my colleagues outside my lab, thank you for your unconditional support over the last five years. Special thanks to Dr Jie Lu and his wife Yuping Li from Dr Yutian Wang’s lab. You are like my parents in academia who have taken care of me since I joined the program. Your advice on life and career development is of great value to me. Thank you, Ms Eva So, for being such a supportive friend, hearing me venting my frustration and dragging me out for Marvel movies when I was “dying” in the lab. That is a lot of “extra work” you have done.
Last but not least, I could not have made this so far without support from my family and friends. I thank my parents for their unconditional love. Thank you for allowing me to stay as a student for the last two decades and supporting every decision I have made. No matter what happens, I will stand strong because I know you are there for me. If the last five years are the hardest years so far in my life, my best friends and mentors, Ms Susan Lin and Mr Wei Lin, are the best things that could ever happen to me. I don’t remember how many times I said thanks to you two, but I am sure this will not be the last time. Wei, a friend and mentor outside of my field, thank you for teaching me the other perspective of the world. You inspired me to push myself and believe that I could succeed if I put my mind into things I am interested in. Thank you for helping me realize my self-worth and focus on the great part of my life. Susan, a brilliant master’s student from my lab, thank you for hugging me six times a day when I failed my experiment for months. Thank you for spending hours talking to me and helping me through the broken-hearted time. Thank you for providing a lot of humors in the lab during the tough time. You are the most beautiful and talented person I have ever met, both inside and out. I hope you will succeed in the future career and be able to do things you are passion about.
To those who still believed in me when my life was not significant at all (p>0.05)
Chapter 1

General introduction

1.1 Down’s syndrome (DS)

1.1.1. DS overview

Down’s syndrome (DS), with an incidence of 1 in 700–1000 live births, is the most common genetic cause of intellectual disabilities (Down, 1866; Jacobs et al., 1959; Lejeune et al., 1959). DS is caused by either an extra copy of entire human chromosome 21 (Hsa21) in all cells (Trisomy-21), in some cells (mosaicism) or only triplication of a part of chromosome by translocation (partial trisomy). Hsa21 is the smallest human autosome and represents around 1-1.5% of the human genome (Hattori et al., 2000), and Trisomy 21 accounts for majority of DS cases. The incidence of Hsa21 is affected by maternal age and genetic background (Canfield et al., 2006; Glasson et al., 2002). The extra copy of Hsa21 may cause increased expression of genes located on the chromosome 21. It is proposed that the imbalance between the expression of Hsa21 and non-Hsa21 genes contributes to the pathogenesis of DS, including cardiac, gastrointestinal, immune system abnormalities, and mental retardation. After middle age, individuals with DS inevitably develop Alzheimer’s disease (AD), characterized by neuritic plaques, neurofibrillary tangles (Glenner & Wong, 1984) and neuronal death (Brooksbank & Balazs, 1984; Busciglio & Yankner, 1995. Sun et al, 2011).
1.1.2 Socioeconomic burden of DS.

As mortality rates for individuals with Down syndrome have improved (Yang et al., 2002), health services must address the increased need of DS individuals for care access. Compared with other children with special health care needs, children with DS have a greater number of co-morbid conditions such as depression and attention deficit/hyperactivity disorder. The average lifespan of DS individuals is now greater than 55 years (O’Nuallain et al., 2007) and most of them develop AD after middle age. The annual societal costs of AD worldwide in 2010 were US$604 billion, or 1% of the aggregated worldwide Gross Domestic Product (GDP). In addition to the financial burden, the disease has imposed heavy social and psychological burden on patients as well as their families.

1.1.3 Phenotypic variability of DS

DS is characterized by extensive phenotypic variability, with most traits occurring in only a fraction of affected individuals. Cognitive impairment, muscle hypotonia at birth, and dysmorphic features occur to some extent in all affected individuals. In contrast, the majority of other traits such as congenital heart defects are only present in a fraction of individuals with trisomy 21 (Roizen & Patterson, 2003). In addition, although cognitive impairment is present in all patients with DS, the severity varies (Pennington et al., 2003). There are several main hypotheses addressing the phenotypic variability in DS, including 1) natural gene-expression variation of HSA21 genes in individuals with trisomy 21 contributes to the phenotypic variability in DS (Prandini et al., 2007); 2) environmental factors interact with genetic factors, allowing certain phenotypes to only occur in a portion of individuals with DS.
1.1.4 Variations in gene expression in trisomy 21

Theoretically, the extra copy of human chromosome 21 would result in a 50% increase in the level of transcripts of all genes on HSA21 and therefore lead to 1.5-fold of protein level changes. However, there is not always a direct correlation between genomic imbalance and transcript level of genes within the aneuploid segment, suggesting that complex molecular mechanisms regulate RNA transcript levels of HSA21 genes (Kahlem et al., 2004; Lyle et al., 2004). At the same time, not all the Hsa21 genes are dosage-sensitive. Therefore, it is essential to understand the genomic content of Hsa21 and evaluate how the altered expression of dosage-sensitive Hsa21 genes contributes to the molecular, cellular and physiological changes underlying DS pathology.

To help explain how variations in gene expression in trisomy 21 contribute to the phenotypic variability, one hypothesis suggests that the effect of some dosage-sensitive genes on the phenotypes are allele-specific that a phenotype is only present if the total transcript/protein level from the combination of the three alleles reaches a crucial amount. Variations in the dosage-sensitive gene expression in each allele contribute to the changes in the total protein expression of those genes from allelic combination, resulting in certain phenotypes to only occur in a portion of individuals. An alternative explanation suggests that certain conserved functional non-genic sequences on HSA21 contribute to the DS phenotypes through regulating dosage-sensitive genes in Hsa21. Finally, dosage-sensitive genes could have some indirect effects on the phenotype. The indirect effects might be due to the interactions of HSA21 genes or gene products with non-HSA21 genes or gene-products, that contribute to the susceptibility of specific phenotypes in DS.
1.2 Alzheimer’s disease

1.2.1. Overview

Alzheimer’s disease (AD) is the most common neurodegenerative disease leading to dementia, characterized by neuritic plaques, neurofibrillary tangles and neuronal cell loss. Approximately one in 9 individuals over the age of 65 years and nearly half of the population over 85 years are affected by sporadic AD (Alzheimer’s, 2016). Early-onset Alzheimer’s disease, caused by genetic mutations and inherited in an autosomal dominant manner, may occur much earlier than 65 years. Less than 1% of AD cases are early-onset familial AD (FAD) and those patients develop symptoms from as early as 40 years old (Campion et al., 1999). Individuals with DS inevitably develop characteristic AD after middle age, which is earlier than the general population (Glenner & Wong, 1984).

Clinical symptoms of Alzheimer’s disease. Clinical presentation of AD has four stages: the pre-dementia stage, mild stage, moderate stage and severe stage. Symptoms at the pre-dementia stage include mild impairment in acquiring new information and minor defects in short-term memory (Arnaiz & Almkvist, 2003). At mild dementia stage, learning and memory are further impaired. At the moderate stage, logical reasoning, planning, and organizing abilities significantly deteriorate, along with a progressive loss in speaking, reading and writing skills. At the severe stage of AD, all cognitive and verbal functions are severely impaired. Patients need assistance in their lives. At the terminal stage, patients often die of other conditions but not AD itself (Sadock, 2004).
Pathological features of Alzheimer’s disease  Neuritic plaques, neurofibrillary tangles and neuronal cell loss are the three major features of AD. The central component of neuritic plaques is amyloid β (Aβ) surrounded by dystrophic neurites. Deposition of Aβ in the brain is the unique hallmark of AD pathology (Mattson, 2004). Intracellular neurofibrillary tangles (NFT) are composed of hyperphosphorylated microtubule-associated tau protein. NFT can also be found in many other neurodegenerative diseases, including frontotemporal dementia, Pick Disease, argyrophilic grain disease, progressive supranuclear palsy, and corticobasal degeneration (Rademakers et al., 2004). Neuronal death in neurodegenerative diseases is selective for specific brain regions. In AD, neuronal loss is prominent in the cerebral cortex and the limbic lobe (Arnold et al., 1991; Brun & Englund, 1981).

1.2.2. Amyloid hypothesis

The original amyloid hypothesis, proposed in 1992 by Hardy & Higgins, suggested that the deposition of Aβ, the main component of the plaque, triggered AD pathologies, including neurofibrillary tangles, neuronal loss and dementia (Figure 1.1 black arrow). The biggest counter-evidence is that the number of amyloid plaques in the brain is correlated poorly with the degree of cognitive impairment in AD patients, while NFT pathology correlated better with AD cognitive defects (Arriagada et al., 1992)

In 2002, Hardy and Selkoe proposed revised amyloid oligomer hypothesis aiming to explain the discrepancy between some of the observations and the Aβ plaque hypothesis (Figure 1.1 red arrow). The revised version suggested that instead of amyloid plaques, Aβ oligomers are responsible for initiating the AD pathogenic cascade. Aβ hypothesis has become the most popular in the field, supported by the following scientific observations. First, levels of soluble
Aβ are well correlated with AD cognitive impairment (McLean et al., 1999) and diffusible Aβ oligomers inhibit hippocampal long-term potentiation (LTP) (Lambert et al., 1998), impair synaptic plasticity (McDonald et al., 2010) and memory in rodents (Lesne et al., 2006). Secondly, the majority of the early onset familial ADs (FAD) are caused by missense mutations of genes associated with Aβ production. Those mutations are sufficient to cause AD as early as 40s in FAD patients. On the other hand, mutations of tau, the protein composed of NFTs, are not sufficient to cause AD. Frontotemporal dementia, caused by tau mutation, shows distinct clinical features from AD (Hutton et al., 1998; Spillantini et al., 1998), with no amyloid plaques formed as in AD. At the same time, ApoE, a genetic risk factor associated with AD (Corder et al., 1994; Corder et al., 1993), is associated with Aβ production and clearance (Bu, 2009) instead of NFT formation. This evidence shows that the generation of Aβ and the formation of Aβ oligomers are essential for triggering AD pathogenesis.
Figure 1.1. The amyloid hypothesis of Alzheimer’s disease

The amyloid cascade hypothesis states that the formation of amyloid plaques causes Alzheimer’s pathogenesis (black arrow), followed by other features including neurodegeneration and tau pathology. The revised amyloid oligomer hypothesis stated that instead of Aβ plaques, Aβ oligomers induced neuronal dysfunctioning and neuronal death.
1.2.3. APP processing pathway

**Overview** APP is a type I transmembrane protein encoded by a single gene on HSA21. It undergoes sequential proteolytic cleavage on its extracellular domain and transmembrane domain to produce multiple fragments. Aβ is derived from APP after sequential cleavage by β- and γ-secretase (Selkoe, 2001) (Figure 1.2). Under normal physiological conditions, the majority of APP goes through the non-amyloidogenic pathway, cleaved first by α-secretase, the Aβ domain is then cleaved by γ-secretase, which precludes the generation of Aβ (Esch et al., 1990).

Another non-amyloidogeneic pathway involves θ-secretase (BACE2), which cleaves APP at the θ-secretase site within the Aβ domain that precludes Aβ production (Sun et al., 2006). BACE1 cleaves APP at the minor Asp+1 site (Yan et al., 1999) and a major Glu+11 (Deng et al., 2013) of Aβ to produce membrane-bound C99 and C89 respectively. Further cleavage of C89 by γ-secretase does not yield amyloidogenic Aβ, while cleavage of C99 yields an Aβ fragment of 39 to 43 amino acids (Zhang et al., 2000; Wolfe et al., 1999). The majority of α-cleavage is processed on the plasma membrane while β-cleavage favors acidic intracellular compartments (Wolfe et al., 1999), suggesting that the trafficking and localization of APP affect the preferential cleavage between the α-site and the β-site.
Figure 1.2: APP processing pathways

APP is first cleaved by α-secretase or β-secretase followed γ-secretase. Under physiological conditions, the predominant APP processing is by α-cleavage within the Aβ domain at Leu-17 to produce sAPPα and membrane-bound CTFα C83 (middle purple arrow). C83 is further cleaved by γ-secretase (left orange arrow), producing extracellular fragment P3 and intracellular AICD C57/59. Alternatively, APP is processed by β-secretase (red arrow and left purple arrow). APP is cleaved at Asp-1 site to produce CTFβ C99 and sAPP β, or at Glu-11 site to produce CTFβ C89 and sAPPβ. C99 is subsequently cleaved by γ-secretase to yield the amyloidogenic Aβ and intracellular AICD C57/59 (left blue arrow). C89 is cleaved by γ-secretase to yield the non-amyloidogenic truncated Aβ (tAβ) and C57/59 (right blue arrow). β-secretase preferentially cleaves wild-type APP at Glu-11 site over Asp-1 site, whereas it cleaves APP Swedish mutant mainly at Asp-1 site. A small portion of APP may undergo θ-secretase at Phe-20 site by BACE2 (right purple arrow). CTFθ C80 is subsequently cleaved by γ-secretase to produce P3θ and C57/59 (right orange arrow). Image adapted from Zhang & Song, 2017.
Non-amyloidogenic pathways of APP processing Under normal physiological conditions, APP is mainly cleaved by \( \alpha \)-secretase between Lys-16 and Leu-17 within the A\( \beta \) domain, that yields sAPP\( \alpha \) and CTF\( \alpha \) C83 (Esch et al., 1990). C83 is further processed by \( \gamma \)-secretase to generate p3 and AICD C57/59 (Haass & Selkoe, 1993). Some ADAM (A disintegrin and metalloprotease) family proteins, including ADAM9, ADAM10 and ADAM17, have been proposed to be the \( \alpha \)-secretases. It has been shown that an ADAM inhibitor decreased sAPP\( \alpha \) and CTF\( \alpha \) C83 production in wild-type but not in ADAM17 knockout fibroblasts (Buxbaum et al., 1998), suggesting that other ADAM proteases may compensate for the loss of ADAM17 and more than one protease can act as the \( \alpha \)-secretase. At the same time, different ADAM knockout mice showing different phenotypes suggested that each protease has its own unique physiological function (Black et al., 1997; Hartmann et al., 2002; Guaiquil et al., 2009).

BACE2, sharing 52% amino acid sequence identity with BACE1, was identified as a homolog of BACE1 (Saunders et al., 1999; Acquati et al., 2000). It is located on the Down Syndrome (DS) critical region of chromosome 21. It was suspected that BACE2 contributed to the early-onset FAD syndrome in DS patients, while studies have shown that it cleaved APP at Phe-19 and Phe-20 sites much more efficiently than at the BACE1 cleavage site Asp-1 (Farzan et al., 2000). Our lab further showed that BACE2 functions as a \( \theta \)-secretase and overexpressing BACE2 by lentiviral infection in the primary neurons of APP transgenic mice significantly decreased A\( \beta \) production and AD pathogenesis (Sun et al., 2006). Therefore, BACE2, a \( \theta \)-secretase in vivo, serves as an alternative non-amyloidogenic cleavage enzyme other than \( \beta \)-secretase.
Amyloidogenic pathway of APP processing  
Beta-site APP cleaving enzyme 1 (BACE1) cleaves APP either at the Asp-1 site to yield CTFβ C99, or at the Glu-11 site to produce CTFβ C89. Aβ can be only produced by the γ-secretase’s cleavage of C99, not C89. For human wild-type APP, BACE1 mainly cleaves APP at Glu-11 and produces non-pathogenic truncated Aβ. In some cases of familial Alzheimer’s disease (FAD), APP mutation may shift the preferential β-cleavage sites to Asp-1 site (Deng et al., 2013). The shift between preferential cleavage site results in a higher C99/C89 ratio, and subsequently yields more pathogenic Aβ.

1.2.4 Functions & regulation of the components of Amyloidogenic pathway.

APP  
APP is a type I transmembrane protein that is highly expressed in the brain. In human, it is located on chromosome 21. Depending on the site of alternative splicing, there are three major isoforms of APP- APP695 (Kang et al., 1987) as the main isoforms in neurons, APP751 (Ponte et al., 1988; Tanzi et al., 1987), and APP770 (Kitaguchi et al., 1988). Full-length APP can interact with microtubule motor protein kinesin-I through its KLC subunit to facilitate axonal transport in neurons (Kamal et al., 2000). APP itself also participates in the axonal transport of BACE1 and presenilin 1 (PS1) (Kamal et al., 2001). It is also involved in cell adhesion by interacting with extracellular matrix proteins (Kibbey et al., 1993; Beher et al., 1996; Clarris et al., 1997). Up-regulation of APP promotes spine-formation and specific activation of the GluR2 subunit of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in cultured hippocampal neurons and APP-transgenic mice (Lee et al., 2010). Mice with APP-deficiency had fewer and shorter dendritic spines than their littermate control mice. Increased glutamate synthesis was observed in the brain of APP transgenic mice. Glutamate levels were reduced while GABA (Gamma-amino butyric acid) levels were increased in APP-deficient mice,
suggesting that APP also regulates the balance of excitatory and inhibitory neurotransmitters (Lee et al., 2010). These findings suggest that APP plays an important role in the synaptic formation, intracellular trafficking and regulation of neurotransmitters, therefore its dysregulation may affect learning and memory.

APP is synthesized in the endoplasmic reticulum (ER) and trafficked to the Golgi apparatus for further post-translational modification before reaching the plasma membrane (Koo et al., 1996; Small & Gandy, 2006). It is then rapidly internalized to the endosome, after which the majority is sorted to the lysosome for degradation, while a small fraction is trafficked back to the trans-Golgi network (TGN) (Reviewed by Small & Gandy, 2006). APP maturation involves many post-translational modifications throughout trafficking in the secretory pathway. In the ER, its N-terminus signal peptide is removed and NH2-groups of asparagine residues are modified by N-glycosylation. In the Golgi compartment, APP is further modified by O-glycosylation on its ectodomain. Trafficking into the TGN, APP is subjected to sulfation on tyrosine residues within its ectodomain (Reviewed by Small & Gandy, 2006).

Under normal physiological conditions, the majority of APP is degraded by the lysosome system while a small proportion of APP undergoes proteasome degradation. Lysosome inhibition by either chloroquine or ammonium chloride inhibits the degradation of full-length APP. Under cellular stress, ubiquitin ligase Synoviolin/Hrd1 ubiquitinated misfolded APP and target them for ERAD by the proteasome.
**Beta-secretase 1 (BACE1)** BACE1, the β-secretase *in vivo*, is required for Aβ generation. It is a type I membrane protein with a luminal active site allowing it to cleave APP at the β-secretase cleavage site. Its highest activity was observed in neural tissues (Seubert et al., 1993) while its activity is low in glial cells (Zhao et al., 1996). Under physiological conditions, BACE1 also plays roles in axonal growth and brain development through regulating myelination by cleaving neuregulin-1, which has been known for regulating myelination in both the CNS and PNS (Hu et al., 2006). BACE1 knockout mice have hypomyelinated axons in both central and peripheral nervous system (Hu et al., 2006). At the same time, some of the essential auxiliary subunits of voltage-gated sodium channels (VGSC)- ion channels involved in the initiation and propagation of action potential, are also the substrates of BACE1 and γ-secretase. The regulation of those sodium channels’ expression and turnover by BACE1 may play a role in modulating neuronal activity (Kim et al., 2007). As a result, BACE1 deficient mice exhibited impairment in synaptic plasticity and cognitive function, suggesting that BACE1 plays an essential role in maintaining the normal physiological functioning of the neuron.

BACE1 gene expression is tightly regulated by transcription factor Sp1 (Christensen et al., 2004). Its β-secretase activity depends on its protein levels and maturation (Qing et al., 2004). During its maturation, BACE1 undergoes a complex set of post-translational modifications, including the removal of the 24-amino-acid N-terminal region of the pro-BACE1 (Benjannet et al., 2001; Capell et al., 2000; Haniu et al., 2000) and the N-glycosylation at Asn153, 172, 223, and 352 (Benjannet et al., 2001; Capell et al., 2000; Haniu et al., 2000; Charlwood et al., 2001). Following glycosylation, BACE1 is sulfated and then palmitoylated at its three cysteine residues within the cytosolic tail (Benjannet et al., 2001). Phosphorylation of BACE1 on Ser498 regulates BACE1 recycling between the cell surface and endosomes (Walter
et al., 2001). The mature form of BACE1 is mainly located in compartments with a low pH environment, including TGN and late endosomes. The endosome trafficking signal sequence at the C-terminal of BACE1- DISLL (amino acid 496-500), an acid cluster-dileucine motif (ACDL), controls its intracellular trafficking (Pastorino et al., 2002). Deletion or mutation of the ACDL motif alters the trafficking of BACE1, causing it to be localized more at the cell surface and less within endosomal compartments (Pastorino et al., 2002).

BACE1 can be trafficked to endosomes-lysosomal pathway via either internalization from the plasma membrane or directly from the TGN, then ultimately degraded within lysosomes (Huse et al., 2000). It can also be regulated by autophagy-mediated degradation. It can be recruited into the autophagic vacuoles (AVs) and trafficked to lysosome through AV retrograde transport. Either autophagy activation or enhanced retrograde transport by overexpressing dynein adaptor Snapin facilitates the turnover BACE1 (Feng et al., 2017).
Figure 1.3. Intracellular trafficking of APP and BACE1

APP and BACE1 are type-I transmembrane proteins that sorted through multiple membranous compartments of the cell. Both APP and BACE1 are N-glycosylated in the ER and O-glycosylated in the Golgi apparatus before reaching the plasma membrane. Protein folding properly requires glycosylation. Unless it is folded properly, APP will be degraded through ER-associated degradation (ERAD), executed by proteasome. The sorting triangle that interconnects the trans-Golgi network (TGN), cell surface, and the endosome is critically important for APP and BACE1 protein sorting. They are then rapidly internalized to the endosome, where some are sorted to lysosome for degradation while a small fraction is sent back to TGN. Part of the protein are directly sorted to the endosome without reaching the plasma membrane.
1.2.5. APP processing and trafficking in the secretory pathway

Both APP and BACE1 are sorted through the secretory and endocytotic pathways. The majority of APP is localized in the Golgi, with a portion of it further trafficked to the cell membrane and then internalized into early endosomes (Caporaso et al., 1994). From the endosome, APP can either recycled back to the cell surface, or sorted to the lysosomal degradation pathway (Haass & Selkoe, 1993). Since α-secretase is enriched at the plasma membrane, those APPs sorted to the cell surface are more likely to be cleaved by α-secretase rather than BACE1, which is located mainly in the endosome and TGN (Vassar et al., 1999). Endosome and TGN are acidic compartments, allowing the optimal cleavage activity of β-secretase. Therefore, those APPs trafficked to the endosome and TGN are more likely to be cleaved by β-secretase than α-secretase (Koo & Squazzo, 1994). Gamma-secretase exists in the ER, Golgi/TGN and endosome (Zhang et al., 1998). It is active in the endosome in presynaptic terminal of the neuron (Rechards et al., 2003). Therefore, APP trafficking pathways into endosomes allows Aβ production through β- and γ-cleavage of APP. Blocking endocytosis to reduce APP internalization decreases Aβ production, while promoting endocytosis to facilitate APP sorting into endosomes increases Aβ production (Grbovic et al., 2003). These findings suggest that APP and BACE1 trafficking have significant effects on Aβ production. Retention of BACE1, APP and γ-secretase in the TGN or enhancing their internalization into endosome promotes amyloidogenic pathway (Haass et al., 1992) while enhancing BACE1 and APP trafficking to the cell surface or reducing their internalization facilitates non-amyloidogenic pathway (Koo & Squazzo, 1994).
Figure 1.4. APP and BACE1 protein trafficking.

APP matures through the constitutive secretory pathway from ER to the plasma membrane. In the non-amyloidogenic pathway, the majority of APP (Green arrow pathway) was internalized into early endosomes, either recycled back to the plasma membrane, or degraded in lysosome. Those APPs are more likely to be cleaved by α-secretase than β-secretase, since α-secretase is enriched in the plasma membrane. In the amyloidogenic pathway (Red arrows), APP trafficks through the secretory and recycling pathways where APP interacts with β-secretase, whose activity also relies on the acidic pH of endosome and TGN compartments.
1.3. Alzheimer’s disease, neurogenesis and neurodegeneration

1.3.1 Neurogenesis overview

Neurogenesis, a process of generating functional neurons from precursors, occurs mainly in the subventricular zone (SVZ) and subgranular layer of the dentate gyrus (DG) in the adult brain. It is a dynamic, highly-regulated process that is subjected to modulation by various physiological and pathological stimuli. In both regions, neuronal stem cells (NSCs)- capable of self-renewal and broadly multipotent, can give rise to neural progenitor cells (NPCs). NPCs proliferate quickly and remain capable of becoming glia and neurons within a certain number of progeny. Early in mammalian embryogenesis, the central nervous system (CNS) is derived from the dorsal midline of the embryo- a clearly defined layer of cells called the neural plate. In the early neural plate, growth and proliferation of the cells eventually result in the closure of the developing neural groove. A hollow neural tube is formed, with a cavity and an epithelial layer that contains the neural stem cells (NSCs). NSCs either divide symmetrically to enrich the NSC pool, or asymmetrically to generate more differentiated progeny from which mature cells of neuronal and glial lineages develop. As development continues, differentiated progeny migrate from the ventricular zone into the overlying cortex, while neural stem cells remain attached to the basal lamina, retaining their ability to self-renew and differentiate into different types of cells in the CNS (reviewed by Beddington & Robertson, 1999). In the adult SGZ, proliferating radial and non-radial precursors generate intermediate progenitors that give rises to neuroblasts. Immature neurons, derived from neuroblasts, migrate into the inner granule cell layer and differentiate into dentate granule cells in the hippocampus. Within days, newborn neurons extend dendrites towards the molecular layer and project axons through the hilus toward the CA3, and therefore integrating into the existing circuitry (reviewed by Ming & Song, 2011).
1.3.2. Role of cell cycle in regulating neurogenesis

A cell can be duplicated into two daughter cells through a process called cell cycle that consists of a series of highly coordinated events (reviewed by Schafer, 1998). Starting from a quiescent state (G0), the cell enters the first growth phase (G1) that allows the cell to prepare for DNA replication (S phase). Later, the cell enters into the second growth phase (G2) that allows it to continue to grow, followed by the separation of its two daughter cells. This process is highly regulated by the cyclin-dependent kinase (Cdks) and their corresponding regulatory partner cyclins. In the G1 phase, E2f transcription factor is induced by the Cdk4/6-cyclin D complex to trigger the expression of certain genes involved in the cell cycle progression. Cdk2-cyclin E is responsible for the entry into S phase through activating certain gene transcriptions involved in the G1/S transition, followed by the S/G2 transition regulated by Cdk2/cyclin A complex. In the final step, mitosis and cell division are regulated by Cdk1 and cyclin B complex.

In the adult dentate gyrus, neurogenesis occurs when quiescent neural stem cells (Type 1 NSCs) enter into the cell cycle and give rises to postmitotic neurons that could be integrated into pre-existing neuronal network (Kempermann et al., 2004). The proliferation of adult neural stem cells and the cell cycle progression are strictly regulated by specific CDK proteins. Cyclin D2 is one of the cyclins in the adult neurogenic niches regulating neurogenesis. The induction of cyclin D2 activates the transcription factor E2F, which is essential for cell-cycle progression (Ohtani et al., 1987; Iwanaga et al., 2001). Cyclin D2 ablation almost blocked neurogenesis in the adult dentate gyrus. The induction of cyclin D2 is essential for the commitment of the neural stem cells to neurons (Kowalczyk et al., 2004). The few cells derived within the adult dentate gyrus of cyclin D2-knockout mice belong to the astroglial lineage (Kowalczyk et al., 2004). On the other hand, cyclin D1 overexpression induces the proliferation of stem cells at the expense of
differentiation and promotes the commitment of the neural stem cells to neurons, thus reducing neurogenesis in the adult hippocampus (Artegiani et al., 2011). The balance between the abundance of cyclin D1 and cyclin D2 affects the fates of neuronal stem cell-neuronal or glial.

1.3.3. Defects in neurogenesis in AD

In humans, normal moderate decline in neurogenesis occurs with aging. Declines in adult neurogenesis with age may affect hippocampal functioning, which is particularly impaired in AD. Studies in high resolution fMRI (Brickman et al., 2014), object/pattern recognition (Toner et al., 2009; Stark et al., 2010) and learning/memory (Yassa et al., 2011; Brickman et al., 2012) have revealed that age-related memory loss starts in the DG, whose synaptic contacts onto granular cells also reduce with age (Flood et al., 1996; Geinisman et al., 2004). These results suggest that decline in neurogenesis due to aging increases DG’s susceptibility to memory impairments and contributes to cognitive dysfunction in AD. Some studies suggested that mouse models of FAD have shown reduced neurogenesis during early stage, while other studies revealed that most FAD mouse lines that overexpress APP exhibited enhanced rather than reduced neurogenesis (summarized by Hollands et al., 2016). Soluble APP, a proliferation factor for neural progenitor cells (Demars et al., 2011), may contribute to the enhanced neurogenesis in those FAD mouse lines. During early development, granule cells in those FAD mouse lines showed greater dendritic length and spine density than wild-type controls. However, during later maturation phase, those cells exhibited functional deficits and morphological defects, and received imbalanced GABAergic and glutamatergic inputs (Sun et al., 2009), suggesting that new neurons are less likely to mature and function normally.
1.3.4. Cell death and apoptosis: overview

The active proliferation and differentiation of progenitors is balanced by apoptosis. During development, neurons are produced in excess and their survival requires the establishment of appropriate connections with their targets. Apoptosis is involved in establishing connections between neurons and eliminating excess neurons that fail to contact to their targets (Barde, 1994). Apoptosis can also serve as a defense mechanism once noxious agents have invaded the cell (Norbury & Hickson, 2001). It is conducted in a controlled manner that minimizes damage and disruption of neighboring cells (Kerr et al., 1972) and no inflammatory reaction is triggered. The apoptotic process can be induced either by signals arising within the cell or by external stimuli that activate death receptors. Cells undergoing apoptosis retract from neighboring cells and shrink. They are rounded with dark eosinophilic cytoplasm and tightly packed organelles, followed extensive dynamic plasma membrane blebbing. In the end, fragments of the apoptotic cells are separated into apoptotic bodies and subsequently engulfed by phagocytes (reviewed by Kurosaka et al., 2003).

Apoptosis is executed by caspases. There are two groups of caspases—those that are centrally involved in apoptosis and those that are involved in cytokine processing during inflammatory responses. The ones involved in apoptosis are either initiator caspases, which are responsible for initiating caspase activation cascades, or effector caspases that cleave cellular substrates and dismantle cells (Los et al., 1999). Triggered by external stimuli such as oxidative stress, the activation of initiator caspases converts effector caspases into their active forms (Slee et al., 1999). There are three major caspase activation pathways. Binding of Fas ligand to Fas receptor promotes the activation of caspase-8, which then cleaves pro-caspase-3, leading to further downstream substrate proteolysis (Kruidering & Evan, 2000). Caspase-8 can also initiate
apoptosis through the mitochondrial pathway by promoting cytochrome c release from mitochondria, resulting in the formation of apoptosome and the activation of caspase-9 (Li et al., 1997). Active caspase-9 then processes caspases-3, and -7, which propagates further caspase processing events when caspase-3 is absent or disabled (Inoue et al., 2009). Caspase-2 is activated in response to DNA damage. Instead of triggering apoptosis, the activation of caspase-2 causes cell-cycle arrest and promotes G2/M DNA-damage checkpoints (Ho et al., 2009).

1.3.5. Role of aging and oxidative stress in regulating cell death and neurogenesis

Ageing is featured with progressive decline in physiological functioning, as the consequence of increased free radical-induced damage and decreased ability to counterbalance the damage though endogenous antioxidant defensive mechanisms (Beckman & Ames, 1998). The majority of free radicals are oxygen radicals and other reactive oxygen species derived from mitochondria during cellular energy production (Balaban et al., 2005). The alternative source of free radicals is through peroxisomal β-oxidation of fatty acids that yields H2O2 as a by-product (Beckman & Ames, 1998). In the aging brain, the functioning of normal antioxidant defense mechanisms declines, which increases the susceptibility of the brain towards oxidative damages including changes in mitochondrial DNA (Richter et al., 1988), membrane fatty acid composition (Ulmann et al., 2001) and protein oxidation (Smith et al., 1991). In response to the brain damage due to oxidative stress, microglia induce protective immune responses that usually resolve potential acute pathogenic conditions. However, during chronic inflammation, prolonged activation of microglia releases a variety of pro-inflammatory cytokines including interleukin-1 (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) (Gemma et al., 2002), along
with free radicals such as superoxide and nitric oxide (Chang & Liu, 1999; Colton & Gilbert, 1987), which may further increase the oxidative stress.

Both ageing and neurodegenerative diseases are associated with increased oxidative stress and altered adult neurogenesis. In the short-term, oxidative stress stimulation promotes proliferation and differentiation of neural progenitor cells (Bai et al., 2013). However, the long-term exposure of reactive oxidative species (ROS) damages cells and induces cellular apoptosis. Superoxide dismutase (SODs) deficit mice have long-term ROS accumulation and exhibit decreased neurogenesis in hippocampus after irradiation (Huang et al., 2012). Increasing SOD function or overexpression of SOD can rescue the effect of ROS and prevent the cognitive defects dependent on hippocampal functioning caused by ROS (Zou et al., 2012).

1.3.6. Neurodegeneration in AD

AD is one of the most common aging-dependent neurodegenerative disorders. Examination of postmortem brain in AD has shown atrophy in cortex that is associated with significant reduction in the number of neurons (Ball, 1977; Masliah et al., 1991). Neuronal loss is also accompanied by reduced synaptic density. The extent of neuronal loss in hippocampus and cerebral neocortex is correlated with the clinical manifestation of AD. Alteration of APP processing, enhancing the generation of Aβ and formation of Aβ oligomers, may contribute to neuronal loss in AD. The accumulation of Aβ oligomers in the nerve terminals may lead to synaptic damage and cause neuronal death (Walsh & Selkoe, 2004).

Neuronal death in neurodegenerative diseases is selective for different neuron types. Apoptosis plays an essential role in neuronal cell death in both AD and DS (Sun et al., 2011; Lakhani et al., 2006). It can be initiated either through an intrinsic or extrinsic apoptotic
pathway. Altered expression of apoptotic proteins, such as Bcl family proteins, p53, and caspase 3, has been shown in the AD brain (Sun et al., 2011). Studies have shown that Aβ can activate apoptosis through either the extrinsic pathway by extracellular Aβ (Oddo et al., 2003) or the intrinsic pathway by intracellular Aβ (Glabe, 2001). Extracellular Aβ oligomers binding can initiate apoptosis through disrupting calcium homeostasis, altering glutamatergic transmission and promoting neuroinflammation (Gatta et al., 2011). Accumulation of intracellular Aβ in ER or endosomes can also initiate apoptosis through triggering the unfolded protein response or ER stress. The initiation of neuroinflammation by Aβ may further aggregate apoptosis by enhancing the levels of IL-1 and TNF-α, which promotes the release of cytochrome-C in mitochondria and provokes the activation of caspase 9 (Alvarez et al., 2011). Both IL-1 and TNF-α can also initiate apoptosis through activating NF-κB signaling pathway (as discussed below). The consequences of caspase activation are neuronal loss and cleavage of tau protein. The cleaved form of tau is more readily aggregates into fibrils than full-length tau, which may contribute to the formation of NFT in AD (Gamblin et al., 2003)

1.3.7. Role of NF-κB in regulating neurogenesis and neurodegeneration

**NF-κB activation** NF-κB is a dimeric transcription factor consisting of proteins with highly conserved Rel-homology domains (RHD) including p65, c-Rel, Rel-B, p50 and p52 (Li & Verma, 2002). The Rel-homology domain (RHD) enables NF-κB to interact with the IκB proteins, translocated into the nucleus and bind promoters containing consensus sequence (Beg et al., 1992). The IκB proteins- IκBα, β and ε, regulate NF-κB activation by blocking its nuclear import and inhibit its transcriptional activity. NF-κB activation pathways can be classified as either the canonical or non-canonical pathway. In the predominant canonical pathway, agonists
such as TNFα and IL-1β activate the IκK complex, which phosphorylates IκB proteins at two N-terminal serine residues (Chen et al., 1995). The phosphorylation signaling targets IκB for polyubiquitination and degradation by the proteasome, releasing NF-κB and allowing its translocation into the nucleus (Palombella et al., 1994).

TRAF (TNF receptor associated factor) proteins, a family of ubiquitin E3 ligases (Deng et al., 2000), are essential in initiating NF-κB signaling pathway. Many studies have been focused TRAF2 and TRAF6. TNF receptor activation recruits TRAF2, which interacts with the receptor interacting protein (RIP), a protein kinase signaling in the TNF-NF-κB pathway (Shi & Kehrl, 2003). TRAF6 is responsible for NF-κB activation in both IL-1 and TLR pathways (Naito et al., 1999; Lomaga et al., 1999). IL-1R or TLRs activation recruits MyD88 adaptor protein and protein kinase RAK4 and IRAK1. IRAK1 then triggers the K63-linked polyubiquitination of TRAF6. Ubiquitinated TRAF6 activates TAK1 kinase, which activates IκK and JNK to initiate downstream NF-κB signaling cascades.
NF-κB activation.

NF-κB signaling pathway can be activated via various stimuli, including oxidative stress and agonists such as tumor necrosis factor α (TNFα) or interleukin-1β (IL-1β). In response to ROS, the signal pathway activates the IkK complex by phosphorylation. The phosphorylation signaling targets IkB for polyubiquitination and degradation by the proteasome, which releases P65/P50 complex and allows its nuclear translocation. The activation of NF-κB promotes the expression of genes that typically promotes cellular survival, LTP, apoptosis, learning and memory.
Role of NF-κB in learning and memory. NF-κB is involved in many physiological processes including immunity, inflammation, apoptosis, learning and memory. NF-κB is constitutively activated in glutamatergic neurons, in particular hippocampus and cerebral cortex (Kaltschmidt et al., 1993; Kaltschmidt et al., 1994). Its constitutive activation can be suppressed by glutamate antagonists (Lilienbaum & Israel, 2003), suggests that constitutive NF-κB activation results from physiological basal synaptic transmission. p65 protein can be retrogradely transported from active synaptic sites back to the nucleus (Wellmann et al., 2001; Meffert et al., 2003) after glutamatergic stimulation. It has been shown that NF-κB is involved in multiple types of learning and memory. In a radial arm maze, p65−/− mice made significantly more trial errors than control mice (Meffert et al., 2003), suggesting that p65−/− mice had spatial learning defects. Mice expressing the super-repressor of NF-κB also had long-term potentiation (LTP) and long-term depression (LTD) defects (Kaltschmidt et al., 2006). To demonstrate the underlying mechanism of NF-κB’s regulation on learning and memory, a previous study has shown that repression of NF-κB in transgenic mice resulted in reduced Protein kinase A (PKA) activity, which reduced cAMP response element-binding protein (CREB) phosphorylation and thus raised intracellular cAMP levels (Kaltschmidt et al., 2006). PKA–CREB signaling is essential for regulating LTP, LTD, spatial learning and memory (Kandel, 2001). In human PKA genes’ promoter, functional NF-κB binding element was located, suggesting that NF-κB also regulates PKA at transcriptional level (Kaltschmidt et al., 2006). These findings provide strong evidences suggesting that activation of NF-κB signaling pathway plays an essential role in regulating spatial learning and memory through the PKA/CREB pathway.
Role of NF-κB in neuronal death associated with oxidative stress NF-κB can serve either as an anti-apoptotic or pro-apoptotic transcription factor, depending on the nature of the stimulus. It can induce apoptosis either through its interaction with tumor suppressors (Tergaonkar et al., 2002) or acting as a transcriptional activator of some pro-apoptotic genes such as Fas/CD95 (Wiener et al., 2004) and FasL (Kimura et al., 2003). Activated by TNF-α via its type 1 receptor (TNFR1), NF-κB inhibits apoptosis by inducing the expression of certain genes whose products can inhibit apoptosis (Jones et al., 1997).

In response to ROS, the activation of NF-κB promotes the expression of genes that enhances cell survival, including the gene expression of antioxidant proteins such as Manganese Superoxide Dismutase (SOD2) (Jones et al., 1997; Djavaheri-Mergny et al., 2004) and Copper-Zinc Superoxide Dismutase (SOD1) (Rojo et al., 2004). The alternative way that contributes to the role of NF-κB in cell death response to ROS is the crosstalk between NF-κB and JNK. The activation of NF-κB inhibits sustained JNK activation, therefore prevents both apoptosis and necrosis (Reuther-Madrid et al., 2002; Tang et al., 2002).

ROS has been shown to be able to activate and repress the NF-κB pathway, depending on the regions within the cell. ROS often promotes the NF-κB pathway in the cytoplasm while inhibiting NF-κB activity in the nucleus (Kabe et al., 2005). Oxidation of NF-κB proteins by ROS inhibits its DNA binding ability in the nucleus (Toledano & Leonard, 1991). ROS can also affect the activation of NF-κB through altering its phosphorylation sites of IκB, thus inhibiting its ubiquitination and degradation (Schoonbroodt et al., 2000; Schieven et al., 1993).

In summary, there is a mutual interaction between ROS and NF-κB signaling pathways. Depending on the intracellular location and context, ROS can either activate or inhibit NF-κB
signaling. Through promoting the gene expression of antioxidant proteins, NF-κB affects the intracellular level of ROS.

**Role of NF-κB in neurogenesis and cell cycle regulation** NF-κB regulates both embryonic and adult neurogenesis. During embryonic development, NF-κB pathway regulates both differentiation and pluripotency of embryonic stem cells (ES) through transcription factor Nanog (Yang et al., 2007). The canonical IKKβ/IκBα/p65 pathway is activated to induce ES cell differentiation through inhibiting C/C/EBPβ expression (Zhang et al., 2012). In order to maintain the pluripotency of some ES cells, Nanog binds to NF-κB proteins and cooperates with Stat3 to inhibit NFκB activity and increase expression of proteins responsible for pluripotency (Torres & Watt, 2008) such as miR-290 (Luningschror et al., 2012).

NF-κB also plays an essential role in adult neurogenesis. NFκB family proteins are highly expressed in the zones of active neurogenesis in the adult mouse (Denis-Donini et al., 2008), indicating the potential role of NFκB in regulating the proliferation, migration and differentiation of adult NSCs/NPCs. In p65 and p50 double knockout mice, decreased number of progeny but an increased proportion of neurons was observed (Young et al., 2006). Overexpression of super inhibitor IκBα mutant in NSCs/NPCs inhibits the proliferation of control and TNF-treated NSCs (Widera et al., 2006). These results suggested that NFκB is involved in neuron proliferation. NFκB also plays a role in neuron differentiation. In p50-deficient mice, the differentiation of adult hippocampal NSCs is reduced by half (Denis-Donini et al., 2008). In particular, activation of NFκB signaling regulates the early differentiation of NSCs (Zhang et al., 2012). During the early stage of differentiation, NFκB signaling is activated by TNFα to induce neural differentiation of NSCs/NPCs (Zhang et al., 2012; Lou et al., 2003;
Bernardino et al., 2008). Selective inhibition NFκB signaling through pharmacologic inhibitors, shRNA or over-expression of IκBα can promote NSCs’ self-renewal (Zhang et al., 2012). These results suggest that NFκB signaling is essential for maintaining the balance between neuronal proliferation and differentiation. Moderate activation of NFκB signaling promotes neuronal differentiation while at the same time maintains a continuous pool of NSCs for adult neurogenesis. Over-activation of NFκB signaling in NSCs may reduce the number of NSCs, which may lead to neurogenesis defects in the adult.

NF-κB family proteins regulate cell cycle through affecting CDK/CKI system. It has been shown that NF-κB increases the abundance of cyclin D1 protein and the activity of the cyclin D1 kinase holoenzyme complex (Guttridge et al., 1999). It has also been shown that IκB ‘super repressor’ (IκB -SB) delays S-phase entry by inhibiting NF-κB activity (Guttridge et al., 1999; Hinz et al., 1999). IκB-SR’s effect on cell cycle seems to be specific to cyclin D, since it did not affect the levels of other cyclins including cyclin E (Guttridge et al., 1999). Three putative, non-consensus, NF-κB-binding sites are present in the cyclin D1 promoter and competent to bind NF-κB transcription factors in gel shift assays (Motokura & Arnold, 1993). The nuclear abundance of NF-κB correlates with its binding activity to the −39 cyclin D1 promoter site. Promoter truncation and mutagenesis at −39 base pairs impaired the binding activity of NF-κB to cyclin D1 promoter site and thus reduced the cyclin D1 expression, suggesting that NF-κB-binding site at −39 base pairs is functionally important in the cyclin D1 promoter (Joyce et al., 1999; Guttridge et al., 1999). It has been shown that the cyclin D2 promoter possesses an atypical NF-κB-binding site, which is responsible for Tax induced activation of cyclin D2 promoter, suggesting that NF-κB can also regulate cell-cycle progression through affecting Cyclin D2 gene expression (Iwanaga et al., 2008).
**NF-κB pathway in AD** NF-κB pathway plays an essential role in neuroinflammation and neurodegeneration in AD. In the CNS of AD patients, NF-κB is constantly activated in the neurons and glial cells around the amyloid plaques (Kaltschmidt *et al.*, 1997). TNF-α, a NF-κB activator and proinflammatory cytokine, is significantly increased in AD. There are two TNF-α receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). In neuron, TNF-R1 can induce apoptosis and differentiation, while TNF-R2 exerts a protective effect in general. TNF-R1’s protein level is up-regulated in AD (Fillit *et al.*, 1991), while TNF-R2’s is down-regulated (Taoufik *et al.*, 2007). In FAD mouse lines, TNF-R1 overexpression promotes neuronal death induced by Aβ and deteriorates cognitive functioning (Li *et al.*, 2004), while its knockout improves learning abilities (He *et al.*, 2007). Alternatively, NF-κB can also be activated in neurons in response to cytokines and oxidative stress triggered by stress or injury. Activation of TNF-R2 by TNFα and ceramide confers anti-apoptotic effects on hippocampal neurons treated with oxidative insults and amyloid β-peptide, by inducing Mn-SOD generation, suppressing peroxynitrite formation and inhibiting membrane lipid peroxidation (Marchetti *et al.*, 2004). NF-κB can also regulate APP and BACE1 expression level through regulating their transcriptions. NF-κB binding sites exist in APP and BACE1 gene promoter regions. Stimulation of both TNF-α receptors can enhance both the transcriptional and protein levels of BACE1 and APP, promoting the amyloidogenic pathway of APP processing and increasing Aβ production (Grilli *et al.*, 1996). NF-κB is also involved in the initiation and amplification of inflammation triggered by proinflammatory stimuli such as TNF-α or IL-1, which may further contribute to the neurotoxicity in AD (Tak & Firestein, 2001). These results suggest that dysregulation of NF-κB activation may contribute to the pathogenesis of AD through affecting neuronal survival, neuroinflammation and APP processing.
1.4. Alzheimer’s disease (AD) and Down syndrome (DS)

1.4.1. Overview

Individuals with DS inevitably develop characteristic AD, including neuritic plaques and neurofibrillary tangles, neuronal loss and the eventual onset of dementia after middle ages. The neuropathology of AD in DS patients closely resembles that of AD patients without DS (Zigman & Lott, 2007). Early AD clinical signs in individuals with DS, including confusion, disorientation, progressive cognitive decline and subtle memory loss (Devenny et al., 2002; Evenhuis, 1990), have been observed between 50-60 years of age, and most developed typical AD by 60-70 years of age. Excluding DS individuals from the sample, individuals with intellectual disabilities did not have a higher chance for developing AD than those without intellectual disability (Cole et al., 1994), suggesting that the high incidence of AD in DS is not related to the developmental disability, but to the trisomy 21.

1.4.2. Neuropathology perspectives.

**APP processing in DS.** The levels of Aβ and APP C-terminal fragment C99- the major β-secretase product are both significantly increased in DS (Busciglio et al., 2002). Autopsy studies revealed that by the age of 30, amyloid plaques are present in DS brains, whether these patients have been diagnosed with AD or not (Busciglio et al., 2002). One of the candidates for a dosage-sensitive gene contributing to this phenotype is APP as the substrate whose hydrolysis generates amyloid-β (Aβ), the main constituent of amyloid plaques in AD brains. Although the additional copy of the APP gene is present in most DS cases and the transcription of APP gene was indeed increased in DS (Podlisny et al., 1987), there was no change in the APP protein level corresponding to the fold change of Aβ in DS, suggesting that the gene dosage effect of APP
cannot fully account for the occurrence of AD in DS (Sun et al., 2006). In addition, the onset age of AD in DS varies significantly (Mutton et al., 1996), suggesting that the extra copy of APP on HSA21 alone cannot act as the sole determining factor contributing to AD in DS.

**Tau pathology in DS.** Abnormal protein expression and hyperphosphorylation of Tau are presented in both AD and DS. In DS, the up-regulation of several HSA21 genes may contribute to the increased Tau protein level and its hyperphosphorylation in the DS brain. Dual-specificity tyrosine phosphorylated and regulated kinase 1A (DYRK1A) is located in DS critical region of chromosome 21. DYRK1A phosphorylates tau protein and its overexpression may contribute to early onset neurofibrillary degeneration in DS brains (Wegiel et al., 2011). It also phosphorylates alternate splicing factors, leading to an increase ratio of 3R:4R tau (Iqbal et al., 2013). RCAN1, also known as DSCR1, is located on HSA21 and overexpressed in DS. It inhibits calcineurin and promotes tau phosphorylation by lowering calcineurin phosphatase activity and increasing the protein level of GSK3β, which can phosphorylate tau protein and enhance NFT formation (Cardenas et al., 2012).

**Neurodegeneration in DS.** Both defective neurogenesis and up-regulation of apoptosis contribute to the progression of a variety of neurodegenerative disorders in adults, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and Down syndrome. DS individuals have a widespread reduction of neurons in their hippocampus (Guidi et al., 2008), cerebellum (Wisniewski, 1990) and neocortex (Larsen et al., 2008), with defective synaptogenesis, connectivity and synaptic plasticity. Consistent with DS individuals, during the prenatal and early postnatal stages, DS animal model Ts65Dn mice show reduced cell density in
the neocortex (Chakrabarti et al., 2007) and hippocampus (Insausti et al., 1998). By studying fetal DS brains, it has been shown that their primary neuronal cultures exhibit increased intracellular oxidative stress, which may lead to an enhanced occurrence of apoptosis. Increased number of apoptotic cells in the dentate gyrus (DG) and the germinal zones of the hippocampus and subventricular zone (SVZ) have also been found DS fetuses. One of the candidate genes located on Hsa21 that contribute to AD pathogenesis through affecting neuronal survival is RCAN1. It is significantly elevated in the AD and DS brains. Long-term accumulation of RCAN1 in SH-SY5Y cells facilitates oxidative stress-induced apoptosis via promoting caspase-3 activation (Wu & Song, 2013) and its transgenic mice showed a reduced number of neurons within the hippocampus (Martin et al., 2012), indicating that its overexpression in DS contributes to the pathogenesis of AD by affecting neuronal survival.

1.4.3. Current hypothesis

**Oxidative stress hypothesis.** The accumulation of ROS in neurons can cause DNA damage and plasma membrane structural change, leading neuronal death. The lack of balance between the generation and removal of ROS accompanied by mitochondrial dysfunction (Busciglio & Yankner, 1995), due to a dysregulation of gene expression associated with trisomy 21, may contribute to the pathogenesis of AD in DS (Conti et al., 2007). Among those HSA21 genes, one of the most relevant genes is copper-zinc superoxide dismutase (SOD1). SOD1 plays a major role in antioxidant defense by converting ROS to O2 and H2O2, which will be further degraded to water converted by catalase (CAT) and glutathione peroxidase (GPX) (Benzi & Moretti, 1995). The extra copy of SOD-1 gene in DS patients causes a 50% increase in SOD-1 protein expression, leading to an imbalance in the ratio of SOD-1 to CAT and GPX and causing excess
accumulation of H₂O₂ (de Haan et al., 1995). Mitochondrial impairments also contribute to the accumulation of ROSs in DS (Pallardo et al., 2006). These impairments include mitochondrial DNA (mtDNA) mutations and altered functionality of mitochondrial enzymes involved in ROSs metabolism. Many studies have also shown that both Aβ40 and Aβ42 contribute to the increased oxidative stress in DS. However, oxidative stress itself is not sufficient to explain AD pathogenesis in DS and the usage of antioxidants in treating AD in DS has been proven ineffective (Kamoun et al., 2003).

**Brain development hypothesis** Differences in brain development that exist between people with DS and the general population may help to partially explain the pathogenesis of AD in DS. Postmortem examination revealed growth retardation in DS brains, resulting in smaller frontal and temporal lobes, reduced number and depth of cerebral sulci, and decreased width of the superior temporal gyrus (Teipel & Hampel, 2006). Pyramidal neurons in DS appear to have fewer dendritic arborizations and abnormalities in the size and orientation of spines (Becker et al., 1991; Takashima et al., 1994). Several HSA21 genes may contribute to abnormal brain development in DS. DYRK1A is highly expressed both in the developing and adult brains (Hammerle et al., 2008). Its overexpression can inhibit cell proliferation and promote premature neuronal differentiation through regulating NOTCH signalling (Yabut, et al., 2010; Hammerle et al., 2011). RCAN1 expression is up-regulated in both the fetal and adult brains of people with DS (Fuentes et al., 2000; Ermak et al., 2001). Its overexpression affects brain structure and reduces adult neurogenesis within hippocampus (Martin et al., 2012). In RCAN1-Tg mice, the density of dendritic spines is reduced, similar to the defects seen in DS brain (Martin et al., 2012). However, those changes may only partially explain the neurodegenerative perspective in
AD while fail to explain the generation of amyloid plaques and NFT, suggesting other factors exist and account for the development of AD in DS.

**Questions remain** Brain development and the oxidative stress hypothesis are not sufficient to explain AD pathogenesis in DS. At the same time, the gene dosage effect of APP itself cannot fully account for the occurrence of increased C99 protein level and Aβ, prompting us to look into other HSA21 genes. Since BACE2 is a homologue of BACE1 and mapped to a DS critical region on HSA21, it was speculated that the extra copy of BACE2 contributes to AD in DS. However, our lab reported that BACE2 and BACE1 have distinct function and transcriptional regulation. BACE2 processes APP at a novel θ-secretase site within the Aβ production (Sun et al., 2006). Our lab also showed that although BACE1 transcription was unchanged, both BACE1 protein level and its β-secretase activity were elevated significantly in DS (Sun et al., 2006). Moreover, the ratio of mature to immature forms of BACE1 was significantly higher in DS than in control, suggesting that increased BACE1 accumulation and maturation contribute to the pathogenesis of AD in DS. However, the underlying molecular mechanism of altered BACE1 in DS is not clear. We speculated that extra copy of certain genes located on HSA21 contributes to AD in DS by altering BACE1 protein level and maturation.
1.5. Ubiquitin-proteasome system (UPS) and neurodegenerative disease.

1.5.1 Ubiquitin and protein degradation

The ubiquitin-proteasome system (UPS) and the autophagy-lysosome system are the two major intracellular protein degradation systems. In general, UPS is mainly responsible for the degradation of short-lived proteins, while the autophagy-lysosome system is preferred for the degradation of long-lived proteins and large organelles.

Ubiquitin (Ub), a highly conserved 76 amino acid protein, is covalently conjugated to lysine residues of the substrate (Glickman & Ciechanover, 2002). It is encoded by four genes in the human genome including UBB, UBC, UBA52 and UBAB0 (summarized by Komander et al., 2009)). Ubiquitin precursor needs to be processed into mono-ubiquitin before it can be functional. Ubiquitin is best known for its role in ubiquitin-proteasome system (UPS) in regulating protein degradation (Glickman & Ciechanover, 2002), gene expression (Lipford et al., 2005) and quality control (Meusser et al., 2005) of protein synthesis. The UPS degrades proteins that are polyubiquitinated into small peptides through the 26S proteasome (Baumeister et al., 1998). The ubiquitination system consists of enzymes that link chains of the polypeptide co-factor, Ub, onto proteins to mark them for degradation (Glickman & Ciechanover, 2002; Pickart, 2004). These enzymes include: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E1 binds to the ubiquitin protein with ATP, and then passes the ubiquitin protein to E2. E2 interacts with an E3, which recognizes the targeted substrates and catalyzes the transfer of ubiquitin to them. E3s are substrate-specific, which ensures that only certain proteins are ubiquitinated and degraded at a particular time. This process can be repeated and form polyubiquitin chains associated with tagged proteins. To form a polyubiquitin chain, the glycine-76 of a second ubiquitin can be conjugated to one of the lysine residues of the
ubiquitin that is bound to the substrate. Lys-48 and Lys-63 are the most common residues recruited to form the isopeptide with other ubiquitin. Depending on the type of ubiquitination (mono- or polyubiquitination), and the lysine residues involved, different downstream cellular processes can be triggered.

In addition to its role in the UPS, ubiquitin serves as a signal for many other cellular systems including endocytosis and autophagy-lysosome. It can mark cell-surface proteins for internalization from the plasma membrane and target them for lysosome degradation (Hicke, 1999). Ubiquitin can be also recruited as one of the signals of autophagy-lysosome degradation (Ding et al., 2007). Further, the ubiquitination signal hosts the crosstalk between proteasomal and autophagic degradation, by enhancing autophagic-lysosomal activity when proteasome activity is inhibited (Pandey et al., 2007; Kraft et al., 2010).
Figure 1.6: The ubiquitination process and the roles of deubiquitination enzymes (DUBs).

(A). The activation of ubiquitin is initiated by the ubiquitin-activating enzyme (E1) via binding to the C-terminus. The activated ubiquitin on E1 is then transferred to the cysteine residues of a ubiquitin-conjugating enzyme (E2). The E2 protein acts in conjunction with ubiquitin ligase (E3). E2-E3 complexes bind to protein substrates and catalyze the transfer of ubiquitin to a lysine on the substrate protein. The substrate is thus mono-ubiquitinated. The process can be repeated to form polyubiquitin chains. (B). Role of DUBs: (a). Generation of free ubiquitin from ubiquitin precursor by DUBs. (b). Reversal of ubiquitin signal. (c). Removal of ubiquitin immediately before protein degradation.
1.5.2. Deubiquitinating enzymes

Ubiquitin-signaling regulation involves both conjugation and deconjugation processes to determine the final fate of the modified protein. Deubiquitination is an important regulatory process of ubiquitination through the actions of deubiquitinating enzymes (DUBs). DUBs are proteases that either cleave ubiquitin from proproteins or reverse the ubiquitination (summarized by Reyes-Turcu et al., 2009). There are about 100 DUBs encoded by the human genome, which can be categorized into five different families: ubiquitin carboxyl-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins, and JAB1/MPN/MOV34 metalloenzymes (JAMM or MPN+). Those DUBs serve a variety of functions including processing ubiquitin precursors, rescuing substrate from degradation, removing non-degradative ubiquitin signal, removing ubiquitin immediately before degradation, editing and recycling free ubiquitin and disassembly of unanchored ubiquitin oligomers. The large number of gene families and individual members suggests a high degree of substrate specificity of DUBs. In order to prevent the adventitious cleavage of inappropriate substrates by DUBs, their expressions are highly regulated at the transcriptional level and their activities are controlled by an abundance of substrates and scaffolding proteins.
Figure 1.7. deubiquitinating enzymes (DUBs) and their functioning
1.5.3. Deubiquitinating enzymes’ functions in nervous system.

DUBs play an essential role in nervous system functions by regulating Ub-dependent pathways in neurons. Some of DUBs are essential for synaptic structure and maintaining Long-Term Potentiation (LTP), including Ubiquitin Carboxyl-terminal hydrolase L1 (UCHL1). It stabilizes mono-Ub and increased mono-Ub availability, and its knockout and in-frame deletions causes axonal degeneration (Saigoh et al., 1999), suggests UCHL1 is crucial for neuronal maintenance. Some DUBs associated with the 19S proteasome, including USP14 (Crimmins et al., 2006), recycle Ub at the proteasome. USP14 is also important for NMJ development (Crimmins et al., 2006) and central synaptic function (Lappe-Siefke et al., 2009). Its mutation in mice causes progressive ataxia and tremor (D’Amato & Hicks, 1965), paralysis and early death (Lappe-Siefke et al., 2009). Some DUBs play important roles during development. USP33 is critical for axonal pathfinding and its knockdown results in impaired midline crossing (Yuasa-Kawada et al., 2009). Some DUBs, including Ataxin-3, are implicated in protein quality control by assisting proteasomal targeting of ERAD substrates and editing ubiquitin chains on those substrates (Zhong & Pittman, 2006).

Due to its involvement in a variety of cellular pathways and protein quality control, the UPS is a potential therapeutic target. However, direct proteasome inhibition yields undesirable side effects since it affects all cellular processes regulated by ubiquitin-dependent protein degradation. DUBs are implicated in a variety of neurodegenerative diseases, including Parkinson’s disease, Amyotrophic Lateral Sclerosis, Huntington’s disease, and Alzheimer’s disease. Since they regulate UPS in cell-type and substrate specific manners, DUBs can serve as more attractive targets for neurodegenerative diseases than proteasome inhibition.
1.5.4. Ubiquitination and Alzheimer’s disease

Dysfunctional UPS has been implicated in AD pathogenesis. It has been showed that proteasome activity, along with E1 and E2 activity, are significantly decreased in AD brains (Lopez Salon et al., 2000). Alteration in the autophagy-lysosomal degradation pathway has also been described in normal brain aging and in many age-related neurodegenerative diseases including AD. Many of the proteins involved in Aβ production are at least partially degraded by both the ubiquitin-proteasome and autophagy-lysosomal pathways. Improper clearance of proteins in AD, derived either from defective intracellular degradation pathways or from the accumulation of AD proteins, induces alterations in protein degradation pathways, and may result in neuron dysfunction and loss.

Many proteins involved in AD pathogenesis can be regulated by UPS. Full-length APP can be ubiquitinated under physiological condition (Morel et al., 2013). Several lysine residues within its C-terminal fragment region, including K649 (Morel et al., 2013), K651 (Watanabe et al., 2012) and K688 (El Ayadi et al., 2012), have been shown to be the APP ubiquitination sites. The inhibition of proteasome activity can accumulate ubiquitinated APP in the aggresomes and prevent it from being degraded by the proteasome (Dehvari et al., 2012; Kaneko et al., 2010). These results suggest that APP can be ubiquitinated and its turnover is regulated by UPS. BACE1 can be ubiquitinated and both the UPS (Qing et al., 2004) and autophagy-lysosome pathway (Huse et al., 2000) regulate its degradation. Blocking the ubiquitin-proteasomal pathway by proteasomal inhibitors- either MG132 or lactacystin, inhibited BACE degradation (Qing et al., 2004). Mutations of Lys203 and Lys382 of BACE1, two of its ubiquitination sites, abolished the degradation of the BACE1 ubiquitin-proteasome pathway (Wang et al., 2012). The ubiquitin-proteasome pathway also plays a crucial role in the pathways of NF-κB activation.
Ubiquitination of IκB is carried out by an E2 of the Ubc4/5 family (Chen et al., 1995) and SCF-βTrCP E3 ligase (Yaron et al., 1998). During NF-κB activation, the phosphorylated IκB is polyubiquitinated and selectively degraded by the 26S proteasome, allowing NF-κB itself to be released and imported into the nucleus (Palombella et al., 1994). The ubiquitin-proteasome pathway is also responsible for processing precursors of p50 and p52 (Palombella et al., 1994). Agents such as lipopolysaccharides (LPS) can stimulate post-translational processing of p105 by activating IκK, which phosphorylates p105 at C-terminal domain (Ciechanover et al., 2001). Therefore, dysregulation of UPS may have an effect on the degradation of proteins associated with AD pathogenesis, including APP, BACE1 and NF-κB.

1.5.5. Ubiquitination and Down syndrome

Like AD, DS is also associated with increased oxidative damage, accumulation of unfolded/damaged protein aggregates and dysfunction of intracellular degradative system. In particular, it has been shown that in DS brains, oxidative damage targets many proteins involved in the intracellular quality control system including GRP78, UCH-L1, V0-ATPase, cathepsin D and GFAP, resulting in decreased activity of the proteasome and autophagosome formation (Di Domenico et al., 2013). Those disturbances in the proteostasis network may contribute to the accumulation of protein aggregates involved in AD pathogenesis during the early phase of DS. Studying ubiquitin-immunoreactivity in DS brains has revealed widely distributed ubiquitin-immunoreactive dot-like structures in the white matter corresponding to granular degeneration of myelin and ubiquitin-immunoreactive dystrophic neurites in the hippocampus (Mattiace et al., 1991). Ubiquitin-immunoreactive dystrophic neurites frequently formed aggregates consistent
with senile plaques in the presence of amyloid. These data suggest that amyloid deposition is associated with local accentuation of ubiquitin-immunoreactive neuritic dystrophy in DS.

There are several DUBs located in HSA21 that may contribute to the pathogenesis of AD. USP16, one of HSA21 genes, regulates expansion of neural progenitors and fibroblasts through deubiquitinating Cdkn2a and H2AK129. Overexpression of USP16 reduced the expansions of both fibroblasts and post-natal neural progenitors while its down-regulation rescued those effects, indicating that USP16 is involved in the self-renewal and senescence pathways in DS (Adorno et al., 2013). USP16 also regulates H2A deubiquitination, a process that is essential for ESCs gene expression and differentiation. It binds to the promoter regions of many ESCs genes that are lineage-specific. The binding is inversely correlated with the protein levels of those genes, suggesting that USP16 regulates their transcriptions. USP16 knockout ESCs failed to differentiate and USP16 expression rescued the differentiation defects in Usp16-/- ESCs, suggesting that Usp16 plays an essential role in ESC gene expression and differentiation (Yang et al., 2014).

1.5.6. USP25 as novel target for Down Syndrome

Overview USP25 is located in the DS critical region of human chromosome 21 (Figure 1.9 A) and overexpressed in DS patients. Based on results derived from real-time quantitative PCR, in trisomic fetal brain samples 1.7-fold overexpression of USP25 was shown compared with disomic samples (Valero et al., 2001). It spans over 150 kb at 21q11.2, one of the lowest gene density regions of the human genome (Valero et al., 1999). It is made up of 25 exons and encodes a 1087-aa protein, containing ubiquitin-specific protease (USP) domain, ubiquitin-interacting motif (UIM), SUMO-interacting (SIM) motif, and ubiquitin-associated (UBA)
There are three USP25 isoforms in human, all containing one ubiquitin associated domain (UBA) and two ubiquitin-interacting motifs (UIMs) in its N-terminal region for substrate recognition (Denuc et al., 2009). These isoforms differ in the specificity of tissue and cell types, subcellular localization and substrate recognition. The most abundant isoform transcript contained the in-frame fusion of exons 18 and 20, while the latter isoforms differ from the former by having exons 18–19b-20, and 18–19a-19b-20. The latter isoform was expressed only in skeletal muscle and heart and was therefore called USP25m (USP25 muscle isoform).

USP25 can cleave both lysine 48- and lysine 63-linked polyubiquitin chains and its DUB activity was dependent on a cysteine residue (Cys178) and a histidine residue (His607) (Zhong et al., 2013). USP25 can be monoubiquitinated and able to auto-deubiquitinate itself (Denuc et al., 2009). Its basal expression was observed in all human tissues. In particular, it is highly expressed in skeletal muscle, heart, testis and brain. In situ hybridization in mouse embryonic brains showed a clear correlation of USP25 expression with proliferative neuroepithelial cells and post-mitotic neurons (Valero et al., 1999). It is involved in a variety of cellular processes, including immunity, myogenesis/myogenic differentiation and protein degradation.
Figure 1.8: Human chromosome 21 and USP25.

(A). The location of USP25 gene on human chromosome 21; (B). Protein structure of USP25;
**Protein structure and intramolecular regulations.** At the N-terminal region of USP25 proteins, there are three ubiquitin-binding domains (UBD): one ubiquitin-associated domain (UBA) and two ubiquitin-interacting motifs (UIMs), which enable its interaction with ubiquitin and substrate recognition (**Figure 1.9B**). In addition to the UBDs, at the extended C-terminal region outside of the catalytic domains, a coiled-coil stretch between amino acids 679 to 769 regulates its catalytic activity (Denuc et al., 2009).

USP25’s protease activity can be regulated through its post-translational modification, including ubiquitination and SUMOylation. USP25 itself can be monoubiquitinated at the preferential site lysine 99 (K99) and able to auto-deubiquitinate itself. Conjugation of ubiquitin K99 favors UBDs’ catalytic action, and therefore promotes USP25’s activity (Denuc et al., 2009). Mutation of K99 decreased the rescue effect of USP25 on one of its specific substrates MyBPC1 from proteasome degradation. The ubiquitin interaction motifs (UIMs) of USP25 at the N terminal region are required for efficient hydrolysis of ubiquitin chains. SUMOylation within these regions impairs USP25’s binding to and hydrolysis of ubiquitin chains. The residue of USP25 monoubiquitination is also a target for SUMO and this SUMOylation inhibits USP25 activity (Meulmeester et al., 2008), suggesting K99 is a key residue for regulating the protease activity of USP25.

**Role in immune system** USP25 regulates immunity through interacting with the key proteins in immune signaling pathway. Toll-like receptor (TLR) signaling plays a critical role in innate immunity and USP25 regulates TLR signaling through affecting its ubiquitination (Zhong et al., 2013). After lipopolysaccharide (LPS) stimulation of TLR4, USP25 was recruited to the TLR4 signaling complex and associated with the adaptor proteins tumor necrosis factor receptor—
associated factor 3 (TRAF3) and TRAF6. USP25 reversed the Lys48-linked ubiquitination of TRAF3 and deficit of USP25 facilitated its ubiquitination and degradation. Overexpression of USP25 down-regulated LPS-induced inflammatory cytokines like TNF-α, IL-6 while its silencing mediated by siRNA up-regulated the level of TNF-α, IL-6, and IFN-β through strengthening MAPKs phosphorylation and IκB degradation (Ding et al., 2016). Mice with USP25 deficiency were associated with enhanced production of proinflammatory cytokines and decreased production of interferon-α (Zhong et al., 2013), suggesting that USP25 plays an essential role in maintaining a balanced innate immune response.

It has also been shown that USP25 is involved in regulating the virus-triggered type I interferon (IFN) induction pathway (Lin et al., 2015). It down-regulates the virus-induced activation of IFN-β, IRF3 and NF-κB through deubiquitinating retinoic acid-inducible gene I (RIG-I), TRAF2, and TRAF6 respectively. USP25’s mutant lacking DUB activity and the knockdown affected its ability to block induction of the IFN-β triggered by virus infection.

USP25 can also serve as a downstream effector in immune activation. Spleen Tyrosine Kinase (SYK), a non-receptor tyrosine kinase, is a key effector of immune signaling in hematopoietic cells. The C-terminal region of USP25 interacts with the second SH2 domain of SYK independently of tyrosine phosphorylation. At the same time, SYK can phosphorylate USP25 and regulate its cellular levels protease activity (Cholay et al., 2010). Viral infection and lipopolysaccharide (LPS) treatment can up-regulate the expression of a large number of genes that are essential for regulating host anti-pathogen immunity and inflammation. USP25 is one of the genes that is significantly up-regulated by viral infection and LPS treatment through interferon regulatory factor 7 (IRF7) directly binding to the two conserved IRF binding sites on the USP25 promoter (Ren et al., 2016). Above all, it suggests that USP25 is essential for
downstream innate immune signaling. However, the functional consequences of up-regulation of USP25 protein and its protease activity induced by innate immune activation remain unclear.

**Role in myogenesis/myogenic differentiation** One of the three USP25 isoforms- USP25m, is only expressed in muscular tissues. Its expression is increased during myogenesis, suggesting a potential role in regulating muscle development. USP25m interacts with several sarcomeric proteins, including actin alpha-1 (ACTA1), filamin C (FLNC), and myosin binding protein C1 (MyBPC1), which are critically involved in muscle differentiation and maintenance (Bosch-Comas et al., 2006). In particular, USP25 regulates the turnover of the short-lived protein MyBPC1 and overexpression of USP25m prevents its degradation through UPS. Dysregulation of these sarcomeric proteins has been implicated in the pathogenesis of some severe myopathies.

**Role in Endoplasmic Reticulum (ER)-associated degradation (ERAD).** Protein quality control is essential for maintaining the functioning and homeostasis of many cellular pathways. Protein quality malfunction has been linked to a variety of neurodegenerative disease and metabolic syndromes (Buchberger et al., 2010). In eukaryotic cells, abnormal proteins with a short half-life are degraded and recycled by the ubiquitin-proteasome system. Endoplasmic Reticulum (ER)-associated degradation (ERAD) is part of the protein quality control that recognizes, deglycosylates, ubiquitinates, and extracts misfolded proteins into the cytosol (Claessen et al., 2012). Therefore, ubiquitinated misfolded proteins are recognized by the proteasome and degraded. USP25 is localized at the ER and interacts with ERAD components, including HRD1 as an ER-resident ubiquitin ligase (Kikkert et al., 2004), and AAA ATPase
VCP/p97 (Ballar et al., 2011) that extract ERAD substrates into the cytosol (Blount et al., 2012). USP25 opposes ubiquitin ligase functioning of HRD1 through deubiquitinating the endogenous proteins associated with HRD1 (Jung et al., 2015). It also reduced the ubiquitination status of endogenous species associated with VCP/p97, rescuing several ERAD substrates from proteasomal degradation, including APP.

ER stress can be induced by an increased in the level of intracellular calcium. During the ER stress, proteins associated with ERAD, including HRD1 and proteasome, are up-regulated, while USP25 is down-regulated (Jung et al., 2015). In response to ER stress induced by calcium influx, APP is rapidly ubiquitinated and degraded by UPS. Although APP interacts with USP25, whether decreased USP25 levels during ER stress contributes to the increased level of APP remains unknown.

1.6. Rationale of the study.

To search for the potential candidate proteins responsible for regulating BACE1 in DS, our lab has searched for candidate proteins that interact with BACE1 using a two-hybrid yeast assay. We fished out two candidate proteins- USP25 and UCHL1. We showed that UCHL1 affects BACE1 degradation and downstream APP processing. However, the human UCHL1 gene is not located on HSA21. Alternatively, the human USP25 gene is located in HSA21 and it interacts with BACE1, suggesting that the extra copy of the human USP25 gene may contribute to AD in DS.

We have also generated USP25 transgenic mice from C57BL/6 background mice. USP25 overexpression is driven under the human USP25 promoter in BAC. Therefore, USP25 overexpression in the transgenic mice mimics the expression pattern of USP25 in humans.
Transgenic mice were subjected to the Morris Water Maze (MWM) test at the age of six months. These mice have shown deficiencies in spatial learning and memory spatial learning (explicitly explained in the result section). In the sucrose preference test, USP25 transgenic mice exhibited less preference towards sucrose drinking water than their littermate controls, indicating defects in neurogenesis/death.

Based on the preliminary results above, we hypothesized that **USP25 affects AD pathogenesis in DS by regulating APP processing and neurogenesis**. Specifically, we proposed:

1). **To examine the transcriptional regulation of USP25.** Despite of the presence of an extra copy of HSA21 in most DS patients, the onset age of AD in DS varies significantly, suggesting that the expression of HSA21 genes may vary among DS patients. Previous studies suggested that USP25 may play an important role in DS pathogenesis. Therefore, its upregulation may have a pathogenic relevance in the development of DS. We were therefore interested in studying the underlying mechanism that regulates USP25 expression. In particular, we studied the transcriptional regulation of USP25. Identifying transcription factors that regulate USP25 transcription would help us understand the signaling pathways that contribute to the development of DS through regulating USP25. We cloned the 5’ promoter region of USP25 and identified functional SP1 binding site on it. The expression regulation of USP25 was studied at both protein level and mRNA level.
2). To investigate the role of USP25 in APP processing. We hypothesized that USP25 regulates APP processing. Our preliminary data indicated that increased APP and BACE1 protein level by USP25 overexpression. Since USP25 is a DUB that involves in ubiquitin signalings, we further studied the role of USP25 in APP and BACE1 degradation and trafficking. In this section, we examined the effects of USP25 on APP processing and trafficking by transgene expression of USP25 in vitro and confirmed those effects in USP25 transgenic mice.

3). To examine the role of USP25 in neurogenesis and cell death. In addition to amyloid plaque formation, neuronal loss is also a prominent feature of AD and DS pathology. Apoptosis and defective neurogenesis were shown in both the DS and AD brain. We looked into whether USP25 facilitates apoptosis under oxidative stress and affects neurogenesis through regulating the cell cycle. In this section, we also study the long-term effects of USP25 overexpression on the development of DS and AD pathology through behavioral testing.

The overall goal of this thesis was to investigate the transcriptional regulation of USP25, and the role of USP25 in APP processing and neuronal loss. We also aimed to examine the long-term effects of USP25 on AD and DS pathogenesis in vivo, which could provide insights about its potential as a treatment strategy for AD in DS.
SP1 regulates USP25 gene expression

2.1. Introduction

USP25 is expressed in all human tissue. It is overexpressed in DS fetal brain samples (Valero et al., 2001). The expression of USP25 was moderately high in testis, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart (Valero et al., 1999). In situ hybridization in mouse embryonic brains showed a clear correlation of expression with proliferative neuroepithelial cells and post-mitotic neurons (Valero et al., 1999). It has been shown that viral infection and lipopolysaccharide (LPS) treatment can up-regulate the expression of USP25 through interferon regulatory factor 7 (IRF7) directly binding to the two conserved IRF binding sites on the USP25 promoter (Ren et al., 2016). However, there have been few studies on the mechanism of USP25 tissue-specific expression and endogenous transcriptional regulation of the USP25 gene.

In this chapter, we cloned and analyzed the USP25 gene promoter region. We identified a functional Sp1 binding site within the promoter region. Sp1 was found to up-regulate USP25 expression and its inhibitor down-regulated USP25 expression.
2.2. Method.

2.2.1 Cloning and Plasmids

The 5’-flanking regions of the human USP25 gene were amplified by PCR from human BAC DNA clone (BACPAC Resources Center, CHORI, Oakland, CA, USA). Primers were designed to include restriction enzyme sites such that PCR products could be cloned into cloning sites of pGL3-Basic (Promega, Madison, WI, USA). Eleven fragments covering the 5’-flanking region of USP25 gene from -2031 bp upstream to +226 bp downstream of the transcription start site at +1 (adenine) were amplified by PCR and inserted in front of the luciferase reporter gene (Luc) in the pGL3-basic expression vector. The primers, including restriction enzyme sites, were synthesized as follows: forward 1) Xho12075 5’-ccgctcgagaggaggacacgccattcc; 2) HindIII1976 5’-cccaagcttgacctcgcggcgccg; 3) Xho11967 5’-ccgctcgagcaatgtagggtagggcgg; 4) Bam1742 5’-ttggttaagctaggatccgac; 5) Nh1211 5’-ctagctacgttccagatttagctcctg; 6) Nh57 5’-ctagctacgttaatgttagcagattagtt; Reverse 1) Hind2313 5’-cacaagcttaaacgccgactgtgagg; 2) Bam1781 5’-ctgcgcccagggcttgctggatc; 3) BI1298 5’-gaagatggaattgtaaggaaatct; 4) BI2313 5’-gaagatgctaaaacgccgactgtgagg; 5). HindII12169 5’-cccaagctttcgacgttctctcctttg; 6) HindII2095 5’-cccaagctttccctcgcgagtctcctc;

2.2.2 Switching mechanism at 5’end of RNA transcription (SMART) RACE cDNA amplification

Total RNA was extracted from HEK293 cells using TRI reagent (Sigma) following the manufacturer’s protocol. SMART-RACE was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech) following the user’s manual. Simply, the first strand cDNA was synthesized from total RNA extracted from HEK293 cells with oligo (dT) primer in the presence
of SMARTer IIA oligonucleotide (5’-aagcagtgtatatcaacgcagagtacxxxxx (X is undisclosed base in the proprietary SMARTer oligo sequence). The SMARTer IIA oligonucleotide is able to anneal to the 5’-end of the first strand cDNA and serves as template to extend the 5’-end cDNA tail. A USP25 reverse primer (5’-cgtggccctcacagtcg) was specifically designed to recognize the +201 to +221 bp of human USP25 gene downstream of the translation start site (ATG). The PCR products containing USP25 promoter region were amplified using SMARTer IIA oligonucleotide and USP25 reverse primer and the first strand cDNA as template. The resulting PCR products were cloned into pcDNA4/myc-His A vector for sequencing and the first nucleotide linking with the adapter sequence was identified as the transcription start site of the human USP25 gene.

2.2.3 Cell culture, transfection, and luciferase assays

HEK293, SH-SY5Y and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 mmol/L of sodium pyruvate, 2 mmol/L of L-glutamine and 50 units of penicillin and 50 µg of streptomycin (Invitrogen Carlsbad, CA, USA). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were seeded onto 24-well plates or 35-mm-diameter plate 1 day prior to transfection and grown to approximately 25% confluence by the day of transfection. Cells were transfected with 0.5 µg of plasmid DNA per well using 1.5 µL or 2 µg plasmid DNA per 35-mm-diameter plate for RNA extraction by 6 µL PEI reagent (Cat#. 23966, Polysciences Inc.) The Renilla luciferase vector pRLuc was co-transfected to normalize the transfection efficiency of various luciferase reporter constructs. Cells were harvested at 48 h after transfection and lysed with 100 µL passive lysis buffer (Promega) per well. Firefly luciferase activities and Renilla luciferase activities were measured sequentially using the Dual-luciferase reporter assay system (Promega). The firefly luciferase activity was
normalized according to Renilla luciferase activity and expressed as relative luciferase units to reflect the promoter activity.

2.2.4 Electrophoretic mobility shift assay (EMSA)

HEK293 cells were transiently transfected with SP1 plasmid and nuclear extracts were collected. Cells were rinsed and harvested with 1 X Phosphate-buffered saline. After centrifugation, cell pellets were resuspended with 5 x volume of buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were pipetted up and down gently and maintained on ice for 15 minutes. The cell suspension was transferred to a Kontes all glass Dounce tissue grinder and ruptured by 10 strokes. 10% NP40 was added into the cell suspension for a final concentration of 0.5%. The samples were placed on ice for 15 minutes and stroked 5 more times. Crude nuclei were collected by centrifugation at 2000 g for 10 minutes and washed three times with buffer A containing 0.5% NP40 and resuspended in buffer C [20 mM HEPES pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% Glycerol] at 4°C for 15 min. The samples were centrifuged at 12000 g for 4 min at 4°C, and the supernatant containing nuclear proteins was collected. Oligonucleotides containing the putative SP1 binding site on human USP25 promoter region were synthesized for detecting the binding ability of SP1 to USP25 promoter. The sequences of the oligonucleotides were USP25 -80 -53, forward, 5’-gaggagccgccgggggatg and reverse, 5’-catccccgccccgccggtcctcac; The probes corresponding to SP1 binding sites were labeled with IRDye-700 (IDT). Prior to incubation with nuclear extract, oligonucleotide probes were heated at 98°C for 5 minutes and annealed at 65°C for 10 minutes. Sp1 consensus oligo 0.5 pmol of
annealed probes were incubated with 2 μl of nuclear extract for 20 minutes at 22°C and the reaction mixtures were separated on a 4% Tris-glycine-EDTA gel in darkness. The mobility of probes on the gel was visualized using the LI-COR Odyssey (LI-COR Biosciences). For the competition assay, wild-type SP1 consensus oligonucleotides and mutant SP1 oligonucleotides were used as positive and negative controls, respectively.

2.2.5 Semi-quantitative RT-PCR

HEK293, HeLa, SH-SY5Y cells were harvested 48 hours after the treatments and total RNA was extracted using TRI reagent (Sigma). Reverse transcription was sequentially performed using ThermoScript™ RT-PCR system kit following the manufacturer’s protocol (Invitrogen). The USP25 gene specific primer F-tgacacccagatactacagc and USP25 R- gccacactctctagcccaacg were used to amplify a 433 bp fragment of human USP25 gene. The GAPDH gene was also amplified using the forward primer (tgccacaccaactgcttagc) and reverse primer ggcattaccgtgttagc, which produced a 87 bp fragment. The PCR products were analyzed on 1.5% agarose gels.

2.2.6 Immunoblotting.

HeLa cells were lysed in triton lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 50 mM Tris-HCl (pH 8.0) and protease inhibitor cocktail (Roche), followed by brief sonication. Protein concentration was measured by Bradford assay (Bio-rad) and 4x sodium dodecyl sulfate (SDS) sample buffer was added to each sample. Cell lysates were resolved by 7.5% Tris–glycine SDS–polyacrylamide gel electrophoresis (PAGE) for detecting USP25 and Sp1-HA, respectively. Rabbit anti-USP25 polyclonal antibody, mouse anti-β-actin monoclonal antibody AC-15 (Sigma) were used as primary antibodies. Blots were enhanced with a
chemiluminescence detection reagent kit (Fisher, 32106) and visualized with a Bio-Rad imager and Quantity One software. Signal intensities from each band were quantified with Bio-Rad Image Lab software, and the bands were analyzed relative to their controls from the same membrane and experiment.

2.2.7 Statistical analysis

All results were presented as mean ± SEM and analyzed by 2-tailed Student’s t-test, one-way ANOVA or two-way ANOVA. Statistical significance is accepted when p<0.05 (*p<0.05, **p<0.01, ***p<0.001).
2.3. Results

2.3.1 Cloning the human USP25 gene promoter and mapping the transcription start site of the USP25 gene.

Human USP25 gene spans a large area (> 150 000 bp) on the chromosome 21q11.2 region. The gene is located at 17102344 - 17252377 on Chromosome 21. Based on the predicted coding sequence of this gene in the Ensembl genome database (Ensembl ID: ENSG00000155313), the human USP25 gene contains 21 exons and has 10 spliced variants. We extracted human genomic DNA from HEK293 cells and cloned a 2,500- bp 5’ flanking region of the USP25 gene. The DNA fragment was sequenced (Figure 2.1A). To identify the transcription initiation site of the human USP25 gene, 5’ RLM-RACE was performed. Products of the nested PCRs (Figure 2.1B) were sequenced and the transcription initiation site was identified (Figure 2.1C). The transcription initiation site, an adenine labeled as +1, is located 251-bp upstream of the translation start site (Figure 2.1D). Sequence analysis revealed that the human USP25 gene promoter has a very high GC content (> 70%) in the region upstream of the transcription initiation site. A transcription factor binding site searching engine MatInspector2.2 (Genomatrix, Oakland, CA, USA) revealed that the 5’ flanking region of USP25 contains several putative regulatory elements, indicating that USP25 gene transcription is under tight regulation. Predicted putative transcription factors that may participate in the transcriptional regulation of USP25 gene promoter including NF-AT1, c-Jun, p53 and SP1.
Figure 2.1. Identification of transcription start site (TSS) of the USP25 gene.

(A). 2.5-kb fragment of the 5′flanking region of the human USP25 gene. The adenine +1 represents the TSS. (B). Smarter RACE cDNA amplification kit was used to amplify full-length cDNA from HEK293 cells. Nested PCR was performed and the product was resolved on 1.5% agarose gel; (C). Sequencing results of (b). * represents the TSS. (D). Sequence of the 5′UTR region upstream of the USP25 gene translation start site. Mapped transcription initiation site (+1) is labeled by an arrow.
2.3.2. Functional analysis of the human USP25 gene promoter

A series of nested deletions of the cloned 5’ UTR fragment were subcloned to analyze the activity of the USP25 gene promoter. Fragments were cloned into pGL3-Basic for the luciferase reporter assay. The inserts were verified by gel analysis following restriction enzyme digestion and sequencing (Figure 2.2A). These fragments, with various lengths and orientations, cover different parts of the cloned 5’ UTR region. We transfected these reporter plasmids into HEK293 cells and performed luciferase assays to examine their promoter activities. The pGL3-Basic vector lacks a functional eukaryotic promoter and thus has little luciferase expression when transfected into cells. However, once a fragment with functional promoter activity is inserted into its multiple cloning sites, the construct will express luciferase proteins and the level of expression reflects the promoter activity of the inserted fragment. After controlling for transfection efficiency using a co-transfected pRLuc plasmid, the promoter activities of the nine fragments were assayed (Figure 2.2B). The construct containing the region from -2031 to +226 (USP25p-A) showed significant promoter activity, compared with the negative control (pGL3-basic empty vector), indicating that this region contains the functional promoter of human USP25 gene. Further deletions from both 5’ and 3’ (USP25p-C, E and K) did not show significantly reduced promoter activity, indicating that these deletion regions lack important regulatory elements that are required for optimum promoter function. A region from -122 to +226 is required for a minimum promoter activity (3.0±0.18-fold, p < 0.01), while deletion of a 135 bp fragment from -122 to -13 USP25p-H resulted in no significant promoter activity, suggesting the fragment from -122 to -13 contains important regulatory elements.
Figure 2.2 Functional analysis of USP25 promoter

(A) Schematic diagram of the USP25 promoter constructs consisting of the 5’ flanking region with serial deletions cloned into the pGL3-basic vector. Arrow shows the direction of transcription. The numbers represents the end points of each construct. (B) USP25 promoter constructs were verified by restriction enzyme digestion and the digested products were resolved on 1.5% agarose gel, which was further confirmed by sequencing; The deletion plasmids were cotransfected with pCMV-Luc into HEK293 cells. 24 h after the transfection, the luciferase activity was measured and expressed in relative luciferase units (RLU). The pCMV-Luc was used to normalize for transfection efficiency. The pGL3-Basic plasmid served as the negative control and the reverse of USP25p-I (+226 to -122) was used for normalizing the result. The values represent means ± SEM. n = 3, *p < 0.01, by one-way ANOVA followed by post hoc Tukey’s multiple comparisons test.
2.3.3. The *USP25* gene promoter contains SP1 binding sites

A 5' deletion from -122 to -13 significantly reduced USP25 gene promoter activity. This suggests that this region contains an important cis-acting element that strongly regulates the USP25 gene promoter activity. By analyzing the DNA sequence of this region, we found putative SP1 elements. The element is located from -80 to -53 (5’gaggagccgcgggccgggccgggatg). This sequence is homologous to the SP1 consensus sequence 5’-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' as the GC box element (Figure 2.3 A). To examine this putative binding sites, we performed a gel-shift assay (Figure 2.3 B). A 27-bp double-stranded oligonucleotide probe corresponding to the USP25 gene promoter -80 to -53 was synthesized. The probe corresponding to SP1 was labeled with infrared-dye. The probe showed shifted bands (Figure 2.3 B, lane 2) after incubation with the nuclear extract of HEK293 cells overexpressing SP1. The intensity of shifted band was remarkably reduced if a 10-fold excess competitor (SP1 consensus oligo) was added to the incubation system and further reduced if a 100-fold excess competitor was added (Figure 2.3B, lanes 3 and 4). These shifted bands are the same as those of USP25-SP1 consensus oligo (Figure 2.3B, lane 5 and 6). However, there was little competition effect when we used a 10- and 100-fold concentration of mutated SP1 oligo containing two successive G to C mutations at the beginning of the binding site, as a competitor (Figure 2.3B, lane 7 and 8). Taken together, our gel-shift assay reveals a physical binding between SP1 and the human USP25 gene promoter.
Figure 2.3 Functional SP1 sites within USP25 promoter.

(A). 122 to -13 region contain several putative SP1 elements. (B) Gel shift assays with SP1 consensus probe for examining the interaction between SP1 and the USP25 gene promoter. Lane 1 is labeled probe alone without protein extract. Incubation of the probe with SP1 enriched nuclear extracts forms a shifted DNA-protein complex band (lane 2). Competition assays were performed by further adding different competition oligonucleotides;
2.3.4 Sp1 regulates **USP25** promoter activity and transcription

USP25 promoter deletions constructs in Figure 2, plasmids pGL3-A, C, E, I were co-transfected with either the SP1 expression vector or the empty vector into HEK293. Mithramycin A (MTM), a SP1 inhibitor, was added 24 h after transfection (**Figure 2.4A**). For each fragment with active USP25 gene promoter, promoter activity was elevated by overexpression SP1 and inhibited by the treatment of 75 nM MTM (A: 207.6±1.28% and 45.21±2.067%; C: 248.6±8.308% and 49±1.927%; E: 229.8±1.759% and 507.6±2.657%; I: 247.9±17.61% and 40.1±2.498% respectively, p<0.05) The semi-quantitative RT-PCR was performed to test whether endogenous USP25 mRNA level was affected by Sp1 overexpression or SP1 inhibitor. Using the human GAPDH as an internal control, Sp1 overexpression increased endogenous human USP25 transcription 1.33 ± 0.10 folds (**Figure 2.4B**). MTM significantly decreased endogenous human USP25 transcription to 67 ± 9% (p < 0.01) (**Figure 2.4C**), in SH-SY5Y cells.

2.3.5 Sp1 regulates **USP25** gene expression

Since USP25 protein level is unable to be detected in HEK293 and SH-SY5Y cells, in order to examine the effect of Sp1 on USP25 endogenous protein level, HeLa cells were used due to their detectable USP25 endogenous protein by western blot. The semi-quantitative RT-PCR was performed to confirm in HeLa cells that endogenous USP25 mRNA level was affected by Sp1 overexpression and Sp1 inhibitor in a similar manner compared with SH-SY5Y (**Figure 2.5A, B**). Sp1 overexpression significantly increased USP25 protein level by 1.933 ± 0.8819 folds (p< 0.001, **Figure 2.5C, E**). Endogenous USP25 protein expression was also detected in HeLa cells after 75 nM MTM. Consistent with Sp1’s effect on USP25 gene expression, inhibition of Sp1’s activity by MTM decreased USP25 protein level to 54 ± 5.89% (p< 0.01, **Figure 2.5D, F**).
Figure 2.4 SP1 upregulates USP25 promoter activity and endogenous mRNA expression; (A). To examine whether the USP25 gene promoter activity is regulated by Sp1, the USP25p-A, C, E, I plasmid was co-transfected with either the Sp1 expression vector or the corresponding empty vector into HEK293. 75 nM Mithramycin A (Sp1 inhibitor) was added 24 h after transfection. Luciferase assay was performed. The pGL3-Basic plasmid served as the negative control and was used for normalizing the result. The values represent means ± SEM. n = 3, *p < 0.01, by Student’s t-test; (B). SP1 overexpression increases the endogenous human USP25 mRNA level in SH-SY5Y cells (left panel) and quantification (right); (C). Mithramycin A decreases the endogenous human USP25 mRNA level. SH-SY5Y cells were transfected with either the SP1 expression vector or empty vector pcDNA4. RT-PCRs were performed using either primers specific to the human USP25 coding sequence or the human GAPDH coding sequence. Quantification of mRNA was performed by ImageJ software. The values in this figure represent means ± SEM. n =6, *p < 0.05, analyzed by Student’s t-test.
SP1 overexpression (A) and Mithramycin A (B) have similar effect on endogenous human USP25 mRNA level in HeLa cells than SH-SY5Y cells; (C). SP1 overexpression increases the endogenous human USP25 protein level in HeLa cells; (D). 75 nM Mithramycin A (Sp1 inhibitor) decreased the endogenous human USP25 protein level in HeLa cells; (E).
Quantification of USP25 and β-actin protein levels in HeLa cell were completed by ImageJ software in C; (F). Quantification of USP25 and β-actin protein levels in HeLa cell were completed by ImageJ software in D; The values in this figure represent means ± SEM. n =3, **p < 0.01, ***p<0.001, analyzed by Student’s t-test.
2.4. Discussion

Despite the presence of an extra copy of HSA21 in most DS patients, the onset age of AD in DS varies significantly, suggesting that the expression of HSA21 genes may vary among DS patients. We cloned the human USP25 gene promoter and mapped the transcription initiation site at 251 bp 5’ upstream of the translation start site. Deletion analysis revealed that a region from -122 to +226 is required for minimum promoter activity while deletions a 135 bp fragment from -122 to -13 resulted in no significant promoter activity, suggesting the fragment from -122 to -13 contains important regulatory elements. Sequence analysis of the fragment from -122 to -13 revealed putative sites for transcription factors SP1. We showed that the USP25 gene promoter activity is regulated by SP1. USP25 promoter activity is increased when SP1 is overexpressed and decreased when SP1 inhibitor is applied. Physical interaction between the human USP25 gene promoter and the SP1 molecule was confirmed by gel-shift assays. Overexpressing SP1 increased endogenous human USP25 transcription and protein expression. SP1 inhibitor Mithramycin A significantly decreased endogenous human USP25 transcription and protein expression.

Understanding the interaction between the USP25 gene promoter and SP1 is informative for the study of the regulation of HSA21 genes and DS pathogenesis. Sp1 is a ubiquitously expressed zinc finger-containing DNA binding protein that binds GC-rich motifs with high affinity and enhances transcription with one of the two glutamine-rich domains (Ray et al., 1989). Sp1 is involved in regulating cell growth/differentiation (Black et al., 1999), embryogenesis (Marin et al., 1997), and preventing CpG islands from methylation (Brandeis et al., 1994). SP1 proteins contain zinc fingers allowing sequence-specific DNA binding, and two domains located at the N-terminal for transcription activation. It can bind to GC-box and
GT/CACCC-box. Its consensus binding sequence is (G/T)GGCGG(G/A)(G/A)(C/T) (Giglioni, Comi et al., 1989). Mithramycin A was discovered to bind to GC-rich sequence with high affinity (Van Dyke & Dervan, 1983), and it competitively binds to Sp1 consensus binding site working as a site-specific inhibitor (Ray et al., 1989). Our data shows that in human USP25 promoter contains one functional putative binding site in the fragment from -122 to -13. Application of MTM resulted in significantly reduced USP25 promoter activity, gene transcription and expression.

It has been shown that Sp1 regulates the transcriptions of many genes involved in neurodegeneration. Our lab has shown that BACE1, the major β-secretase involved in cleaving APP, is regulated by Sp1 at the transcriptional level, which may contribute to APP generating Aβ in Alzheimer's disease (Christensen et al., 2004). Sp1 also enhanced the gene transcription of huntingtin and Mithramycin A reduced huntingtin gene expression, suggesting that the dysregulation of Sp1-mediated huntingtin transcription may partially contribute to the pathogenesis of Huntington’s disease (Wang et al., 2012). Leucine-rich repeat kinase 2 (LRRK2) gene is one of the mutations associated with familial Parkinson’s disease that contributes to the dopaminergic neurodegeneration in the nigrostriatal pathway. Our lab has shown that Sp1 signaling regulates human LRRK2 gene expression and controls LRRK2 level. Manipulating Sp1 signaling may be beneficial to attenuate PD-related neuropathology (Wang & Song, 2016).

Here we revealed another pathway by which Sp1 contributes to neurodegenerative diseases. Sp1 signaling may up-regulate USP25 expression, further promoting its protein levels in DS. As discussed in the introduction section, the dysregulation of USP25 may affect a variety of cellular processes, including immunity, myogenesis/myogenic differentiation and protein
degradation. We wondered whether the overexpression of USP25 also contributes to AD pathogenesis in DS. Those results will be covered in the next two chapters.

2.5. Conclusion.

In summary, we identified transcriptional start site (TSS) and functional SP1 response element on the 5’ flanking region of the human USP25 genes. We demonstrated that SP1 overexpression increased USP25 gene transcription and protein expression. Moreover, inhibition of SP1 signaling by MTM resulted in decreased USP25 mRNA and protein levels. Those results suggested that USP25 gene transcription was regulated by SP1 signaling.
Chapter 3

The role of USP25 in APP processing

3.1 Introduction

Intracellular proteins are degraded mainly through either the UPS or autophagy-lysosomal pathways. Many AD-associated proteins, including BACE1 and APP protein could be degraded by the UPS (Morel et al., 2013; Qing et al., 2004; Wang et al., 2012). When the proteasome is dysfunctional, autophagy-lysosome can be recruited to degrade ubiquitinated proteins. APP can be ubiquitinated at several lysine residues within its C-Terminal fragment regions as its sites of ubiquitination (Morel et al., 2013, Watanabe et al., 2012; El Ayadi et al., 2012). BACE1 can be ubiquitinated and blocking the ubiquitin-proteasomal pathway by proteasomal inhibitors- either MG132 or lactacystin inhibited BACE degradation (Qing et al., 2004). Mutations of BACE1 ubiquitination sites abolished its degradation via ubiquitin-proteasome pathway (Wang et al., 2012). Besides degradation, UPS can also regulate the trafficking of BACE1. BACE1 can be sorted from plasma membrane to the endosomes and lysosomes by interacting with specific trafficking molecule through its signals present in the carboxyl-terminal fragment (Kang et al., 2012).

The levels of Aβ and C99 are both significantly increased in DS (Busciglio et al., 2002). To search for the potential candidate proteins that are responsible for regulating BACE1 in DS, our lab has found that USP25 and UCHL1 affect BACE1. USP25 is located in the human chromosome 21 and overexpressed in DS patients (Valero et al., 1999). USP25 can cleave both lysine 48- and lysine 63-linked polyubiquitin chains, suggesting that it can regulate both protein degradation and trafficking.
In the current study, we have shown that USP25 affects the accumulation and degradation of APP and BACE1, both in vitro and in vivo. Its overexpression also promotes the trafficking of BACE1 from plasma membrane to the TGN. As a result, USP25 facilitates the generation of CTF. Our work is the first to suggest that USP25 dysregulation may contribute to the AD pathogenesis in DS through affecting APP processing.

3.2 Method.

3.2.1 cDNA constructs, cell cultures and transfection.

Human cDNA library was generated from mRNA extracted from HEK29e cells by RT-PCR. Human USP25 cDNA was amplified and cloned into mammalian expression vector pcDNA4-mycHisA (Invitrogen) at the BamHI and XhoI sites to generate the mammalian expression plasmid pZ-USP25-st. Human embryonic kidney (HEK) 293 cells, human neuroblastoma SH-SY5Y cells and mouse neuroblastoma N2A cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1%l-glutamine and 1% penicillin / streptomycin (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Transient transfections of plasmids were performed using Polyethylenimine (PEI) method.

3.2.2 Pharmacological treatment

Protein half-life was determined by using a 100 μg/mL CHX chase for 0, 6, 12 or 24 h. Chloroquine (100 μM) was used to inhibit lysosomal protein degradation. MG132 (5 μM) was used to inhibit proteasomal protein degradation. LY2886721 (20 nM) was used as BACE1 inhibitor.
3.2.3 Immunoblotting

HEK cells and mouse brain tissues were lysed in triton lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 50 mM Tris-HCl (pH 8.0) and protease inhibitor cocktail (Roche), followed by brief sonication. Protein concentration was measured by Bradford assay (Bio-rad) and 4x sodium dodecyl sulfate (SDS) sample buffer was added to each sample. For western blot analysis, sample were diluted in 4X SDS-sample buffer and loaded onto 8% tris-glycine, 12% tris-glycine or 16% Tris-Tricine SDS-PAGE and transferred to PVDF-FL membranes to detect APP, BACE1 and CTF. Rabbit anti-USP25 polyclonal antibody, mouse anti-β-actin monoclonal antibody AC-15 (Sigma), mouse anti-myc antibody (9E10) that recognized BACE1-myc and rabbit C20 that recognized last 20 C-terminal amino acids of APP were used as primary antibodies. IRDye 680-labelled goat anti-rabbit and IRDye 800CW-labelled goat anti-mouse antibodies were from LI-COR Biosciences. Blots were visualized on the Odyssey system (LI-COR Bioscience). Signal intensities from each band were quantified using ImageJ, and the bands were analyzed relative to their controls from the same membrane and experiment.

3.2.4 Animals and genotyping

USP25 transgenic mice were generated from C57BL/6 background mice. USP25 overexpression is driven under the human USP25 promoter in BAC. Therefore, USP25 overexpression in the transgenic mice mimics the expression pattern of USP25 in human. All transgenic mice were genotyped at one month of age. Mice were anesthetized with isoflurane and earmarked. The tissue was digested in 300 μL of lysis buffer (10 mM Tris-HCL pH 8.0, 10 mM EDTA pH 8.0, 150 mM NaCl, 0.5% SDS) with proteinase K (100 ng/mL (New England Biolabs) at 55 °C overnight. The next day, samples were centrifuged at 16,000g for 10 min and supernatants were
transferred to new tubes. Genomic DNA was extracted with phenol-chloroform, precipitated with 0.7X volume of isopropanol, and pelleted with centrifugation at 16,000g for 15 min. DNA was then washed twice with 70% ethanol, air dried and re-suspended in sterile deionized water. For the genotyping of USP25 expression, genomic DNA was subjected to PCR to amplify USP25 Transgene using forward primer USP25 5’-CAAACCACAGCATTGTTACAC and reverse primer USP25 5’-CTGTTGTACTGAAGAACATTGAG.

3.2.5. Subcellular fractionation

Cells were harvested in 1 ml PBS, centrifuged down at 1000 X g for 60 sec and resuspended in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25M sucrose (8.56%), and Roche protease inhibitor Complete). The collected cells were homogenized with 15 strokes of a Kontes Dounce homogenizer and centrifuged at 1000 Xg for 15 min to produce a post-nuclear supernatant (PNS). PNS was loaded on top a decreasing sucrose gradient consisting of 0.8 mL 2 M, 1.2 mL 1.3 M, 1.2 mL 1.0 M, and 0.8 mL 0.5 M sucrose in HB. Tubes were balanced and spun at 280,000 X g for 2 h at 4°C. Nuclei and intact cells were precipitated in pallet by low-speed centrifugation at 1000 x g for 10 mins. Samples were manually collected from the top of the tube and concentrated by TCA/Acetone protein precipitation, then subjected to Western Blot analysis.

3.2.6. Biotin plasma membrane labeling

Cell were washed with PBS three times and 1 ml of 1 mg/ml Sulfo-NHS-LC-Biotin in PBS was added and incubated with shaking at 4°C for 30 minutes. Reaction was quenched by washing cells 2x with ice cold PBS with 1M glycine. Cells were washed once with PBS, before lysed in 600 μL of Lysis Buffer (1%NP-40, 150mM NACL, 10mM Tris HCL pH7.4 +0.1% Protease
Inhibitor). 200 μL of prewashed Streptavidin was added to the supernatant. The mixture was incubated overnight at 4°C on orbital shaker. Resin was washed 3 times in PBS and boiled for 10 mins in 2X SDS loading buffer to elute biotin labeled protein. The eluted supernatant was subjected to Western Blot analysis.

3.2.7. Immunohistochemistry (IHC)

COS-7 cells were seeded onto glass coverslips in 24 well plates. Forty-eight hours after the transfection, cells were rinsed in PBS, fixed in 4% PFA for 20 min, permeabilized with PBS-Tx for 30 min, blocked in 5% bovine serum albumin (BSA) in PBS-Tx for 30 min, and incubated with primary antibodies in 1% BSA in PBS-Tx overnight at 4°C. The next day cells were rinsed, incubated for 1 h with goat anti-rabbit Alexa Fluor 488 (green) and goat anti-mouse Alexa Fluor 568 (red), and mounted using Fluoromount-G (Southern Biotech). Cells were imaged with a 63× objective lens on a Leica confocal microscope.

3.2.11. Statistics

Data were expressed as mean ± SEM. Data were analyzed by Student’s t-test for pairwise comparisons, by one-way ANOVA when there is one measurement variable and one factor, or by two-way ANOVA with the post hoc Bonferroni test when one measurement variable and two factors are involved.
3.3. Results

3.3.1 USP25 affects the degradation of APP both *in vivo* and *in vitro*

APP can be degraded through both lysosomal pathway and ubiquitin-proteasome pathway. In order to investigate the effect of USP25 overexpression on APP protein level, 12 weeks old-USP25 transgenic mice and their littermates were sacrificed. Whole brain lysis were subjected to Western Blot analysis. USP25 overexpression significantly increased both immature and mature APP protein level by 1.52 ± 0.12 (p<0.05) and 1.40 ± 0.053 (p<0.05) folds respectively (*Figure 3.1 A, B & C*). To investigate whether USP25 affected APP degradation, we first confirmed the effect of USP25 on APP *in vitro*, using stable HEK cell lines over-expressing wild-type APP (HAW). Transfection of USP25 plasmid in HAW cells for 48h increased both mature APP level and immature APP (*Figure 3.1 D, E & F*). Blocking the lysosomal degradation by chloroquine abolished the effect of USP25 overexpression on APP, implied that USP25 affect the turnover of APP (*Figure 3.1 D*). To eliminate the possible involvement of USP25 in APP synthesis and to confirm its role in the degradation, we examined its effect on the half-life of APP. Cycloheximide (CHX) is a protein synthesis inhibitor derived from the *Streptomyces griseus* bacterium (Kay & Korner, 1966; Kerridge, 1958). Stopping protein synthesis with CHX treatment, the amount of protein remaining after a certain time interval can be measured, thus the protein half-life being calculated. We found that USP25 overexpression significantly increased the half-life of APP and affected its degradation (p<0.05, group comparison by two-way ANOVA) (*Figure 3.1 G & H*), elevating the accumulation of APP after 60 mins CHX treatment by 2.291± 0.4713 folds (p<0.05) (*Figure 3.1 G & H*).
Figure 3.1: Overexpressing USP25 significantly increased the protein level of APP by affecting its degradation.

(A). Brain lysis of USP25-transgenic mice and little mate controls were subjected to western blot analysis. APP was detected by C20 antibody; (B) Quantification of (A), immature APP protein; (C) Quantification of (A), mature APP protein; N=3, *p<0.05 by student t-test; (D). HAW cells (HEK stably overexpressing wild-type APP) were transfected with pz-USP25L-st or control plasmid for 48 h. Cells were treated either with chloroquine or DMSO for 12 h. Cell lysates were subjected to western blot analysis. APP was detected by C20 antibody; (E). Quantification of D, immature APP protein; (F). Quantification of D, mature APP protein N=3, *p<0.05 by student t-test; (G). HAW cells were transiently transfected either with control plasmids or pz-USP25L-st for 48 h before treated with 100 μg/mL CHX at the same time for 0, 4, 8, and 12h. APP was detected by C20 antibody. (H). Quantification of G; APP level was plotted as a percentage of the amount at 0 h. N=3, *p<0.05, by two-way ANOVA group comparison.
3.3.2 USP25 affects the degradation of BACE1 both in vivo and in vitro

BACE1 can be ubiquitinated and degraded through ubiquitin-proteasome pathway. To investigate whether USP25 affected APP degradation, we first investigated the effect of USP25 in vivo, using 12 week-old USP25 transgenic mice and their littermates. Their whole brain lysis was subjected to Western Blot analysis. USP25 overexpression significantly increased BACE1 protein level by 1.48 ± 0.07 folds (p<0.01) (Figure 3.2 A & D). To confirm the effect of USP25 on BACE1 in vitro, we used stable cell lines overexpressing BACE1 and transfection of USP25 plasmid in 2EB2 cells increased BACE1 protein level by 1.48 ± 0.05 folds (p<0.05) (Figure 3.2 B & E) while site-directed mutagenesis at three BACE1 ubiquitination sites abolished the effect (Figure 3.2 C & F), suggesting that USP25 affects BACE1 protein level through affecting its ubiquitination. To eliminate the involvement of USP25 in BACE1 synthesis and to confirm its role in the degradation, we examined its effect on the half-life of BACE1 using CHX. We found that USP25 significantly increased the half-life of BACE1 (p<0.01, two-way ANOVA), by increasing the the accumulation of BACE1 after 12 hours CHX treatment by 1.557±0.007143 folds (p<0.01) (Figure 3.1 G & I). MG132 inhibits proteasome-dependent protein degradation (Rock et al., 1994), while chloroquine inhibits lysosomal protein degradation through altering lysosomal pH (Gonzalez-Noriega et al., 1980). To examine whether USP25 affects BACE1 protein level through interfering its lysosomal or proteasomal degradation, 2EB2 cells with USP25 overexpression were treated with MG132 and chloroquine. MG132 (79.67±3.93%, p<0.05) and chloroquine (97.67±8.452, p>0.05) either reversed or blocked the effect of USP25 overexpression on BACE1 suggesting that USP25 regulates BACE1 through both lysosomal and proteasomal degradation pathways (Figure 3.1 H & J).
Figure 3.2: Overexpressing USP25 affects BACE1 degradation.

(A). Brain lysates of USP25-transgenic mice and little mate controls were subjected to western blot analysis. BACE1 was detected by BACE1 antibody; (B). 2EB2 cells (HEK stably overexpressing BACE1-myc and APPswe) were transfected with pz-USP25L-st or control plasmid for 48 h. Cell lysates were subjected to western blot analysis. BACE1-myc was detected by 9E10; (C). HEK cells was transfected with pz-BACE1(M4)-myc (m168+m203+m382). 24 hours after the transfection, either pz-USP25L-st or control plasmid were transfected. 24 hours after the second round of transfection, cells were harvested and subjected to Western Blot analysis. BACE1-M4-myc was detected by 9E10. (D). Quantification of A; (E). Quantification of B; (F). Quantification of C; N=3, *p<0.05 by student t-test. 2EB2 cells were transfected with pz-USP25L-st or control plasmid for 48 h and then treated with (G) 100 μg/mL CHX at the same time for 0, 4, 8, and 12 h, or (H) Tunicamycin, MG132, or chloroquine for 12h. Cell lysates were subjected to western blot analysis. BACE1-myc was detected by 9E10. (I) Quantification of G, BACE1 level was plotted as a percentage of the amount at 0 h. N=3, *p<0.05 by two-way ANOVA group comparison; (J). Quantification of H. N=3, *p<0.05 by student t-test.
3.3.3 USP25 overexpression altered the trafficking of BACE1.

USP25 can regulate BACE1 degradation through lysosomal degradation pathway, suggesting that it may affect the internalization and trafficking of BACE1. We transfected COS-7 cells with Pz-BACE1-Myc plasmid, followed by transfection of USP25-OE or PcDNA4 control plasmid. We detected BACE1-myc by mouse anti-myc antibody, USP25 by rabbit anti-USP25 antibody and Golph 4 (TGN Marker) by goat anti-Golph 4. The merged image shows that USP25 altered the trafficking of BACE1 by accumulating BACE1 in TGN (Figure 3.3 A). We quantified the fluorescence intensity of BACE1-myc signal that co-localized with Golgi marker. The results suggest that USP25 overexpression significantly increased the relative TGN-BACE1 protein level by 3.59±0.46 folds (p<0.01) (Figure 3.3 B). To confirm the effect of USP25 in BACE1 trafficking into the Golgi, we performed sucrose gradient subcellular fractionation that extract Golgi protein. USP25 overexpression altered the subcellular pattern of BACE1 distribution and accumulate it in the Golgi (Figure 3.3 D). To confirm the effect of USP25 in BACE1 internalization, we performed biotin plasma membrane labeling to isolate plasma membrane and cytosol proteins. The results from western blot analysis suggested that USP25 overexpression significantly reduced the relative amount of BACE1 protein level on the cell surface to 21.3±6.88% (Figure 3.3 C & E), hence enhanced the internalization of BACE1. This evidence suggests that USP25 overexpression affects the subcellular trafficking of BACE1, by enhancing the internalization of BACE1 from the cell surface and accumulation of BACE1 protein in TGN.
Figure 3.3 Overexpressing USP25 affects the trafficking BACE1 protein

(A). Cos-7 cells was co-transfected with pBACE1-myc plasmid and p-USP25L-st or control plasmid. 48 hours after the transfection. Cells were fixed in 4% PFA. 9E10 and USP25 antibody were used to detect the BACE1-myc and USP25. Anti-GOLPH4 was used as a Golgi Marker. Confocal image was taken at 63X. (B). Quantification of fluorescence intensity of A N=5, **p<0.01 by student t-test; 2EB2 cells were transfected with pz-USP25L-st or control plasmid for 48 h; (C). Biotin plasma membrane labeling was performed to isolate plasma membrane and cytosol proteins; (D). Fractions from 10~50% sucrose density gradient were collected and concentrated by TCA precipitation; Those proteins samples were subjected to western blot analysis. 9E10 antibody was used to detect BACE1-Myc in Western blot analysis. (E). Quantification of C. USP25 significantly decreased the amount of BACE1 on the cell surface. N=4, ***p<0.001 by student t-test.
3.3.4 USP25 promotes APP CTF production through affecting BACE1

Since USP25 up-regulated BACE1 and APP levels, we then asked whether it would affect the production of APP CTFs. For this purpose we first examined CTF levels produced from USP25 transgenic mice. USP25 significantly increased C89 levels by 1.363 ± 0.0841 folds (p<0.05, Figure 3.4 A & B) folds but not C83 levels in vivo. In 2EB2 cells overexpressing APP swe and BACE1, USP25 significantly increase C99 level by 1.743 ± 0.171 folds (p<0.05) but not C83 (Figure 3.4 C & D), suggesting that USP25 may affect CTFs levels through BACE1 actions. To confirm that, 2EB2 cells were treated with BACE1 inhibitors to determine whether the the effect of USP25 on C89/C99 was due to its effect on BACE1 cleavage of APP. BACE1 inhibitor reversed the effect of USP25 overexpression (Figure 3.4 C).

Figure 3.4. USP25 promotes CTF and Aβ production through affecting BACE1

(A). Brain lysis of USP25-transgenic mice and little mate controls were subjected to western blot analysis. CTF was detected by C20 antibody; (B). Quantification of A. N=4, *p<0.05 by student t-test; (C). 2EB2 cell were transfected either by pZ-USP25-st plasmid or empty vector. 48 hours after the transfection, cells were treated either with DMSO or BACE1 inhibitor. Cell lysis were subjected to western blot analysis. (D). Quantification of C. N=4, *p<0.05 by student t-test;
3.4. Discussion

As a DUB enzyme, USP25 has been shown to regulate UPS-mediated protein degradation, including TRAF3, TRAF6, and MyBPC. Its role in ERAD suggested that USP25 can potentially regulate protein trafficking (Jung et al., 2015). Here we identified two additional proteins, APP and BACE1, whose protein expression levels can be regulated by USP25. The results suggested that USP25 affects the degradation of BACE1 and APP, both of which contribute to the pathogenesis of AD. It can also regulate the trafficking of BACE1 by altering its subcellular location - enhance internalization from the surface and accumulation in the Golgi.

We have shown that USP25 overexpression significantly enhanced the protein levels of APP, BACE1 and CTF, both *in vivo* and *in vitro*. It was also reported that USP25 is involved in Endoplasmic Reticulum (ER)-associated degradation (ERAD) and regulates the turnover of several ERAD substrates including APP (Jung et al., 2015). We confirmed the role of USP25 in regulating APP turnover using cycloheximide chase assay. APP is mainly degraded via the lysosomal pathway. It can be also ubiquitinated and degraded by proteasome (Morel et al., 2013). Ubiquitination can act as a target signal used by many proteins for lysosomal degradation. Chloroquine, a lysosomal inhibitor, prevented USP25-mediated degradation of APP, suggesting that USP25’s effect on APP degradation is mainly via lysosomal pathway. The molecular mechanisms of USP25-regulated APP degradation remain unclear, since we did not detect a direct interaction between USP25 and APP degradation. The lack of interaction was not likely due to a deficiency in protein amount, since we had overexpressed both USP25 and APP in the cell. We speculate that there are intermediate players between USP25 and APP. Further experiments are required to explore the intermediate proteins.
We also found that USP25 affects the degradation of BACE1. BACE1 can be ubiquitinated and targeted for both UPS (Qing et al., 2004) and autophagy-lysosome degradation (Huse et al., 2000). Its degradation by UPS can be blocked by both proteasomal inhibitors MG132 (Qing et al., 2004) and lysosomal inhibitor chloroquine (Huse et al., 2000). Our initial two-hybrid yeast experiment suggested that USP25 interact with BACE1 physically. Our experiment further showed that USP25 overexpression affected BACE1 degradation through regulating its ubiquitination. We further confirmed the functioning of USP25 in BACE1 degradation using CHX chase assay. At the same time, both Chloroquine and MG132 prevented USP25-mediated degradation of BACE1, suggesting that USP25 affects BACE1 degradation through both proteasomal and lysosomal pathway.

In addition to degradation, UPS can also regulate the trafficking of BACE1. BACE1 can be sorted from plasma membrane to the endosomes and lysosomes by interacting with specific trafficking molecule through its signals present in the carboxyl-terminal fragment. Lysosomal inhibition prevented USP25-regulated degradation of BACE1 suggesting that USP25 may also affect how BACE1 is sorted from plasma membrane to the endosomes and lysosomes. Therefore, we examined the effect of USP25 overexpression in BACE1 trafficking. Isolations of Golgi proteins and plasma membrane proteins from cytosol ones allow us to examine the relative distributions of BACE1 in its secretory pathway. Our results suggested that USP25 overexpression accumulated BACE1 in Golgi and enhanced its internalization, thus altering its subcellular distribution. However, we have not tested which trafficking molecules serve as intermediate proteins that connect USP25 and BACE1. Further investigations are needed in order to better understand the underlying molecular mechanism for how USP25 regulated BACE1 trafficking.
Since USP25 upregulated both APP and BACE1 levels, we went on to explore its effect on APP processing downstream products, APP CTF. As expected, USP25 affects CTF production through its regulation of BACE1, since BACE1 inhibitor blocked the effect of USP25 overexpression on CTF. Taken together, we propose several possible mechanisms by which USP25 increased CTF. Firstly, USP25 deubiquitinates BACE1 and the intermediate proteins that regulate the degradation of APP, therefore enhancing products’ generation by increasing enzymes and substrates’ levels. Secondly, USP25 facilitates the sorting of BACE1 into TGN and endosome. Endosome and TGN are acidic, allowing for optimal cleavage activity of β-secretase in those compartments. Therefore, BACE1 trafficking into endosomes enhances CTF production through β-cleavage of APP.

3.5. Conclusion

In summary, in this chapter we examined the role of USP25 in APP processing, both in vitro and in vivo. We found that USP25 enhanced CTF production by affecting the degradation of APP and BACE1. Moreover, USP25 promoted BACE1 trafficking into Golgi and its internalization from plasma membrane, thus promoting APP amyloidogenic pathway.
Chapter 4

The role of USP25 in regulating cell cycle and apoptosis

4.1 Introduction

DS individuals have widespread hypocellularity - reduced number of neurons, in their hippocampus (Guidi et al., 2008), cerebellum (Wisniewski, 1990) and neocortex (Larsen et al., 2008), along with defective synaptogenesis, connectivity and synaptic plasticity. Consistent with DS individuals, the DS mouse model Ts65Dn show reduced cell density in the neocortex (Chakrabarti et al., 2007) and hippocampus (Insausti et al., 1998) during prenatal and early postnatal stages. Primary neuronal cultures derived from fetal DS brains exhibited increased intracellular oxidative stress, leading to an enhanced occurrence of apoptosis in the dentate gyrus (DG) and the germinal zones of the hippocampus and subventricular zone (SVZ).

The active proliferation and differentiation of progenitors are balanced by apoptosis. Both defective neurogenesis and up-regulation of the apoptotic pathway contribute to the progression of neurodegeneration in DS. The apoptotic process can be triggered by signals arising from within the cell or by extrinsic death activators. One of the key regulators of cell proliferation and death is NF-κB. NF-κB can serve either as an anti-apoptotic or pro-apoptotic transcription factor, depending on the nature of the stimulus. It can inhibit apoptosis through being activated by TNF-α via its type 1 receptor (TNFR1), which induces the expression of certain genes whose products can inhibit apoptosis. NF-κB also inhibits apoptosis though the mitochondria-dependent pathway by affecting DNA damaging agents such as Bcl-2 protein family.

The ubiquitin-proteasome pathway plays a crucial role in the pathways of NF-κB activation. One of the major roles of UPS in NF-κB activation is the degradation of IκB. When the NF-κB signal pathways are induced, IκB is polyubiquitinated and selectively degraded by the
26S proteasome, while p65 itself is released and imported into the nucleus (Palombella et al., 1994). Deubiquitination- the reverse reaction of ubiquitination, is an important regulatory process of ubiquitination through the actions of DUBs. There have been few studies on the effect of USP25’s overexpression in DS pathogenesis, which may contribute to the cognitive impairment present in all patients with DS. It down-regulated the virus-induced activation of nuclear factor-kappa B (NF-κB) through deubiquitinating retinoic acid-inducible gene I (RIG-I), TRAF2, and TRAF6 (Lin et al., 2015).

To better define the role of USP25 in DS pathogenesis, we have cloned USP25 plasmid and generated USP25 transgenic mice. We found that overexpression of USP25 facilitated oxidative stress-induced cell death and caspase-3 activation. USP25 regulates the activation of NF-κB both in vitro and in vivo. and NF-κB mediates the regulation of cell death by USP25. USP25-Tg mice showed behavioral changes in sucrose preference task, novelty suppressed feeding and Morris water maze, indicating defects in neurogenesis. Our study suggested that overexpression of USP25 contributes to the progression of neurodegeneration by affecting neurogenesis and upregulating apoptotic pathway. It could be considered a potential pharmaceutical target for intervening in cognitive impairment in DS.
4.2. Method

4.2.1. Mice

Mice were housed on a 12 h light:dark cycle and provided with food and water ad libitum. To generate USP25 transgenic mice and embryo, heterozygous USP25-Tg mice were bred with wild-type C57Bl/6. Genotyping was performed according to the protocol described above. Heterozygous USP25-Tg and wild type controls littermates were used for comparison. During breeding, observation of a vaginal plug was marked as embryonic day 0.5 (E0.5). At the time of sacrifice, pregnant dams were euthanized with an intraperitoneal injection of Euthanyl followed by cervical dislocation. For the bromodeoxyuridine (BrdU) and 5-ethynyl-2′-deoxyuridine (EdU) birth-dating, pregnant dams received an intraperitoneal injection of BrdU 0.5 hours and EdU 2 hours prior to euthanasia. Following euthanasia of the dam, embryos were collected and submersion fixed overnight in 4% paraformaldehyde.

4.2.2. Western blotting

Cells and brain tissue were lysed with RIPA-Doc buffer containing: 50 mm Tris–HCl (pH 7.2), 150 mm NaCl, 1% deoxycholate, 1% Triton X-100, 1% sodium dodecyl sulphate and protease inhibitor cocktail Complete (Roche Molecular Biochemicals, Indianapolis, IN, USA) The samples were diluted in 4× SDS-sample buffer, boiled and loaded onto 7.5%,10% glycine or 16% tricine gels, followed by transfer to Polyvinylidene fluoride (PVDF-FL) membranes. For immunoblotting analysis, membranes were blocked for 1 hour in PBS containing 5% nonfat dried milk, followed by overnight incubation at 4°C in primary antibodies diluted in the blocking medium. Rabbit anti-Caspase 3 polyclonal antibody was used to detect both holo-caspase 3 and cleaved caspase 3 (Cell Signaling Technology, 1:1000). The NF-κB p65 subunit was determined
using rabbit anti-p65 (Cell Signaling Technology, 1:1000). IκB was detected by rabbit anti-IκB (Cell Signaling Technology, 1:1000). Cyclin D2 was detected using mouse monoclonal Anti-cyclin D2 antibody (Abcam, 1:200). Cyclin D1 was detected using rabbit anti-cyclin D1 antibody (Abcam, 1:10000). Cyclin E1 was detected mouse anti-cyclin E1 antibody (Abcam, 1:1000). Cyclin A2 was detected by mouse anti-cyclin A2 (Cell Signaling Technology, 1:2000), Cyclin B1 was detected by mouse anti-cyclin B1 (abcam, 1:1000). Internal control β-actin was analyzed using monoclonal antibody AC-15 (Sigma-Aldrich, 1:10000).

4.2.3. Cell viability assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed following manufacturer’s instruction (Promega) to measure cell viability after H2O2. The absorbance of the formazan product at 490 nm was measured. Cell media were collected and LDH (lactate dehydrogenase) assay was performed (Clontech) to measure cell toxicity. A red, formazan-class dye, which was converted by LDH that was leaked to cell media, was measured by absorbance at 492 nm.

4.2.4. cDNA constructs, cell cultures and transfection

Human cDNA library was generated from mRNA extracted from HEK293 cells by reverse transcription-polymerase chain reaction (RT-PCR). The forward primer 5’- and reverse primer were designed to amplify human USP25 cDNA and the PCR product was cloned into mammalian expression vector pcDNA4-myc-His (Invitrogen) at the BamHI and XhoI sites to generate the mammalian expression plasmid pZ-USP25-st. Human embryonic kidney (HEK) 293 cells, human neuroblastoma SH-SY5Y cells and mouse neuroblastoma N2a cells were cultured
in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1%l-glutamine and 1% penicillin / streptomycin (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Transient transfections of plasmids were performed using Polyethylenimine (PEI) method.

4.2.5. Luciferase assay

Cells were seeded onto 24-well plates or 35-mm-diameter plate 1 day prior to transfection and grown to approximately 25% confluence by the day of transfection. Cells were transfected with 0.5 μg of plasmid DNA per well using 1.5 μL or 2 μg plasmid DNA per 35-mm-diameter plate for RNA extraction by 6 μL PEI reagent (Cat#. 23966, Polysciences Inc.) The Renilla luciferase vector pRLuc was co-transfected to normalize the transfection efficiency of various luciferase reporter constructs. Cells were harvested at 48 h after transfection and lysed with 100 μL passive lysis buffer (Promega) per well. Firefly luciferase activity and Renilla luciferase activity were measured sequentially using the Dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized according to Renilla luciferase activity and expressed as relative luciferase units to reflect the promoter activity.

4.2.6. Immunohistochemistry (IHC)

Adult mice were sacrificed and perfused with PBS. Half of the brains was fixed overnight in 4% paraformaldehyde, and cryoprotected in 30% sucrose prior to freezing. Embryos were collected in E13 and fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose prior to freezing. The fixed brain or embryo was sectioned with a Leica Cryostat to 30 μm thickness after embedding into O.C.T. solution. Every 6th slice with the same reference position was mounted
onto the slides for staining. Immunohistochemistry was performed on 30 μm coronal sections. Tissue samples were fixed by perfusion with paraformaldehyde, permeabilized with 1% Triton X, blocked with 5% serum for 1 hour at room temperature and antigen retrieval was by heat mediation in citrate buffer, pH 6.0. The sample was incubated with primary antibody at 4°C for 12 hours. Alexa Fluor® 488 or 594 conjugate goat polyclonal (1/200) was used as the secondary antibody. Primary antibodies include rat anti-BrdU antibody (Abcam, 1:200).

4.2.7. Statistics

Data were expressed as mean ± SEM. Data were analyzed by Student’s t-test for pairwise comparisons, by one-way ANOVA with the post hoc Newman–Keuls test when there is one measurement variable and one factor, or by two-way ANOVA with the post hoc Bonferroni test when one measurement variable and two factors are involved.

4.2.8. Animal Behavioral test

**Morris Water Maze test.** This behavioral test was performed in a 1.5-meter diameter pool with a 10-cm diameter platform placed in the southwest quadrant of the pool. The procedure consisted of one day of visible platform tests and 4 days of hidden platform tests, followed by a probe trial 24 hours after the last hidden platform test. In the visible platform test, the platform was placed 1 cm above the clear water surface. The position of the platform and the starting direction varied across trials. Mice were tested for 5 continuous trials with an inter-trial interval of 75 minutes. Mice were allowed to swim for at most 60 seconds before climbing onto the platform. Failed mice were gently guided to the platform and remained there for 20 seconds before they were sent back to their home cages. In the hidden platform tests, the platform was submerged 1 cm below
an opaque water surface in a fixed position in the southwest/third quadrant. Mice were trained for 5 trials with an inter-trial interval of 75 minutes. Mice were allowed to swim for at most 60 seconds before climbing onto the platform, and again, failed mice were gently guided to the platform and allowed to remain there for 20 seconds. In the probe trial, the platform was removed and mice were allowed to swim for 60 seconds. Mouse behavior including speed and escape latency was video-recorded by an automated video tracking system (ANY-maze, Stoelting).

**Sucrose preference** Mice were individually housed and given two water bottles containing water with the left/right location balanced across animals. After 7 days of habituation, both bottles were removed and replaced with a water bottle containing water and a second with 4% sucrose with the left/right location balanced across animals. The bottles were weighted and recorded before being given to the mice. After 24 hours, the bottles were weighed again and the weight difference before and after it was given to the mice was recorded. Sucrose preference was expressed as \((\Delta\text{weight sucrose})/(\Delta\text{weight sucrose} + \Delta\text{weight water}) \times 100\).

**Novelty-suppressed feeding.** The test was performed in an open field after the mice were food-deprived for 24 hours before the test. Food was removed from the cage 24 h before testing. For testing, mice were placed for 10 minutes in the open field (same one mentioned above) with a food pellet in the center on a slightly (0.5 cm) elevated platform. Mice were placed in the arena directly from their home cage and their behavior was videotaped. The latency for each mouse to begin feeding was scored, offline, by an experimenter blind to the genotype and condition of each mouse. Upon returning to their home cage, the total amount of food consumed during a 5-min period was analyzed to test whether feeding differences in the novel environment were due to differences in hunger/motivation.
4.3. Result

4.4.1. Physiological characterization of USP25 transgenic (Tg) mice

Overall, most of the USP25 transgenic mice remained healthy for the 6 months of the study. No obvious differences were observed during animal husbandry in regarding to animals suffering from serious illnesses which resulted in sacrifice or death. USP25-Tg mice grew as well as control mice during the 6 months study period and there were no significant differences between body (38.98±1.908 vs 36.32±3.659 g) (Figure 4.1A) and brain weights (0.4834±0.0102 vs 0.5052±0.002035 g) (Figure 4.1B). However, comparison of brain cortexes of 6 month-old USP25-Tg mice and those of their wild-type littermates around the similar cortical region, USP25-Tg overexpression had significantly reduced the thickness of the cortex to 77.8±0.399% (p<0.01) (Figure 4.1C & D), suggesting potential defects in neuronal survival and neurogenesis. We speculated that impaired neurogenesis, reduced neuronal survival, or both, affect the development of USP25-Tg mouse neuronal cortex.
Figure 4.1 Physiological characterization of USP25 Transgenic mice

At 6-months ages, USP25 transgenic mice’ body and brain weights were recorded. (A), No significant difference was found in brain weight; (B), No significant difference was found in body weight; (C). Brains were cryosectioned and nuclei were stained with DAPI. Image was taken at 2.5X on mice cortex. (D). Thickness of the cortex were measured (marked in yellow) and quantified, suggesting USP25 transgenic mice’ cortexes were significantly thinner than their littermate controls’. N=5, ***p < 0.001, by Student’ t-test.
4.4.2. USP25 facilitates oxidative stress-induced cell death and caspase-3 activation

To examine the role of USP25 in oxidative stress-induced cell death, N2a control cells transfected with vector pcDNA4 and those transfected with p-USP25L plasmid were treated with H2O2 in different dosages for 24 hours, and the MTS assay was performed to determine the cell viability. MTS assay showed that H2O2 treatment decreased the cell survival ratio in cells with USP25L overexpression cells, in a dosage-dependent manner (p<0.05, two-way ANOVA) (Fig 4.2A). USP25 overexpression significantly decreased the cell viability when treated with 50 μM (92.27 ± 0.902%, p<0.0.5), 100 μM (92.56 ± 3.156%, p<0.05) and 400 μM (79.43±3.0005%, p<0.05) H2O2 treatment. To further determine whether USP25 affects the cell cytotoxicity under oxidative stress, LDH assay was performed with control cells and USP25-overexpressing cells after different dosages of H2O2 treatment for 12 hours. The result showed that USP25-overexpression increased the cell cytotoxicity (p<0.05, two-way ANOVA) (Figure 4.2B). USP25 overexpression significantly increase cytotoxicity when treated with 100 μM (1.126 ± 0.00666 folds, p<0.0.5) and 200 μM (1.167 ± 0.01154 folds, p<0.05) H2O2.

To investigate if the decreased cell viability and increased cytotoxicity induced by USP25 overexpression is associated with the caspase signaling pathway, western blot assays were performed to detect the cleavage of caspase-3 in N2a cells transfected by empty vector or USP25L in the presence of H2O2 treatment. Generation of the cleaved caspase-3 was markedly increased in USP25-overexpressing cells compared with controls in a dosage-dependent manner (p<0.01, two-way ANOVA) (Figure 4.2 C, D) (200 μM: 1.791±0.2266 folds; 400 μM: 2.094 ±0.3032 folds, p<0.05)
Figure 4.2. USP25 facilitates oxidative stress-induced cell death and Caspase-3 activation

N2A cells were transiently transfected with empty vector or USP25L. Both cells were treated with different dosage of H2O2 48 hours after transfection. After 24 hours H2O2 treatment, (A) MTT assay was performed. H2O2 treatment significantly reduced the cell survival of USP25 overexpressing cells compared with control cells, in a dosage dependent manner; (B) LDH assay was performed, H2O2 treatment significantly increased the cytotoxicity of USP25 overexpressing cells compared with control cells, in a dosage-dependent manner. (C) After 6 hours H2O2 treatment at different dosages, Western blot assays were performed. (D) H2O2 treatment significantly increased the protein level of cleaved caspase 3 of USP25 overexpressing cells compared with control cells, in a dosage-dependent manner; n = 3. *p < 0.05 **p<0.01; Two-way ANOVA.
4.4.3. USP25 overexpression inhibits NF-κB activation

To investigate the mechanism underlying the effect of USP25 on cell death, we sought candidate proteins that could be correlated with caspase-3 activation during oxidative stress. ROS often promotes the NF-κB pathway in the cytoplasm, while inhibiting NF-κB activity in the nucleus. We found that corresponding to the increased caspase-3 activation, USP25 inhibited the accumulation of phosphorylated-p65 protein during H2O2 treatment (p<0.05, two-way ANOVA) (Figure 4.3 A & B) to 59.45 ± 3.756% (p<0.05) after 12 h LPS treatment. To further examine USP25’s effect NF-κB activity in the nucleus, we co-transfected N2a cells with pNF-κB–Luc and USP25 or an empty vector, followed by treatment with H2O2. Compared with control, USP25 overexpression altered the pattern of pNF-κB-Luc promoter activity changes due to H2O2 (p<0.05, two-way ANOVA) (Figure 4.3 C), by increasing the promoter activity by 2.423 ± 0.1312 folds (p<0.01) for 6 hours and 1.597 ± 0.1912 folds (p<0.05) for 12 hours LPS treatment. These results indicate that USP25 affects the activation of NF-κB signaling during oxidative stress.

To confirm the role of USP25 on NF-κB-signaling, we further examined the effect of USP25 on pathways of LPS-induced NF-κB activation in neuronal cell lines (Figure 4.3 D). USP25 overexpression increased the baseline protein level of p65. Treatment with LPS increased the protein level of phosphorylated-p65 in a time-dependent manner, while USP25 overexpression inhibited the accumulation of phosphorylated-p65 (p<0.001, two-way ANOVA) to 43.39 ± 3.218% (p<0.01) after 16h LPS treatment. (Figure 4.3 E).
Figure 4.3: USP25 overexpression down-regulates NF-κB signaling pathway;

(A). N2a cells were transiently transfected with empty vector or USP25L. 48 hours after transfection, both cells were treated with 200 μM H2O2 treatment. After 0, 6 and 12 hours 200 μM H2O2 treatment, cell lysis was collected and Western blot assays were performed. Phospho-p65 was detected using Phospho-p65 antibody; (B). Change in the protein level of p65 from 0, 6 to 12 hours. Phospho-p65 level was plotted as a fold change of the amount in 0h; N=4, **p < 0.01, by two-way ANOVA group comparison; (C). N2a cells were transfected with pNF-κB-Luc and pCMV. After 12 hours of transfection, cells were split and transfected either with USP25L or vector plasmid. 36 hours after the second transfection, both cells were treated with 0, 6 and 12 hours 200 μM H2O2. Luciferase assay was performed and Renilla luciferase was used to normalize for transfection efficiency. NF-κB promoter activity was plotted as a fold change of the amount in 0h; N=3, *p<0.05, ***p < 0.001, by two-way ANOVA group comparison; (D). N2a cells were transiently transfected with empty vector or USP25L. 48 hours after transfection, both cells were treated with 30 ng/ml LPS for 0h, 4h, 8h and 16h. Cell lysis was collected and Western blot assays were performed. Phospho-p65 was detected using Phospho-p65 antibody. (E). Quantification of D; N=3, *p<0.05, ***p < 0.001, by two-way ANOVA group comparison.
4.4.4. USP25 affects LPS-induced IκB turnover and p65 nuclear trafficking

To further examine the effect of USP25 on NF-κB activation, N2a cells were co-transfected with pNF-κB-Luc and USP25 or an empty vector, followed by 16h treatment of LPS. USP25 overexpression decreased the pNF-κB-Luc promoter activity both under LPS (27.9 ± 1.054%, p<0.001) and vehicle treatment (55 ± 1.528%, p<0.01), and it also down-regulated the increasing trend of pNF-κB-Luc promoter activity induced by LPS (p<0.05, two-way ANOVA) (Figure 4.4 A). Potent NF-κB activators, such as TNF-a, IL-1 and LPS, cause degradation of IκB (especially IκBa). This process is mediated by the 26S proteasome, which releases NF-κB and allows its nuclear translocation (Palombella et al., 1994). USP25 overexpression decreased the baseline protein level of IκB to 68.83 ± 1.111% (p<0.05) and the treatment of LPS decreased the protein level of IκB while USP25 overexpression altered the effect of LPS on IκB (p<0.05, by two-way ANOVA) (Figure 4.5 C & D). Further, USP25 altered the trafficking of p65 into the nucleus during LPS treatment (Figure 4.5 B). These results further suggested that USP25 overexpression affects NF-κB activation through affecting IκB turnover and p65 nuclear trafficking.
Figure 4.4. USP25 affects LPS-induced IκB turnover and p65 nuclear trafficking

(A). N2a cells were transfected with pNF-κB-Luc and pCMV. After 12 hours of transfection, cells were split and transfected either with USP25L or vector plasmid. 36 hours after the second transfection, both cells were treated with either DMSO or 30 ng/ml LPS. Luciferase assay was performed and Renilla luciferase was used to normalize for transfection efficiency. (B). Cos-7 cells were co-transfected with pNK-κB plasmid and p-USP25L-st or control plasmid. 48 hours after the transfection, both cells were treated with 30 ng/ml LPS. Cells were fixed in 4% PFA. Phospho-p65 and USP25 antibody were used to detect the p65 and USP25. Confocal image was taken at 63X. (C) N2a cells were transiently transfected with empty vector or USP25L. 48 hours after transfection, both cells were treated with 30 ng/ml LPS for 12 hours. Cell lysis was collected and Western blot assays were performed. IκB was detected using IκB antibody. (D). Quantification of C. N=3 *p<0.05, by student’ t-test and two-way ANOVA group comparison.
4.4.5. NF-κB mediates the regulation of cell death by USP25.

To confirm the role of the NF-κB signaling pathway in the role of USP25 in cell proliferation, we transfected NF-κB p65 knockout RelA-KO cells and wild-type control Mouse Embryonic Fibroblasts cells (MEF) with pc-USP25L-st and pcDNA4 plasmid. After 48 hours USP25 overexpression followed by 12 hours H2O2 treatment, MTT and LDH assay were performed. H2O2 treatment significantly reduced the cell viability of USP25 overexpressing wild-type MEF cells compared with those transfected with empty vector (p<0.05, two-way ANOVA) at 100 μM (60.45 ± 3.202%, p<0.05) and 200 μM (78.78 ± 3.223%, p<0.05) H2O2 treatment (Figure 4.5 A), while the effect was reversed by NF-κB knockout (p<0.05, two-way ANOVA) at 200 μM H2O2 treatment (129.7 ± 5.021%, p<0.05) (Figure 4.5 B). H2O2 treatment significantly increased the cytotoxicity of USP25 overexpressing wild-type MEF cells compared with control cells under the treatment of different dosage of H2O2 (p<0.05, two-way ANOVA) (Figure 4.5 C) at 400 μM H2O2 treatment (116 ± 3.726%, p<0.05) while it failed to affect the cytotoxicity of USP25 overexpressing RelA-KO cells (400 μM: 1.038 ± 0.02472 folds, p>0.05) compared with those transfected with empty vector (p>0.05, two-way ANOVA) (Figure 4.5 D). These results suggest that USP25 affects cell survival through mediating NF-κB.
Figure 4.5: USP25 regulation of cell proliferation and death is dependent on NF-κB p65 expression

After 48 hours USP25 overexpression followed by 12 hours H2O2 treatment, MTT and LDH assay was performed for RelA-KO cells and its wild-type control cell (MEF). (A) H2O2 treatment significantly reduced the cell viability of USP25 overexpressing wild-type MEF cells compared with those transfected with empty vector. n = 3. *P < 0.05; Two-way ANOVA. (B) H2O2 treatment significantly increased the cell viability of USP25 overexpressing RelA-KO cells compared with those transfected with empty vector, in a dosage dependent manner. n = 3. *P < 0.05; Two-way ANOVA; (C). H2O2 treatment significantly increased the cytotoxicity of USP25 overexpressing wild-type MEF cells compared with control cells under the treatment of different dosage of H2O2. n = 3. *P < 0.05; Two-way ANOVA; (D). H2O2 treatment failed to affect the cytotoxicity of USP25 overexpressing RelA-KO cells compared with those transfected with empty vector. n = 3. *P > 0.05. Two-way ANOVA.
4.4.6. USP25 overexpression affects NF-κB signal pathways \textit{in vivo}

To confirm the effect of USP25 overexpression on NF-κB signaling pathway \textit{in vivo}, we isolated the hippocampi from the cortex of USP25 transgenic mice. The hippocampi were dissected and lysed in RIPA buffer for Western Blot analysis. TRAF6, NF-κB p65, IκB and active caspase-3 were detected by Anti-TRAF6, Anti-p65, anti-IκB and anti-Cleaved caspase 3 (Figure 4.6 A). The elevated protein level of active caspase 3 (1.583 ± 0.0100 folds, p<0.01) indicated increased apoptosis in hippocampus (Figure 4.6 E), consistent with the effect of USP25 overexpression \textit{in vitro}. USP25-overexpression significantly reduced TRAF6 protein levels to 62.8 ± 2.53% (p<0.01) (Figure 4.6 B), IκB by 1.88 ± 0.0633 folds (p<0.01) (Figure 4.6 C) and increased the level of p65 by 1.58 ± 0.146 folds (p<0.05) (Figure 4.6 D), suggesting that UPS25 affects NF-κB signaling pathway \textit{in vivo}.
Figure 4.6. USP25 overexpression affects NF-κB signal pathways in vivo

(A). The hippocampi of USP25-Tg mice and their littermate were subjected to western blot analysis. USP25 overexpression significantly increased the protein levels of active caspase 3, suggesting that it enhanced hippocampal apoptosis. USP25 overexpression also increased p65 and IκB while decreased TRAF6 protein levels, indicating altered NF-κB signaling. (B). Quantification of TRAF6 protein levels; (C). Quantification of IκB protein levels; (D). Quantification of p65 protein levels; (E). Quantification of activate caspase 3 protein levels; n=3. *P < 0.05. Student’s t-test.
4.4.7. USP25 overexpression affects cell cycle regulation

Incorporation of labeled DNA precursors into cellular DNA during the S phase of the cell cycle allows us to label proliferating cells. To assess the effect of USP25 on cell proliferation \textit{in vitro}, BrdU cell proliferation assay was performed in SH-SY5Y cells. The rate of BrdU uptake between control cells and those with p-USP25L transiently transfected was measured. After incubation for 2 hours with BrdU, SH-SY5Y cells with USP25 overexpression significantly increased the number of cells labeled with BrdU compared to SH-SY5Y cells transfected with empty vector by 1.53 ± 0.008 folds (Figure 4.7 A & B). However, SH-SY5Y cells with USP25 overexpression yielded less Ki67 (60.2 ± 6.19%, p<0.01) and PCNA (65.9 ± 1.57%, p<0.01), positive cells compared to those transfected with empty vector (Figure 4.7 C, D & E).

Ki-67 protein is present during all active phases of the cell cycle G1, S, G2, and mitosis, but it is absent from the resting cells stage G0. PCNA is presented mainly during G1, S, and G2. BrdU was incorporated into the genome DNA during S phase (Summarized in Figure 4.7 A). Increased number of BrdU positive cells associated with decreased number of proliferating cells may indicate increased cell cycle arrest due to USP25 overexpression. We quantified the number of PCNA/BrdU positive cells to determine the effect of USP25 in the number of proliferating cells during different cell phases. Overexpression of USP25 increased the number of PCNA/BrdU positive cells by 2.53 ± 1.83 folds, (p<0.01), suggested that more cells were arrest in S phase compared with the control group (Figure 4.7 F & G). This finding demonstrates that USP25 overexpression decreases cell proliferation by affecting cell cycle regulation in SH-SY5Y cells.
Figure 4.7. USP25 overexpression affects cell cycle regulation

(A). Overview of cycle cell and markers Ki67, PCNA and BrdU; (B). BrdU cell proliferation assay was performed in SH-SY5Y cells; (C). SH-SY5Y cells with USP25 overexpression significantly increased the number of cells labeled with BrdU, n=4. *P < 0.05. Student’s t-test; (D). Immunostaining of SH-SY5Y cells with anti-ki67; (E). USP25 overexpression significantly decreased the number of PCNA and Ki67 positive cells n=4. **P < 0.01. ***P < 0.001. Student’s t-test; (F). Immunostaining of SH-SY5Y cells with anti-BrdU and anti-PCNA; (G) USP25 overexpression significantly increased the number of BrdU/PCNA positive cells n=4 **P < 0.01. ****P < 0.0001. Student’s t-test.
4.4.8. USP25 overexpression affects cell cycle during embryonic neurogenesis

In the developing brain, proliferating neuronal precursors (NPCs) cells are located within the ventricular zone and subventricular zone (SVZ). To determine whether USP25 affects proliferation status and cell cycle regulation of NPCs, dual labeling with different thymidine analogues, 5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU), was used to examine cell cycle kinetics. At E13.5, pregnant dams received an intraperitoneal injection of EdU 2 hours before the sacrifice, followed by an injection of BrdU 1.5 hours after the EdU injection. Embryos were collected 2 hours after EdU injection. BrdU and EdU signals were detected by immunohistochemistry with an anti-BrdU antibody and the click EdU reaction (followed manufacturer’s protocol Click-iT EdU Alexa Fluor 594), respectively. Quantification of BrdU-positive and EdU-positive cells revealed that USP25 overexpression increased the number of cells co-labelled with BrdU and EdU by 1.61 ± 0.0459 folds (p<0.0001) (Figure 4.8 A, B). This finding suggests USP25 overexpression altered the cell cycle kinetics by affecting G1/S and S/G2 checkpoints.

In both the developing and adult brain, Doublecortin (DCX) is expressed in cells that are committed to the neuronal lineage (Francis et al., 1999). Its expression peaks during the early stages of corticogenesis around E14 (des Portes et al., 1998). The NeuN protein, localized in nuclei and perinuclear cytoplasm of most neurons in the central nervous system, has been actively used for targeting mature neurons (Mullen et al., 1992). To examine the consequences of affected cell cycle exit induced by USP25 in embryonic neurogenesis, immunohistochemistry was performed with antibodies to BrdU, DCX and NeuN, followed by DAPI nuclei staining (Figure 4.8 C & D). No significant was found in the total number of EdU (1.209±0.0994 folds, p>0.05), NeuN (77.85±6.917%, p>0.05) or DCX-positive cells (80.69±11.47%, p>0.05) (Figure
4.8 E & F). However, the ratios of DCX and BrdU-positive cells were significantly reduced in the ventricular zone of USP25-Tg embryos to 63.3 ± 1.20% compared with wild-type embryos (p<0.01). The ratio of NeuN and BrdU-positive cells were altered in a similar manner (60.7 ± 3.28%, p<0.01), suggesting that fewer cells in neuronal lineage were yielded per EdU-positive proliferating cell and that each for proliferating cell, less neurons were generated in USP25-Tg E13 embryos.
Figure 4.8. USP25 overexpression affects cell cycle during embryonic neurogenesis.

Immunohistochemistry was performed on E13 embryonic sagittal section. (A). Immunostaining of lateral ventricle with anti-BrdU, followed by EdU and nuclei staining; (B). USP25 overexpression significantly increased the number of BrdU/EdU+ cells in lateral ventricle; (C). Immunostaining of lateral ventricle with anti-NeuN, followed by EdU and nuclei staining; (D). USP25 overexpression significantly decreased the number of NeuN/EdU+ cells in lateral ventricle; (E). Immunostaining of lateral ventricle with anti-DCX, followed by EdU and nuclei staining; (F). USP25 overexpression significantly decreased the number of DCX/EdU+ cells in lateral ventricle; n=5 **P < 0.01, Student’ t-test.
4.4.9. USP25 overexpression affects adult cortical development though altering cyclin balance.

Most of the cerebral cortex is neocortex, which has six layers. Each layer contains neurons with different shapes, sizes and density (reviewed by Gilmore & Herrup, 1997). These layers can be divided into three parts. The supragranular layers (layer I, II and III) permit communication between different regions of the cortex. Internal granular layer (layer IV) receives thalamocortical connection and infragranular layers (layer V and VI) connect the cerebral cortex with subcortical regions. Each layer is formed by radial glial cells in the ventricular zone, which migrate to their final layer destination.

During corticogenesis, there is a slowing down of the rate of cell-cycle progress, mainly due to a lengthening of the G1 phases, allowing the increase of neuron production and differentiation (Savatier et al., 1996). A prolonged G1 phase allows both cell-fate determining extrinsic signals and inherited factors to act over a sufficient time period (Gotz & Huttner, 2005; Calegari & Huttner, 2003). Shortening of the G1 phase can prevent stem cells from being affected by signals that induce differentiation (Burdon et al., 2002). The rate of cell-cycle progression is determined by the abundance of positive and negative regulators, including cyclin D, cyclin E, cyclin A and cyclin B.

In order to examine the effect of USP25 on the structure of the cerebral cortex, we conducted immunohistochemistry on the lateral ventricle of 3 month-old USP25 Tg mice with anti-CITP2 and anti-TBR1 (Figure 4.9 A). CITP2 is a marker for cells in the deeper cortical layers (layer V and VI) while TBR1 stains layers II, III, V, and VI. We quantified the number of CITP2+ cells (layer V and VI) and CITP2-/TBR1+ cells (II, III), and the results showed that USP25 significantly reduced the ratio of CITP2-/TBR1+ cells and CITP2+ cells (Figure 4.9 B)
to 60.1 ± 3.90% (p<0.01), suggesting that USP25 overexpression altered the distribution of cortical layers.

Previous results have shown that USP25 overexpression affected cell cycle regulation both in vitro and in embryonic neurogenesis. We hypothesized that USP25 overexpression would also affect the cell cycle progression in cerebral cortex and alter the balance between positive and negative regulators in corticogenesis. Western blot analysis (Figure 4.9 D) showed that USP25 overexpression significantly increased the protein level of cyclin D1 by 2.13 ± 0.233 folds (p<0.01) (Figure 4.9 E), cyclin E1 by 1.307 ± 0.0717 folds (p<0.05) (Figure 4.9 F), reduction in the cyclin A2 level to 70.9 ± 7.07% (Figure 4.9 G), while there was no change in the cyclin B1 level (p>0.05) (Figure 4.9 H). These results suggest that USP25 altered the balance of cyclins which regulate different cell phases, thus playing a role in regulating cell cycle progression in corticogenesis. USP25 overexpression had the opposite effect on cyclin D1/E1 and cyclin A2, suggesting that USP25 overexpression may promote cells to exit the G1 phase, while inhibiting them from exiting the entire cell cycle.
Figure 4.9. USP25 overexpression affects adult cortical development.

(A). Immunohistochemistry was performed on 3-month coronal brain section. Immunostaining of lateral ventricle with anti-CITP2 and anti-TBR1, followed by nuclei staining; (B). USP25 significantly reduced the ratio of CITP2+/TBR1+ cells and CITP2+ cells; (C). Overview of cyclin-regulated cell phase; (D). Western blot analysis of cortex lysis; USP25 overexpression significantly increased the protein level of cyclin D1, cyclin E1, cyclin A2, while no change in the cyclin B1 level. n=4. *P < 0.05, **P<0.01. Student’s t-test.
4.4.10. NF-κB mediates the regulation of proliferation by USP25.

In order to confirm the role of the NF-κB signaling pathway and the role of USP25 in cell proliferation, we transfected NF-κB p65 knockout RelA-KO cells and wild-type control cells with pc-USP25-L-st and pcDNA4 plasmid. After 2 hours incubation with BrdU, wild-type control cells with USP25 overexpression had significantly increased the number of cells labeled with BrdU by 1.14 ± 0.039 folds (Figure 4.10 A, C), whereas overexpression of USP25 had no effect on the number of BrdU-positive RelA-KO cells (P > 0.05) (Figure 4.10 B, C). This suggests the disruption of NF-κB p65 expression in RelA-KO cells abolished USP25’s effect on cell proliferation.

Figure 4.10. USP25 overexpression affects cell proliferation through NF-κB dependent pathway; Wild-type MEFs (A) and RelA-KO MEFs (B) that are dysfunctional for NF-κB activity, were either transfected with USP25 or its vector plasmid. 48 hours after the transfection, the cells were fixed in 4% PFA and staining for BrdU. (C) Quantification of A & B n=4. *P < 0.05, Student’ t-test.
4.4.11. Upregulation of USP25 promotes the commitment of the neural stem cells to glial cell fates and suppress neuronal cell fate

The proliferation and differentiation of adult neural stem cells can be also regulated by CDK proteins, through their role in cell cycle regulation. The induction of cyclin D2 is essential for the commitment of neural stem cells to neurons (Kowalczyk et al., 2004). Alternatively, cyclin D1 overexpression induces the proliferation of stem cells at the expense of differentiation and promotes the commitment of neural stem cells to neurons, thus reducing neurogenesis in the adult hippocampus (Artegiani et al., 2011). The balance between the abundance of cyclin D1 and cyclin D2 affects the fate of neuronal stem cell- neuronal or glial.

In order to examine whether the effects of USP25 on cell cycle also apply to hippocampal neurogenesis, we performed western blot analysis of the lysis of USP25-Tg mice’ hippocampus (Figure 4.11 A & B). Overexpression of USP25 significantly increased the protein level of cyclin D1 by 2.06 ± 0.102 folds (Figure 4.11 C) and reduced the abundance of cyclin D2 protein to 70.9 ± 7.07% (Figure 4.11 E), suggesting altered cell cycle regulation in USP25-Tg mice. USP25 overexpression also upregulated the expression of glial marker GFAP by 1.60 ± 0.150 folds (Figure 4.11 F)- neuronal marker and decreased the expression of NeuN to 69.0 ± 9.06% (Figure 4.11 D), suggesting the altered cell cycle regulation by USP25 overexpression is correlated with changes in neuronal cell fates in hippocampus.
Figure 4.11. Upregulation of USP25 promotes the commitment of the neural stem cells to glial cell fates and suppresses neuronal cell fate.

(A). Western blot analysis of hippocampal lysis for cyclin D1 and cyclin D2; (B). Quantification of A. USP25 overexpression significantly increased the protein level of cyclin D1 while decrease the protein level of cyclin D2 in hippocampi, n=4. **P < 0.01, Student’ t-test; (C). Western blot analysis of hippocampal lysis for GFAP and NeuN; (D). USP25 overexpression significantly increased the protein level of GFAP while decreased the protein level of NeuN in hippocampi. n=4. *P < 0.05, **P<0.01. Student’ t-test;
4.4.12. USP25 transgenic mice with defective neurogenesis showed increased anxiety/depression-like behaviors.

The results discussed above indicated dysregulated neurogenesis in USP25-Tg mice. Anhedonia is a symptom associated with affected adult neurogenesis. We subjected USP25-Tg mice to sucrose preference test. We habituated both USP25 Tg mice and their wild-type littermate control mice to freely-available water and 4% sucrose for 3 days. Following water and sucrose deprivation, the bottles were re-introduced and preference was measured during a 24-hour period. The decreased sucrose preference in USP25-Tg mice to 91.9 ± 3.28% (Figure 4.12A) suggests that anhedonia and decreased neurogenesis are associated with USP25-overexpression.

We next examined whether adult neurogenesis dysregulated by USP25 overexpression affects the behavioral response to stress in the novelty-suppressed feeding (NSF) test. Food-deprived mice were introduced to a novel open field containing a food pellet at its center. The latency to begin feeding was measured and USP25 Tg mice showed longer feeding latencies (1.41 ± 0.11 folds, n=9) (Figure 4.12B). There were no significant differences in body weight (Figure 4.1A). Mice from all groups consumed food after returning to their home cage, suggesting that decreased motivation to eat did not contribute to the behavioral change observed in the NSF test. These results suggest that USP25 upregulation altered the behavioral response to stress, which is associated with defective neurogenesis.
4.4.13. Learning deficiency in USP25 transgenic mice

To examine if USP25 overexpression exacerbates memory deficit, we subjected transgenic mice and their littermates to the Morris Water Maze (MWM) test at the age of three months. In the visible platform test on day 1, there was no difference in the escape latency (1.021±0.0562 folds, p>0.05) (Figure 4.13 A) or swimming speed (1.054±0.0435, p>0.05) (Figure 4.13 B) between USP25 transgenic mice and WT control mice, suggesting that USP25 overexpression did not affect motor ability or vision. In the hidden platform test from day 2 to day 5, USP25 transgenic mice exhibited longer escape latency (Day 5: 2.667±27.27 folds, p<0.05) (Figure 4.13 C) and longer swimming path length (Day 5:2.075±0.2608 folds) (Figure 4.13 D) (p<0.05, two-way ANOVA). In the probe trial test on day 6, USP25 transgenic mice spent less time in the target quadrant to 55.88 ± 3.16%, p<0.05 (Figure 4.13 E), compared to the control subjects. Those results demonstrate that USP25 overexpression affects spatial learning and memory in vivo.
Figure 4.13. Overexpression of USP25 significantly affects memory ability.

Morris water maze test consists of one day of visible platform trials, 4 days of hidden platform trials and a probe trial 24 h after the last hidden platform trial. USP25 transgenic mice were subjected to Morris water-maze test at the age of 8 weeks old. On the first day of visible platform test, USP25 transgenic mice display similar escape latency (A) and swimming speed (B) as the control mice. N=12, p>0.05 by Student’s t-test; During day 2 day 5 of hidden platform test, USP25 transgenic mice exhibited longer escape latency (C) and longer path lengths (D) compared to control mice. N=12, *p<0.05 by two-way ANOVA group comparison; (E) On day 6 in the probe trial, USP25 transgenic mice spent less time in the target quadrant than control. N=12, *p<0.05 by Student’s t-test. The values are expressed as mean ± SEM.
4.5 Discussion

Hypo-cellularity in hippocampus and neocortex has been observed in both DS individuals and animal models indicating enhanced neuronal death, affected neurogenesis or both. The active proliferation and differentiation of neuronal progenitors are balanced by apoptosis. One of the key regulators of cell proliferation and death is NF-κB, whose activation can be regulated by UPS. In the current study, we showed USP25 overexpression contributes to the hypo-cellularity in USP25-Tg mice through enhancing apoptosis and affecting neuronal cell cycle exit. It facilitated oxidative stress-induced cell death and caspase-3 activation by inhibiting NF-κB activation, affecting IκB turnover and p65 nuclear trafficking. USP25 upregulation affected cell cycle regulation during embryonic neurogenesis and cortical development during adulthood through altering cyclin balance. Its overexpression in the hippocampus promoted the commitment of neural stem cells to neuroglial cell fates and suppressed neuronal cell fate by altering the balance between the abundance of cyclin D1 and cyclin D2, thus reducing neurogenesis in the adult hippocampus. Defective neurogenesis in USP25-Tg mice was also implied in their anxiety/depression-like behaviors shown in both the novelty-suppressed feeding test and sucrose-preference test, and learning deficits in the Morris water maze. These results suggested that USP25 overexpression affected cell cycle and neuronal survival, contributing to defective neurogenesis in USP25-Tg mice. Our study suggests the up-regulation of USP25 may play a role in the hypocellularity in DS individual’s hippocampus and neocortex.

Several studies have also shown that DUBs play an essential role in cell cycle regulation and proliferation. They control cell cycle progression by regulating ubiquitin ligases with cell cycle function (Everett et al., 1997), cell cycle-specific transcription (Popov et al., 2007), growth factor signaling (Niendorf et al., 2007) and cell cycle checkpoints (Zhang et al., 2006). In the
present study, we have found another DUB that is involved in regulating cell cycle and proliferation. USP25-Tg mice exhibited learning deficits and increased anxiety/depression-like behaviors, indicating defective neurogenesis. In embryonic lateral ventricle, USP25 overexpression altered cell cycle kinetics and inhibited cell cycle exit. In adulthood, USP25 overexpression affected cortical development and hippocampal neurogenesis. These results suggest that USP25 overexpression affects cell cycle exit and neurogenesis both during development and in adulthood, which may contribute to their learning deficits and anxiety behaviors.

Ubiquitination plays an essential role in regulating cell survival and cell death. Many DUBs are involved in the process of switching from pro-survival signaling to cell death signaling when cells encounter stressors that triggers cell death. The timing of DUB activity and the specificity of ubiquitin associated with its substrates during apoptosis are essential to ensure apoptosis is conducted in a coordinated manner. USP7 plays a key role in stabilizing p53, which actively regulates cell cycle progression (Saridakis et al., 2005). USP9 regulates apoptosis through deubiquitinating apoptosis signal-regulating kinase1 (ASK1), which mediates oxidative stress-induced cell death through activating JNK and p38 MAPK pathways (Cummins & Vogelstein, 2004). USP16 deubiquitinates proteins such as histones H2A and H2B that are involved in the condensation of mitotic chromosomes during apoptosis (Adorno et al., 2013). In the present study, we have identified another DUB involved in regulating apoptosis and cell survival. It facilitated oxidative stress-induced cell death and caspase-3 activation by influencing NF-κB activation, which may help explain the enhanced apoptosis and hypo-cellularity in hippocampus and neocortex observed in DS brains.
One of the signaling pathways that connects cell cycle regulation and cell death is NF-κB activation. Many NF-κB-responsive genes are involved in apoptosis, cellular proliferation and differentiation. In the current study, we have shown that overexpression of USP25 promoted apoptosis and inhibited the activation of NF-κB *in vitro*. Knockout NF-κB abolished the effects of USP25 overexpression on cell proliferation and apoptosis. These results suggested that USP25 affects neuronal survival and proliferation by regulating NF-κB.

Although USP25 overexpression inhibited NF-κB activation, we have shown that it elevated NF-κB baseline protein levels both *in vivo* and *in vitro*, which may alter the abundance of cyclins that are responsible for cell cycle regulation. The progression of cell cycle, which consists of four discrete phases, G1, S, G2 and M, is monitored at checkpoints to ensure that conditions will allow the cell to enter the next phase. Cell-cycle phase transition is initiated by the phosphorylation of distinct substrates by cyclin-dependent kinase (CDKs). Cyclins are synthesized and degraded at particular times during the cell cycle progression. They bind CDKs and become fully active by CDK-activating kinases. UPS plays an essential role in regulating the cell cycle progression. Fully active cyclin-CDK complexes phosphorylate certain substrates and target them for UPS degradation. The timing of those substrates’ degradation is essential for advancing the cell cycle to enter the next phase (reviewed by Lim *et al.*, 2016)). It has been shown that NF-κB increases the abundance of cyclin D1 protein (Guttridge *et al.*, 1999) through regulating its transcription (Motokura & Arnold, 1993). Alternatively, cyclin D2 promoter possesses an-atypical NF-κB-binding site and Tax induces the activation of cyclin D2 promoter through NF-κB, suggesting that NF-κB can also affect cell-cycle progression by influencing Cyclin D2 gene expression (Iwanaga *et al.*, 2008). We have demonstrated that in USP25-Tg mice, the balance between the levels of cyclin D1 and cyclin D2 was altered in hippocampi, thus
promoting the commitment of neural stem cells to neuroglial cell fates and suppressing neuronal cell fate.

Prolonged G1 phase during corticogenesis is essential for neuron production and differentiation. (Savatier et al., 1996). It also allows extrinsic signals and inherited factors to act over a sufficient time period in order to influence cell fate (Gotz & Huttner, 2005; Calegari & Huttner, 2003). Shortening of the G1 phase can prevent stem cells from being affected by signals that induce differentiation, thus leading to defects in cortical development in adulthood (Burdon et al., 2002). During cell cycle progression, reduction of cyclin D1 is required for DNA synthesis in S phase (Baldin et al., 1993). Cyclin D1 is increased once again when it enters the G2 phase. In this way, cyclin D1 serves as an active switch in regulating cell cycle progression (Guo et al., 2002). Cyclin D1 overexpression in progenitors affects cell cycle progression by preventing G1 lengthening, resulting in a thicker subventricular zone and delayed neurogenesis (Lange et al., 2009). Our results suggest that USP25 overexpression up-regulated the expression of cyclin D1, both in cortex and hippocampus. We also observed altered cortical development and relative distribution of different cortical layers in USP25-Tg mice, consistent with cyclin D1 overexpression in neural progenitors.

Besides cyclin D1, we also observed changes in cyclin A2, and cyclin E1, but no change in cyclin B. These results suggest that USP25 changes the balance of cyclins that regulate different cell phases. However, the underlying mechanism of USP25’s effects on those cyclins remain unknown. One possibility is that USP25 directly regulates the expression of those proteins, thus altering the cell cycle progression. The other possibility is that USP25 directly regulates one of the cyclins that influences cell cycle progression, thus altering the levels of other
cyclins. Further experiments are needed to determine the molecular mechanism underlying the effect of USP25 on cyclins.

4.6. Conclusion

In summary, in this chapter we found that USP25 overexpression enhanced apoptosis and affect neurogenesis by inhibiting NF-κB activation. It also affects cell cycle regulation during embryonic neurogenesis and cortical development during adulthood by altering the levels of cyclins. Its overexpression in the hippocampus of USP25-Tg mice also promotes the commitment of neural stem cells to neuroglial cell fates and suppresses neuronal cell fate by altering the balance between the levels of cyclin D1 and cyclin D2, thus reducing neurogenesis in the adult hippocampus. The enhanced cell death and defective neurogenesis induced by USP25 overexpression had behavioral implications in USP25-Tg mice, including anxiety behavior and learning deficits. Our study is the first to show that USP25 plays a role in regulating cell cycle and apoptosis, and its overexpression may contribute to defective neurogenesis and enhanced apoptosis in the DS brain.
Chapter 5

Conclusion and discussion

5.1 Conclusion

5.1.1 Chapter 2: SP1 signaling regulates USP25 gene expression.

Previous studies have shown that increased USP25 levels in DS may contribute to the pathogenesis of DS. To determine whether there are extra factors up-regulating USP25 protein levels we studied the transcriptional regulation of USP25. We cloned the human USP25 gene promoter region and identified a functional SP1 binding site on it. We demonstrated that SP1 signaling promoted USP25 gene transcription. Moreover, inhibition of SP1 signaling by MTM resulted in decreased USP25 mRNA and protein levels. Taken together, we demonstrated that SP1 signaling promoted USP25 gene expression. This discovery implies that SP1 signaling up-regulation in the aging brains may serve as an additional factor that accounting for USP25 overexpression in DS beside Trisomy 21.

5.1.2 Chapter 3: Role of USP25 in APP processing

USP25 has been shown to regulate the turnover of several proteins, including TRAF3, RIG-I and MyBPC. In this chapter we identified another two proteins, APP and BACE1, whose expression levels are also regulated by USP25. We demonstrated that USP25 promoted the accumulation of APP and BACE1 by altering their degradation. It also influenced the trafficking of BACE1 by enhancing its internalization and accumulation in the TGN. Overexpression of USP25 further promoted CTF production, indicating its potential role in promoting amyloidogenic pathway in AD pathogenesis.
**Future experiments.** In this chapter we examined the effect of USP25 in APP processing both *in vivo* and *in vitro*, suggesting that USP25 is a potential target that contributes to AD pathogenesis in DS. We tried to explore the underlying molecular mechanisms of how USP25 altered BACE1 degradation and trafficking. We also want to further explore whether USP25 can serve as a potential therapeutic target that can be tested in our AD mouse model. Those aspects are what we would like to investigate but have not had the chance yet. Here we present future experiments that we have planned.

We have detected the physical binding of BACE1 and USP25 through two-hybrid yeast. We also showed that USP25 altered BACE1 degradation by altering its ubiquitination, since mutations on the ubiquitin sites of BACE1 abolished the effect of USP25. However, we do not know whether USP25 deubiquitinates BACE1 directly or exerts its effect through regulating another enzyme that interacts with BACE1. To understand the interaction between USP25 and BACE1, we will perform domain analysis to determine which domains of USP25 are required for regulating APP, CTF and Aβ production. If a certain domain is required for the regulatory role of USP25 on CTF and Aβ production, mutating the domain may either decrease or abolish the effect of USP25 on regulating APP, CTF and BACE1 trafficking. We can further identify the lysine residues that are deubiquitinated by USP25 using different mutated BACE1 plasmids.

To investigate the role of USP25 on APP processing and AD pathogenesis using an AD mouse models, we will manipulate USP25 expression in model mice in two ways. USP25 will be overexpressed by breeding APP23 mice with USP25 transgenic mice. The APP23 AD mouse model carries human APP751 cDNA with the Swedish mutation at position 670/671(KM/NL) under control of the murine Thy-1.2 promoter. At six months old, APP23 mice will start to develop a small amount of amyloid neuritic plaques. We will investigate whether USP25
overexpression increases the number of plaques and exacerbates memory deficits in APP23 mice. USP25 will be knocked down in APP23/PS45 mice by breeding APP23/PS45 mice with USP25 knockout mice. APP23/PS45 was derived from crossing APP23 and PS45 mice. PS45 is a mouse strain that carries the human familial AD-associated G384A mutant PS1. The introduction of PS45 mutation facilitates Aβ plaque deposition and learning and memory deficits in APP23 mice, which shortened the breeding time prior to the experiments. The double transgenic mice develop detectable neuritic plaques in the neocortex and hippocampus as early as one months of age. At three months old, the neuritic plaque formation in APP23/PS45 mice is intense. By breeding APP23/PS45 mice with USP25 knockout mice, APP23/USP25-hemi-KO expresses approximately 50% of USP25 protein compared to APP23/PS45 or wild-type C57BL/6 mice. We will investigate whether the USP25 knockdown will decrease the number of Aβ plaques and improve memory deficits in APP23/PS45 mice.

For both mice, the Morris water maze will be carried out at six months of age to examine learning deficits, after which mice will be sacrificed for histochemical and biochemical analysis. Morris water maze test consists of one day of visible platform trial, 4 days of hidden platform trials and a probe trial 24 h after the last hidden platform trial. Escape latency during the hidden platform test and the amount of time subject spend in the quadrant where the hidden platform is placed during the probe test will be measured to indicate the memory abilities. We will also examine the effect of USP25 on APP processing \textit{in vivo}. One batch of dissected hippocampi will be lysed for western blot analysis and Aβ ELISA assay. The other hemisphere will be processed for immunohistochemistry staining of plaques with 1% thioflavin-S. We will quantify the number of Aβ plaques in the hippocampal region. If USP25 affects AD pathogenesis \textit{in vivo}, overexpression of USP25 will exacerbate memory deficits in APP23 transgenic mice and partial
loss of USP25 will rescue memory deficits in APP23/PS45 transgenic mice. Overexpression of USP25 in APP23 transgenic mice will increase the number of Aβ plagues, APP CTF and Aβ production, while USP25 knock-down in APP23/PS45 mice will reduce the number of Aβ plagues, APP CTF and Aβ production.

5.1.3 Chapter 4: The role of USP25 in regulating cell cycle and apoptosis

Hypo-cellularity in hippocampus and neocortex observed in both DS individuals and animal models suggests altered neuronal survival and defective neurogenesis. In this chapter, we showed that USP25 overexpression contributes to the hypo-cellularity in the cortex of USP25-Tg mice through promoting apoptosis and affecting neuronal cell cycle exit. It facilitated oxidative stress-induced cell death and caspase-3 activation through inhibiting NF-κB activation, affecting IκB turnover and p65 nuclear trafficking. On the other hand, USP25 upregulation affected neurogenesis during embryonic development and corticogenesis during adulthood by altering cyclin balance. At the same time, its overexpression in hippocampus also promoted the commitment of neural stem cells to a neuroglial cell fate and suppressed neuronal cell fate by altering the balance between the abundance of cyclin D1 and cyclin D2, thus reducing neurogenesis in the adult hippocampus. The defective neurogenesis in USP25-Tg mice was also implied in their anxiety/depression-like behaviors shown in both the novelty-suppressed feeding test and sucrose-preference test, and learning deficits shown in Morris Water Maze. These results suggest that USP25 overexpression affects cell cycle and neuronal survival, both of which contribute to defective neurogenesis in USP25-Tg mice. Our study implied that USP25 upregulation may contribute to the hypocellularity in DS individual’s hippocampus and neocortex.
**Future experiments** We will use mice with duplication spanning the entire human chromosome 21 syntenic region on mouse chromosome 16 as the model of Down syndrome (Dp(16)1Yey/+), which is a more complete trisomic mouse model compared with the segmental trisomy 16 (Ts65Dn) mouse, containing a reciprocal translocation that can be used to produce segmental trisomy for distal chromosome 16 corresponding to human chromosome 21q21-22.3. It includes the majority of the genes located on DS critical region, however it does not cover the region where USP25 is located.

We will first confirm that USP25 is overexpressed in Dp(16)1Yey/+ compared with wild-type mice using both RT-PCR and Western Blot. USP25 will be knocked down by intracranially injecting AAV1 containing human USP25 siRNA to the hippocampal regions of Dp(16)1Yey/+ mice. Human USP25 siRNA will be cloned into the pAAV-GFP-cDNA6 vector. Driven by a separate CMV promoter, USP25 and eGFP will be expressed in individual transduced neurons. USP25 knockdown will be verified by examining lysed hippocampal tissue. To examine the effect of USP25 knockdown on the memory abilities of mice, eight weeks post-injection of AAV-USP25 siRNA-GFP or control AAV in Dp(16)1Yey/+ mice will be subjected to the Morris water maze test. We will also verify the effect of USP25 knock-down on APP processing and NF-κB signaling pathway in vivo by subjecting hippocampal tissues from USP25 knock-down and control subjects for western blot analysis to detect relevant proteins along those pathways.

If USP25 contributes to AD pathogenesis in DS in vivo, knockdown of USP25 in Dp(16)1Yey/+ mice will rescue memory deficits in Dp(16)1Yey/+ mice. USP25 knockdown by siRNA will affect the protein levels of APP, BACE1, CTF, TRAF6, p65, IκB and active caspase in Dp(16)1Yey/+ mice. We have shown that USP25 overexpression affected cell cycle exists and cyclin equilibrium. However, the mechanism underlying the effect of USP25 regulating cell
cycle exit remains unknown. In order to understand the effect of USP25 on those cyclins, we will adapt similar approaches to those employed in Chapter 2 and Chapter 3 to study cyclin proteins. We will first check the mRNA level of those cyclin proteins after USP25 overexpression, to determine whether USP25 regulates the gene expression of those cyclin genes and what is the underlying mechanism of those effects. If USP25 overexpression does not alter their mRNA levels, then we determine whether it affects their degradation. We will clone those genes and overexpress those plasmids in HEK cells. We will then examine whether overexpression of USP25 will affect their protein levels. If USP25 does affect their protein abundance we will investigate whether USP25 physically interacts with those cyclins and affects their degradation using cycloheximide pulse chase. If so, we can further determine the underlying mechanism of the regulatory effect of USP25 on those cyclins through the lysosomal pathway or the proteasomal pathway, using the same method of examining the degradation of APP and BACE1.

5.2 Significance of the study

USP25 is a relatively poorly studied gene. Previous studies have suggested its role in immunity, myogenesis and quality control in protein synthesis. In particular, its interaction with APP indicates its potential role in regulating APP processing. Correlational analysis has shown that it is expressed at a high level in postmitotic neurons, indicating its potential role in neurogenesis. However, whether a causal relationship exists between USP25 and AD development in DS remains unknown. Our research is the first to investigate the effect of USP25 overexpression in AD pathogenesis in vitro and in vivo.

First, we found that USP25 transcriptional expression is regulated by SP1 signaling. This finding indicated that during ageing, the up-regulation of SP1 signaling may account for the
upregulation of USP25. Combined with results from Chapter 3 and Chapter 4, it implied a potential pathway by which upregulated SP1 signaling during ageing increases USP25 transcriptional expression, contributing to the development of AD pathogenesis, both in normal health population and DS individuals.

This is the first report to examine the role of USP25 in APP processing. Our results have shown that USP25 regulates APP processing by affecting the degradation of APP and BACE1. We also discovered that USP25 altered the intracellular trafficking of BACE1, which further promotes the amyloidogenic pathway of APP processing, resulting in increased CTF production.

Finally, we demonstrated that overexpression of USP25 contributed to neurodegeneration by promoting neuronal deaths and influencing cell cycle progression. It facilitated oxidative stress-induced cell death and caspase-3 activation through inhibition of NF-κB activation. In USP25-Tg mice, defective neurogenesis in hippocampus and altered corticogenesis were also implied in novelty-suppressed feeding test, sucrose-preference test, and Morris Water Maze. The comprehensive approach used in this project demonstrated the role of USP25 in the development of AD pathogenesis, from aging, APP processing to neuronal loss. Our results provide important insights into the studying the underlying mechanism of AD pathogenesis in DS, and information that may assist in developing the pharmaceutical potential of targeting USP25 for treating AD in DS.
5.3 Further implication on AD in DS

In the last two decades, the amyloid hypothesis has evolved from Aβ plaques hypothesis to the Aβ oligomer hypothesis. Aβ oligomers have been shown to induce neuronal toxicity and memory impairment \textit{in vivo} (Lesne et al., 2006; Shankar et al., 2008). Its levels also correlated with the severity of AD cognitive impairment (McLean et al., 1999). The hypothesis suggested the formation of amyloid oligomer initiates Alzheimer’s pathogenesis, followed by other features including tau pathology and neuronal loss.

Despite mounting evidences supporting amyloid oligomer hypothesis in sporadic and FADs, current DS research suggests the amyloid cascade may not apply to AD in DS. At the same time, there are limitations in the current researches on studying AD in DS, which are mainly due to the lack of DS mice model that also develops amyloid plaques. We will discuss these issues in this section. Finally, we will include results from our experiments in this section.

5.3.1. Limitation in the current research of AD in DS

One of the problems with studying AD in DS is that none of the current DS animal models develop amyloid plaques. Many DS animal models, including Ts65Dn, Dp(16)1Yey/+ , Ts1Cje, all exhibit learning deficits and defective neurogenesis, but none of them develop amyloid plaques due to a lack of the human APP transgene. Therefore, whether the neurological features demonstrated in DS models are related to amyloid generation remains unknown. A DS model with a human APP knock-in would be a useful model for us to study amyloid pathogenesis in DS model. Unfortunately, such models do not yet exist.
5.3.2. Implication from current researches on AD in DS

Individuals with DS inevitably develop AD, characterized by neuritic plaques, neurofibrillary tangles and neuronal death after middle age. However, whether amyloid hypothesis validated in sporadic AD and FAD is also valid in DS remains unknown. Instead of one HSA21 gene initiating the amyloid cascade in DS, it is more likely that multiple genes contribute to the AD pathogenesis through different pathways. Some genes may affect neuron survival and promote apoptosis. Some genes may be responsible for tau pathology. Those pathological features can be initiated separately from amyloid pathogenesis.

**APP** One of the dosage-sensitive genes contributing to amyloid pathogenesis is APP, as the substrate whose hydrolysis generates amyloid-β (Aβ). The additional copy of the APP gene is present in the majority of DS cases and the transcription of APP gene was increased (Podlisny et al., 1987). The APP protein level was not changed corresponding to the fold change of Aβ in DS, indicating that additional factors are involved in amyloid pathogenesis in DS. In the current study, we demonstrated that USP25 regulates APP and BACE1, contributing to the development of AD. These results suggest that the extra copy of APP and USP25 may contribute to AD pathogenesis by promoting amyloidogenic pathway in DS.

**DYRK1A.** In DS, the up-regulation of DYRK1A contributes to an increase in Tau protein levels and its hyperphosphorylation in the DS brain. DYRK1A is located in the DS critical region of chromosome 21. Its overexpression may contribute to early onset neurofibrillary degeneration in DS brains by phosphorylating tau protein (Wegiel et al., 2011) and increasing the ratio of 3R:4R tau (Iqbal et al., 2013).
**RCAN1.** RCAN1, a gene located on HSA21, contributes to AD pathogenesis by affecting tau phosphorylation and neuronal survival. RCAN1 is significantly elevated in AD and DS brains. It promotes tau phosphorylation by lowering calcineurin phosphatase activity and increasing the protein level of GSK3β, which can phosphorylate tau protein and thus enhance NFT formation (Cardenas et al., 2012). Long-term accumulation of RCAN1 in SH-SY5Y cells facilitates oxidative stress-induced apoptosis via promoting caspase-3 activation (Wu & Song, 2013). RCAN1-Tg mice exhibit a reduced number of neurons within the hippocampus and memory deficits in hippocampal-dependent learning (Martin et al., 2012), indicating that RCAN1 overexpression in DS contributes to the pathogenesis of AD by affecting neuronal survival.

**USP16.** In addition to USP25, USP16, another DUB located on HSA21, regulates neurogenesis through de-ubiquitinating Cdkn2a and H2AK129. Its upregulation reduced the expansions of both fibroblasts and post-natal neural progenitors while its down-regulation rescued those effects (Adorno et al., 2013). USP16 also binds to the promoter regions of many lineage-specific ESCs genes and regulates H2A deubiquitination, a process that is essential for ESCs gene expression and differentiation (Yang et al., 2014). ThEse results suggested that USP16 plays an essential role in regulating neurogenesis and proliferation and its overexpression in DS may contribute to the neurodegeneration associated with the condition in DS.
5.3.3. Implications from this project

In the current study, we have shown that USP25 contributes to the pathogenesis of AD in DS by influencing APP processing, facilitating neuronal death and reducing neurogenesis. Based on our results, it is likely that its overexpression causes different neurological features through separated pathways. It affects APP processing through regulating the degradation and trafficking of APP and BACE1. Its overexpression contributes to the hypo-cellularity in DS by promoting apoptosis and affecting neuronal cell cycle exit, by inhibiting NF-κB activation and altering the expression of cyclins during cell proliferation. The graph below summarizes the effects of the HSA21 genes mentioned above on the development of AD in DS, which may indicate a new perspective of understanding AD pathogenesis in DS.

![Diagram of HSA21 genes in AD pathogenesis]

Figure 5.1. HSA21 genes in AD pathogenesis.
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