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

by

Beibei Song

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

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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

Examining Committee:

Weihong Song

Supervisor

Yutian Wang

Supervisory Committee Member

Xin-Ming, Li

External Examiner

Honglin Luo

University Examiner

Christian Kastrup

University Examiner

Additional Supervisory Committee Members:

Ann Marie Craig

Supervisory Committee Member

Jason Snyder

Supervisory Committee Member

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.

Table of Contents

Abstract ..... **iii**

Lay Summary ..... **iv**

Preface ..... **v**

Table of Contents ..... **vi**

List of Figures ..... **xii**

List of Abbreviations ..... **xiv**

Acknowledgements ..... **xviii**

Dedication ..... **xx**

Chapter 1: General introduction **1**

1.1 Down's syndrome (DS): Trisomy 21 (Hsa21) 1

1.1.1 DS overview 1

1.1.2 Socioeconomic burden of DS 2

1.1.3 Phenotypic variability of DS 2

1.1.4 Variations in gene expression in Trisomy 21 3

1.2 Alzheimer's disease, amyloid hypothesis and APP processing 3

1.2.1 Overview 4

1.2.2 Amyloid hypothesis 5

1.2.3 APP processing pathway 8

1.2.4 Functions & regulation of the components of amyloidogenic pathway 11

1.2.5 APP processing and trafficking in the secretory pathway 16

1.3 Alzheimer's disease, neurogenesis and neurodegeneration 18
1.3.1 Neurogenesis overview 18
1.3.2 Role of cell cycle in regulating neurogenesis 19
1.3.3 Defects in neurogenesis in AD 20
1.3.4 Cell death and apoptosis: overview 21
1.3.5 Role of aging and oxidative stress in regulating cell death and neurogenesis 23
1.3.6 Neurodegeneration in AD 23
1.3.7 Role of NF-κB in regulating neurogenesis and neurodegeneration 24
1.4 Alzheimer's disease and Down Syndrome (DS) 32
1.4.1 Overview 32
1.4.2 Neuropathology perspectives 32
1.4.3 Current hypothesis 34
1.5 Ubiquitin-proteasome system (UPS) and neurodegenerative disease 37
1.5.1 Ubiquitin and protein degradation 37
1.5.2 Deubiquitination enzymes 40
1.5.3 Deubiquitinating enzymes' functions in nervous system 42
1.5.4 Ubiquitination and Alzheimer's disease 43
1.5.5 Ubiquitination and Down syndrome 44
1.5.6 USP25 as a novel target for Down syndrome 45
1.6 Rationale of the study 51
Chapter 2: SP1 regulates USP25 gene expression 54
2.1 Intoduction 54
2.2 Method 55

2.2.1 Cloning and plasmids 55
2.2.2 SMART RACE cDNA amplification 55
2.2.3 Cell culture, transfection, and luciferase assays 56
2.2.4 Electrophoretic mobility shift assay (EMSA) 57
2.2.5 Semi-quantitative RT-PCR 58
2.2.6 Immunoblotting 58
2.2.1 Statistical analysis 59
2.3 Results 60
2.3.1 Cloning the human USP25 gene promoter and mapping the transcription start site of the USP25 gene 60
2.3.2 Functional analysis of the human USP25 gene promoter 62
2.3.3 The USP25 gene promoter contains SP1 binding sites 64
2.3.4 Sp1 regulates USP25 promoter activity and transcription 66
2.3.5 Sp1 regulates USP25 gene expression 66
2.4 Discussion 69
2.5 Conclusion 71
Chapter 3: Role of USP25 in APP processing 72
3.1 Introduction 72
3.2 Method 73
3.2.1 cDNA constructs, cell cultures and transfection 73
3.2.2 Pharmacological treatment 73
3.2.3 Immunoblotting 74

3.2.4 Animals and genotyping 74
3.2.5 Subcellular fractionation 75
3.2.6 Biotin plasma membrane labeling 75
3.2.7 Immunohistochemistry (IHC) 76
3.2.8 Statistics 76
3.3 Results 77
3.3.1 USP25 affects the degradation of APP both *in vivo* and *in vitro* 77
3.3.2 USP25 affects the degradation of BACE1 both in vivo and in vitro 79
3.3.3 USP25 alters the trafficking of BACE1 81
3.3.4 USP25 promotes CTF and Aβ production through affecting BACE1 84
3.4 Discussion 84
3.5 Conclusion 86
Chapter 4: The Role of USP25 in regulating cell cycle and apoptosis 73
4.1 Introduction 87
4.2 Method 89
4.2.1 Mice 89
4.2.2 Western blotting 89
4.2.3 Cell viability assay 90
4.2.4 cDNA constructs, cell cultures and transfection 90
4.2.5 Luciferase assay 91
4.2.6 Immunohistochemistry 91
4.2.7 Statistics 92
4.2.8 Animal behavioral test 92

4.3 Result 94
4.3.1 Physiological characterization of USP25 transgenic (Tg) mice 94
4.3.2 Overexpression of USP25 facilitates oxidative stress-induced cell death 96
4.3.3 USP25 overexpression inhibits NF-κB activation 98
4.3.4 USP25 affects LPS-induced IκB turnover and p65 nuclear trafficking 100
4.3.5 NF-κB mediates the regulation of cell death by USP25 102
4.3.6 USP25 overexpression affects NF-κB signal pathways *in vivo* 104
4.3.7 USP25 overexpression affects cell cycle regulation 106
4.3.8 USP25 overexpression affects cell cycles during embryonic neurogenesis 108
4.3.9 USP25 overexpression affects adult cortical development 111
4.3.10 NF-κB mediates the regulation of proliferation by USP25 114
4.3.11 Upregulation of USP25 promotes the commitment of the neural stem cells to neuronsglial cell fates and suppress neuronal cell fate 115
4.3.12 USP25 transgenic mice with defective neurogenesis showed increased anxiety/depression-like behaviors 117
4.3.13 Learning deficiency in USP25 transgenic mice 118
4.4 Discussion 119
4.5 Conclusion 120
Chapter 5: Conclusion and discussion 125
5.1 Conclusion 125
5.1.1 Chapter 2: SP1 signaling regulates USP25 gene expression 125
5.1.2 Chapter 3: Role of USP25 in APP processing 125
5.1.3 Chapter 4: The role of USP25 in regulating cell cycle and apoptosis 128

5.2 Significance of the research 130

5.3 Further implication on AD in DS 132

5.3.1 Limitation in the current research of AD in DS 132

5.1.1 Implication from current researches on AD in DS 136

5.1.1 Implications from this project 135

References 136

List of Figures

Figure 1.1 The amyloid hypothesis of Alzheimer's disease 7

Figure 1.2 APP processing pathways 9

Figure 1.3 Intracellular trafficking of APP and BACE1 15

Figure 1.4 APP and BACE1 protein trafficking 17

Figure 1.5 NF-κB activation 26

Figure 1.6 The ubiquitination process and the roles of deubiquitination enzymes (DUB) 39

Figure 1.7 Deubiquitinating enzyme (DUBs) and their functioning 41

Figure 1.8 Human chromosome 21 and USP25 47

Figure 2.1 Identify the transcription start site (TSS) of the USP25 gene 61

Figure 2.2 USP25 promoter deletion constructs consist of a 5' flanking region 63

Figure 2.3 Functional SP1 sites within USP25 promoter 65

Figure 2.4 SP1 upregulates USP25 promoter activity and endogenous mRNA expression 67

Figure 2.5 SP1 upregulates USP25 endogenous protein level 68

Figure 3.1 Overexpressing USP25 increased APP by affecting its degradation 78

Figure 3.2 Overexpressing USP25 increased BACE1 and affecting its degradation 80

Figure 3.3 Overexpressing USP25 affects the trafficking BACE1 protein 82

Figure 3.4 USP25 promotes CTF and Aβ production through affecting BACE1 83

Figure 4.1 Physiological characterization of USP25 Transgenic mice 95

Figure 4.2. USP25 facilitates oxidative stress-induced cell death and Caspase-3 activation 97

Figure 4.3: USP25 overexpression down-regulates NF-κB signaling pathway 99

Figure 4.4. USP25 affects LPS-induced IκB turnover and p65 nuclear trafficking 101

Figure 4.5: USP25 regulation of cell proliferation and death is dependent on NF-κB 103

Figure 4.6. USP25 overexpression affects NF-κB signal pathways *in vivo* 105

Figure 4.7. USP25 overexpression affects cell cycle regulation..... 107

Figure 4.8. USP25 overexpression affects cell cycle during embryonic neurogenesis 110

Figure 4.9. USP25 overexpression affects adult cortical development..... 113

Figure 4.10. USP25 overexpression affects cell proliferation through NF-κB..... 114

Figure 4.11. Upregulation of USP25 promotes the commitment of the neural stem cells to neuronsglial cell fates and suppress neuronal cell fate 116

Figure 4.12. Behavioral implication associated USP25 overexpression: neurogenesis 118

Figure 4.13. Overexpression of USP25 significantly affects memory ability..... 119

Figure 5.1. HSA21 genes in AD pathogenesis 135

List of Abbreviations

5'UTR	5'-untranslated region
5'RACE	Rapid amplification of 5' complementary DNA ends
AA	Amino acid
AAV	Adeno-associated virus
ACTA1	Actin alpha-1
ANOVA	Analysis of variance
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid β-precursor-like protein
Aβ	β-amyloid
Aph-1	Anterior pharynx factor-1
ApoE	Apolipoprotein E
AVs	Autophagic vacuoles
BACE1	β-site APP cleaving enzyme 1
BACE2	β-site APP cleaving enzyme 2
bp	base pair
BrdU	5-BRomo-2-DeoxyUridine
BSA	Bovine serum albumin
CA3	Cornu Ammonis 3
CAT	Catalase
Cdks	Cyclin-dependent kinase
CHX	Cycloheximide
CREB	cAMP response element-binding protein
DG	Dentate gyrus
CNS	Central nervous system
CTF	C-terminal fragment
DCX	Doublecortin
DUB	De-uqibuitinating enzyme
DS	Down syndrome
DSCAM	Down syndrome cell adhesion molecule

DSCR	Down Syndrome Critical Region
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
DTT	Dithiothreitol
EdU	5-ethynyl-2'-deoxyuridine
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ES	Embryonic stem cells
FAD	Familial Alzheimer's disease
FLNC	Filamin
fMRI	functional Magnetic Resonance Imaging
FBS	Fetal Bovine Serum
GABA	Gamma-amino butyric acid
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDP	Gross Domestic Product
GFAP	glial fibrillary acidic protein
GPX	Glutathione peroxidase
GSA	gel-shifting assay
HRD1	ERAD-associated E3 ubiquitin-protein ligase HRD1
HSA21	Human chromosome 21
IκB -α	NF-κB inhibitor, α
IκK	IκB kinase
IL	interleukin
IRF	Interferon regulatory factor
JAMM	JAB/MPN/MOV34 metalloenzymes
KCNJ6	Potassium Voltage-Gated Channel Subfamily J Member 6
KO	knockout
LDH	lactate dehydrogenase
LPS	Lipopolysaccharide
LTP	Long-term poentiation
MEF	mouse embryonic fibroblast
mtDNA	Mitochondrial DNA

MTM	Mithramycin A
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium)
MWM	Morris Water Maze
MyBPC1	Myosin binding protein C1
NeuN	Neuronal nuclear antigen
NFT	neurofibrillary tangles
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NSCs	Neuronal stem cells
NSF	Novelty-suppressed feeding
OTUs	Ovarian tumor proteases
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
PEI	Polyethylenimine
PKA	Protein kinase A
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PS1	Presenilin 1
PS2	Presenilin 2
PNS	Post-nuclear supernatant
PVDF-FL	polyvindylidine fluoride
RCAN1	Regulator of Calcineurin1
RHD	Rel-homology domains
RIG-I	Retinoic acid-inducible gene I
RIPA	radio-immunoprecipitation assay deoxycholate
RIP	Receptor interacting protein
ROS	Reactive oxidative species
RT-PCR	reverse transcription polymerase chain reaction

SAD	sporadic Alzheimer's disease
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SODs	Superoxide dismutase
SVZ	Subventricular zone
TF	transcription factor
TLR	Toll-like receptors
TGN	trans-Golgi network
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TSS	transcription start site
TSA21	Trisomy 21
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Ub	Ubiquitin
UCH	ubiquitin carboxyl-terminal hydrolase
UCHL1	ubiquitin carboxyl-terminal hydrolase L1
UPS	ubiquitin proteasome system
USP	ubiquitin-specific protease
VGSC	Voltage-gated sodium channels (VGSC)
WT	wildtype

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 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.

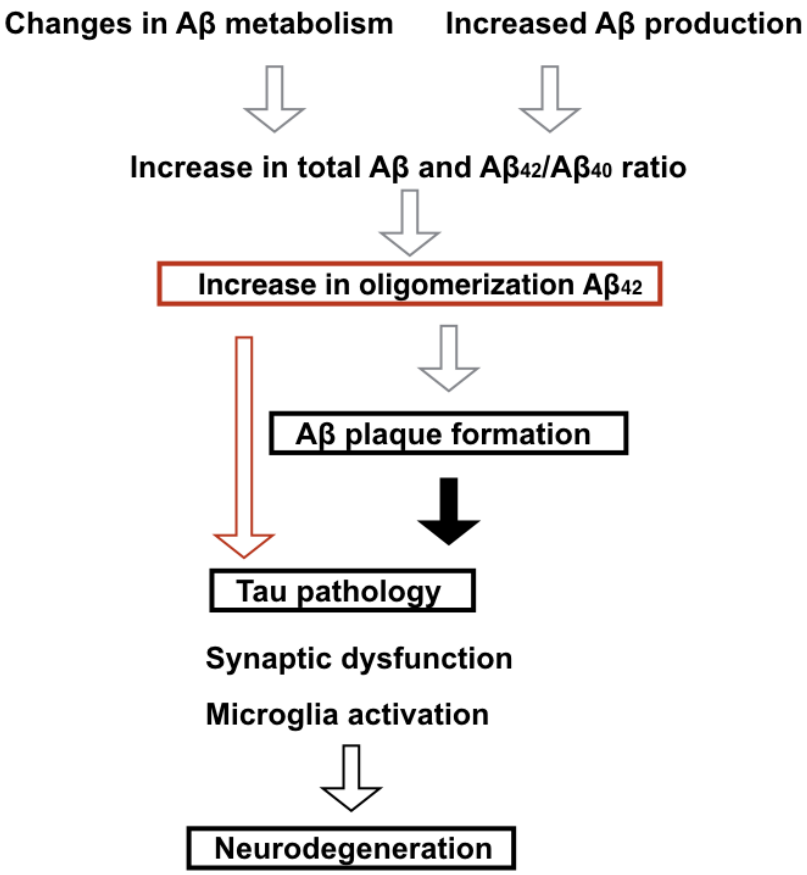

Figure 1.1. The amyloid hypothesis of Alzheimer's disease

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.

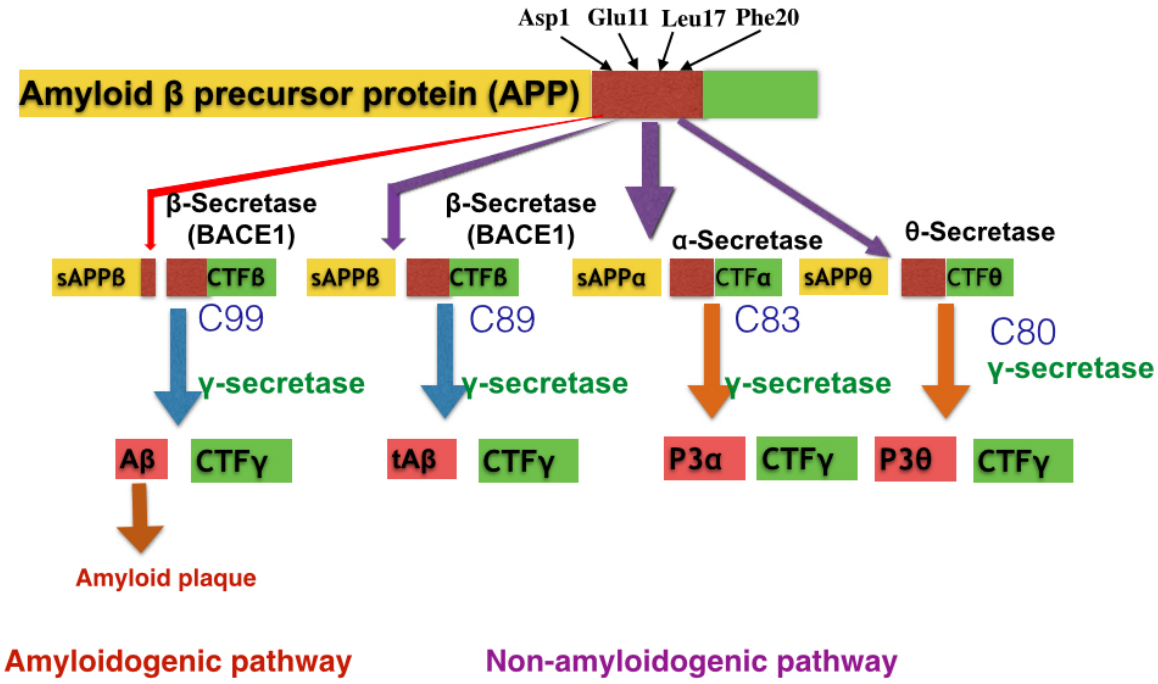

Figure 1.2: APP processing pathways

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 α-secretase between Lys-16 and Leu-17 within the Aβ domain, that yields sAPPα and CTFα C83 (Esch et al., 1990). C83 is further processed by γ-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 α-secretases. It has been shown that an ADAM inhibitor decreased sAPPα and CTFα 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 α-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 θ-secretase and overexpressing BACE2 by lentiviral infection in the primary neurons of APP transgenic mice significantly decreased Aβ production and AD pathogenesis (Sun *et al.*, 2006). Therefore, BACE2, a θ-secretase *in vivo,* serves as an alternative non-amyloidogenic cleavage enzyme other than β-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).

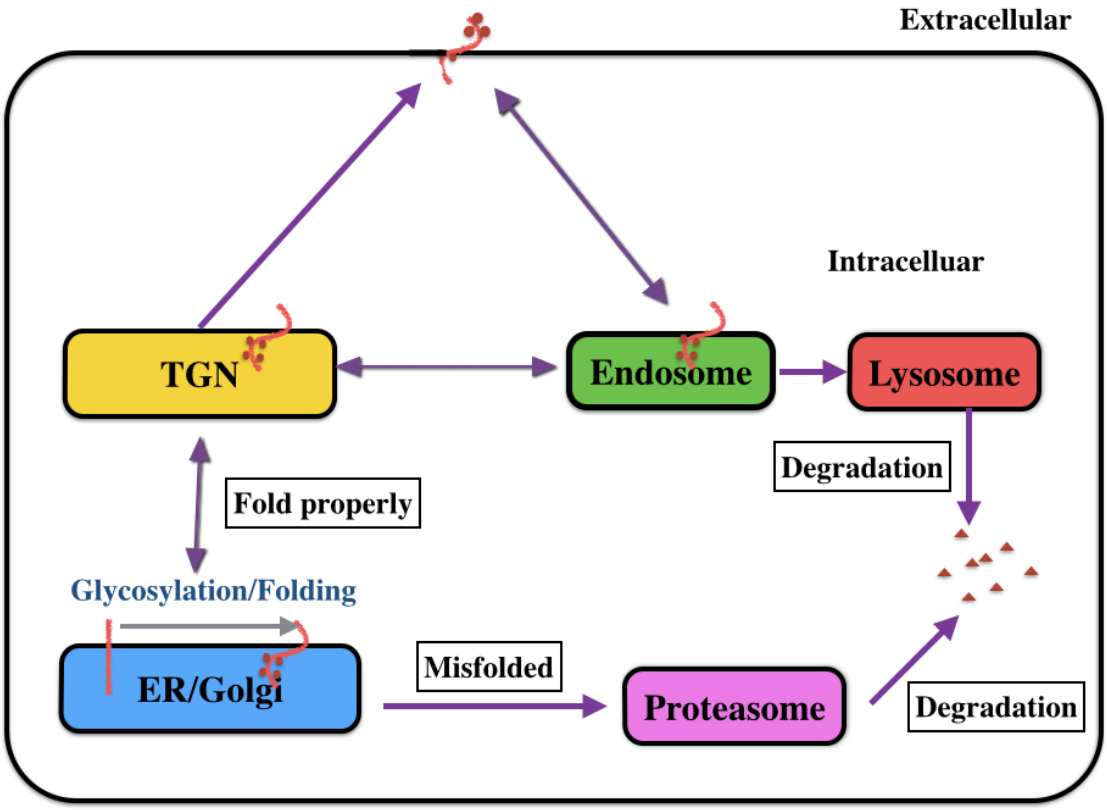

Figure 1.3. Intracellular trafficking of APP and BACE1

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).

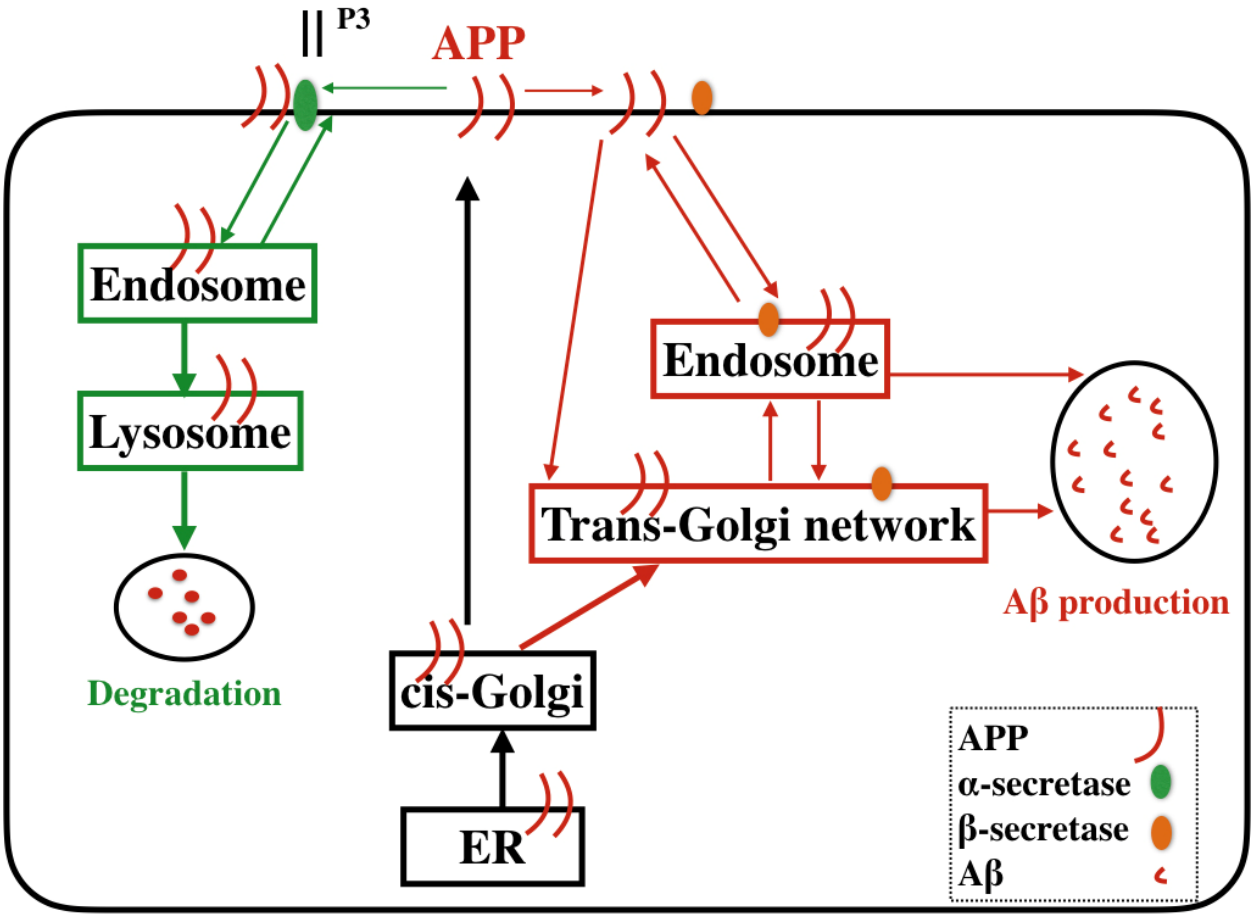

Figure 1.4. APP and BACE1 protein trafficking.

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 H_2O_2 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.

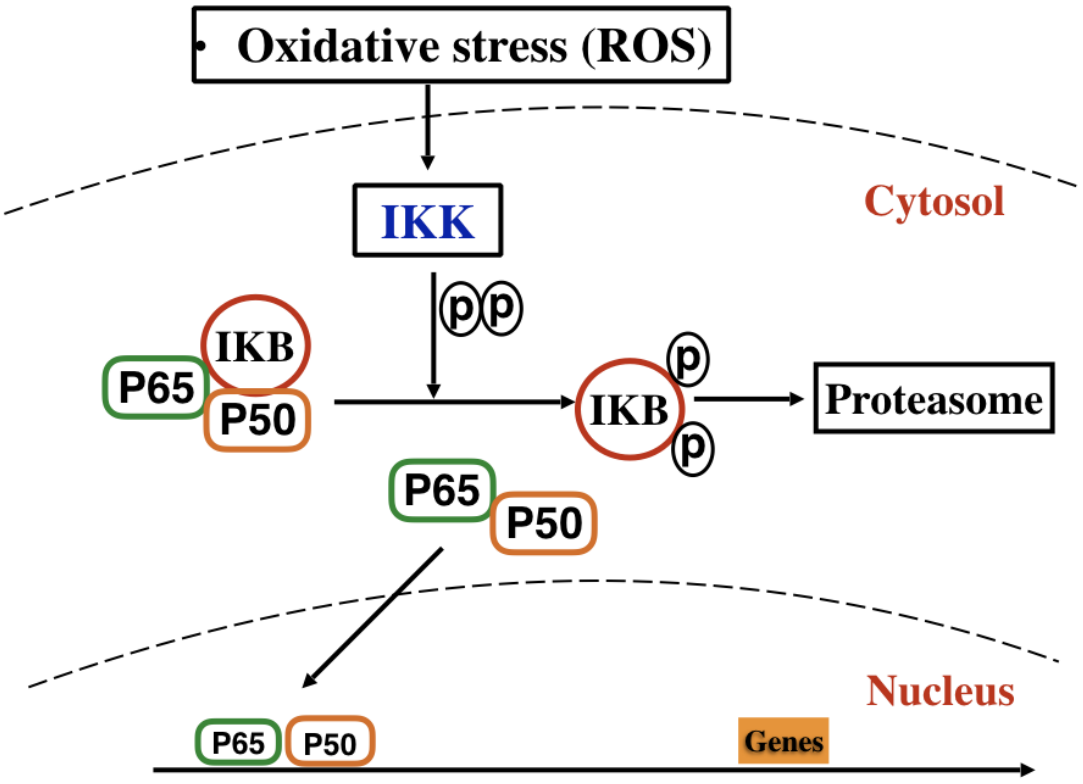

Figure 1.5. NF-κB activation.

NF-κB signaling pathway can be activated via various stimuli, including oxidative stress and agonists such as tumor necrosis factorα (TNFα) or interleukin-1β (IL-1β). In response to ROS, the signal pathway activates the IκK complex by phosphorylation. The phosphorylation signaling targets IκB 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 H_2O_2, 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 H_2O_2 (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).

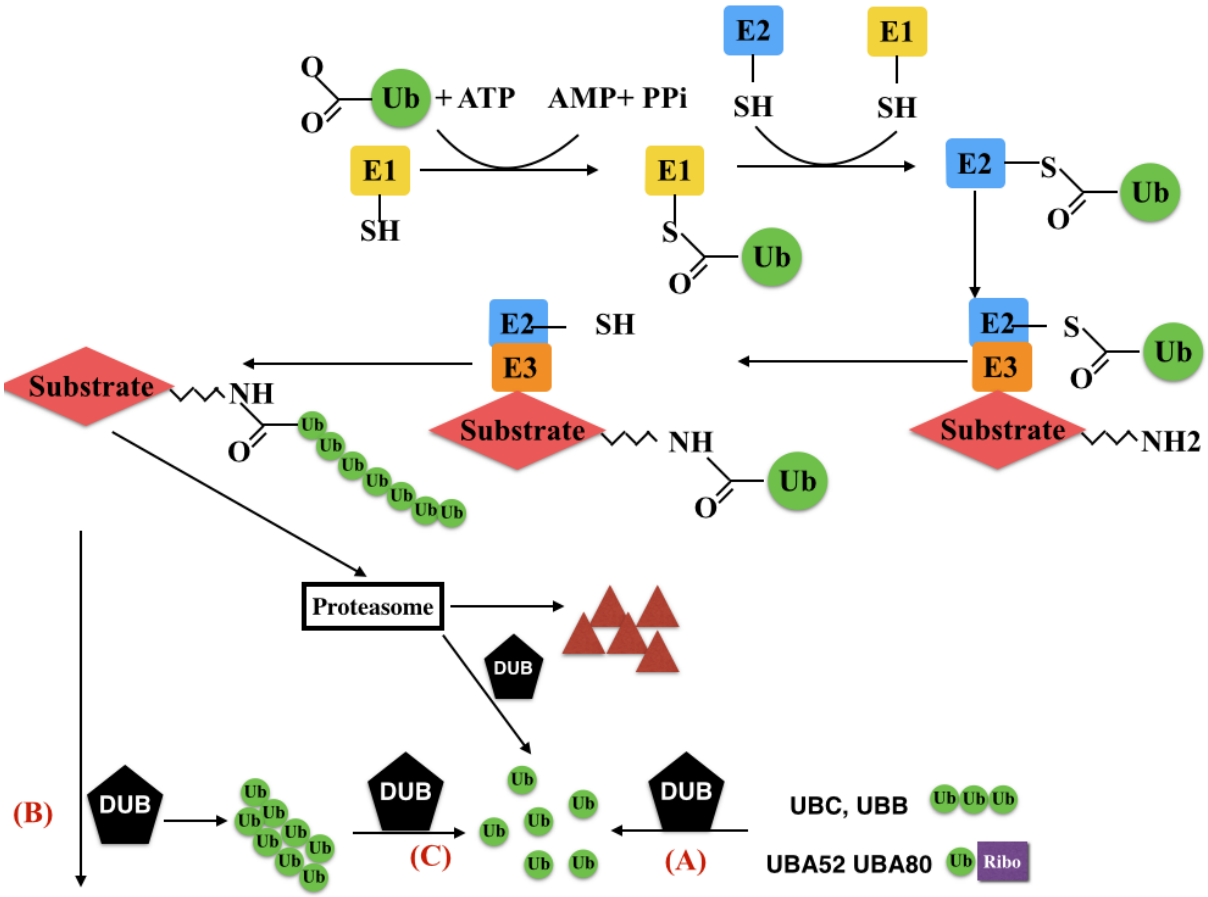

Figure 1.6: The ubiquitination process and the roles of deubiquitination enzymes (DUBs).

(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.

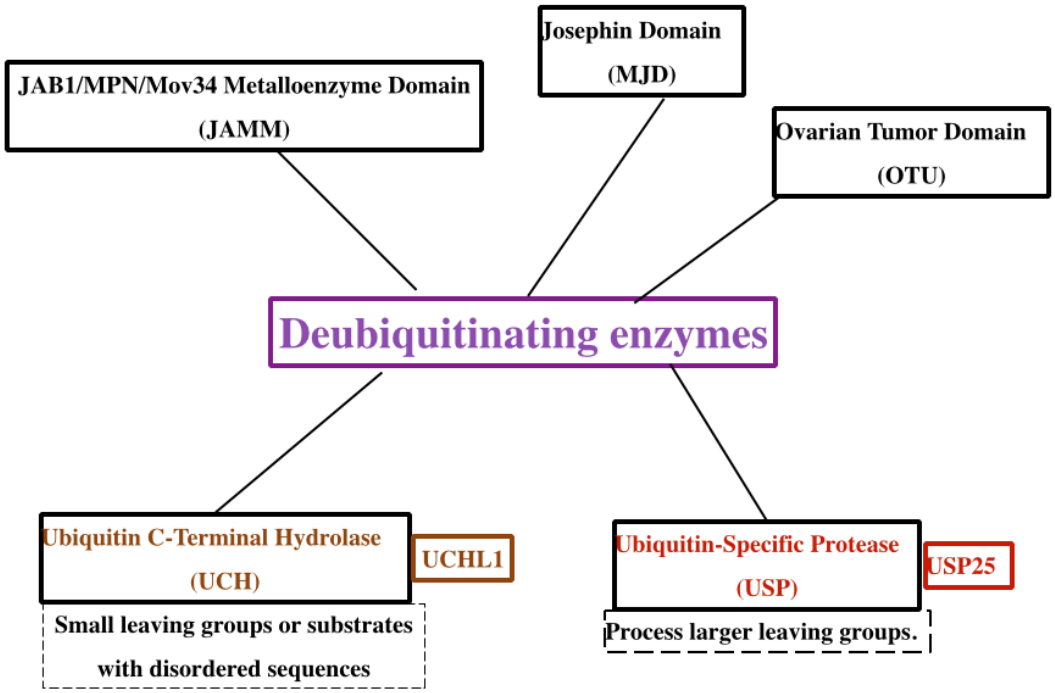

Figure 1.7. deubiquitinating enzymes (DUBs) and their functioning

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)

domain (Shi *et al.*, 2014)**.** 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.

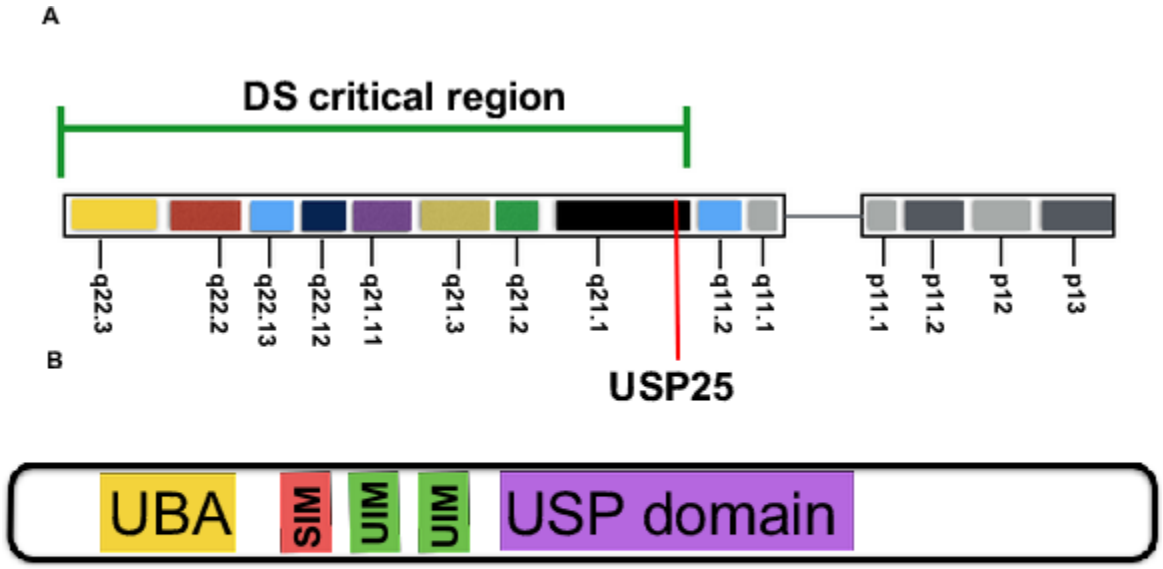

Figure 1.8: Human chromosome 21 and USP25.

(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.

Chapter 2

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'-ccgctcgagaggaggacaacgccattcc; 2) HindIII1976 5'-cccaagcttgtgaccctccgggcggc; 3) Xho11967 5'-ccgctcgagcaatgtagggttagggcgg; 4) Bam1742 5'-tttgggtaagctagggatccgac; 5) Nh1211 5'-ctagctagcgttcccagattagctcctg; 6) Nh57 5'-ctagctagcgtaatgtagcagtttagaagtt; Reverse 1) Hind2313 5'-cacaagcttaaacgccgactgtgagg; 2) Bam1781 5'-ctgcgcccagggcttgtcggatc; 3) BI1292 5'-gaagatctggaattgtaaggaaaatct; 4) BI2313 5'-gaagatctaaacgccgactgtgagg; 5). HindIII2169 5'-cccaagcttgttcgcacgttcctttg; 6) HindIII2095 5'-cccaagctttccctccgcgagtcctc;

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’-aagcagtggtatcaacgcagagtacxxxxx (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℃ 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 × 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'-gaggagccgccgggcgggggcggggatg and reverse, 5'-catccccgcccccgcccggcggctccac; 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- gccaacattctttagcccaacg were used to amplify a 433 bp fragment of human USP25 gene. The GAPDH gene was also amplified using the forward primer (tgcaccaccaactgcttagc) and reverse primer ggcatggactgtggtcatgag), 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.

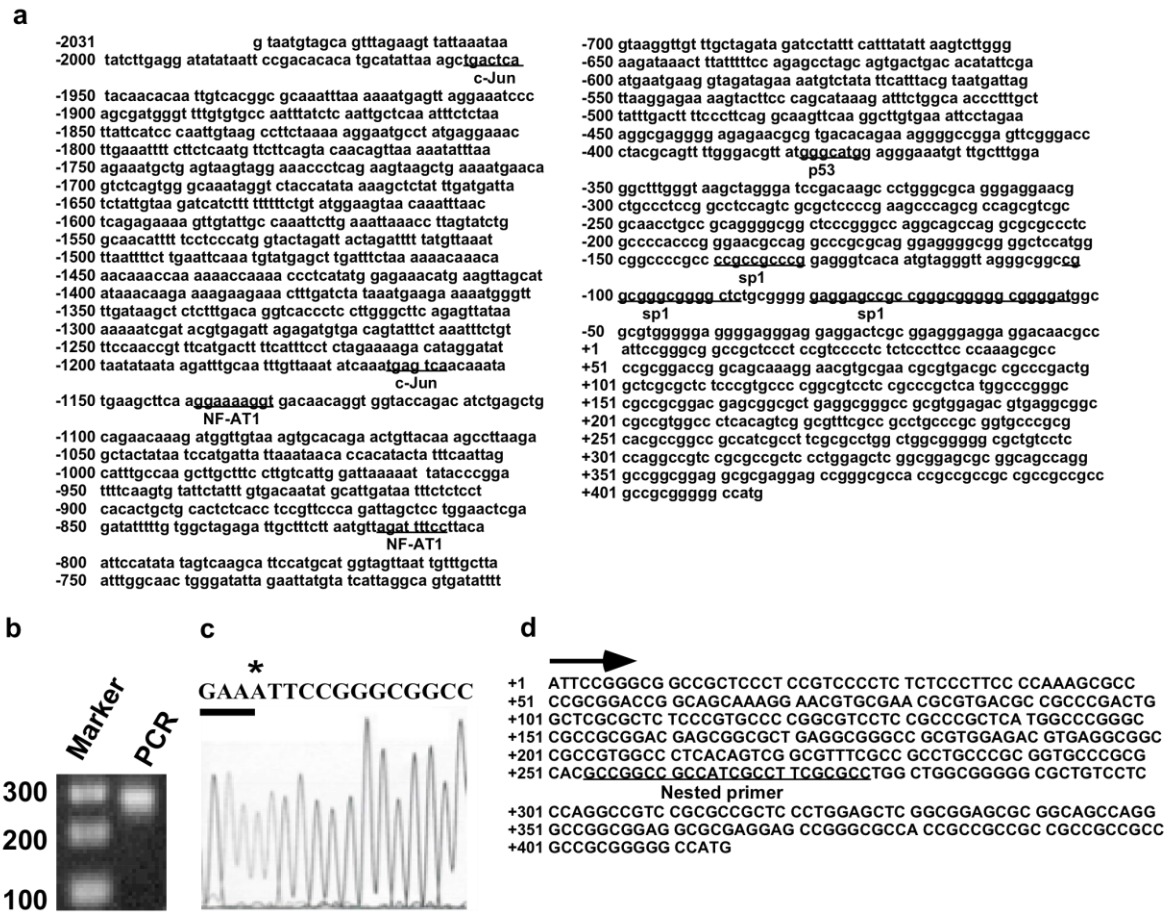

Figure 2.1. Identification of transcription start site (TSS) of the USP25 gene.

(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.

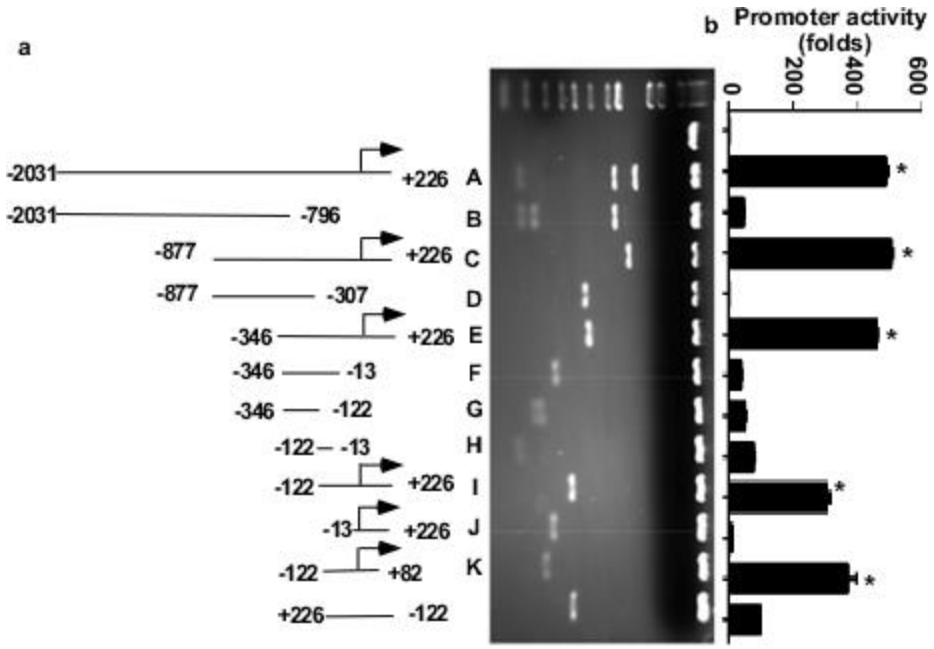

Figure 2.2 Functional analysis of USP25 promoter

(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’gaggagccgccgggcgggggcggggatg). 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.

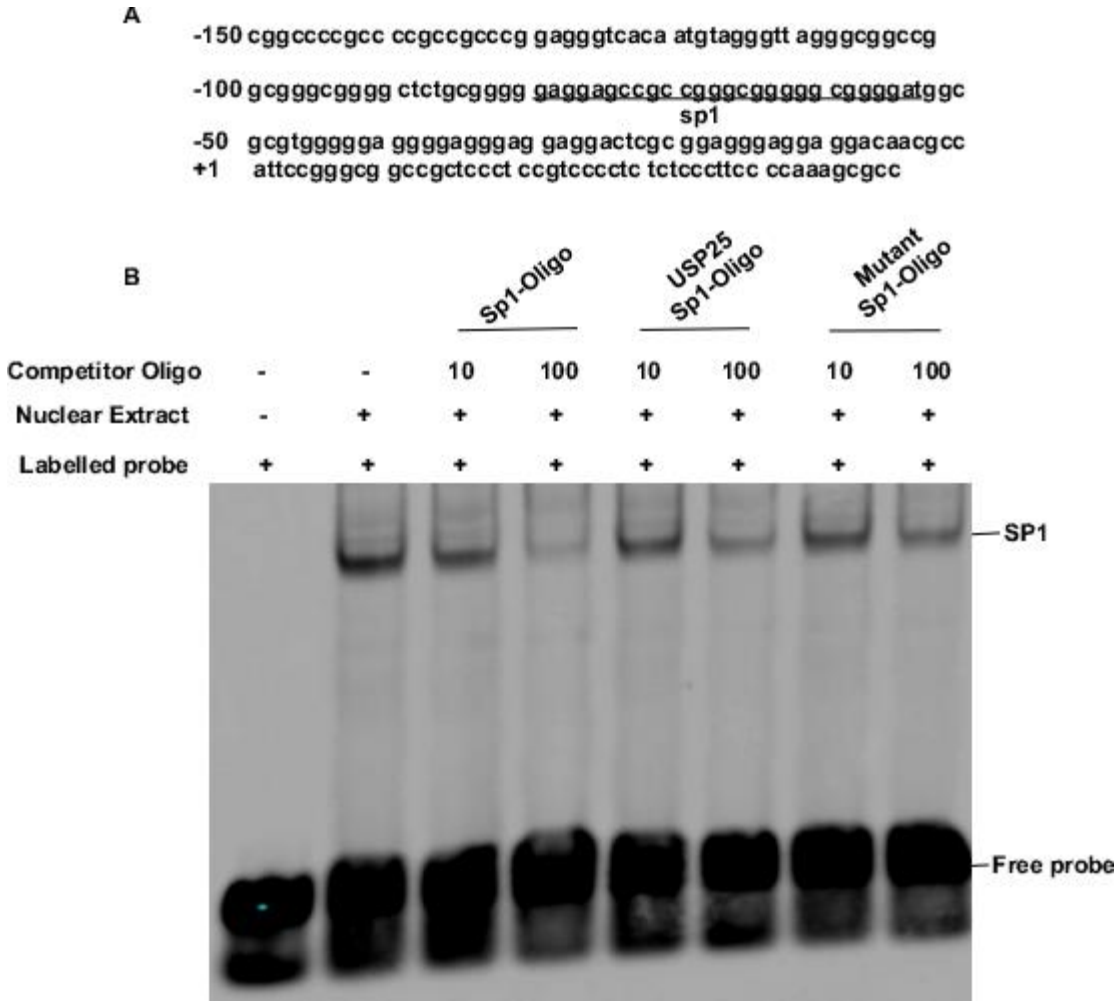

Figure 2.3 Functional SP1 sites within USP25 promoter.

(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**).

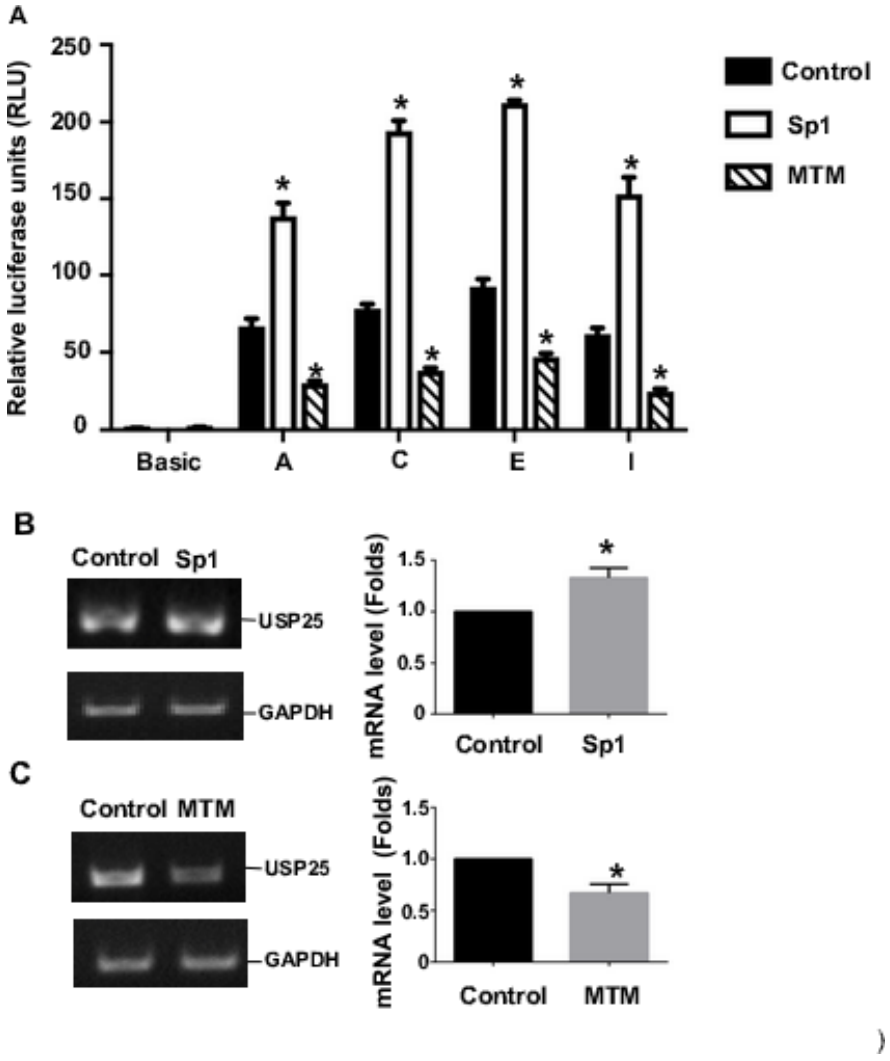

Figure 2.4 SP1 upregulates USP25 promoter activity and endogenous mRNA expression;

(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.

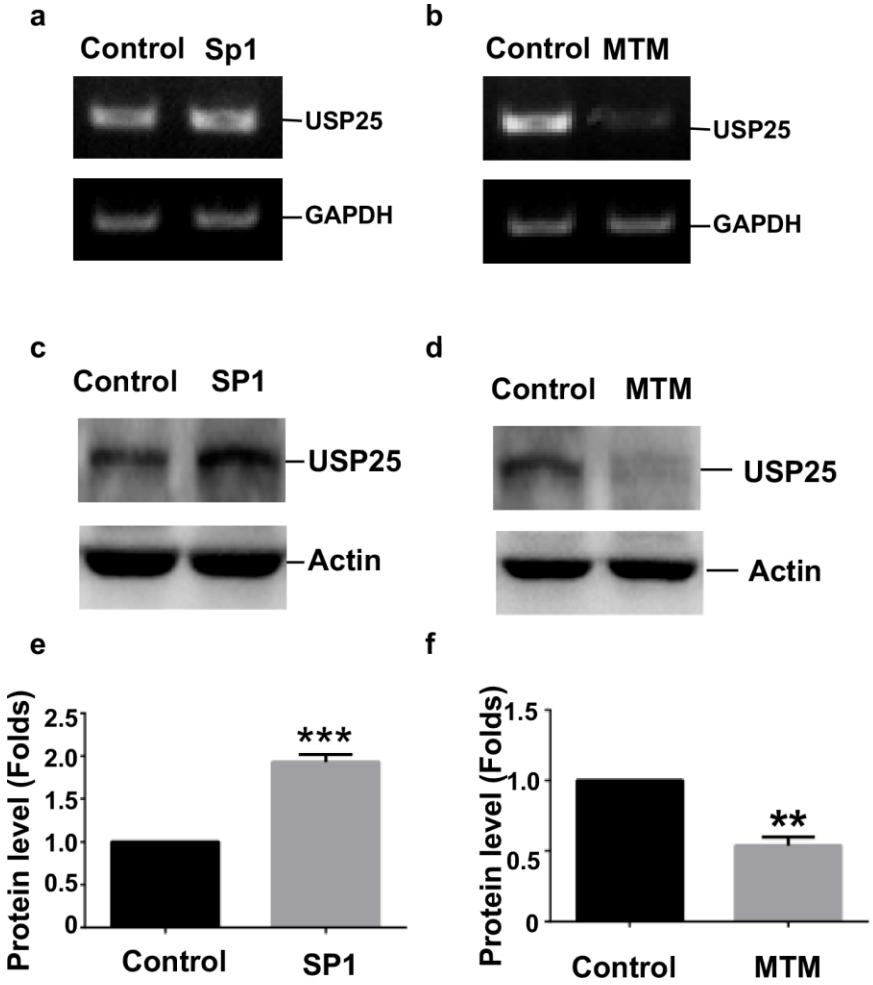

Figure 2.5 SP1 upregulates USP25 endogenous protein level;

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)GGGCGG(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 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**).

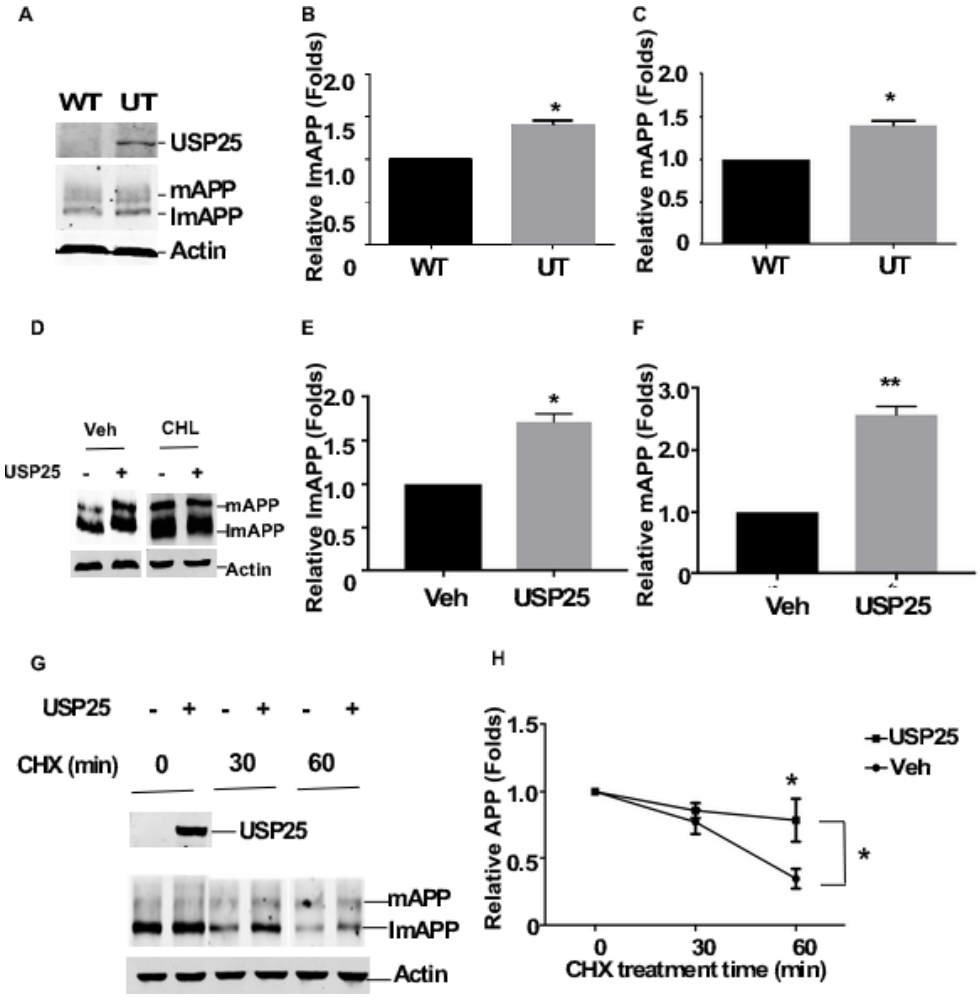

Figure 3.1: Overexpressing USP25 significantly increased the protein level of APP by affecting its degradation.

(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**).

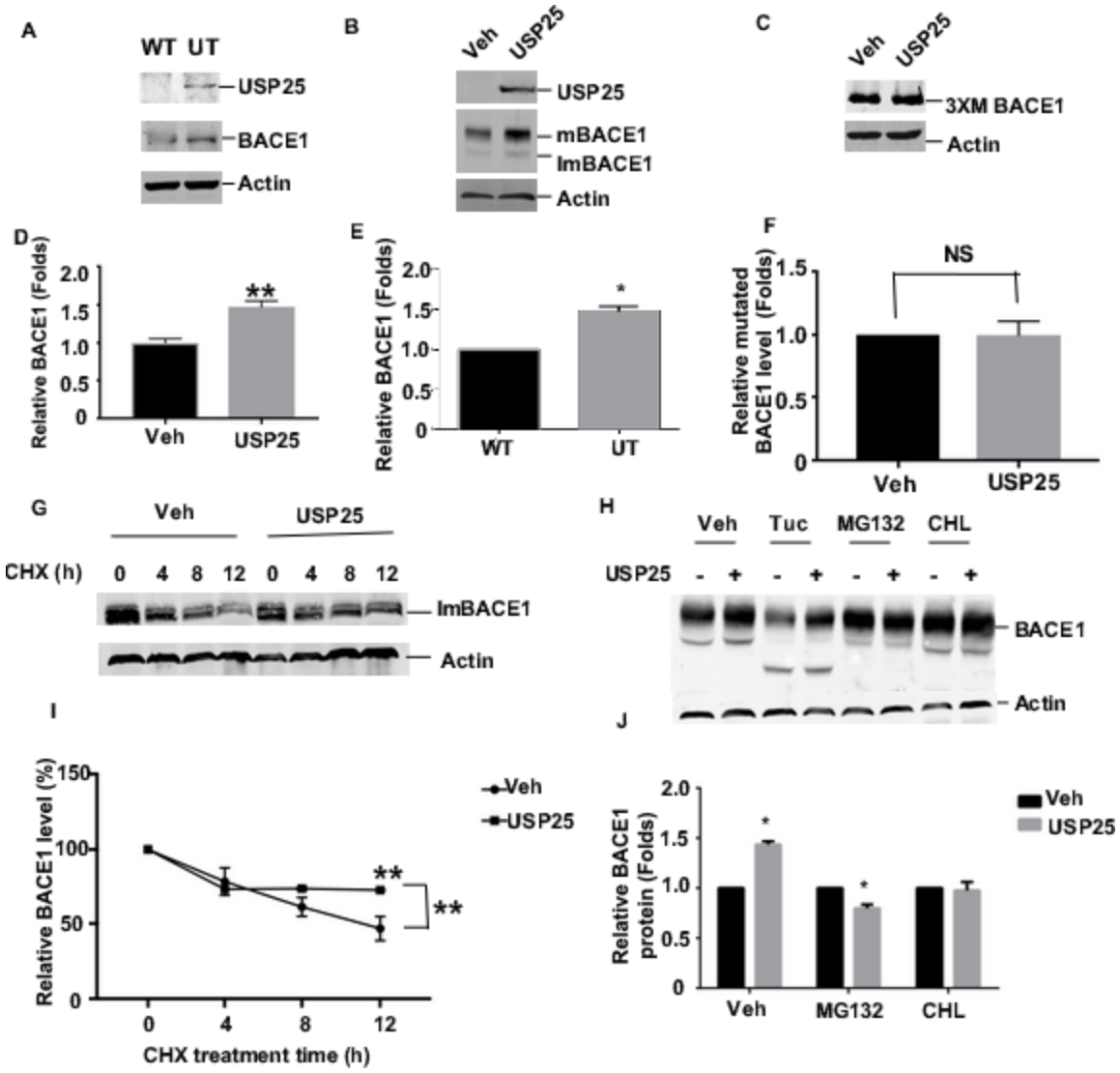

Figure 3.2: Overexpressing USP25 affects BACE1 degradation.
(A). Brain lysis of USP25-transgenic mice and little mate controls were subjected to western blot analysis. BACE1 was detected by BACE1 antibody; **(B).** 2EB2 cells (HEK stably overexpressing BACE1-myc and APPswe) were transfected with pz-USP25L-st 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.

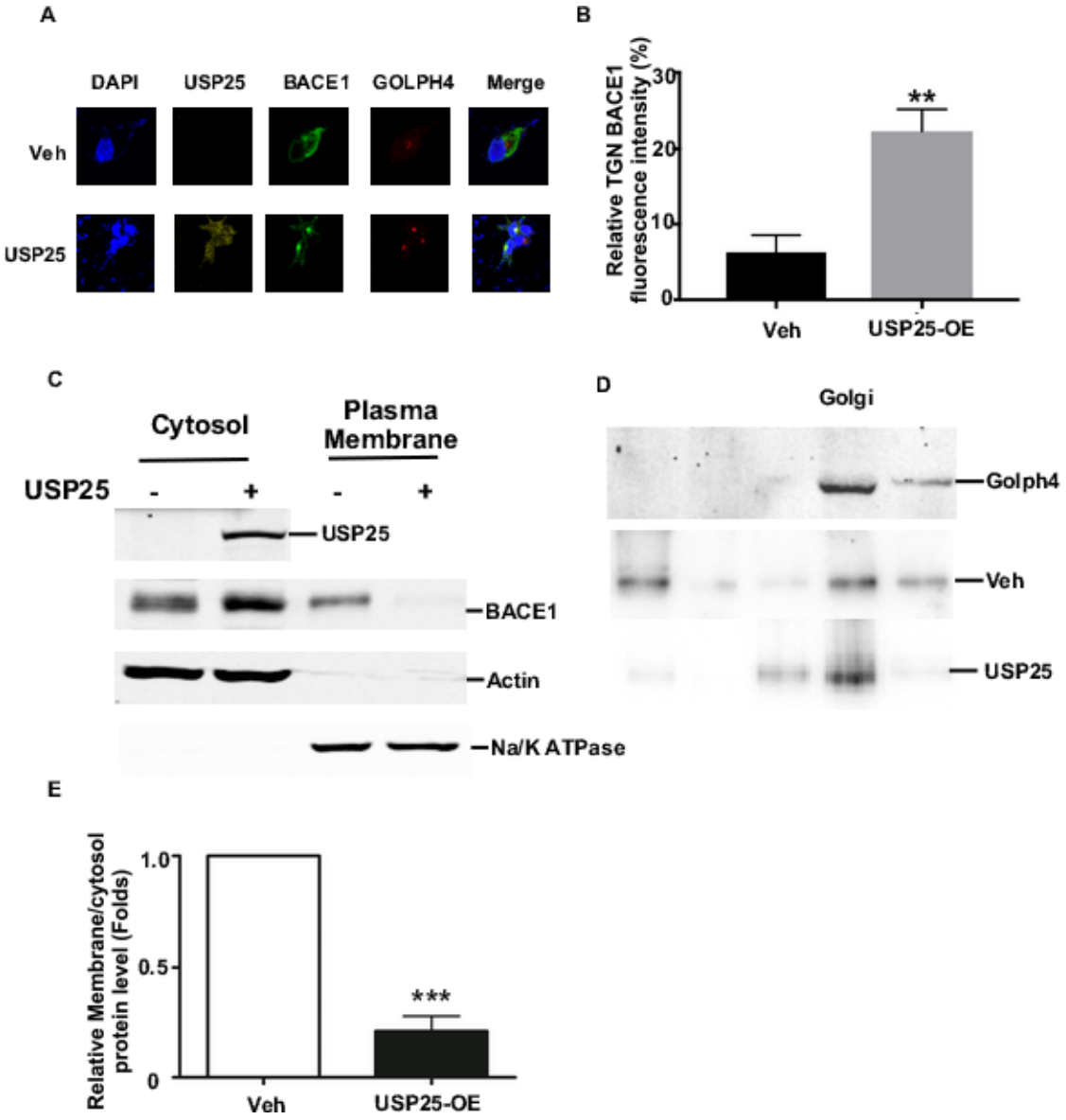

Figure 3.3 Overexpressing USP25 affects the trafficking BACE1 protein

(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)**.

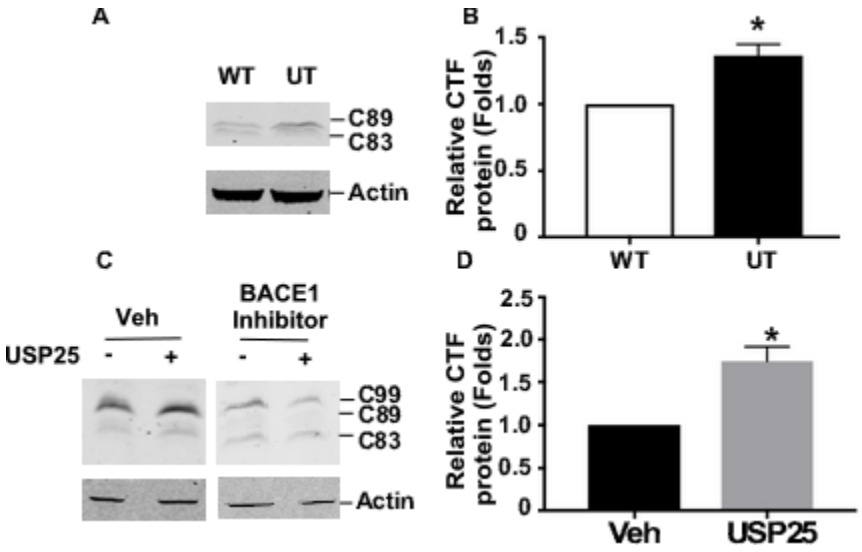

Figure 3.4. USP25 promotes CTF and Aβ production through affecting BACE1

(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 activitiy 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 (Δweight sucrose)/(Δweight sucrose + Δweight water) × 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.

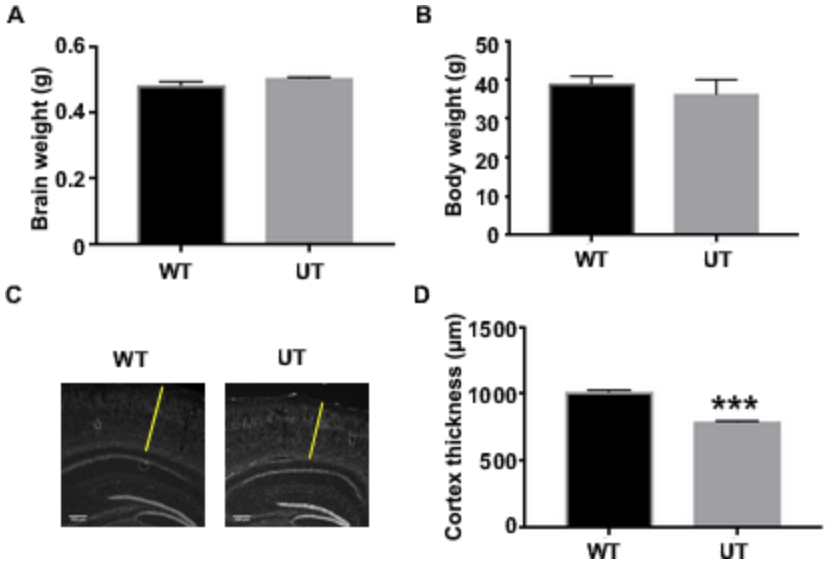

Figure 4.1 Physiological characterization of USP25 Transgenic mice

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 H_2O_2 in different dosages for 24 hours, and the MTS assay was performed to determine the cell viability. MTS assay showed that H_2O_2 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%, $p<0.05$) H_2O_2 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 H_2O_2 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, $p<0.05$) H_2O_2.

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 H_2O_2 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, $p<0.05$)

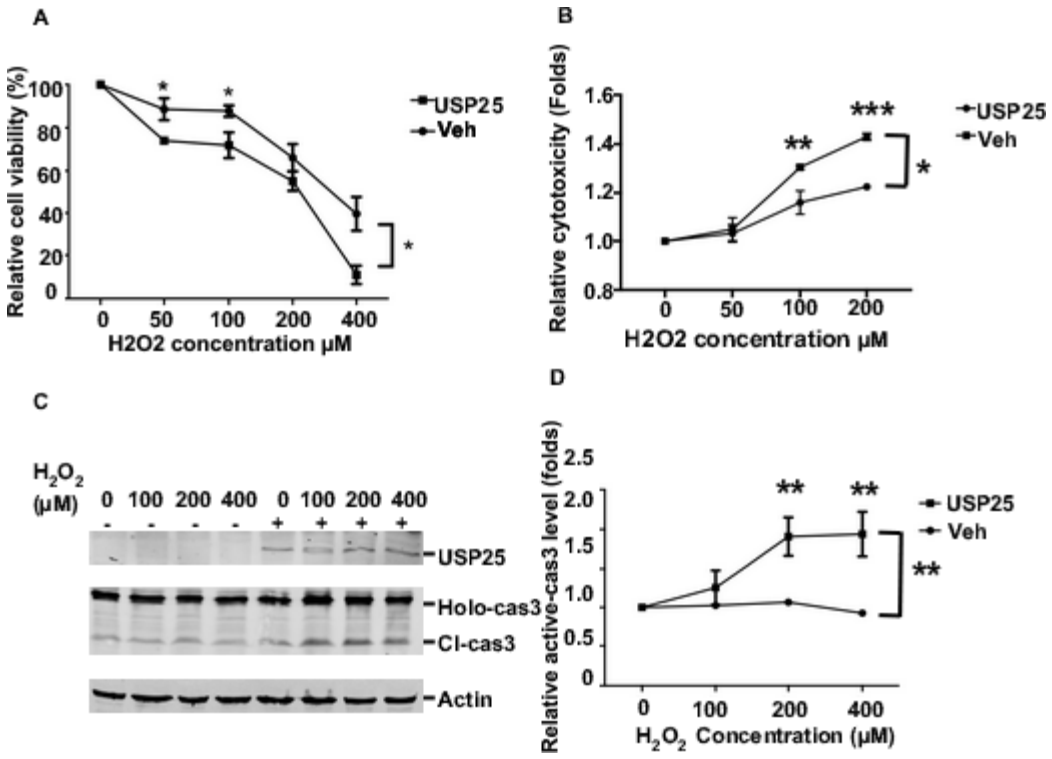

Figure 4.2. USP25 facilitates oxidative stress-induced cell death and Caspase-3 activation

N2A cells were transiently transfected with empty vector or USP25L. Both cells were treated with different dosage of H_2O_2 48 hours after transfection. After 24 hours H_2O_2 treatment, **(A)** MTT assay was performed. H_2O_2 treatment significantly reduced the cell survival of USP25 overexpressing cells compared with control cells, in a dosage dependent manner; **(B)** LDH assay was performed, H_2O_2 treatment significantly increased the cytotoxicity of USP25 overexpressing cells compared with control cells, in a dosage-dependent manner. **(C)** After 6 hours H_2O_2 treatment at different dosages, Western blot assays were performed. **(D)** H_2O_2 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 H_2O_2 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 H_2O_2. Compared with control, USP25 overexpression altered the pattern of pNF-κB-Luc promoter activity changes due to H_2O_2 ($p<0.05$, 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**).

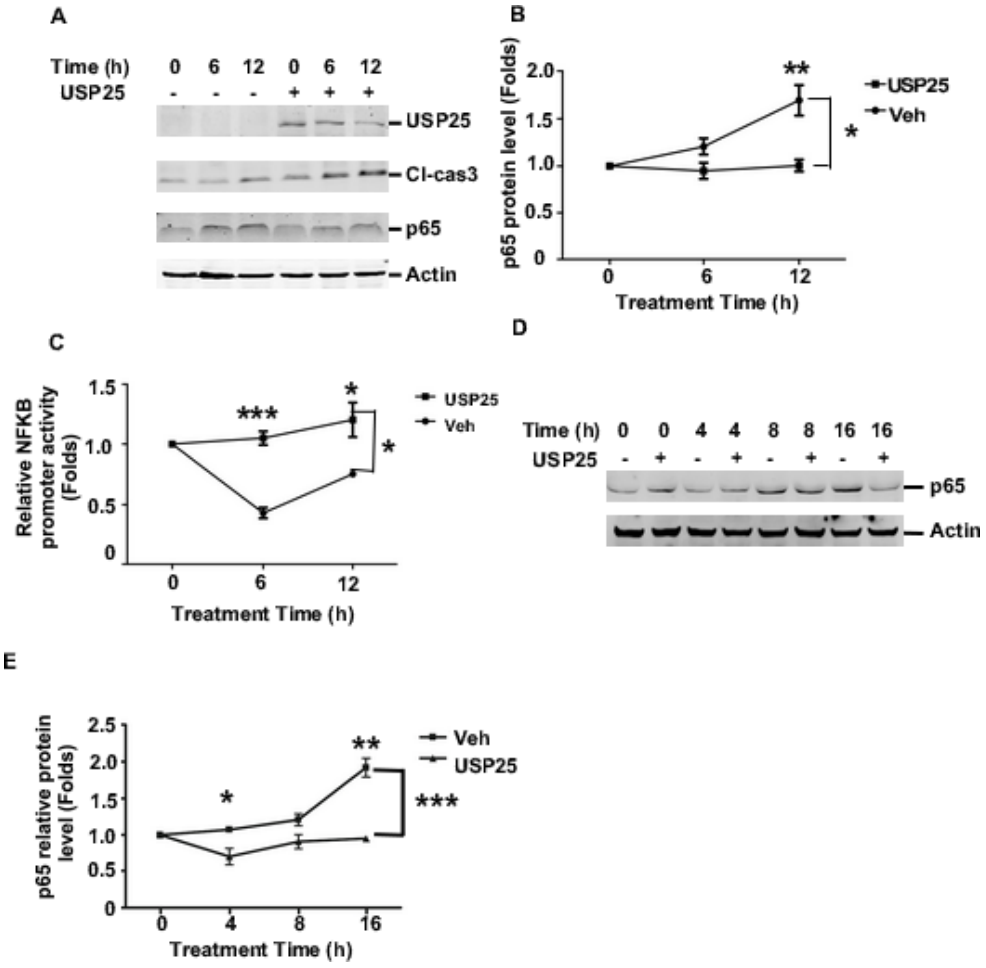

Figure 4.3: **USP25 overexpression down-regulates NF-κB signaling pathway;**

(A). N2a cells were transiently transfected with empty vector or USP25L. 48 hours after transfection, both cells were treated with 200 μM H_2O_2 treatment. After 0, 6 and 12 hours 200 μM H_2O_2 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 H_2O_2. 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.

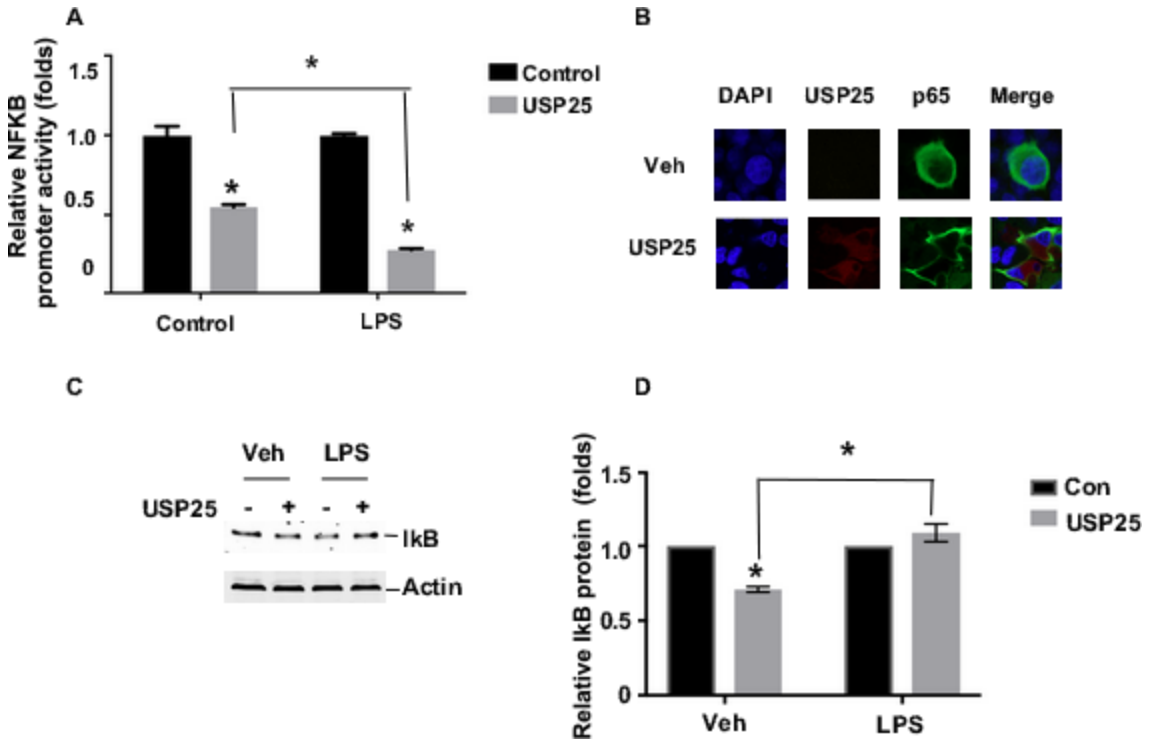

Figure 4.4. USP25 affects LPS-induced IκB turnover and p65 nuclear trafficking

(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 H_2O_2 treatment, MTT and LDH assay were performed. H_2O_2 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%, $p<0.05$) H_2O_2 treatment (**Figure 4.5 A**), while the effect was reversed by NF-κB knockout ($p<0.05$, two-way ANOVA) at 200 μM H_2O_2 treatment (129.7 ± 5.021%, $p<0.05$) (**Figure 4.5 B**). H_2O_2 treatment significantly increased the cytotoxicity of USP25 overexpressing wild-type MEF cells compared with control cells under the treatment of different dosage of of H_2O_2 ($p<0.05$, two-way ANOVA) (**Figure 4.5 C**) at 400 μM H_2O_2 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.

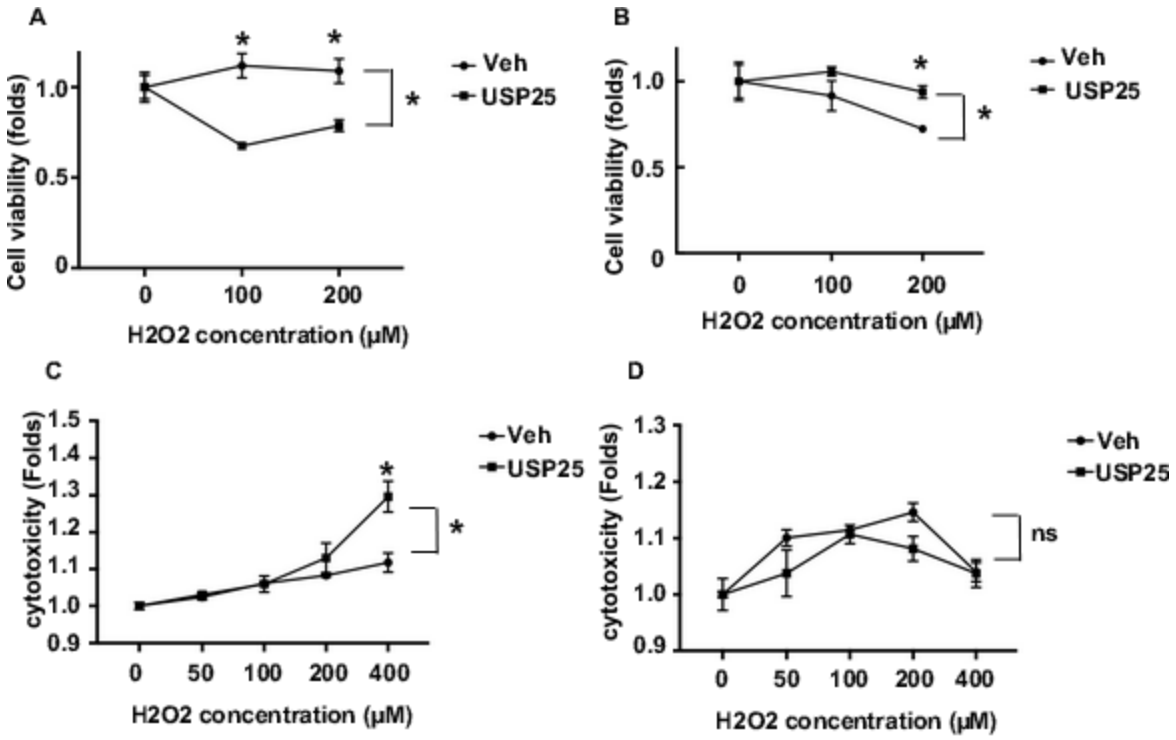

Figure 4.5: USP25 regulation of cell proliferation and death is dependent on NF-κB p65 expression

After 48 hours USP25 overexpression followed by 12 hours H_2O_2 treatment, MTT and LDH assay was performed for RelA-KO cells and its wild-type control cell (MEF). **(A)** H_2O_2 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)** H_2O_2 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).** H_2O_2 treatment significantly increased the cytotoxicity of USP25 overexpressing wild-type MEF cells compared with control cells under the treatment of different dosage of H_2O_2. n = 3. *P < 0.05; Two-way ANOVA; **(D).** H_2O_2 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 *in vivo*

To confirm the effect of USP25 overexpression on NF-κB signaling pathway *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 *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 *in vivo.*

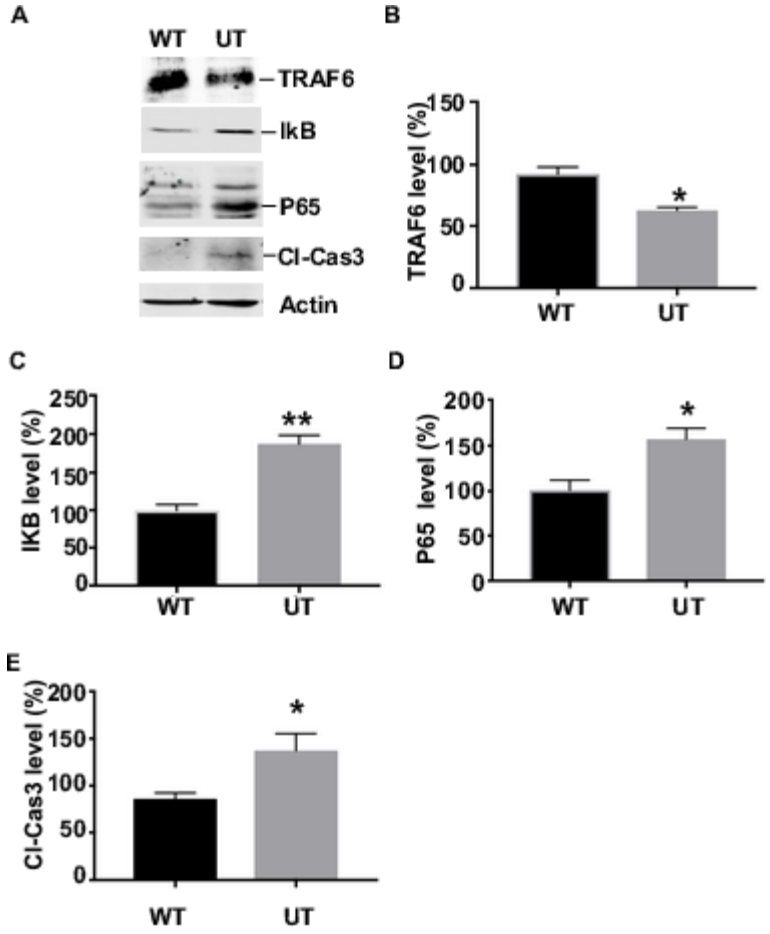

Figure 4.6. USP25 overexpression affects NF-κB signal pathways *in vivo*

(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' 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 *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.

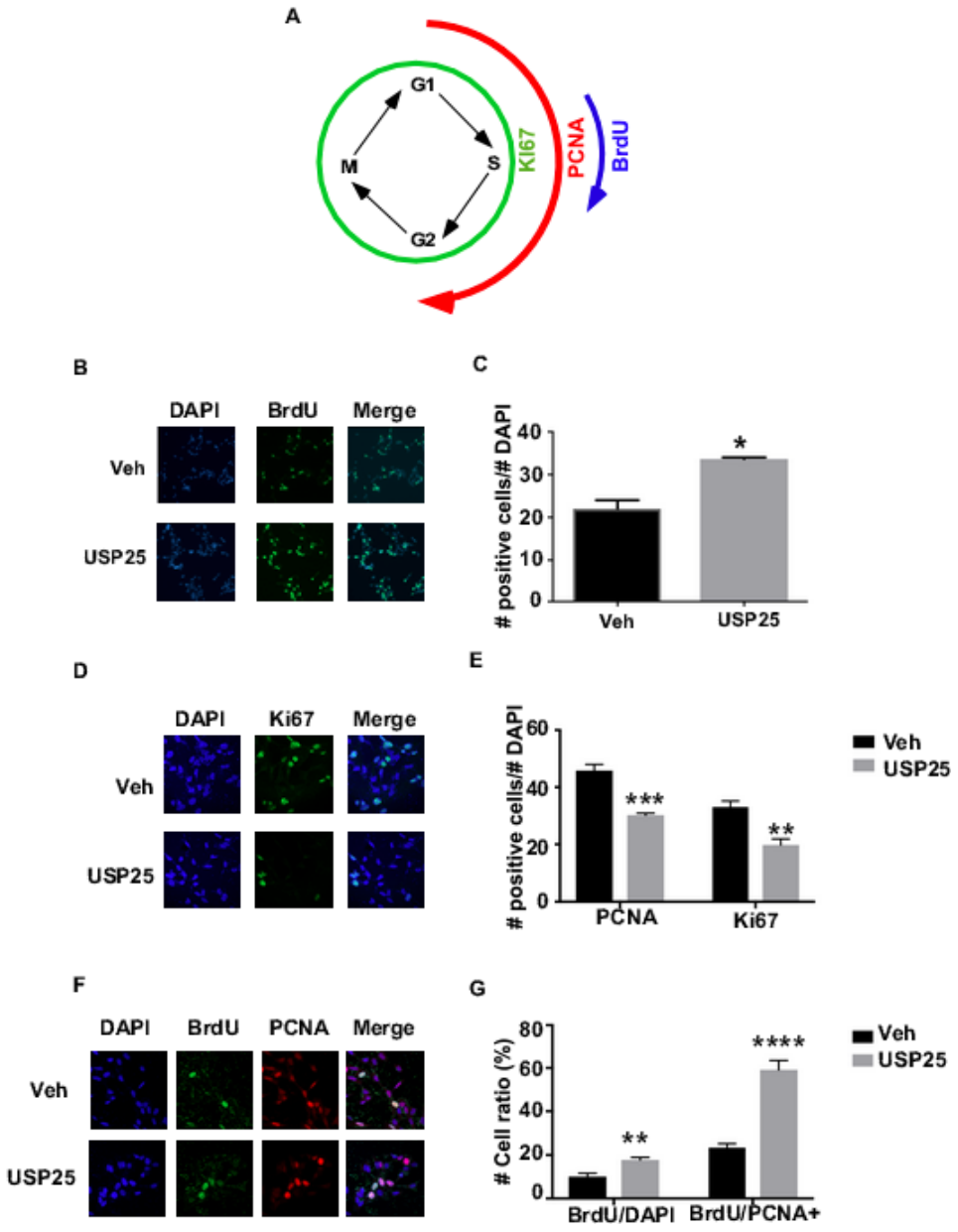

Figure 4.7. USP25 overexpression affects cell cycle regulation

(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' 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' 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' 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 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.

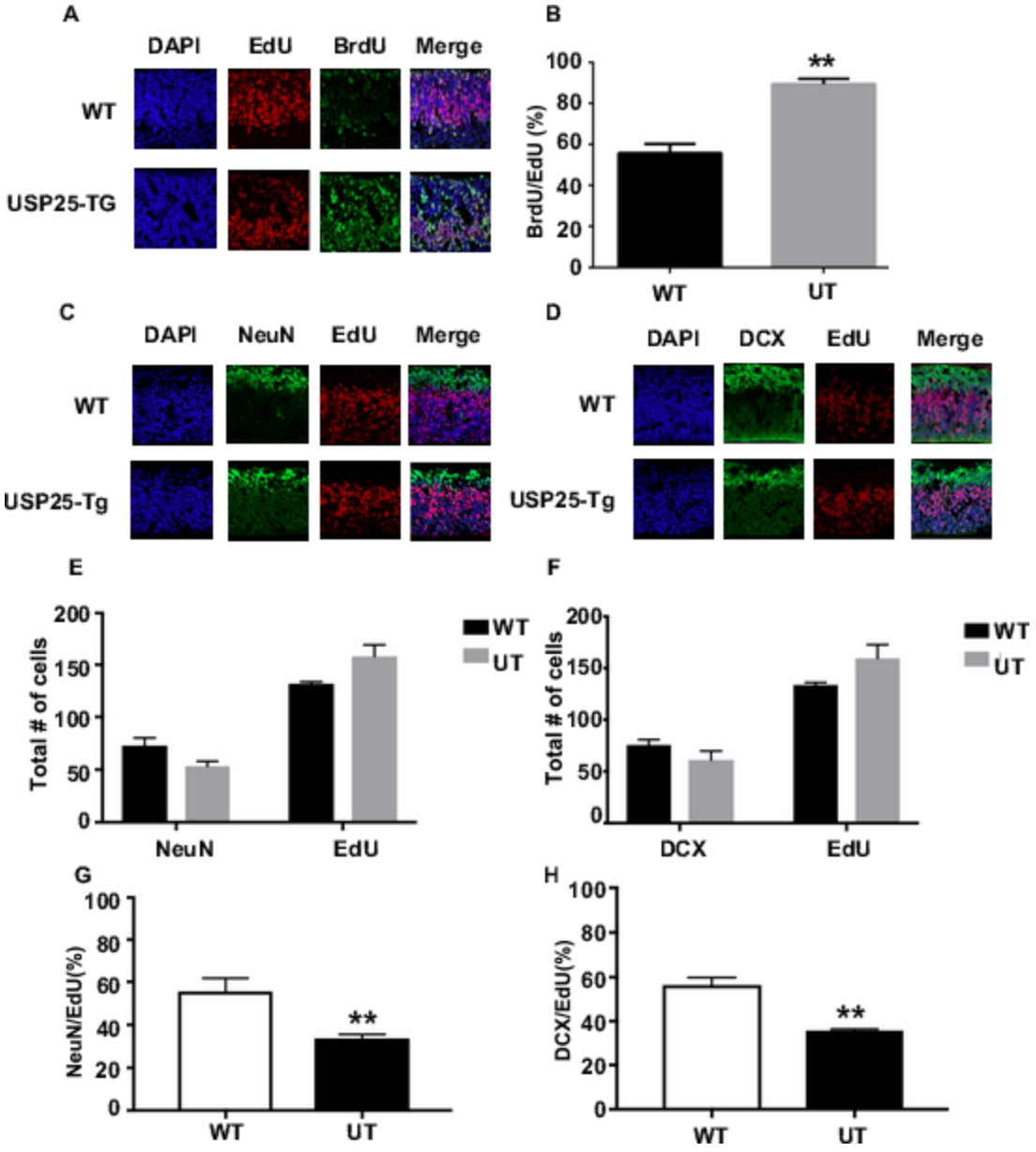

Figure 4.8. USP25 overexpression affects cell cycle during embryonic neurogenesis.

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-/TRR1+ cells (II, III), and the results showed that USP25 significantly reduced the ratio of CITP2-/TRR1+ 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.

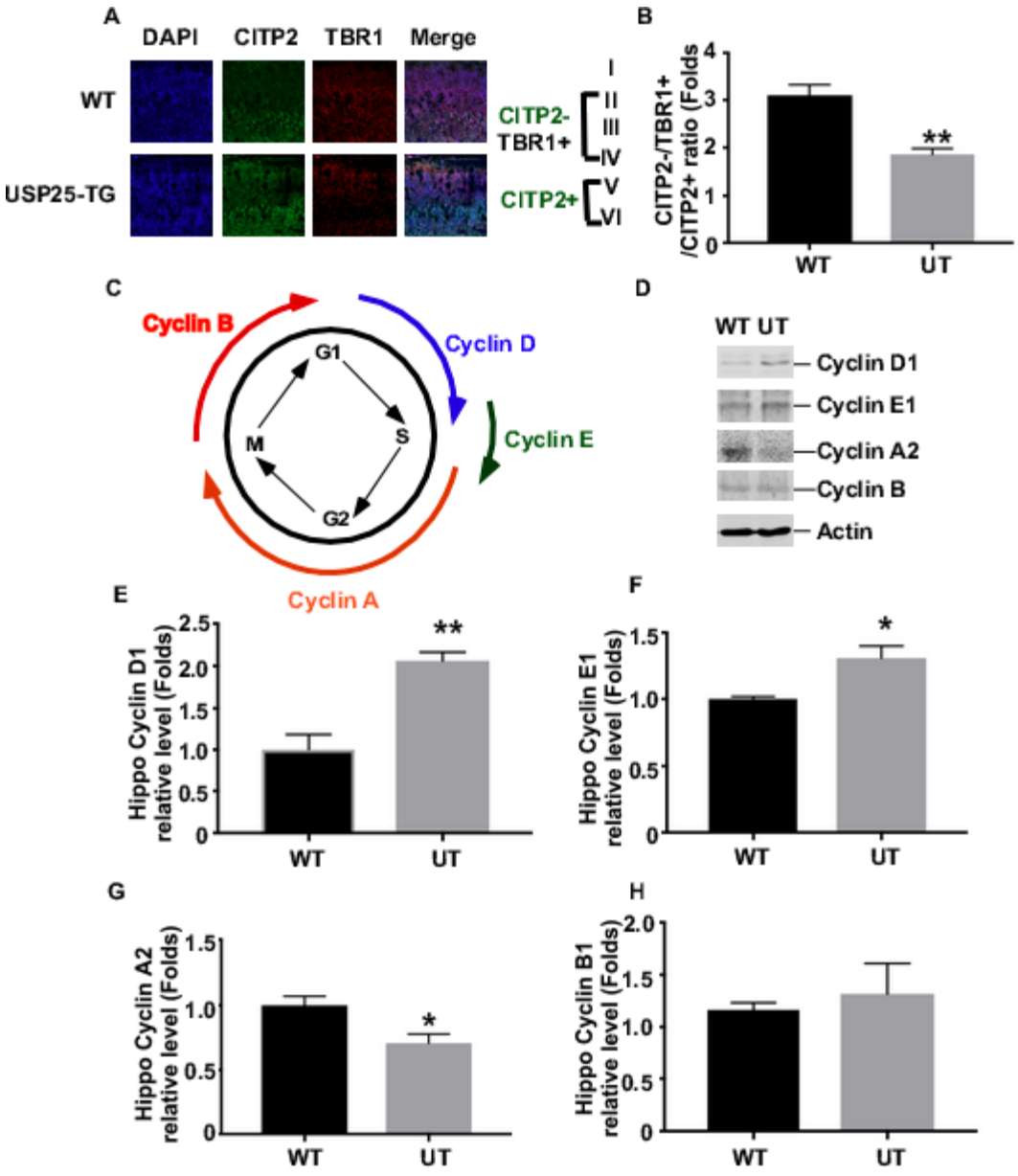

Figure 4.9. USP25 overexpression affects adult cortical development.

(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 **(E)**, cyclin E1 **(F),** cyclin A2 **(G),** while no change in the cyclin B1 level **(H)**. n=4. *P < 0.05, **P<0.01. Student' 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-USP25L-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.

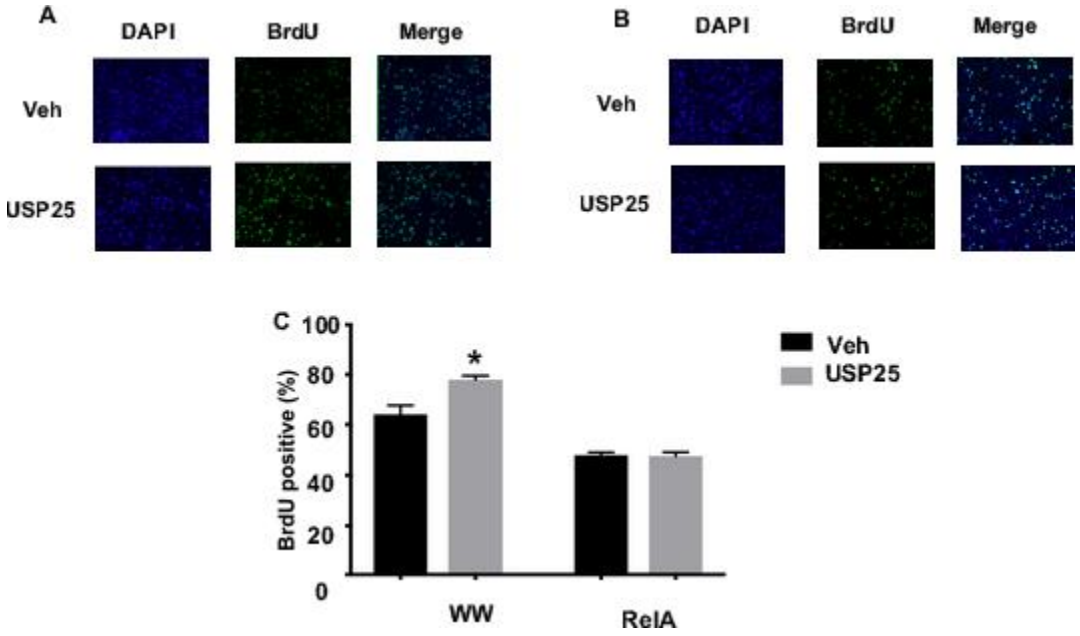

Figure 4.10. USP25 overexpression affects cell proliferation through NF-κB dependent pathway; Wild-type MEFs **(A)** and RelA-KO MEFs **(B)** that are dysfunctional for NF-κB activity, were either transfected with USP25 or its vector plasmid. 48 hours after the transfection, the cells were fixed in 4% 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 up-regulated 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.

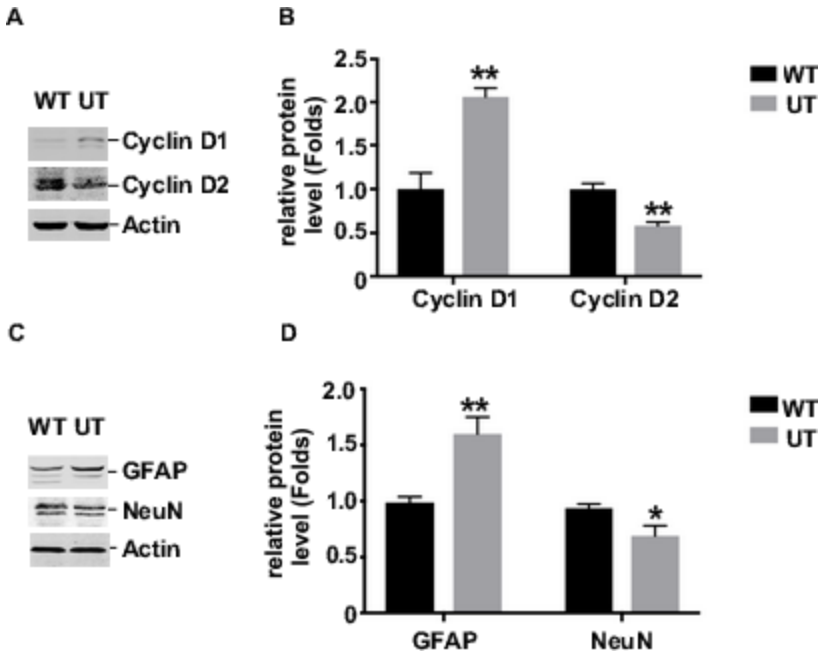

Figure 4.11. Upregulation of USP25 promotes the commitment of the neural stem cells to glial cell fates and suppresses neuronal cell fate.

(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 4.12 A**) 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 4.12 B)**. 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.

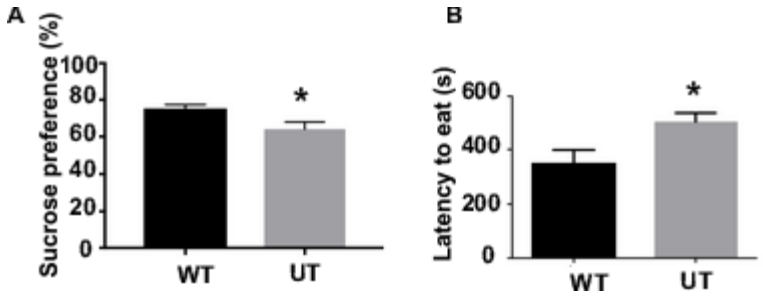

Figure 4.12. Behavioral implication associated USP25 overexpression: neurogenesis. **(A).** In the novelty-suppressed feeding (NSF) test, USP25 transgenic mice showed increased latency to feed in a novel environment following restraint stress. n=9. *P < 0.05, Student' t-test; (**B).** In sucrose preference tests, neurogenesis-deficient USP25-Tg mice showed reduced preference for sucrose compared with their littermate wild-type mice, n=7. *P < 0.05, Student' t-test.

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*.

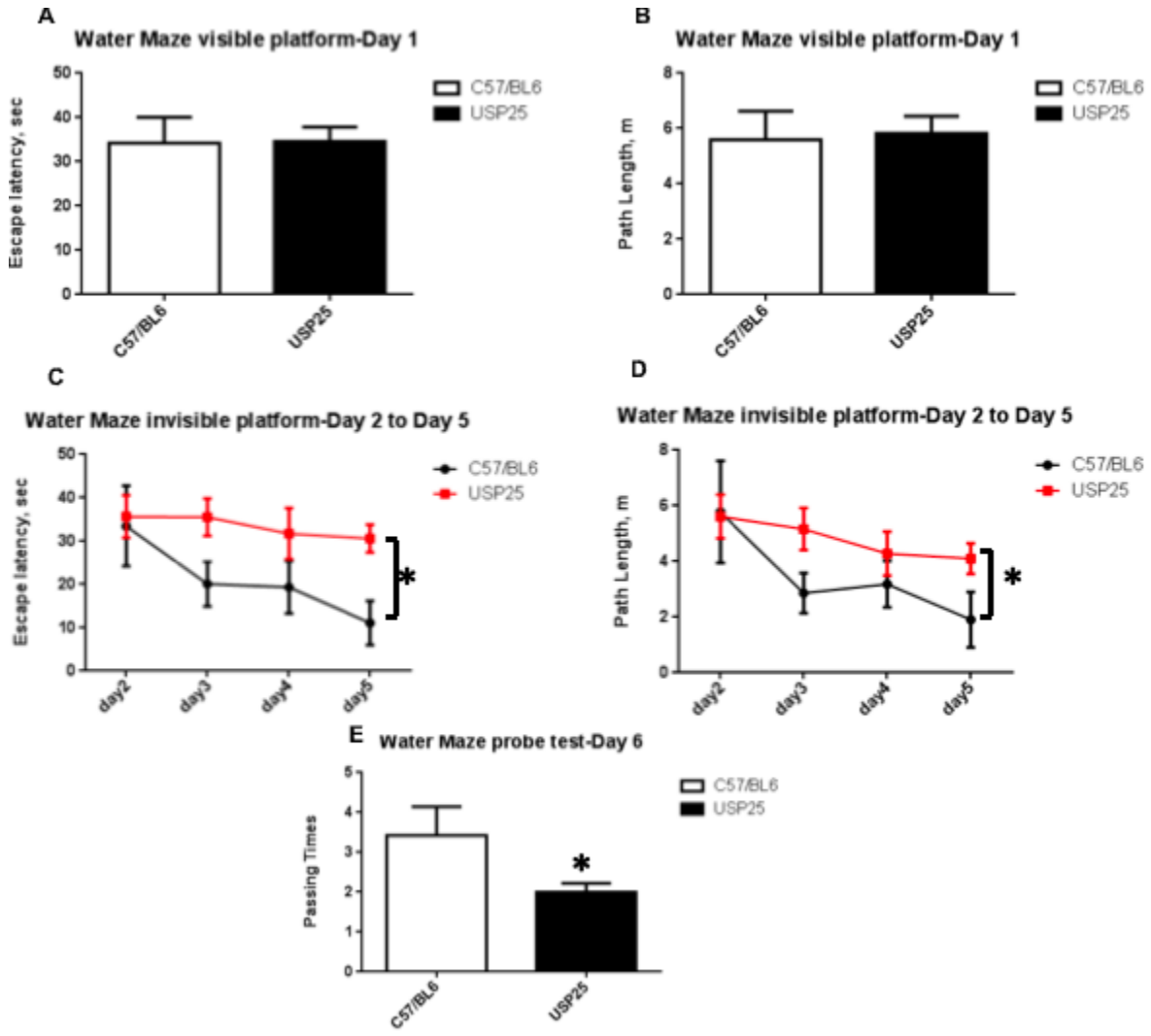

Figure 4.13. Overexpression of USP25 significantly affects memory ability.

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 *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 *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 *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.

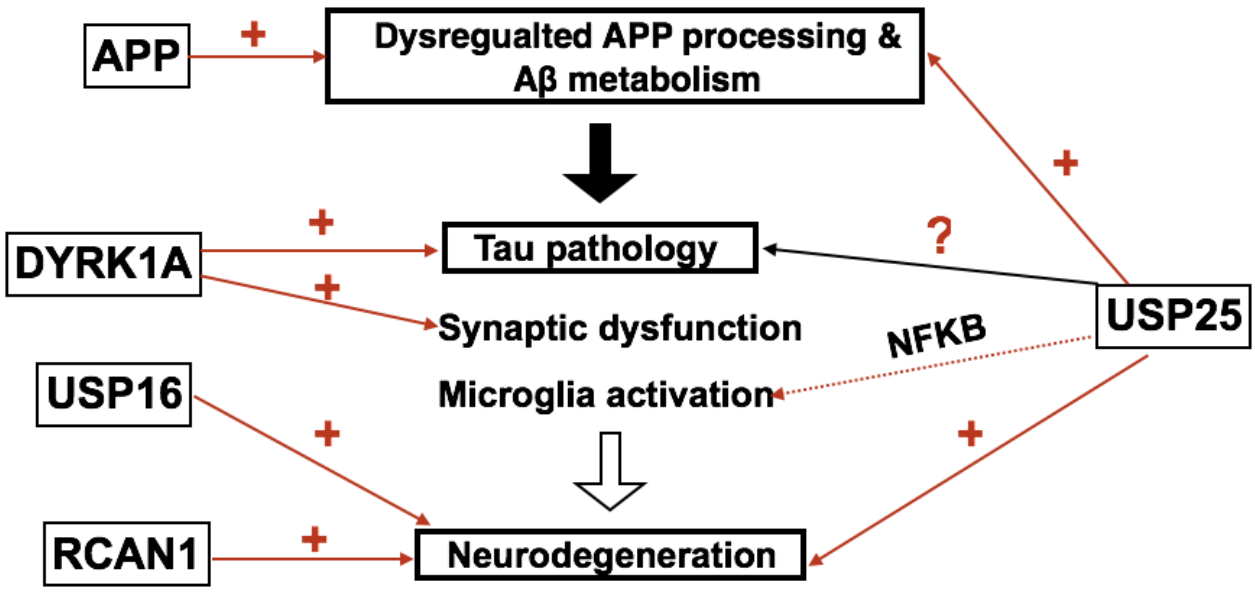

Figure 5.1. HSA21 genes in AD pathogenesis.

References

2011 Alzheimer's disease facts and figures. (2011). Alzheimers Dement, 7(2), 208-244. doi:10.1016/j.jalz.2011.02.004

Acquati, F., Accarino, M., Nucci, C., Fumagalli, P., Jovine, L., Ottolenghi, S., & Taramelli, R. (2000). The gene encoding DRAP (BACE2), a glycosylated transmembrane protein of the aspartic protease family, maps to the down critical region. FEBS Lett, 468(1), 59-64.

Adorno, M., Sikandar, S., Mitra, S. S., Kuo, A., Nicolis Di Robilant, B., Haro-Acosta, V., . . . Clarke, M. F. (2013). Usp16 contributes to somatic stem-cell defects in Down's syndrome. Nature, 501(7467), 380-384. doi:10.1038/nature12530

Alvarez, S., Blanco, A., Fresno, M., & Munoz-Fernandez, M. A. (2011). TNF-alpha contributes to caspase-3 independent apoptosis in neuroblastoma cells: role of NFAT. PLoS One, 6(1), e16100. doi:10.1371/journal.pone.0016100

Arnaiz, E., & Almkvist, O. (2003). Neuropsychological features of mild cognitive impairment and preclinical Alzheimer's disease. Acta Neurol Scand Suppl, 179, 34-41.

Arnold, S. E., Hyman, B. T., Flory, J., Damasio, A. R., & Van Hoesen, G. W. (1991). The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. Cereb Cortex, 1(1), 103-116.

Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., & Hyman, B. T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology, 42(3 Pt 1), 631-639.

Artegiani, B., Lindemann, D., & Calegari, F. (2011). Overexpression of cdk4 and cyclinD1 triggers greater expansion of neural stem cells in the adult mouse brain. J Exp Med, 208(5), 937-948. doi:10.1084/jem.20102167

Bai, X., Yan, Y., Canfield, S., Muravyeva, M. Y., Kikuchi, C., Zaja, I., . . . Bosnjak, Z. J. (2013). Ketamine enhances human neural stem cell proliferation and induces neuronal apoptosis via reactive oxygen species-mediated mitochondrial pathway. Anesth Analg, 116(4), 869-880. doi:10.1213/ANE.0b013e3182860fc9

Balaban, R. S., Nemoto, S., & Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell, 120(4), 483-495. doi:10.1016/j.cell.2005.02.001

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., & Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev, 7(5), 812-821.

Ball, M. J. (1977). Neuronal loss, neurofibrillary tangles and granulovacuolar degeneration in the hippocampus with ageing and dementia. A quantitative study. Acta Neuropathol, 37(2), 111-118.

Ballar, P., Pabuccuoglu, A., & Kose, F. A. (2011). Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). Int J Biochem Cell Biol, 43(4), 613-621. doi:10.1016/j.biocel.2010.12.021

Barde, Y. A. (1994). Neurotrophins: a family of proteins supporting the survival of neurons. Prog Clin Biol Res, 390, 45-56.

Baumeister, W., Walz, J., Zuhl, F., & Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. Cell, 92(3), 367-380.

Becker, L., Mito, T., Takashima, S., & Onodera, K. (1991). Growth and development of the brain in Down syndrome. Prog Clin Biol Res, 373, 133-152.

Beckman, K. B., & Ames, B. N. (1998). The free radical theory of aging matures. Physiol Rev, 78(2), 547-581.

Beddington, R. S., & Robertson, E. J. (1999). Axis development and early asymmetry in mammals. Cell, 96(2), 195-209.

Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., & Baldwin, A. S., Jr. (1992). I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. Genes Dev, 6(10), 1899-1913.

Beher, D., Hesse, L., Masters, C. L., & Multhaup, G. (1996). Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. J Biol Chem, 271(3), 1613-1620.

Benjannet, S., Elagoz, A., Wickham, L., Mamarbachi, M., Munzer, J. S., Basak, A., . . . Seidah, N. G. (2001). Post-translational processing of beta-secretase (beta-amyloid-converting enzyme) and its ectodomain shedding. The pro- and transmembrane/cytosolic domains affect its cellular activity and amyloid-beta production. J Biol Chem, 276(14), 10879-10887. doi:10.1074/jbc.M009899200

Benzi, G., & Moretti, A. (1995). Are reactive oxygen species involved in Alzheimer's disease? Neurobiol Aging, 16(4), 661-674.

Bernardino, L., Agasse, F., Silva, B., Ferreira, R., Grade, S., & Malva, J. O. (2008). Tumor necrosis factor-alpha modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures. Stem Cells, 26(9), 2361-2371. doi:10.1634/stemcells.2007-0914

Black, A. R., Jensen, D., Lin, S. Y., & Azizkhan, J. C. (1999). Growth/cell cycle regulation of Sp1 phosphorylation. J Biol Chem, 274(3), 1207-1215.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., . . . Cerretti, D. P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature, 385(6618), 729-733. doi:10.1038/385729a0

Blount, J. R., Burr, A. A., Denuc, A., Marfany, G., & Todi, S. V. (2012). Ubiquitin-specific protease 25 functions in Endoplasmic Reticulum-associated degradation. PLoS One, 7(5), e36542. doi:10.1371/journal.pone.0036542

Bosch-Comas, A., Lindsten, K., Gonzalez-Duarte, R., Masucci, M. G., & Marfany, G. (2006). The ubiquitin-specific protease USP25 interacts with three sarcomeric proteins. Cell Mol Life Sci, 63(6), 723-734. doi:10.1007/s00018-005-5533-1

Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., . . . Cedar, H. (1994). Sp1 elements protect a CpG island from de novo methylation. Nature, 371(6496), 435-438. doi:10.1038/371435a0

Brickman, A. M., Khan, U. A., Provenzano, F. A., Yeung, L. K., Suzuki, W., Schroeter, H., . . . Small, S. A. (2014). Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. Nat Neurosci, 17(12), 1798-1803. doi:10.1038/nn.3850

Brickman, A. M., Meier, I. B., Korgaonkar, M. S., Provenzano, F. A., Grieve, S. M., Siedlecki, K. L., . . . Zimmerman, M. E. (2012). Testing the white matter retrogenesis hypothesis of cognitive aging. Neurobiol Aging, 33(8), 1699-1715. doi:10.1016/j.neurobiolaging.2011.06.001

Brooksbank, B. W., & Balazs, R. (1984). Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. Brain Res, 318(1), 37-44.

Brun, A., & Englund, E. (1981). Regional pattern of degeneration in Alzheimer's disease: neuronal loss and histopathological grading. Histopathology, 5(5), 549-564.

Bu, G. (2009). Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. Nat Rev Neurosci, 10(5), 333-344. doi:10.1038/nrn2620

Buchberger, A., Bukau, B., & Sommer, T. (2010). Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Mol Cell, 40(2), 238-252. doi:10.1016/j.molcel.2010.10.001

Burdon, T., Smith, A., & Savatier, P. (2002). Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol, 12(9), 432-438.

Busciglio, J., Pelsman, A., Wong, C., Pigino, G., Yuan, M., Mori, H., & Yankner, B. A. (2002). Altered metabolism of the amyloid beta precursor protein is associated with mitochondrial dysfunction in Down's syndrome. Neuron, 33(5), 677-688.

Busciglio, J., & Yankner, B. A. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature, 378(6559), 776-779. doi:10.1038/378776a0

Buxbaum, J. D., Liu, K. N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., . . . Black, R. A. (1998). Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem, 273(43), 27765-27767.

Calegari, F., & Huttner, W. B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J Cell Sci, 116(Pt 24), 4947-4955. doi:10.1242/jcs.00825

Campion, D., Dumanchin, C., Hannequin, D., Dubois, B., Belliard, S., Puel, M., . . . Frebourg, T. (1999). Early-onset autosomal dominant Alzheimer disease: prevalence, genetic

heterogeneity, and mutation spectrum. Am J Hum Genet, 65(3), 664-670. doi:10.1086/302553

Canfield, M. A., Honein, M. A., Yuskiv, N., Xing, J., Mai, C. T., Collins, J. S., . . . Kirby, R. S. (2006). National estimates and race/ethnic-specific variation of selected birth defects in the United States, 1999-2001. Birth Defects Res A Clin Mol Teratol, 76(11), 747-756. doi:10.1002/bdra.20294

Capell, A., Steiner, H., Willem, M., Kaiser, H., Meyer, C., Walter, J., . . . Haass, C. (2000). Maturation and pro-peptide cleavage of beta-secretase. J Biol Chem, 275(40), 30849-30854. doi:10.1074/jbc.M003202200

Caporaso, G. L., Takei, K., Gandy, S. E., Matteoli, M., Mundigl, O., Greengard, P., & De Camilli, P. (1994). Morphologic and biochemical analysis of the intracellular trafficking of the Alzheimer beta/A4 amyloid precursor protein. J Neurosci, 14(5 Pt 2), 3122-3138.

Cardenas, A. M., Ardiles, A. O., Barraza, N., Baez-Matus, X., & Caviedes, P. (2012). Role of tau protein in neuronal damage in Alzheimer's disease and Down syndrome. Arch Med Res, 43(8), 645-654. doi:10.1016/j.arcmed.2012.10.012

Chakrabarti, L., Galdzicki, Z., & Haydar, T. F. (2007). Defects in embryonic neurogenesis and initial synapse formation in the forebrain of the Ts65Dn mouse model of Down syndrome. J Neurosci, 27(43), 11483-11495. doi:10.1523/jneurosci.3406-07.2007

Chang, J. Y., & Liu, L. Z. (1999). Manganese potentiates nitric oxide production by microglia. Brain Res Mol Brain Res, 68(1-2), 22-28.

Charlwood, J., Dingwall, C., Matico, R., Hussain, I., Johanson, K., Moore, S., . . . Camilleri, P. (2001). Characterization of the glycosylation profiles of Alzheimer's beta -secretase

protein Asp-2 expressed in a variety of cell lines. J Biol Chem, 276(20), 16739-16748. doi:10.1074/jbc.M009361200

Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., & Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev, 9(13), 1586-1597.

Cholay, M., Reverdy, C., Benarous, R., Colland, F., & Daviet, L. (2010). Functional interaction between the ubiquitin-specific protease 25 and the SYK tyrosine kinase. Exp Cell Res, 316(4), 667-675. doi:10.1016/j.yexcr.2009.10.023

Christensen, M. A., Zhou, W., Qing, H., Lehman, A., Philipsen, S., & Song, W. (2004). Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1. Mol Cell Biol, 24(2), 865-874.

Ciechanover, A., Gonen, H., Bercovich, B., Cohen, S., Fajerman, I., Israel, A., . . . Orian, A. (2001). Mechanisms of ubiquitin-mediated, limited processing of the NF-kappaB1 precursor protein p105. Biochimie, 83(3-4), 341-349.

Claessen, J. H., Kundrat, L., & Ploegh, H. L. (2012). Protein quality control in the ER: balancing the ubiquitin checkbook. Trends Cell Biol, 22(1), 22-32. doi:10.1016/j.tcb.2011.09.010

Clarris, H. J., Cappai, R., Heffernan, D., Beyreuther, K., Masters, C. L., & Small, D. H. (1997). Identification of heparin-binding domains in the amyloid precursor protein of Alzheimer's disease by deletion mutagenesis and peptide mapping. J Neurochem, 68(3), 1164-1172.

Cole, G., Neal, J. W., Fraser, W. I., & Cowie, V. A. (1994). Autopsy findings in patients with mental handicap. J Intellect Disabil Res, 38 (Pt 1), 9-26.

Colton, C. A., & Gilbert, D. L. (1987). Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett, 223(2), 284-288.

Conti, A., Fabbrini, F., D'Agostino, P., Negri, R., Greco, D., Genesio, R., . . . Nitsch, L. (2007). Altered expression of mitochondrial and extracellular matrix genes in the heart of human fetuses with chromosome 21 trisomy. BMC Genomics, 8, 268. doi:10.1186/1471-2164-8-268

Corder, E. H., Saunders, A. M., Risch, N. J., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Jr., . . . et al. (1994). Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet, 7(2), 180-184. doi:10.1038/ng0694-180

Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., . . . Pericak-Vance, M. A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science, 261(5123), 921-923.

Crimmins, S., Jin, Y., Wheeler, C., Huffman, A. K., Chapman, C., Dobrunz, L. E., . . . Wilson, S. M. (2006). Transgenic rescue of ataxia mice with neuronal-specific expression of ubiquitin-specific protease 14. J Neurosci, 26(44), 11423-11431. doi:10.1523/jneurosci.3600-06.2006

Cummins, J. M., & Vogelstein, B. (2004). HAUSP is required for p53 destabilization. Cell Cycle, 3(6), 689-692.

D'Amato, C. J., & Hicks, S. P. (1965). Neuropathologic alterations in the ataxia (paralytic) mouse. Arch Pathol, 80(6), 604-612.

de Haan, J. B., Cristiano, F., Iannello, R. C., & Kola, I. (1995). Cu/Zn-superoxide dismutase and glutathione peroxidase during aging. Biochem Mol Biol Int, 35(6), 1281-1297.

Dehvari, N., Mahmud, T., Persson, J., Bengtsson, T., Graff, C., Winblad, B., . . . Behbahani, H. (2012). Amyloid precursor protein accumulates in aggresomes in response to proteasome inhibitor. Neurochem Int, 60(5), 533-542. doi:10.1016/j.neuint.2012.02.012

Demars, M. P., Bartholomew, A., Strakova, Z., & Lazarov, O. (2011). Soluble amyloid precursor protein: a novel proliferation factor of adult progenitor cells of ectodermal and mesodermal origin. Stem Cell Res Ther, 2(4), 36. doi:10.1186/scrt77

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., . . . Chen, Z. J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell, 103(2), 351-361.

Deng, Y., Wang, Z., Wang, R., Zhang, X., Zhang, S., Wu, Y., . . . Song, W. (2013). Amyloid-beta protein (Abeta) Glu11 is the major beta-secretase site of beta-site amyloid-beta precursor protein-cleaving enzyme 1(BACE1), and shifting the cleavage site to Abeta Asp1 contributes to Alzheimer pathogenesis. Eur J Neurosci, 37(12), 1962-1969. doi:10.1111/ejn.12235

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., Quadrato, G., . . . Grilli, M. (2008). Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. J Neurosci, 28(15), 3911-3919. doi:10.1523/jneurosci.0148-08.2008

Denuc, A., Bosch-Comas, A., Gonzalez-Duarte, R., & Marfany, G. (2009). The UBA-UIM domains of the USP25 regulate the enzyme ubiquitination state and modulate substrate recognition. PLoS One, 4(5), e5571. doi:10.1371/journal.pone.0005571

des Portes, V., Pinard, J. M., Billuart, P., Vinet, M. C., Koulakoff, A., Carrie, A., . . . Chelly, J. (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. Cell, 92(1), 51-61.

Devenny, D. A., Zimmerli, E. J., Kittler, P., & Krinsky-McHale, S. J. (2002). Cued recall in early-stage dementia in adults with Down's syndrome. J Intellect Disabil Res, 46(Pt 6), 472-483.

Di Domenico, F., Coccia, R., Cocciolo, A., Murphy, M. P., Cenini, G., Head, E., . . . Perluigi, M. (2013). Impairment of proteostasis network in Down syndrome prior to the development of Alzheimer's disease neuropathology: redox proteomics analysis of human brain. Biochim Biophys Acta, 1832(8), 1249-1259. doi:10.1016/j.bbadis.2013.04.013

Ding, C., Li, F., Long, Y., & Zheng, J. (2016). Chloroquine attenuates lipopolysaccharide-induced inflammatory responses through upregulation of USP25. Can J Physiol Pharmacol, 1-11. doi:10.1139/cjpp-2016-0303

Ding, W. X., Ni, H. M., Gao, W., Yoshimori, T., Stolz, D. B., Ron, D., & Yin, X. M. (2007). Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. Am J Pathol, 171(2), 513-524. doi:10.2353/ajpath.2007.070188

Djavaheri-Mergny, M., Javelaud, D., Wietzerbin, J., & Besancon, F. (2004). NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells. FEBS Lett, 578(1-2), 111-115. doi:10.1016/j.febslet.2004.10.082

Down, J. L. H. (1866). Observation on an ethnic classification of idiots. London Hospital Reports, 3, 259-262.

El Ayadi, A., Stieren, E. S., Barral, J. M., & Boehning, D. (2012). Ubiquilin-1 regulates amyloid precursor protein maturation and degradation by stimulating K63-linked polyubiquitination of lysine 688. Proc Natl Acad Sci U S A, 109(33), 13416-13421. doi:10.1073/pnas.1206786109

Ermak, G., Morgan, T. E., & Davies, K. J. (2001). Chronic overexpression of the calcineurin inhibitory gene DSCR1 (Adapt78) is associated with Alzheimer's disease. J Biol Chem, 276(42), 38787-38794. doi:10.1074/jbc.M102829200

Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., . . . Ward, P. J. (1990). Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science, 248(4959), 1122-1124.

Evenhuis, H. M. (1990). The natural history of dementia in Down's syndrome. Arch Neurol, 47(3), 263-267.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M., & Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. Embo j, 16(7), 1519-1530. doi:10.1093/emboj/16.7.1519

Farzan, M., Schnitzler, C. E., Vasilieva, N., Leung, D., & Choe, H. (2000). BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. Proc Natl Acad Sci U S A, 97(17), 9712-9717. doi:10.1073/pnas.160115697

Feng, T., Tammineni, P., Agrawal, C., Jeong, Y. Y., & Cai, Q. (2017). Autophagy-mediated Regulation of BACE1 Protein Trafficking and Degradation. J Biol Chem, 292(5), 1679-1690. doi:10.1074/jbc.M116.766584

Fillit, H., Ding, W. H., Buee, L., Kalman, J., Altstiel, L., Lawlor, B., & Wolf-Klein, G. (1991). Elevated circulating tumor necrosis factor levels in Alzheimer's disease. Neurosci Lett, 129(2), 318-320.

Flood, J. F., Harris, F. J., & Morley, J. E. (1996). Age-related changes in hippocampal drug facilitation of memory processing in SAMP8 mice. Neurobiol Aging, 17(1), 15-24.

Forstl, H., & Kurz, A. (1999). Clinical features of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci, 249(6), 288-290.

Francis, F., Koulakoff, A., Boucher, D., Chafey, P., Schaar, B., Vinet, M. C., . . . Chelly, J. (1999). Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron, 23(2), 247-256.

Fuentes, J. J., Genesca, L., Kingsbury, T. J., Cunningham, K. W., Perez-Riba, M., Estivill, X., & de la Luna, S. (2000). DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet, 9(11), 1681-1690.

Gamblin, T. C., Chen, F., Zambrano, A., Abraha, A., Lagalwar, S., Guillozet, A. L., . . . Cryns, V. L. (2003). Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proc Natl Acad Sci U S A, 100(17), 10032-10037. doi:10.1073/pnas.1630428100

Gatta, V., Drago, D., Fincati, K., Valenti, M. T., Dalle Carbonare, L., Sensi, S. L., & Zatta, P. (2011). Microarray analysis on human neuroblastoma cells exposed to aluminum, beta(1-42)-amyloid or the beta(1-42)-amyloid aluminum complex. PLoS One, 6(1), e15965. doi:10.1371/journal.pone.0015965

Geinisman, Y., Ganeshina, O., Yoshida, R., Berry, R. W., Disterhoft, J. F., & Gallagher, M. (2004). Aging, spatial learning, and total synapse number in the rat CA1 stratum radiatum. Neurobiol Aging, 25(3), 407-416. doi:10.1016/j.neurobiolaging.2003.12.001

Gemma, C., Mesches, M. H., Sepesi, B., Choo, K., Holmes, D. B., & Bickford, P. C. (2002). Diets enriched in foods with high antioxidant activity reverse age-induced decreases in cerebellar beta-adrenergic function and increases in proinflammatory cytokines. J Neurosci, 22(14), 6114-6120. doi:20026622

Giglioni, B., Comi, P., Ronchi, A., Mantovani, R., & Ottolenghi, S. (1989). The same nuclear proteins bind the proximal CACCC box of the human beta-globin promoter and a similar sequence in the enhancer. Biochem Biophys Res Commun, 164(1), 149-155.

Gilmore, E. C., & Herrup, K. (1997). Cortical development: layers of complexity. Curr Biol, 7(4), R231-234.

Glabe, C. (2001). Intracellular mechanisms of amyloid accumulation and pathogenesis in Alzheimer's disease. J Mol Neurosci, 17(2), 137-145. doi:10.1385/jmn:17:2:137

Glasson, E. J., Sullivan, S. G., Hussain, R., Petterson, B. A., Montgomery, P. D., & Bittles, A. H. (2002). The changing survival profile of people with Down's syndrome: implications for genetic counselling. Clin Genet, 62(5), 390-393.

Glenner, G. G., & Wong, C. W. (1984a). Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem Biophys Res Commun, 122(3), 1131-1135.

Glenner, G. G., & Wong, C. W. (1984b). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun, 120(3), 885-890.

Glickman, M. H., & Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev, 82(2), 373-428. doi:10.1152/physrev.00027.2001

Gonzalez-Noriega, A., Grubb, J. H., Talkad, V., & Sly, W. S. (1980). Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J Cell Biol, 85(3), 839-852.

Gotz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. Nat Rev Mol Cell Biol, 6(10), 777-788. doi:10.1038/nrm1739

Grbovic, O. M., Mathews, P. M., Jiang, Y., Schmidt, S. D., Dinakar, R., Summers-Terio, N. B., . . . Cataldo, A. M. (2003). Rab5-stimulated up-regulation of the endocytic pathway increases intracellular beta-cleaved amyloid precursor protein carboxyl-terminal fragment levels and Abeta production. J Biol Chem, 278(33), 31261-31268. doi:10.1074/jbc.M304122200

Grilli, M., Goffi, F., Memo, M., & Spano, P. (1996). Interleukin-1beta and glutamate activate the NF-kappaB/Rel binding site from the regulatory region of the amyloid precursor protein gene in primary neuronal cultures. J Biol Chem, 271(25), 15002-15007.

Guaiquil, V., Swendeman, S., Yoshida, T., Chavala, S., Campochiaro, P. A., & Blobel, C. P. (2009). ADAM9 is involved in pathological retinal neovascularization. Mol Cell Biol, 29(10), 2694-2703. doi:10.1128/mcb.01460-08

Guidi, S., Bonasoni, P., Ceccarelli, C., Santini, D., Gualtieri, F., Ciani, E., & Bartesaghi, R. (2008). Neurogenesis impairment and increased cell death reduce total neuron number in the hippocampal region of fetuses with Down syndrome. Brain Pathol, 18(2), 180-197. doi:10.1111/j.1750-3639.2007.00113.x

Guo, Y., Stacey, D. W., & Hitomi, M. (2002). Post-transcriptional regulation of cyclin D1 expression during G2 phase. Oncogene, 21(49), 7545-7556. doi:10.1038/sj.onc.1205907

Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., & Baldwin, A. S., Jr. (1999). NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol, 19(8), 5785-5799.

Haass, C., Koo, E. H., Mellon, A., Hung, A. Y., & Selkoe, D. J. (1992). Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. Nature, 357(6378), 500-503. doi:10.1038/357500a0

Haass, C., & Selkoe, D. J. (1993). Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell, 75(6), 1039-1042.

Hammerle, B., Elizalde, C., & Tejedor, F. J. (2008). The spatio-temporal and subcellular expression of the candidate Down syndrome gene Mnb/Dyrk1A in the developing mouse brain suggests distinct sequential roles in neuronal development. Eur J Neurosci, 27(5), 1061-1074. doi:10.1111/j.1460-9568.2008.06092.x

Hammerle, B., Ulin, E., Guimera, J., Becker, W., Guillemot, F., & Tejedor, F. J. (2011). Transient expression of Mnb/Dyrk1a couples cell cycle exit and differentiation of neuronal precursors by inducing p27KIP1 expression and suppressing NOTCH signaling. Development, 138(12), 2543-2554. doi:10.1242/dev.066167

Haniu, M., Denis, P., Young, Y., Mendiaz, E. A., Fuller, J., Hui, J. O., . . . Citron, M. (2000). Characterization of Alzheimer's beta -secretase protein BACE. A pepsin family member with unusual properties. J Biol Chem, 275(28), 21099-21106. doi:10.1074/jbc.M002095200

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., . . . Saftig, P. (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet, 11(21), 2615-2624.

Hattori, M., Fujiyama, A., Taylor, T. D., Watanabe, H., Yada, T., Park, H. S., . . . Yaspo, M. L. (2000). The DNA sequence of human chromosome 21. Nature, 405(6784), 311-319. doi:10.1038/35012518

He, P., Zhong, Z., Lindholm, K., Berning, L., Lee, W., Lemere, C., . . . Shen, Y. (2007). Deletion of tumor necrosis factor death receptor inhibits amyloid beta generation and prevents learning and memory deficits in Alzheimer's mice. J Cell Biol, 178(5), 829-841. doi:10.1083/jcb.200705042

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol, 9(3), 107-112.

Hinz, M., Krappmann, D., Eichten, A., Heder, A., Scheidereit, C., & Strauss, M. (1999). NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol, 19(4), 2690-2698.

Ho, L. H., Taylor, R., Dorstyn, L., Cakouros, D., Bouillet, P., & Kumar, S. (2009). A tumor suppressor function for caspase-2. Proc Natl Acad Sci U S A, 106(13), 5336-5341. doi:10.1073/pnas.0811928106

Hollands, C., Bartolotti, N., & Lazarov, O. (2016). Alzheimer's Disease and Hippocampal Adult Neurogenesis; Exploring Shared Mechanisms. Front Neurosci, 10, 178. doi:10.3389/fnins.2016.00178

Hu, X., Hicks, C. W., He, W., Wong, P., Macklin, W. B., Trapp, B. D., & Yan, R. (2006). Bace1 modulates myelination in the central and peripheral nervous system. Nat Neurosci, 9(12), 1520-1525. doi:10.1038/nn1797

Huang, T. T., Zou, Y., & Corniola, R. (2012). Oxidative stress and adult neurogenesis--effects of radiation and superoxide dismutase deficiency. Semin Cell Dev Biol, 23(7), 738-744. doi:10.1016/j.semcdb.2012.04.003

Huse, J. T., Pijak, D. S., Leslie, G. J., Lee, V. M., & Doms, R. W. (2000). Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. J Biol Chem, 275(43), 33729-33737. doi:10.1074/jbc.M004175200

Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., . . . Heutink, P. (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature, 393(6686), 702-705. doi:10.1038/31508

Inoue, S., Browne, G., Melino, G., & Cohen, G. M. (2009). Ordering of caspases in cells undergoing apoptosis by the intrinsic pathway. Cell Death Differ, 16(7), 1053-1061. doi:10.1038/cdd.2009.29

Insausti, A. M., Megias, M., Crespo, D., Cruz-Orive, L. M., Dierssen, M., Vallina, I. F., . . . Florez, J. (1998). Hippocampal volume and neuronal number in Ts65Dn mice: a murine model of Down syndrome. Neurosci Lett, 253(3), 175-178.

Iqbal, K., Gong, C. X., & Liu, F. (2013). Hyperphosphorylation-induced tau oligomers. Front Neurol, 4, 112. doi:10.3389/fneur.2013.00112

Iwanaga, R., Ohtani, K., Hayashi, T., & Nakamura, M. (2001). Molecular mechanism of cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I. Oncogene, 20(17), 2055-2067. doi:10.1038/sj.onc.1204304

Iwanaga, R., Ozono, E., Fujisawa, J., Ikeda, M. A., Okamura, N., Huang, Y., & Ohtani, K. (2008). Activation of the cyclin D2 and cdk6 genes through NF-kappaB is critical for cell-cycle progression induced by HTLV-I Tax. Oncogene, 27(42), 5635-5642. doi:10.1038/onc.2008.174

Jacobs, P. A., Baikie, A. G., Court Brown, W. M., & Strong, J. A. (1959). The somatic chromosomes in mongolism. Lancet, 1(7075), 710.

Jones, P. L., Ping, D., & Boss, J. M. (1997). Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. Mol Cell Biol, 17(12), 6970-6981.

Joyce, D., Bouzahzah, B., Fu, M., Albanese, C., D'Amico, M., Steer, J., . . . Pestell, R. G. (1999). Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. J Biol Chem, 274(36), 25245-25249.

Jung, E. S., Hong, H., Kim, C., & Mook-Jung, I. (2015). Acute ER stress regulates amyloid precursor protein processing through ubiquitin-dependent degradation. Sci Rep, 5, 8805. doi:10.1038/srep08805

Kabe, Y., Ando, K., Hirao, S., Yoshida, M., & Handa, H. (2005). Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. Antioxid Redox Signal, 7(3-4), 395-403. doi:10.1089/ars.2005.7.395

Kahlem, P., Sultan, M., Herwig, R., Steinfath, M., Balzereit, D., Eppens, B., . . . Yaspo, M. L. (2004). Transcript level alterations reflect gene dosage effects across multiple tissues in a

mouse model of down syndrome. Genome Res, 14(7), 1258-1267. doi:10.1101/gr.1951304

Kaltschmidt, B., Ndiaye, D., Korte, M., Pothion, S., Arbibe, L., Prullage, M., . . . Memet, S. (2006). NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. Mol Cell Biol, 26(8), 2936-2946. doi:10.1128/mcb.26.8.2936-2946.2006

Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P. A., & Kaltschmidt, C. (1997). Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. Proc Natl Acad Sci U S A, 94(6), 2642-2647.

Kaltschmidt, C., Kaltschmidt, B., & Baeuerle, P. A. (1993). Brain synapses contain inducible forms of the transcription factor NF-kappa B. Mech Dev, 43(2-3), 135-147.

Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H., & Baeuerle, P. A. (1994). Constitutive NF-kappa B activity in neurons. Mol Cell Biol, 14(6), 3981-3992.

Kamal, A., Almenar-Queralt, A., LeBlanc, J. F., Roberts, E. A., & Goldstein, L. S. (2001). Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature, 414(6864), 643-648. doi:10.1038/414643a

Kamal, A., Stokin, G. B., Yang, Z., Xia, C. H., & Goldstein, L. S. (2000). Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron, 28(2), 449-459.

Kamoun, P., Belardinelli, M. C., Chabli, A., Lallouchi, K., & Chadefaux-Vekemans, B. (2003). Endogenous hydrogen sulfide overproduction in Down syndrome. Am J Med Genet A, 116a(3), 310-311. doi:10.1002/ajmg.a.10847

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science, 294(5544), 1030-1038. doi:10.1126/science.1067020

Kaneko, M., Koike, H., Saito, R., Kitamura, Y., Okuma, Y., & Nomura, Y. (2010). Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid-beta generation. J Neurosci, 30(11), 3924-3932. doi:10.1523/jneurosci.2422-09.2010

Kang, E. L., Biscaro, B., Piazza, F., & Tesco, G. (2012). BACE1 protein endocytosis and trafficking are differentially regulated by ubiquitination at lysine 501 and the Di-leucine motif in the carboxyl terminus. J Biol Chem, 287(51), 42867-42880. doi:10.1074/jbc.M112.407072

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., . . . Muller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature, 325(6106), 733-736. doi:10.1038/325733a0

Kay, J. E., & Korner, A. (1966). Effect of cycloheximide on protein and ribonucleic acid synthesis in cultured human lymphocytes. Biochem J, 100(3), 815-822.

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. Trends Neurosci, 27(8), 447-452. doi:10.1016/j.tins.2004.05.013

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer, 26(4), 239-257.

Kerridge, D. (1958). The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. J Gen Microbiol, 19(3), 497-506. doi:10.1099/00221287-19-3-497

Kibbey, M. C., Jucker, M., Weeks, B. S., Neve, R. L., Van Nostrand, W. E., & Kleinman, H. K. (1993). beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. Proc Natl Acad Sci U S A, 90(21), 10150-10153.

Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., . . . Wiertz, E. (2004). Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J Biol Chem, 279(5), 3525-3534. doi:10.1074/jbc.M307453200

Kim, D. Y., Carey, B. W., Wang, H., Ingano, L. A., Binshtok, A. M., Wertz, M. H., . . . Kovacs, D. M. (2007). BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat Cell Biol, 9(7), 755-764. doi:10.1038/ncb1602

Kimura, M., Haisa, M., Uetsuka, H., Takaoka, M., Ohkawa, T., Kawashima, R., . . . Naomoto, Y. (2003). TNF combined with IFN-alpha accelerates NF-kappaB-mediated apoptosis through enhancement of Fas expression in colon cancer cells. Cell Death Differ, 10(6), 718-728. doi:10.1038/sj.cdd.4401219

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., & Ito, H. (1988). Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. Nature, 331(6156), 530-532. doi:10.1038/331530a0

Komander, D., Clague, M. J., & Urbe, S. (2009). Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol, 10(8), 550-563. doi:10.1038/nrm2731

Koo, E. H., & Squazzo, S. L. (1994). Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J Biol Chem, 269(26), 17386-17389.

Koo, E. H., Squazzo, S. L., Selkoe, D. J., & Koo, C. H. (1996). Trafficking of cell-surface amyloid beta-protein precursor. I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody. J Cell Sci, 109 (Pt 5), 991-998.

Kowalczyk, A., Filipkowski, R. K., Rylski, M., Wilczynski, G. M., Konopacki, F. A., Jaworski, J., . . . Kaczmarek, L. (2004). The critical role of cyclin D2 in adult neurogenesis. J Cell Biol, 167(2), 209-213. doi:10.1083/jcb.200404181

Kraft, C., Peter, M., & Hofmann, K. (2010). Selective autophagy: ubiquitin-mediated recognition and beyond. Nat Cell Biol, 12(9), 836-841. doi:10.1038/ncb0910-836

Kruidering, M., & Evan, G. I. (2000). Caspase-8 in apoptosis: the beginning of "the end"? IUBMB Life, 50(2), 85-90. doi:10.1080/713803693

Kurosaka, K., Takahashi, M., Watanabe, N., & Kobayashi, Y. (2003). Silent cleanup of very early apoptotic cells by macrophages. J Immunol, 171(9), 4672-4679.

Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr., Booth, C. J., Mehal, W. Z., . . . Flavell, R. A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science, 311(5762), 847-851. doi:10.1126/science.1115035

Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., . . . Klein, W. L. (1998). Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A, 95(11), 6448-6453.

Lange, C., Huttner, W. B., & Calegari, F. (2009). Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell, 5(3), 320-331. doi:10.1016/j.stem.2009.05.026

Lappe-Siefke, C., Loebrich, S., Hevers, W., Waidmann, O. B., Schweizer, M., Fehr, S., . . . Kneussel, M. (2009). The ataxia (axJ) mutation causes abnormal GABAA receptor turnover in mice. PLoS Genet, 5(9), e1000631. doi:10.1371/journal.pgen.1000631

Larsen, K. B., Laursen, H., Graem, N., Samuelsen, G. B., Bogdanovic, N., & Pakkenberg, B. (2008). Reduced cell number in the neocortical part of the human fetal brain in Down syndrome. Ann Anat, 190(5), 421-427. doi:10.1016/j.aanat.2008.05.007

Lee, K. J., Moussa, C. E., Lee, Y., Sung, Y., Howell, B. W., Turner, R. S., . . . Hoe, H. S. (2010). Beta amyloid-independent role of amyloid precursor protein in generation and maintenance of dendritic spines. Neuroscience, 169(1), 344-356. doi:10.1016/j.neuroscience.2010.04.078

Lejeune, J., Turpin, R., & Gautier, M. (1959). [Mongolism; a chromosomal disease (trisomy)]. Bull Acad Natl Med, 143(11-12), 256-265.

Lesne, S., Koh, M. T., Kotilinek, L., Kayed, R., Glabe, C. G., Yang, A., . . . Ashe, K. H. (2006). A specific amyloid-beta protein assembly in the brain impairs memory. Nature, 440(7082), 352-357. doi:10.1038/nature04533

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, 91(4), 479-489.

Li, Q., & Verma, I. M. (2002). NF-kappaB regulation in the immune system. Nat Rev Immunol, 2(10), 725-734. doi:10.1038/nri910

Li, R., Yang, L., Lindholm, K., Konishi, Y., Yue, X., Hampel, H., . . . Shen, Y. (2004). Tumor necrosis factor death receptor signaling cascade is required for amyloid-beta protein-induced neuron death. J Neurosci, 24(7), 1760-1771. doi:10.1523/jneurosci.4580-03.2004

Lilienbaum, A., & Israel, A. (2003). From calcium to NF-kappa B signaling pathways in neurons. Mol Cell Biol, 23(8), 2680-2698.

Lim, K. H., Song, M. H., & Baek, K. H. (2016). Decision for cell fate: deubiquitinating enzymes in cell cycle checkpoint. Cell Mol Life Sci, 73(7), 1439-1455. doi:10.1007/s00018-015-2129-2

Lin, D., Zhang, M., Zhang, M. X., Ren, Y., Jin, J., Zhao, Q., . . . Zhong, B. (2015). Induction of USP25 by viral infection promotes innate antiviral responses by mediating the stabilization of TRAF3 and TRAF6. Proc Natl Acad Sci U S A, 112(36), 11324-11329. doi:10.1073/pnas.1509968112

Lipford, J. R., Smith, G. T., Chi, Y., & Deshaies, R. J. (2005). A putative stimulatory role for activator turnover in gene expression. Nature, 438(7064), 113-116. doi:10.1038/nature04098

Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., . . . Mak, T. W. (1999). TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev, 13(8), 1015-1024.

Lopez Salon, M., Morelli, L., Castano, E. M., Soto, E. F., & Pasquini, J. M. (2000). Defective ubiquitination of cerebral proteins in Alzheimer's disease. J Neurosci Res, 62(2), 302-310. doi:10.1002/1097-4547(20001015)62:2<302::aid-jnr15>3.0.co;2-l

Los, M., Wesselborg, S., & Schulze-Osthoff, K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity, 10(6), 629-639.

Lou, S. J., Gu, P., Xu, H., Xu, X. H., Wang, M. W., He, C., & Lu, C. L. (2003). [Effect of tumor necrosis factor-alpha on differentiation of mesencephalic neural stem cells and proliferation of oligodendrocytes in the rat]. Sheng Li Xue Bao, 55(2), 183-186.

Luningschror, P., Stocker, B., Kaltschmidt, B., & Kaltschmidt, C. (2012). miR-290 cluster modulates pluripotency by repressing canonical NF-kappaB signaling. Stem Cells, 30(4), 655-664. doi:10.1002/stem.1033

Lyle, R., Gehrig, C., Neergaard-Henrichsen, C., Deutsch, S., & Antonarakis, S. E. (2004). Gene expression from the aneuploid chromosome in a trisomy mouse model of down syndrome. Genome Res, 14(7), 1268-1274. doi:10.1101/gr.2090904

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K., & Eisel, U. L. (2004). Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. J Biol Chem, 279(31), 32869-32881. doi:10.1074/jbc.M311766200

Marin, M., Karis, A., Visser, P., Grosveld, F., & Philipsen, S. (1997). Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell, 89(4), 619-628.

Martin, K. R., Corlett, A., Dubach, D., Mustafa, T., Coleman, H. A., Parkington, H. C., . . . Pritchard, M. A. (2012). Over-expression of RCAN1 causes Down syndrome-like hippocampal deficits that alter learning and memory. Hum Mol Genet, 21(13), 3025-3041. doi:10.1093/hmg/dds134

Masliah, E., Terry, R. D., Alford, M., DeTeresa, R., & Hansen, L. A. (1991). Cortical and subcortical patterns of synaptophysinlike immunoreactivity in Alzheimer's disease. Am J Pathol, 138(1), 235-246.

Mattiace, L. A., Kress, Y., Davies, P., Ksiezak-Reding, H., Yen, S. H., & Dickson, D. W. (1991). Ubiquitin-immunoreactive dystrophic neurites in Down's syndrome brains. J Neuropathol Exp Neurol, 50(5), 547-559.

Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. Nature, 430(7000), 631-639. doi:10.1038/nature02621

Mc Donald, J. M., Savva, G. M., Brayne, C., Welzel, A. T., Forster, G., Shankar, G. M., . . . Walsh, D. M. (2010). The presence of sodium dodecyl sulphate-stable Abeta dimers is strongly associated with Alzheimer-type dementia. Brain, 133(Pt 5), 1328-1341. doi:10.1093/brain/awq065

McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., . . . Masters, C. L. (1999). Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol, 46(6), 860-866.

Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., & Baltimore, D. (2003). NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci, 6(10), 1072-1078. doi:10.1038/nn1110

Meulmeester, E., Kunze, M., Hsiao, H. H., Urlaub, H., & Melchior, F. (2008). Mechanism and consequences for paralog-specific sumoylation of ubiquitin-specific protease 25. Mol Cell, 30(5), 610-619. doi:10.1016/j.molcel.2008.03.021

Meusser, B., Hirsch, C., Jarosch, E., & Sommer, T. (2005). ERAD: the long road to destruction. Nat Cell Biol, 7(8), 766-772. doi:10.1038/ncb0805-766

Ming, G. L., & Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron, 70(4), 687-702. doi:10.1016/j.neuron.2011.05.001

Morel, E., Chamoun, Z., Lasiecka, Z. M., Chan, R. B., Williamson, R. L., Vetanovetz, C., . . . Di Paolo, G. (2013). Phosphatidylinositol-3-phosphate regulates sorting and processing of amyloid precursor protein through the endosomal system. Nat Commun, 4, 2250. doi:10.1038/ncomms3250

Motokura, T., & Arnold, A. (1993). Cyclin D and oncogenesis. Curr Opin Genet Dev, 3(1), 5-10.

Mullen, R. J., Buck, C. R., & Smith, A. M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development, 116(1), 201-211.

Mutton, D., Alberman, E., & Hook, E. B. (1996). Cytogenetic and epidemiological findings in Down syndrome, England and Wales 1989 to 1993. National Down Syndrome Cytogenetic Register and the Association of Clinical Cytogeneticists. J Med Genet, 33(5), 387-394.

Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., . . . Inoue, J. (1999). Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes Cells, 4(6), 353-362.

Niendorf, S., Oksche, A., Kisser, A., Lohler, J., Prinz, M., Schorle, H., . . . Knobeloch, K. P. (2007). Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol Cell Biol, 27(13), 5029-5039. doi:10.1128/mcb.01566-06

Norbury, C. J., & Hickson, I. D. (2001). Cellular responses to DNA damage. Annu Rev Pharmacol Toxicol, 41, 367-401. doi:10.1146/annurev.pharmtox.41.1.367

O'Nuallain, S., Flanagan, O., Raffat, I., Avalos, G., & Dineen, B. (2007). The prevalence of Down syndrome in County Galway. Ir Med J, 100(1), 329-331.

Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., . . . LaFerla, F. M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron, 39(3), 409-421.

Ohtani, K., Nakamura, M., Saito, S., Noda, T., Ito, Y., Sugamura, K., & Hinuma, Y. (1987). Identification of two distinct elements in the long terminal repeat of HTLV-I responsible for maximum gene expression. Embo j, 6(2), 389-395.

Pallardo, F. V., Degan, P., d'Ischia, M., Kelly, F. J., Zatterale, A., Calzone, R., . . . Pagano, G. (2006). Multiple evidence for an early age pro-oxidant state in Down Syndrome patients. Biogerontology, 7(4), 211-220. doi:10.1007/s10522-006-9002-5

Palombella, V. J., Rando, O. J., Goldberg, A. L., & Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell, 78(5), 773-785.

Pandey, U. B., Nie, Z., Batlevi, Y., McCray, B. A., Ritson, G. P., Nedelsky, N. B., . . . Taylor, J. P. (2007). HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature, 447(7146), 859-863. doi:10.1038/nature05853

Pastorino, L., Ikin, A. F., Nairn, A. C., Pursnani, A., & Buxbaum, J. D. (2002). The carboxyl-terminus of BACE contains a sorting signal that regulates BACE trafficking but not the formation of total A(beta). Mol Cell Neurosci, 19(2), 175-185. doi:10.1006/mcne.2001.1065

Pennington, B. F., Moon, J., Edgin, J., Stedron, J., & Nadel, L. (2003). The neuropsychology of Down syndrome: evidence for hippocampal dysfunction. Child Dev, 74(1), 75-93.

Pickart, C. M. (2004). Back to the future with ubiquitin. Cell, 116(2), 181-190.

Podlisny, M. B., Lee, G., & Selkoe, D. J. (1987). Gene dosage of the amyloid beta precursor protein in Alzheimer's disease. Science, 238(4827), 669-671.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., . . . Fuller, F. (1988). A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. Nature, 331(6156), 525-527. doi:10.1038/331525a0

Popov, N., Wanzel, M., Madiredjo, M., Zhang, D., Beijersbergen, R., Bernards, R., . . . Eilers, M. (2007). The ubiquitin-specific protease USP28 is required for MYC stability. Nat Cell Biol, 9(7), 765-774. doi:10.1038/ncb1601

Prandini, P., Deutsch, S., Lyle, R., Gagnebin, M., Delucinge Vivier, C., Delorenzi, M., . . . Antonarakis, S. E. (2007). Natural gene-expression variation in Down syndrome modulates the outcome of gene-dosage imbalance. Am J Hum Genet, 81(2), 252-263. doi:10.1086/519248

Qing, H., Zhou, W., Christensen, M. A., Sun, X., Tong, Y., & Song, W. (2004). Degradation of BACE by the ubiquitin-proteasome pathway. Faseb j, 18(13), 1571-1573. doi:10.1096/fj.04-1994fje

Rademakers, R., Cruts, M., & van Broeckhoven, C. (2004). The role of tau (MAPT) in frontotemporal dementia and related tauopathies. Hum Mutat, 24(4), 277-295. doi:10.1002/humu.20086

Ray, R., Snyder, R. C., Thomas, S., Koller, C. A., & Miller, D. M. (1989). Mithramycin blocks protein binding and function of the SV40 early promoter. J Clin Invest, 83(6), 2003-2007. doi:10.1172/jci114110

Rechards, M., Xia, W., Oorschot, V. M., Selkoe, D. J., & Klumperman, J. (2003). Presenilin-1 exists in both pre- and post-Golgi compartments and recycles via COPI-coated membranes. Traffic, 4(8), 553-565.

Ren, Y., Zhao, Y., Lin, D., Xu, X., Zhu, Q., Yao, J., . . . Zhong, B. (2016). The Type I Interferon-IRF7 Axis Mediates Transcriptional Expression of Usp25 Gene. J Biol Chem, 291(25), 13206-13215. doi:10.1074/jbc.M116.718080

Reuther-Madrid, J. Y., Kashatus, D., Chen, S., Li, X., Westwick, J., Davis, R. J., . . . Baldwin Jr, A. S., Jr. (2002). The p65/RelA subunit of NF-kappaB suppresses the sustained, antiapoptotic activity of Jun kinase induced by tumor necrosis factor. Mol Cell Biol, 22(23), 8175-8183.

Reyes-Turcu, F. E., Ventii, K. H., & Wilkinson, K. D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem, 78, 363-397. doi:10.1146/annurev.biochem.78.082307.091526

Richter, C., Park, J. W., & Ames, B. N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci U S A, 85(17), 6465-6467.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., . . . Goldberg, A. L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, 78(5), 761-771.

Roizen, N. J., & Patterson, D. (2003). Down's syndrome. Lancet, 361(9365), 1281-1289. doi:10.1016/s0140-6736(03)12987-x

Rojo, A. I., Salinas, M., Martin, D., Perona, R., & Cuadrado, A. (2004). Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and

nuclear factor-kappaB. J Neurosci, 24(33), 7324-7334. doi:10.1523/jneurosci.2111-04.2004

Sadock, B.J.S., Virginia A., ed. (Kaplan & Sadock's Comprehensive Textbook of Psychiatry (Lippincott Williams & Wilkins)

Saigoh, K., Wang, Y. L., Suh, J. G., Yamanishi, T., Sakai, Y., Kiyosawa, H., . . . Wada, K. (1999). Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. Nat Genet, 23(1), 47-51. doi:10.1038/12647

Saridakis, V., Sheng, Y., Sarkari, F., Holowaty, M. N., Shire, K., Nguyen, T., . . . Frappier, L. (2005). Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. Mol Cell, 18(1), 25-36. doi:10.1016/j.molcel.2005.02.029

Saunders, A. J., Kim, T.-W., & Tanzi, R. E. (1999). <div xmlns="http://www.w3.org/1999/xhtml">BACE Maps to Chromosome 11 and a BACE Homolog, BACE2, Reside in the Obligate Down Syndrome Region of Chromosome 21</div&gt. Science, 286(5443), 1255.

Savatier, P., Lapillonne, H., van Grunsven, L. A., Rudkin, B. B., & Samarut, J. (1996). Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. Oncogene, 12(2), 309-322.

Schafer, K. A. (1998). The cell cycle: a review. Vet Pathol, 35(6), 461-478. doi:10.1177/030098589803500601

Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A., & Uckun, F. M. (1993). Reactive oxygen intermediates activate NF-kappa B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56lck and p59fyn tyrosine kinases in human lymphocytes. Blood, 82(4), 1212-1220.

Schoonbroodt, S., Ferreira, V., Best-Belpomme, M., Boelaert, J. R., Legrand-Poels, S., Korner, M., & Piette, J. (2000). Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I kappa B alpha in NF-kappa B activation by an oxidative stress. J Immunol, 164(8), 4292-4300.

Selkoe, D. J. (2001). Alzheimer's disease: genes, proteins, and therapy. Physiol Rev, 81(2), 741-766.

Seubert, P., Oltersdorf, T., Lee, M. G., Barbour, R., Blomquist, C., Davis, D. L., . . . et al. (1993). Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. Nature, 361(6409), 260-263. doi:10.1038/361260a0

Shi, C. S., & Kehrl, J. H. (2003). Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). J Biol Chem, 278(17), 15429-15434. doi:10.1074/jbc.M211796200

Shi, L., Wen, Y., & Zhang, N. (2014). (1)H, (1)(3)C and (1)(5)N backbone and side-chain resonance assignments of the N-terminal ubiquitin-binding domains of USP25. Biomol NMR Assign, 8(2), 255-258. doi:10.1007/s12104-013-9495-1

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., . . . Martin, S. J. (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical

activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol, 144(2), 281-292.

Small, S. A., & Gandy, S. (2006). Sorting through the cell biology of Alzheimer's disease: intracellular pathways to pathogenesis. Neuron, 52(1), 15-31. doi:10.1016/j.neuron.2006.09.001

Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A., & Markesbery, W. R. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc Natl Acad Sci U S A, 88(23), 10540-10543.

Spillantini, M. G., Bird, T. D., & Ghetti, B. (1998). Frontotemporal dementia and Parkinsonism linked to chromosome 17: a new group of tauopathies. Brain Pathol, 8(2), 387-402.

Stark, S. M., Yassa, M. A., & Stark, C. E. (2010). Individual differences in spatial pattern separation performance associated with healthy aging in humans. Learn Mem, 17(6), 284-288. doi:10.1101/lm.1768110

Sun, B., Halabisky, B., Zhou, Y., Palop, J. J., Yu, G., Mucke, L., & Gan, L. (2009). Imbalance between GABAergic and Glutamatergic Transmission Impairs Adult Neurogenesis in an Animal Model of Alzheimer's Disease. Cell Stem Cell, 5(6), 624-633. doi:10.1016/j.stem.2009.10.003

Sun, X., He, G., & Song, W. (2006). BACE2, as a novel APP theta-secretase, is not responsible for the pathogenesis of Alzheimer's disease in Down syndrome. Faseb j, 20(9), 1369-1376. doi:10.1096/fj.05-5632com

Sun, X., Tong, Y., Qing, H., Chen, C. H., & Song, W. (2006). Increased BACE1 maturation contributes to the pathogenesis of Alzheimer's disease in Down syndrome. Faseb j, 20(9), 1361-1368. doi:10.1096/fj.05-5628com

Sun, X., Wu, Y., Chen, B., Zhang, Z., Zhou, W., Tong, Y., . . . Song, W. (2011). Regulator of calcineurin 1 (RCAN1) facilitates neuronal apoptosis through caspase-3 activation. J Biol Chem, 286(11), 9049-9062. doi:10.1074/jbc.M110.177519

Tak, P. P., & Firestein, G. S. (2001). NF-kappaB: a key role in inflammatory diseases. J Clin Invest, 107(1), 7-11. doi:10.1172/jci11830

Takashima, S., Iida, K., Mito, T., & Arima, M. (1994). Dendritic and histochemical development and ageing in patients with Down's syndrome. J Intellect Disabil Res, 38 (Pt 3), 265-273.

Tang, F., Tang, G., Xiang, J., Dai, Q., Rosner, M. R., & Lin, A. (2002). The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. Mol Cell Biol, 22(24), 8571-8579.

Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A., St George-Hyslop, P., Van Keuren, M. L., . . . Neve, R. L. (1987). Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science, 235(4791), 880-884.

Taoufik, E., Valable, S., Muller, G. J., Roberts, M. L., Divoux, D., Tinel, A., . . . Probert, L. (2007). FLIP(L) protects neurons against in vivo ischemia and in vitro glucose deprivation-induced cell death. J Neurosci, 27(25), 6633-6646. doi:10.1523/jneurosci.1091-07.2007

Teipel, S. J., & Hampel, H. (2006). Neuroanatomy of Down syndrome in vivo: a model of preclinical Alzheimer's disease. Behav Genet, 36(3), 405-415. doi:10.1007/s10519-006-9047-x

Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., & Verma, I. (2002). p53 stabilization is decreased upon NFkappaB activation: a role for NFkappaB in acquisition of resistance to chemotherapy. Cancer Cell, 1(5), 493-503.

Toledano, M. B., & Leonard, W. J. (1991). Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. Proc Natl Acad Sci U S A, 88(10), 4328-4332.

Toner, C. K., Pirogovsky, E., Kirwan, C. B., & Gilbert, P. E. (2009). Visual object pattern separation deficits in nondemented older adults. Learn Mem, 16(5), 338-342. doi:10.1101/lm.1315109

Torres, J., & Watt, F. M. (2008). Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3. Nat Cell Biol, 10(2), 194-201. doi:10.1038/ncb1680

Ulmann, L., Mimouni, V., Roux, S., Porsolt, R., & Poisson, J. P. (2001). Brain and hippocampus fatty acid composition in phospholipid classes of aged-relative cognitive deficit rats. Prostaglandins Leukot Essent Fatty Acids, 64(3), 189-195. doi:10.1054/plef.2001.0260

Valero, R., Bayes, M., Francisca Sanchez-Font, M., Gonzalez-Angulo, O., Gonzalez-Duarte, R., & Marfany, G. (2001). Characterization of alternatively spliced products and tissue-specific isoforms of USP28 and USP25. Genome Biol, 2(10), Research0043.

Valero, R., Marfany, G., Gonzalez-Angulo, O., Gonzalez-Gonzalez, G., Puelles, L., & Gonzalez-Duarte, R. (1999). USP25, a novel gene encoding a deubiquitinating enzyme, is located in the gene-poor region 21q11.2. Genomics, 62(3), 395-405. doi:10.1006/geno.1999.6025

Van Dyke, M. W., & Dervan, P. B. (1983). Chromomycin, mithramycin, and olivomycin binding sites on heterogeneous deoxyribonucleic acid. Footprinting with (methidiumpropyl-EDTA)iron(II). Biochemistry, 22(10), 2373-2377.

Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., . . . Citron, M. (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science, 286(5440), 735-741.

Walsh, D. M., & Selkoe, D. J. (2004). Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept Lett, 11(3), 213-228.

Walter, J., Fluhrer, R., Hartung, B., Willem, M., Kaether, C., Capell, A., . . . Haass, C. (2001). Phosphorylation regulates intracellular trafficking of beta-secretase. J Biol Chem, 276(18), 14634-14641. doi:10.1074/jbc.M011116200

Wang, J., & Song, W. (2016). Regulation of LRRK2 promoter activity and gene expression by Sp1. Mol Brain, 9, 33. doi:10.1186/s13041-016-0215-5

Wang, R., Luo, Y., Ly, P. T., Cai, F., Zhou, W., Zou, H., & Song, W. (2012). Sp1 regulates human huntingtin gene expression. J Mol Neurosci, 47(2), 311-321. doi:10.1007/s12031-012-9739-z

Wang, R., Ying, Z., Zhao, J., Zhang, Y., Wang, R., Lu, H., . . . Qing, H. (2012). Lys(203) and Lys(382) are essential for the proteasomal degradation of BACE1. Curr Alzheimer Res, 9(5), 606-615.

Watanabe, T., Hikichi, Y., Willuweit, A., Shintani, Y., & Horiguchi, T. (2012). FBL2 regulates amyloid precursor protein (APP) metabolism by promoting ubiquitination-dependent APP degradation and inhibition of APP endocytosis. J Neurosci, 32(10), 3352-3365. doi:10.1523/jneurosci.5659-11.2012

Wegiel, J., Kaczmarski, W., Barua, M., Kuchna, I., Nowicki, K., Wang, K. C., . . . Gong, C. X. (2011). Link between DYRK1A overexpression and several-fold enhancement of

neurofibrillary degeneration with 3-repeat tau protein in Down syndrome. J Neuropathol Exp Neurol, 70(1), 36-50. doi:10.1097/NEN.0b013e318202bfa1

Wellmann, H., Kaltschmidt, B., & Kaltschmidt, C. (2001). Retrograde transport of transcription factor NF-kappa B in living neurons. J Biol Chem, 276(15), 11821-11829. doi:10.1074/jbc.M009253200

Widera, D., Mikenberg, I., Elvers, M., Kaltschmidt, C., & Kaltschmidt, B. (2006). Tumor necrosis factor alpha triggers proliferation of adult neural stem cells via IKK/NF-kappaB signaling. BMC Neurosci, 7, 64. doi:10.1186/1471-2202-7-64

Wiener, Z., Ontsouka, E. C., Jakob, S., Torgler, R., Falus, A., Mueller, C., & Brunner, T. (2004). Synergistic induction of the Fas (CD95) ligand promoter by Max and NFkappaB in human non-small lung cancer cells. Exp Cell Res, 299(1), 227-235. doi:10.1016/j.yexcr.2004.05.031

Wisniewski, K. E. (1990). Down syndrome children often have brain with maturation delay, retardation of growth, and cortical dysgenesis. Am J Med Genet Suppl, 7, 274-281.

Wolfe, M. S., De Los Angeles, J., Miller, D. D., Xia, W., & Selkoe, D. J. (1999). Are presenilins intramembrane-cleaving proteases? Implications for the molecular mechanism of Alzheimer's disease. Biochemistry, 38(35), 11223-11230. doi:10.1021/bi991080q

Wu, Y., & Song, W. (2013). Regulation of RCAN1 translation and its role in oxidative stress-induced apoptosis. Faseb j, 27(1), 208-221. doi:10.1096/fj.12-213124

Yabut, O., Domogauer, J., & D'Arcangelo, G. (2010). Dyrk1A overexpression inhibits proliferation and induces premature neuronal differentiation of neural progenitor cells. J Neurosci, 30(11), 4004-4014. doi:10.1523/jneurosci.4711-09.2010

Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., . . . Gurney, M. E. (1999). Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature, 402(6761), 533-537. doi:10.1038/990107

Yang, L., Tao, L. Y., & Chen, X. P. (2007). Roles of NF-kappaB in central nervous system damage and repair. Neurosci Bull, 23(5), 307-313.

Yang, Q., Rasmussen, S. A., & Friedman, J. M. (2002). Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet, 359(9311), 1019-1025.

Yang, W., Lee, Y. H., Jones, A. E., Woolnough, J. L., Zhou, D., Dai, Q., . . . Wang, H. (2014). The histone H2A deubiquitinase Usp16 regulates embryonic stem cell gene expression and lineage commitment. Nat Commun, 5, 3818. doi:10.1038/ncomms4818

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., . . . Ben-Neriah, Y. (1998). Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. Nature, 396(6711), 590-594. doi:10.1038/25159

Yassa, M. A., Mattfeld, A. T., Stark, S. M., & Stark, C. E. (2011). Age-related memory deficits linked to circuit-specific disruptions in the hippocampus. Proc Natl Acad Sci U S A, 108(21), 8873-8878. doi:10.1073/pnas.1101567108

Young, K. M., Bartlett, P. F., & Coulson, E. J. (2006). Neural progenitor number is regulated by nuclear factor-kappaB p65 and p50 subunit-dependent proliferation rather than cell survival. J Neurosci Res, 83(1), 39-49. doi:10.1002/jnr.20702

Yuasa-Kawada, J., Kinoshita-Kawada, M., Wu, G., Rao, Y., & Wu, J. Y. (2009). Midline crossing and Slit responsiveness of commissural axons require USP33. Nat Neurosci, 12(9), 1087-1089. doi:10.1038/nn.2382

Zhang, D., Zaugg, K., Mak, T. W., & Elledge, S. J. (2006). A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. Cell, 126(3), 529-542. doi:10.1016/j.cell.2006.06.039

Zhang, J., Kang, D. E., Xia, W., Okochi, M., Mori, H., Selkoe, D. J., & Koo, E. H. (1998). Subcellular distribution and turnover of presenilins in transfected cells. J Biol Chem, 273(20), 12436-12442.

Zhang, Y., Liu, J., Yao, S., Li, F., Xin, L., Lai, M., . . . Hu, W. (2012). Nuclear factor kappa B signaling initiates early differentiation of neural stem cells. Stem Cells, 30(3), 510-524. doi:10.1002/stem.1006

Zhang, Y., & Song, W. (2017). Islet amyloid polypeptide: Another key molecule in Alzheimer's disease pathogenesis? Prog Neurobiol, 153, 100-120. doi:10.1016/j.pneurobio.2017.03.001

Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A., & Yankner, B. A. (2000). Presenilins are required for gamma-secretase cleavage of beta-APP and transmembrane cleavage of Notch-1. Nat Cell Biol, 2(7), 463-465. doi:10.1038/35017108

Zhao, J., Paganini, L., Mucke, L., Gordon, M., Refolo, L., Carman, M., . . . McConlogue, L. (1996). Beta-secretase processing of the beta-amyloid precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. J Biol Chem, 271(49), 31407-31411.

Zhong, B., Liu, X., Wang, X., Liu, X., Li, H., Darnay, B. G., . . . Dong, C. (2013). Ubiquitin-specific protease 25 regulates TLR4-dependent innate immune responses through deubiquitination of the adaptor protein TRAF3. Sci Signal, 6(275), ra35. doi:10.1126/scisignal.2003708

Zhong, X., & Pittman, R. N. (2006). Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates. Hum Mol Genet, 15(16), 2409-2420. doi:10.1093/hmg/ddl164

Zigman, W. B., & Lott, I. T. (2007). Alzheimer's disease in Down syndrome: neurobiology and risk. Ment Retard Dev Disabil Res Rev, 13(3), 237-246. doi:10.1002/mrdd.20163

Zou, Y., Corniola, R., Leu, D., Khan, A., Sahbaie, P., Chakraborti, A., . . . Huang, T. T. (2012). Extracellular superoxide dismutase is important for hippocampal neurogenesis and preservation of cognitive functions after irradiation. Proc Natl Acad Sci U S A, 109(52), 21522-21527. doi:10.1073/pnas.1216913110