Proteomics analysis of an UBE3A knockout mouse modelling Angelman syndrome

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**NANYANG
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**PROTEOMICS ANALYSIS OF AN *UBE3A* KNOCKOUT
MOUSE
MODELLING ANGELMAN SYNDROME**

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**School of Biological Sciences
2014**

**Proteomics Analysis of
an *UBE3A* knockout Mouse
Modelling Angelman Syndrome**

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Abbreviation

2-D DIGE	Two-dimensional fluorescence difference gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA receptor	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor
ARC	Activity-regulated cytoskeleton-associated protein
AS	Angelman Syndrome
ATP	Adenosine triphosphate
ATP5a1	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit
BAG1	BCL2-associated athanogene 1
bHLH	basic helix-loop-helix
bp	base pair
CaBP	Calcium Binding Protein 28k Dalton
CAL	Cystic fibrosis transmembrane regulator-associated ligand
CaM	Calmodulin protein
CaMKII	Calcium-calmodulin kinase type 2
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHIP	Carboxyl terminus of Hsc 70-interacting protein
CNS	Central nervous system
Da	Dalton
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Media
DNA	deoxyribonucleic acid

DTT	Dithiothreitol
Dube3a	Ube3a orthologue in <i>Drosophila</i>
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E6AP/Ube3a	E3 ubiquitin protein ligase
E6APM/Ube3aM	E3 ubiquitin protein ligase with mutated active site C833A
ECL	Enhanced chemiluminescence
ECT2	Epithelial Cell Transforming sequence 2 oncogene
EDTA	Ethylenediaminetetraacetic acid
Eg5	Kinesin 5
FBS	Fetal Bovine Serum
GABA	Gamma-aminobutyric
GluR2	AMPA receptor GluA2
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSTs-Mu1	Glutathione S-transferase class Mu1
GST	Glutathione S-transferase
HECT domain	Homologous to E6AP C-terminus
HRP	horseradish peroxidase
HSF1	Heat shock factor 1
Hsp70	<i>Mus musculus</i> Heat shock protein 70kDa
HSP70	<i>Homo sapiens</i> Heat shock protein 70kDa
IAA	3-indoleacetic acid
ICAT	isotope-coded affinity tags
IEF	isoelectric point focusing
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
K29	Lysine 29
K48	Lysine 48

K63	Lysine 63
kDa	Kilodaltons
LC-MS/MS	Liquid chromatography in conjunction with tandem mass spectrometry
LDH	Lactate dehydrogenase
LTP	long-term potentiation
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time of flight mass spectrometry
Mash1	Mammalian achaete scute homolog1
Mb	Megabase
MDH	Malate dehydrogenase
NAD ⁺	Nicotinamide adenine dinucleotide
NeuroD	Neurogenic differentiation factor
NSF	N-ethylmaleimide sensitive fusion protein
OPCs	oligodendrocyte precursors
PCR	polymerase chain reaction
PD	Parkinson's disease
Plk2	Polo like kinase 2
PNECs	Pulmonary neuroendocrine cells
PTM	post-translational modification
PVDF	Polyvinylidene fluoride
PWS	Prader-Willi Syndrome
REDOX	Reduction-Oxidation
RING domain	Really Interesting New Gene domain
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoid X receptors
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel

	electrophoresis
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SOD1	Copper-Zinc superoxide dismutase
SOD2	Manganese-superoxide dismutase
SOD3	Extracellular Copper-Zinc superoxide dismutase
SRS	Spontaneous recurrent seizure
TBS	Tris-Buffered Saline
TCM	Traditional Chinese medicine
TE	Tris-EDTA
TFA	Trifluoroacetic Acid
TGF- β R-II	Transforming growth factor β receptor type II
TPI	Triosephosphate isomerase 1
Ub	Ubiquitin
UPD	Uniparental disomy
UPS	Ubiquitin proteasome system
UVR	Ultra violet radiation
VDR	Vitamin D receptor
WDS	Wet dog shakes

Summary

Angelman syndrome is a neurobehavioral disease associated with the loss of maternally expressed E3 ubiquitin protein ligase, Ube3a. *Ube3a* gene is biallelically expressed in all tissue except cerebellum, Purkinje cells and hippocampus, in which *Ube3a* is expressed exclusively from maternally inherited chromosome. By using an *Ube3a* knockout mouse model, the effect of loss of Ube3a in brain has been studied using 2-D DIGE method. Due to the fact that Ube3a is involved in ubiquitin-related proteasomal degradation, its substrates and downstream targets are expected to be differentially expressed in knockout and wild-type models. A total of 94 proteins from cerebellum and 74 proteins from hippocampus were found differentially expressed in the *Ube3a* knockout mice using 2-D DIGE, 14 of them were statistically significant. Western blot and Real Time RT PCR were then employed to examine the protein level and mRNA level of those proteins, respectively. CaBP was found downregulated at mRNA as well as protein level; its function as calcium ion buffer may play an inductive role in the seizure observed in AS mouse model and patients.

Despite substantial progress made in the treatment of neurological disorders, epilepsy remains a significant therapeutic challenge; epilepsy is also one of the common features observed in Angelman syndrome. Currently available antiepileptic drugs neither cure nor prevent relapse, and are often accompanied by debilitating adverse effects. *Ube3a* knockout mice have been subjected to

Traditional Chinese herbs, 60% of the tested mice responded well to the herbs administrated. In these mice, CaBP protein level has been restored to the same level as wild type mice and susceptibility to audiogenic seizures has been reduced. Chaperone protein Hsp70 was found to be accumulated at protein level and remain unchanged at mRNA level, these results suggested that Hsp70 might be a substrate of Ube3a. *In-vitro* and *In-vivo* interaction studies have been conducted to confirm that the interaction between Hsp70 and Ube3a facilitates protein degradation; *In-vitro* ubiquitination assay has been conducted and the result suggested that Ube3a was able to ubiquitinate Hsp70, presumably for its degradation. Few proteins that are involved in REDOX have been identified in 2-D DIGE, including LDH, MDH, GSTs-Mu1, SOD2, ATP5a1; the result suggested that the loss of Ube3a may also lead to mitochondrial dysfunction which eventually lead to cell death. *NSF* involved in neuronal signal transmission is reduced at protein level but not affected at the mRNA level. However, differential expression of NSF protein is tissue specific and needs further investigation to determine its effect to AS patients. In summary, loss of Ube3a lead to differential expression of multiple proteins which may contribute to the pathological symptoms observed in the AS mouse model and patients.

Chapter I Introduction

1.1 Angelman syndrome (AS)

Angelman syndrome (AS) is a genetic disorder with an incidence of 1 in 15,000 and it was first described in 1965 by Harry Angelman (1,2). It is characterized by a severe developmental delay together with mental and movement disorder, and behavioral abnormalities. Early severe epilepsy, sleep alteration, ataxia of gait, absence of language and craniofacial dysmorphism are phenotypic characteristics used as diagnostic criteria of AS (3).

Several genetic mechanisms are known to associate with the development of AS including the deletion of 4 Mb regions in chromosome 15q11-13, uniparental disomy (UPD), imprinting centre defects, and point mutations in *UBE3A* gene (1,4). Genomic imprinting refers to the differential marking in the germline and imprinted genes retaining molecular memory of their parental origin, thus resulting in differential allelic expression during development (4). The loss of expression of imprinted genes causes multiple human genetic disorders, including AS and Prader-Willi syndrome (PWS). Although these two diseases are associated with the lack of gene expression from the same chromosome 15q11-q13 region, the clinical features of two disorders are distinct. Deletion or loss of paternally inherited gene expression results in PWS; while loss of maternally inherited gene expression causes AS (4).

Multiple mouse models have been developed for AS study (Table 1). The first reported AS mouse model generated was a mouse with paternal UPD for chromosome 7 (5), followed by another mouse model generated by radiation-induced deletion of *p* locus and *Ube3a* (6). However, these two models carried a large deletion of mouse chromosome 7C that could affect multiple loci (1). Therefore, in the current study, we used a mouse model which carried an exon 2 deletion of the *Ube3a* gene resulting in a shift in the reading frame, resulting in all putative isoforms of Ube3a being inactivated (7). If the offspring mice inherited the mutated *Ube3a* allele from the maternal origin, the mice will have no Ube3a expression in the cerebellum, Purkinje cells and hippocampus since *Ube3a* on paternal chromosome is silenced by genomic imprinting. This mice model exhibits symptoms similar to that of AS patients, including motor dysfunction, seizures, context-dependant learning deficit and severely impaired long-term potentiation (LTP) (7). Another *Ube3a* mutant mice has been initiated by the same group (8) with 1.6-Mb chromosomal deletion from *Ube3a* to *Gabrb3*; the deletion inactive *Ube3a*, *Gabrb3* and deleted *Atp10a* gene. Homozygous mutation didn't caused embryonic lethality but such mice died in prenatal period due to cleft palate resulting from null mutation of GABA receptor *Gabrb3*. Maternal deletion mice displayed significant impaired motor function, increased seizure attack and having difficult in learning and memory task. Function of *Atp10a* in mammal is remains unknown, however, the observation suggest that it do not have essential function in embryonic

development but studies have indicated that it may have an important role in insulin resistant and glucose metabolism. Another similar mouse model (9) has been generated by targeted inactivation of Ube3a; the inactive allele contains a lacZ reporter gene for brain specific imprinting analysis. Maternal inheritance of Ube3a mutant allele impaired the motor function, spatial learning, displayed abnormal EEG recording.

Mice carried mutation in $\beta 3$ subunit of γ -Aminobutyric acid type A receptors (GABA_A-Rs) exhibited clinical features observed in AS mice (10). Those features are hyperactive and hypersensitive to human contact or sensory stimuli; display frequent myoclonus, lack of coordination and epileptic seizure. GABA_A-Rs are heteromeric ligand gated chloride channels; it's the binding sites for many clinically important drugs; it also mediates bulk of rapid inhibitory synaptic transmission in the CNS. β^3 subunit is an essential component in GABA_A-Rs; when it's inactivated, GABA_A-R density is halved in brain of β^3 -deficient mice and its function is severely impaired. Most β^3 deficient mice died at neonatal stage, some but not all are accompanied by cleft palate.

There are AS mouse models that are generated through mutation in imprinting centre. Imprinting centre is established by imprinting through methylation; since PWS-IC is a positive regulatory element required for bi-directional activation of a number of paternally expressed genes; by replacing the mouse

PWS-IC with human PWS-IC (11), maternal imprinting defect result in expression of maternal *Ube3a* AS and repression of *Ube3a* in Cis.

Families with deletions causing AS imprinting defects localize the AS-IC to 35 kb upstream of the PWS-IC, and two mouse mutations resulting in defects similar to that seen in AS patients with deletion of the AS-IC have been reported (12). Those two mutations are 1) an insertion/duplication mutation 13 kb upstream of *Snrpn* exon 1 resulted in lack of methylation at the maternal *Snrpn* promoter, activated maternally repressed genes, and decreased expression of paternally repressed genes. 2) an 80-kb deletion extending upstream of the first mutation, caused a similar imprinting defect with variable penetrance. Another AS mouse model (13) that is generated by transgene insertion and caused a complete deletion of the 4-Mb PWS/AS-homologous region; maternal transmission of the deletion result in loss of imprinted expression of *Ube3a* gene.

Table 1. Angelman syndrome mouse models explain the function of those genes

	Mutation	Phenotype	Reference
1	<i>Ube3a</i> exon 2 deletion	AS	(7)
2	LacZ insertion inactivation of <i>Ube3a</i>	AS	(9)
3	insertion/duplication located 13 kb upstream of <i>Snrpn</i> exon 1	AS imprinting mutation	(12)
4	80-kb deletion located upstream of <i>Snrpn</i> exon 1	AS imprinting mutation	(12)
5	<i>Ube3a-Gabrb3 –</i> <i>Atp10a</i> deletion	AS	(8)
6	Replacement of mouse PWS-IC with human PWS-IC	PWS and AS imprinting mutation	(11)
7	UPD	AS	(5)
8	GABRB3 inactivation	Some clinical features of AS; 90% of $\beta 3^{-/-}$ mice die within 24 h of birth, survived mice exhibit hyperactive, epileptic seizures, neurological impairments	(10)
9	Transgenic insertion induced deletion; <i>Zfp127-Herc2</i> deletion	PWS/AS	(13)

1.2 *UBE3A* & Ubiquitination

UBE3A is located at human chromosome 15q11-13, which contains a bipartite imprinting centre and a cluster of imprinted genes [Figure 1]. *UBE3A* encodes UBE3A/E6AP which is expressed biallelically in all somatic cells, except for Purkinje cells, hippocampal neurons and mitral cells of the olfactory bulb, in which only the maternal allele is expressed (4). UBE3A is one of the E3-ligases responsible for protein ubiquitination that was thought to be mainly responsible for protein degradation. Studies showed that loss of maternal expression of *UBE3A* is associated with AS and autism spectrum disorders (ASDs). There are approximately 10% of AS cases caused by mutation in *UBE3A* gene (14).

Ubiquitination is the process of conjugating a 76 amino acids ubiquitin moiety to the lysine residues of target substrate proteins. Three enzymes are involved in the reaction including ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. E3 is responsible for transferring ubiquitin from E2 to the substrates. The major role of ubiquitination is to control the turnover of substrate proteins, whereby the ubiquitin-tagged substrates are targeted to 26S proteasome for degradation (15). However, ubiquitination is also recently found to associate with degradation-independent pathways such as regulation of signalling pathway by co-activating the transcription activity of human progesterone receptor and other members of nuclear hormone receptors superfamily (16,17), and regulation of endocytotic pathways by ubiquitination

of plasma membrane proteins (18).

In humans, there are two E1 activating enzymes with preference for different E2 enzymes, dozens of E2 conjugating enzymes and hundreds of E3 ligases. Ubiquitination specificity is determined principally by E3 ligases, as each E3 ligase targets a specific set of substrates only (19). E3 ligases belong to a large, structurally and functionally diverse protein family. E3 ligases can be grouped into 2 major classes based on a common structural motif, the HECT (Homologous to E6AP C-terminus) class and RING/RING-like class. RING-like class of E3s, which contain a U-box domain, are structurally related but lack the zinc ligands in RING domain. The major differences between HECT and RING classes of E3s are 1) structural and acting domain difference as well as 2) the mechanism to mediate substrates ubiquitination. HECT E3 ligases such as Ube3a/E6AP, formed a thioester intermediate with ubiquitin before transferring it to the substrates; while RING E3 ligases such as Parkin, do not form such intermediate [Figure 2]. Due to its significant and diverse functions, E3 ligases are involved in many diseases such as cancers development and neurological disorders (20).

Ubiquitin is a 8.5kDa protein with seven lysine residues that can form linkages with target substrates. Each of the ubiquitin lysine linkage appears to translate into different biological functions. The most common linkage is the lysine 48 linkage, which is normally necessary for protein substrates degradation. On the

other hand, lysine 29 and lysine 63 linkages played a role in protein-protein interactions that are mediating signal transduction, substrate translocation and DNA repair other than proteasomal degradation (21). Ubiquitination of proteins can result in diverse functions depending on the topology of the ubiquitin chain.

Protein ubiquitination offers versatile biological function in different physiological conditions. Following ischemia, ubiquitination may regulate autophagic process of damage-induced protein aggregates or mediate inflammatory responses initiated from brain injury (22). On the other hand, knockdown of Ube3a in cell-lines has been known to dramatically enhance the polyglutamine protein aggregation which in turn induces cell death. Overexpression of Ube3a has been shown to protect cells against the toxicity of polyglutamine proteins aggregation in neurodegenerative disease (23).

Ube3a deficiency in AS mice exhibits severe neurological impairment and motor abnormalities. Although no abnormal dendritic branching and neuronal loss in cerebellar Purkinje cell is observed, loss of maternal E6AP does affect the morphology of dendritic spines on cerebellar Purkinje cells, hippocampal and cortical pyramidal neurons (24). It is possible that the loss of maternal E6AP may stabilize some of its target substrates, which are involved in cellular senescence and tumorigenesis (25). Interestingly, it has recently been shown that mice with *UBE3A* deficiency shows abnormal mitochondria and exhibits partial oxidative phosphorylation defect (26).

Figure 1. Genetic map of human chromosome 15q11-13

Genes are imprinted in this region, which means that they have parental specific expression. For example, *NECDIN* (NDN) has paternal specific expression, while *UBE3A* has expression from maternal allele only. (+, expressed; -, not expressed) Figure adapted from (4).

Figure 2. The ubiquitin-proteasome pathway

Degradation of a misfolded protein via the ubiquitin-proteasome pathway (UPS) involves chaperons, covalent attachment of multiple ubiquitin molecules and degradation of the tagged protein by the 26S proteasome. Molecular chaperones function in guiding protein folding and in the degradation of misfolded species. By associating with exposed hydrophobic domain, chaperones Hsp70/40 promote the folding of the newly synthesized proteins and favour their refolding. Alternatively, they can facilitate the recognition of abnormal proteins, leading to their ubiquitination by carboxyl terminus of Hsc 70-interacting protein (CHIP), the E3, and their degradation by the 26S proteasome. The red circles represent ubiquitin. Figure adapted from (27).

Mice with a maternal null mutation in *Ube3a* have defects in long term potentiation (LTP), manifest motor and behavioral abnormalities that parallel findings in AS in spite of normal brain architecture. The neurological deficits have been directly linked to the postsynaptic calcium-calmodulin kinase type 2 (CaMKII) signaling pathway (28) and lack of β_3 subunit of the GABA_A receptor (29). CaMKII signaling pathway in the pyramidal neurons of the hippocampus and cortex shows increasing inhibitory phosphorylation of α CaMKII in the brain (28). Moreover, recent work has demonstrated that neurological deficits in these AS mice can be rescued through a mutation in CaMKII that prevent it from inhibitory phosphorylation (30). Lack of β_3 subunit of the GABA_A receptor is sufficient to cause neurological phenotypes observed in AS mice, such as seizure, memory and learning impairments (29). Accumulating evidence reveals that there is a link between synaptic plasticity and adult hippocampal neurogenesis. It is found that adult neurogenesis and plasticity are affected by *Ube3a* mutation (31). In addition, studies show that Ube3a ubiquitinates and degrades Arc promoting AMPAR expression at synapses. Deficiency of maternally inherited *Ube3a* is found to enhance the cellular level of Arc and lead to a decrease in the number of AMPA receptors at excitatory synapse which may contribute to cognitive dysfunction in AS and ASDs (31,32).

Dube3a (Ube3a orthologue in *Drosophila*) affects the neurite outgrowth in post-mitotic cells. These changes may be mediated through Rho-GEF Pebble (Pbl)

and its regulation of actin-binding GTPase Rac2, which regulates dynamic remodeling of actin cytoskeleton in response to all known neurite stimulus. Epithelial cell transforming sequence 2 oncogene (ECT2, Rho-GEF Pebble (Pbl) orthologue in mammal) is regulated by UBE3A and is redistributed in ectopic regions of cerebellum and hippocampus. This may attribute to general learning difficulties and behavior defect in AS patients (33).

1.3 Project scope

In this project, I intended to investigate the effects of the loss of UBE3A proteins by using *Ube3a* knockout mice, especially those effects in brain where expression of Ube3a is diminished. Since Ube3a is one of the E3-ligases who responsible for the protein degradation, the proteomic profile of *Ube3a* knockout mice should be altered as substrate of the Ube3a might accumulate as the result of knockout. Therefore, 2-D DIGE method was used to identify candidate substrates for Ube3a in cerebellum and hippocampus. After that, western blot analysis has been performed to confirm the observation in 2-D DIGE. Real-Time PCR has been applied to study those affected genes at transcriptional level. Protein-protein interaction studies have been conducted to study the linkage between Ube3a and protein that was differentially expressed.

1.4 Proteomic Techniques

As proteins are responsible for most of the important tasks in almost all cellular processes (34), it is important to understand the protein profile of AS mouse model which might help in the discovery of biomarkers that are crucial in disease development as well as putative targets of Ube3a. This is relevant to our study as no functional Ube3a is expressed in the mouse model that we used. Due to the functionality of UBE3A as E3-ligases, it is believed that the turnover of many proteins will be affected. Hence, we employed proteomic technique to study the protein profile of AS. Currently, there are two common approaches in proteomic study, gel-based and gel-independent methodology. Isoelectric focusing followed by two-dimensional gel electrophoresis (2-DE) is the conventional gel-based methodology. This method has been developed for years and is able to resolve few hundreds to thousands of proteins, including post-translational modified proteins in one routine run. Advance digital image analysis and database software has been developed to ease the post-separation analysis. However, gel based methods have drawbacks as they require a large quantity of sample, are difficult to reproduce due to gel-to-gel variation in spot patterns, proteins that are extremely acidic or basic are impossible to detect by conventional 2-D gel electrophoresis, and it also suffers in poor dynamic range of present post-staining methods (34). Recent technique developments such as 2-D DIGE method used in this study has avoided the problem of high gel to gel variation, and its fluorescent dye improves the dynamic range in samples

comparison (35). Fluorescence two-dimensional differential gel electrophoresis or 2-D DIGE is designed to solve such limitation by allowing different samples to be resolved on the same gel. It is achieved by labelling sample with three different fluorophores (Cy2, Cy3 and Cy5) that are identical in size (500Da) and charge (+1). These fluorophores are also structurally similar but spectrally distinct [Figure 3]. The covalent labelling can be achieved by a nucleophilic substitution reaction with lysine to form an amide. Same as fluorophores, the lysine also carries a positive charge, so that the substitution will not affect the charge distribution of the protein. Two of the fluorophores will be used to label control and treated samples, while a third fluorophore will be used to label the internal control, ideally in a pool comprising equal amount of control and treated samples. This pooled standard is used to normalize protein abundance measurements across multiple gels in an experiment. The fluorophores labelled proteins are then separated according to their isoelectric focusing point on IEF strip, and with the help of carrier ampholytes, proteins will migrate to either end of electric field (+ or -), until they are no longer carrying any charge, thus reaching their isoelectric point. Next, the proteins are resolved according to their molecular weight by gel electrophoresis. The size separated proteins are then visualized by using the Typhoon imager (Amersham), and this is achieved by excitation of fluorophores with different wavelengths generated by different lasers. By comparing 2-D spot patterns from different samples using DeCyder software (Amersham), for example, wild type and disease sample to internal control, changes in protein expression can be detected and quantified. This

allows us to identify biomarkers of a particular disease state or the physiological state in cells or tissue.

Figure 3. The structure of cyanine dyes

All of the CyDye are approximately 500 Da, and carry a +1 charge. It undergoes a nucleophilic attack to substitute a lysine residue in protein. [Figure adapted from Tonge et al. (2001), Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics*, **1**, 377-396]

Liquid Chromatography in conjunction with tandem mass spectrometry (LC-MS/MS) and isotope-coded affinity tags (ICAT) are gel-independent methods employed to conduct proteomic study. Protein sample sets are labelled separately with linker containing either eight hydrogen or eight deuterium atoms at cysteine residues. Thus, mass difference has been introduced before samples are digested and analyzed by LC-MS/MS. The intensity of signal

generated by tagged peptide is translated into the abundance of the protein and the MS/MS profile is used for protein identification (34). The sample amount needed for gel-independent methods is small if compared to 2-DE and its multiplex platform allowed the separation and identification of proteins at the same isoelectric point and molecular mass. The gel-independent platform is also benefited by its ability to mine complex data with higher throughput and good reproducibility (36). Nonetheless, the drawbacks of ICAT technique include inability to quantify proteins lacking cysteines and is limited by chromatographic sample capacity, the analysis time as well as the dynamic range of peptide detection (36,37).

There is another gel-independent proteomics analytical method available called Isobaric Tags for Relative and Absolute Quantification (iTRAQ). Similar to ICAT, it utilized stable isotope labeling and mass spectrometry for quantitative study. However, in contrast to ICAT; its application didn't limited to a binary set of reagents/samples (38). iTRAQ technique was initially used four isobaric reagents (114, 115, 116 and 117 m/z) for stable isotope affinity tagging, allowing the multiplexing of four different samples in single analysis. Recently, the manufacturer has added another four isotope tagging reagents (113, 118, 119, and 121 m/z) in order to extend the range of multiplex platform (38). The 8-plex reagents have been used to study cerebrospinal fluid (CSF) proteins from subjects undergoing intravenous Ig treatment for Alzheimer's disease at different time points (39) as well as comparison studies of activity of six

Leukemogenic Tyrosin Kinases simultaneously (40). The multiplexing capability allows a control sample to be compared with other analytical samples under different circumstances, for example, different points in time of a disease state, different drug treatments (41).

In iTRAQ, protein samples were first reduced and digested by trypsin before labeling. The isobaric mass labels were then placed at the N termini and lysine side chains of peptides in the digestion mix. Each sample was labeled with one of the isotopically labeled tags. Labeled samples were then pooled before subjected to column chromatography for fractionation and further analyzed by LC-MS/MS. The derivatized peptides are indistinguishable in MS profile due to additional balance group was added along with the reporter group, but neutral loss of the balance group occurs during the MS/MS and allowed reporter group to be appeared as distinct masses. The relative quantification is thus performed at the MS/MS stage rather than MS stage in ICAT. Absolute quantification can be achieved by using synthetic peptides tagged with one of the isobaric labels (38).

iTRAQ technique has gained popularity in proteomic studies due to its multiplex ability and sensitivity. However, potential drawbacks of iTRAQ including quantification at MS/MS stage may need more time for data analysis (38), enzymatic digestion prior to labeling which artificially increases sample complexity (42) by introduced errors during sample handling or variable

degrees of tryptic digestion between samples (41). A recent paper has also identified a few pitfalls of iTRAQ technique including, isotopic contamination, background interference and signal-to-noise ratio (43). It showed that 117 reporter ion is most likely to be affected by background as its intensity is lower. The intensity of 121 reporter ion may become partially impaired in low resolution instruments by the close transmission of the phenylalanine immonium ion's isotope in phenylalanine-containing peptides and in turn may dampen the accuracy in quantification (43).

Comparison studies have been performed using gel based and LC-based protein quantitative methods (41,44). In general, ICAT, iTRAQ and 2D-DIGE methods yielded reasonably good accuracy in quantification but iTRAQ and 2D-DIGE both have higher sensitivity when compared to ICAT (44). In this study, we selected 2-D DIGE [Figure 4] to investigate the protein expressions in the *Ube3a* knockout and wild type mice.

Figure 4. Work flow of 2-D DIGE experiment

By comparing protein samples from wild type mice and AS mice in 2-D DIGE study, protein candidates that showed differential expression were found and identified by MALDI-TOF-MS method. Western blot was then employed to confirm the differential expression observed in 2-D DIGE. Real time RT PCR was used to detect any difference at transcriptional level in protein candidates identified. Few proteins were studied for interaction studies by in-vitro and in-vivo methods, followed by ubiquitination assay.

A total of 94 proteins and 74 proteins were initially found differentially expressed in cerebellum and hippocampus of the *Ube3a* knockout mice, respectively. Cerebellum and hippocampus were chosen to study because *Ube3a* is found expressed on maternal allele only in these two tissues; therefore the effect of loss of Ube3a should be distinctive. Next, those protein candidates were tested by filtering criteria such as differential expression observed in all 2-D DIGE runs examined, 1-way ANOVA statistic test and expression level of those proteins must have at least 40% difference when comparing *Ube3a* knockout against wild type mice. Those differentially expressed proteins were then identified by MALDI-TOF with a threshold score of 63. A total of 10 proteins that were successfully identified and fulfilled all the filtering criteria, including CaBP, HSP70, SOD2, LDH, MDH, GSTs-Mu1, NSF, ATP5a1, Cofilin, Triosephosphate isomerase 1. As CaBP play a critical role in calcium signalling in the brain (45), four additional proteins including Mash 1, NeuroD, Pax6 and VDR that were not detected in 2-D DIGE runs were investigated in this project. The biological functions of identified proteins including CaBP, Hsp70 and REDOX related proteins are further elaborated below. Differentially expressed proteins identified by the 2-D DIGE could be due to the direct and/or indirect effect from the loss of Ube3a expression; and Ube3a is known to involve in proteasome-dependent as well as proteasome-independent functions. Therefore, Western blot analysis was performed to verify the differentially expressed proteins identified by 2-D DIGE analysis and further studied at the mRNA level by real-time RT PCR. It helps in understanding whether these

proteins candidates that were affected at protein level might be the result of impaired protein degradation mechanism (4). On the other hand, those proteins may also be affected at transcriptional level as Ube3a is also involved in transcription regulations (16). Next, in-vivo and in-vitro interaction studies were carried out between Ube3a and identified protein, HSP70 that were affected at protein level. HSP70 protein was chosen because it is the only accumulated protein identified in this study, and due to fact that Ube3a is involved in proteosomal pathway, it is interesting to study the relationship between these two proteins. Then, the ubiquitination assay was used to examine the ability of Ube3a to ubiquitinate Hsp70. All mice used in this study were age match (3 months old) as well as gender match to eliminate any bias and difference in development.

1.4 HSP70 (Heat-shock protein 70kda)

The role of ubiquitin proteasome system (UPS) is to remove misfolded and abnormally translated proteins (15). Reports showed that under normal and healthy circumstances, over 30% of newly synthesized proteins are misfolded and aggregated (46). However, little is known about how these abnormal proteins are recognized for degradation. Classical functions of molecular chaperones families include recognition of non-native conformations of proteins, assisting in protein folding, and preventing aggregation of misfolded

or partially folded proteins (27). Members of Hsp70 protein family are highly conserved throughout the evolution process, and they share a common molecular weight and structure. It contains two main functional domains: an N-terminal nucleotide-binding domain with an ATPase domain, in which ATP exchange drive conformational change of Hsp70, as well as a C-terminal substrate binding domain that seems to form a lid over the peptide-binding cleft that flanked both N- and C- termini. The lid formed may function in permitting the binding and release of the substrate. ATP binding and hydrolysis facilitate the conformational change of HSP70, which leads to lid closure and stabilizes the binding of the substrate. The Hsp70 proteins bind to misfolded/partially folded proteins promiscuously during translation or after stress-mediated protein damage (47).

Expression and accumulation of β -amyloid in neuron cells, which are commonly seen in Alzheimer's disease, induces upregulation of Hsp70. Hsp70 participates in the neuroprotective response and rescues neuron cells from toxic effect of protein aggregates (48). Hsp70 overexpression has been shown to prevent the aggregation of mutant ataxins (49) and huntingtin (50), which eventually abrogates cell death. Cytoprotective effect of Hsp70 is related to the chaperone ability to prevent protein aggregation with the cooperation of E3 ligases and chaperones are able to assist with the refolding of denatured or partially folded proteins (51). When molecular handling capacity of chaperone is overwhelmed due to an increase in the amount of damaged proteins; the

“chaperone overload” effect would allow the accumulation in cells of unchaperoned misfolded proteins (52).

Hsp70 is known to interact with E3 ligase such as CHIP and Parkin. CHIP contains a U-box domain which targets protein substrates for proteasome mediated degradation; its targets include several HSP70-client proteins that are involved in neurodegenerative disease (53). CHIP also directly regulates the recovery of cells following heat shock by ubiquitinating stress-induced HSP70 protein once all other clients have been removed (54). CHIP has been shown to have anti-apoptotic function because it and HSP70 suppresses caspase-3 activation to prevent cell death (55). Another E3 ligase Parkin has identified Hsp70 as one of its substrates, but multiple mono-ubiquitination of Hsp70 by Parkin does not lead to proteasomal degradation of Hsp70 (56). Instead, mono-ubiquitinated Hsp70 inactivates JNK-c-Jun pathway that is crucial for Parkin to regulate expression of motor protein Eg5 (57). With the loss of Ube3a in AS patients, its substrates might be damaged and form protein aggregates. However, no inclusion body has been observed or reported in AS patients. Nevertheless, recent evidence suggest that the soluble protein intermediates may not form visible pathological aggregates like the inclusion body observed in neurodegenerative diseases, however such intermediates may still be associated with diseases caused by neurotoxicity (58).

Cytosolic chaperones perform the crucial role within the cell of surveillance

and repair; this is a function that becomes more difficult in non-dividing cells such as neurons. The reason is chaperone activity decreases over lifetime which is the fundamental principle of neurons (58). Heat-shock factor (HSF1) is responsible for activating the transcription of heat-shock genes which encodes chaperones in response to heat or environmental stress. Expression of *hsf-1* has been suggested as the crucial system that enables cells to deal with stress-induced dynamics and low expression of it accelerates tissue aging and shortens life-span of *Caenorhabditis elegans* (59). In *Drosophila melanogaster*, the presence of heat-induced Hsp70 increases its life-span at normal temperature even with only 10-12% more expression than the standard expression level (60).

By interacting with cellular proteins that are responsible for REDOX homeostasis, HSP70 may exhibit protective effects by preventing oxidative stress in cells. Upregulation of HSP70 by gene transfection in mice has shown that it could preserve the activity and content of Mn-superoxide dismutase (SOD2) (61). Another study has shown that under oxidative stress, activity levels of SOD, GSH-Px and catalase are decreased, whereas HSP70 expression is elevated in the masseter muscle of rats. However, the roles of elevated HSP70 still need to be confirmed (62). Since HSP70 is involved in the regulatory steps within apoptotic pathways, it may also maintain cell survival by mediating upstream regulators of apoptosis, for example by dealing with oxidative stress, and Ca^{2+} overload. Given that Ca^{2+} overload has long been implicated in cell death, HSP70 can prevent cell death by stabilizing muscle

cells through maintaining cellular Ca^{2+} homeostasis and by protecting the structure and function of cardiac Ca^{2+} pump (61).

1.5 CaBP1 (Calbindin D-28K)

The Ca^{2+} -buffering in neurons can be achieved by 3 major components: Ca^{2+} transport across the membrane by calcium pump, sequestration by cellular organelles, and by cytosolic proteins such as parvalbumin and Calbindin D-28k (63). Normal levels of Ca^{2+} is essential in signal transduction, controlling the development of its own regulating proteins to modulate calcium ion transients, neurogenesis and many other functions (45). CaBP1 was originally described as a 27 kDa protein induced by vitamin D in the duodenum of the chick (64). In the brain, its synthesis is independent of vitamin-D-derived hormones (65). Lack of cytoplasmic calbindin D-28k severely impairs Ca^{2+} homeostasis and causes nerve cells to be selectively vulnerable to Ca^{2+} related injury (66). Nearly 2/3 of dentate gyrus granule cells lose calbindin D-28k after kindling-induced epilepsy (63).

The neurons in brains of Huntington disease patients are calbindin-depleted. The distinctive loss of an intra-neuronal calcium binding protein (Calbindin D-28k) may severely impair Ca^{2+} homeostasis in these neurons. Calbindin-D 28k mRNA level was also reduced in Alzheimer's hippocampus as compared to Huntington control (67). A decrease in CaBP levels is reported in degenerating

hippocampal neurons of rats and humans. Dentate gyrus granule cells (GCs) have decreased CaBP immunoreactivity following kindling resulting from a genuine decrease in granule cell CaBP; this highly specific loss of CaBP occurs in dentate gyrus during and following kindling-induced epilepsy (63).

Calbindin-D 28k and parvalbumin are both absent in motor neuron population in the early stage of SOD1 transgenic mouse model (68). Another study has shown that motor coordination is severely impaired in calbindin D-28k null mutant mice (69). Mutation in SOD1 is highly related to another neurodegenerative disease, Amyotrophic lateral sclerosis (ALS), in which patients suffer from motor neuron defect (70). Environmental oxidative stress, reactive oxygen species (ROS) and disruption of calcium regulatory mechanism are associated with several neurodegenerative diseases. Oxidized medication causes those proteins to lose their functions (71). Surging free Ca^{2+} level can induce apoptosis and necrosis, disruption of actin, activates caspase-12 and causes damage in CNS and kidney (70).

1.6 NSF (N-ethylmaleimide sensitive fusion protein)

N-ethylmaleimide sensitive fusion protein (NSF) is a homoheptameric ATPase that is primarily involved in membrane fusion of transport vesicles with Golgi cisternae. NSF and other proteins form a complex and fuse to SNAPs (soluble NSF attachment proteins) and the SNARE (SNAP receptors). It serves as a site

for docking and fusion of synaptic vesicles at the plasma membrane during synaptogenesis and is a likely target for regulatory mechanism. NSF is ubiquitously found in the cytoplasm of eukaryotic cells, but is preferentially expressed in the neuron system with the most abundant expression in the hippocampus (72). It is involved in neurotransmitter release and regulates the kinetics of neurotransmitter vesicle fusion (73).

AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors play an important role in neuronal development, synaptic plasticity and neurodegeneration. Dynamic interaction among NSF, Plk2 (Polo like kinase) and GluR2 subunit of AMPA receptors results in a significant, progressive decrement of AMPA receptor-mediated synaptic transmission. It is thought to maintain synaptic plasticity homeostasis that is important in stabilization of neuronal firing (73,74). Study has shown that mutation of comatose (*Drosophila* homolog of NSF) in *Drosophila* causes conditional paralysis at restrictive temperature, presumably due to blockage of synaptic transmission after inactivated NSF causes SNARE (Soluble NSF Attachment Protein Receptor) complexes dissociation(75). AMPA receptors play a pivotal role in neuronal signal transduction (75,76).

Spontaneous recurrent seizure (SRS) has been associated with downregulation of epilepsy-related gene 1 (*ERG1*) transcription. While *ERG1* encodes a *Rattus* homologue of NSF, it has also been identified as an epilepsy related gene.

ERG1/NSF gene expression is also found in specific regions of brain that are seizure sensitive (77). Downregulation of NSF causes a rise in neurite outgrowth in cultured hippocampal neurons and PC12 cells; this phenomenon is similar to the structural change observed after epilepsy (78). NSF expression can be regulated by several mechanisms, including reversible inactivation by phosphorylation, S-nitrosylation and oxidation. The oxidation of NSF can be performed by hydrogen peroxide. It is suggested that NSF could be a REDOX sensor in cell in which its activity is decreased when oxidation state increased (79).

1.7 REDOX reaction

Mitochondria play major roles in cellular metabolisms like ATP production through oxidative phosphorylation, regulating intracellular calcium ion homeostasis, and producing reactive oxygen species (ROS). Increasing mitochondrial calcium overload as a result of excitotoxicity is associated with the generation of ROS and may induce the release of pro-apoptotic mitochondrial proteins, culminating in cell death by apoptosis (80).

REDOX reaction is a process in which electrons are transferred from an electron donor or reducing agent to an electron acceptor or oxidizing agent by oxidoreductase. This reaction often utilizes NADP or NAD as cofactors. Oxidation-reduction reactions are major energy supplying reactions in both

autotrophic and heterotrophic organisms. Superoxide protein family controls the level of superoxide anion which produces hydrogen peroxide. When hydrogen peroxide encounters metal ion, it forms highly reactive hydroxyl radical and may lead to mitochondrial membrane depolarization. If the amount of ROS produced unbalances the amount of antioxidants (catalase, superoxide dismutase, glutathione peroxidase), it may cause oxidative stress, DNA damage and eventually cell death (80,81). Oxidoreductase is a large group of enzymes and can be further classified into subclasses. The classification is mainly based on the targets of oxidoreductase. In our Real Time RT PCR study, these two enzymes oxidoreductase-lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), which is classified in the group that is acting on the CH-OH group of donors, are downregulated in AS patients.

Calcium regulatory proteins including calmodulin, calcium pumps and calcium channels can be damaged by ROS. Increased levels of free Ca^{2+} are able to stimulate oxidative metabolism, for example through activating the dehydrogenases of Krebs cycle, providing ATP needed to support the increased activities of membrane pumps involved in clearing of excess Ca^{2+} . In turn, ROS elevation would oxidize membrane ion pumps and their regulating proteins such as calmodulin, along with the downregulation of the activity of the Ca^{2+} -ATPase and eventually, results in the increase of intracellular free Ca^{2+} . Imbalanced calcium homeostasis and increased reactive oxygen species (ROS) are strongly correlated with neurodegenerative diseases that involve early

aggregates of proteins (81). Alzheimer's disease associated Presenilin 1 induced ER stress response through the suppression of protective UPR signaling results in the formation of β -amyloid. Accumulation of β -amyloid peptide in turn triggers the accumulation of hydroxyl radicals (82,83). β -amyloid induced seizure has also been linked to the depletion of calbindin-D 28k in dentate gyrus (84). Mutation of gene encoded α -synuclein has been implicated in some cases of Parkinson's disease; mutated α -synuclein has been found to accumulate in the brain and liberate the formation of hydroxyl radicals upon the addition of Fe (III). Free radicals damage and oxidative stress are important causes of neuronal cell death in substantia nigra in Parkinson's disease (85). Many proteins involved in mitochondrial function and protection of oxidative stress are reduced in *parkin*^{-/-} mice. These proteins include NADH-ubiquinone oxidoreductase and peroxiredoxin (86). In the cerebral, metabolism is altered in Alzheimer's disease (AD) and the abilities of specific mitochondrial enzyme complexes are reduced; including cytochrome oxidase, pyruvate dehydrogenase complexes and α -ketoglutarate dehydrogenase complex. As a consequence of these alterations, there might be an increase in ROS production (87).

1.7.1 SOD2 (Mn-Superoxide dismutase 2)

There are three forms of superoxide dismutases in humans. SOD1 is a dimer and found in the cytoplasm, SOD2 is a tetramer and exists exclusively in

the mitochondria, while SOD3 is a tetramer containing a signal peptide that directs it to extracellular spaces. SOD1 and SOD3 are copper (Cu) and zinc (Zn) containing enzymes, whereas SOD2 has manganese (Mn) in its reactive centre (88). Superoxide and reactive oxygen radicals are the main reactive oxygen species in cells, and SOD protein family serves as key antioxidants to protect cells from oxidative damage in lipid, protein and nucleic acids. The physiological importance of SOD protein family is emphasized by the severe pathology evident in mice genetically modified to be deficient of these enzymes. Mice with SOD2 (*Sod2*^{-/-}) knockout is lethal and die within 1-18 days after birth from cardiomyopathy or neurodegeneration, mice heterozygous for the SOD2 (*Sod*^{+/-}) amid massive oxidative stress due to reduced mitochondrial SOD activity by at least 50%, and increased cancer incidence (89). Mutation in SOD1 is thought to be one of the causes in ALS development; overexpression of SOD1 has reduced superoxide production in mitochondria of hippocampal pyramidal cells after global cerebral ischemia (90). Its deficiency is involved in a wide range of pathologies, including hepatocellular carcinoma, an acceleration of age-related muscle mass loss, and earlier incidence of cataracts (88). Mice expressing reduced SOD3 do not show any obvious physiological defects but are more sensitive to hypertoxic injury (88). Knockout mice of any SOD enzyme are more sensitive to the lethal effects of superoxide generating drugs, such as herbicide, paraquat and diquat (88,90,91).

Studies of SOD have also been performed on other species. For example, overexpression of SOD1 in *Drosophila melanogaster* has an extended life span, while an absence of SOD1 dramatically shortens its lifespan (92). Similarly, *Drosophila melanogaster* lacking SOD2 die before birth, while an overexpression of SOD2 extends its life span (93). Overexpression of SOD2 increases the chronological life span of yeast; and also shortens the replication potential of yeast cells (94). The transcription of *SOD2* is induced by a large number of compounds including interleukin, lipopolysaccharides and interferon- γ . Most of the oxidative species are converted to hydrogen peroxide by mitochondrial SOD2 and to peroxynitrite by reaction with nitric oxide. Therefore they are the most important line of antioxidant enzyme defense systems against ROS and oxidative radicals. SOD2 has been reported to play a role in cellular differentiation and tumorigenesis (88,91). SOD2 has low or zero expression level in tumor cells. It is shown that life-long reduction in SOD2 activity results in increased DNA damage and cancer incidence. Speculation has been made that it may act as a tumor suppressor. SOD2 overexpression suppresses the malignant phenotype of human melanoma, breast cancer, glioma cells and prostate tumor (89,95,96).

1.7.2 VDR (Vitamin D₃ receptor)

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily, which functions as ligand responsive transcriptional enhancers. Its ligand

vitamin-D₃ controls calcium and phosphorus homeostasis, renal development, urinary calcium excretion and bone formation (97). VDR functions as a ligand-dependent transcriptional regulator; it interacts with specific DNA sequences composed of a hexanucleotide direct repeat, and bind to those DNA sequence as either homodimer or by heterodimerizing with retinoid X receptors (RXRs) (98). VDR and vitamin-D₃ are also directly involved in T cell antigen receptor modulation and induce autophagy that results in anti-inflammation and anti-infection processes. Dysregulation of VDR may lead to exaggerated inflammatory responses, raising the possibility that defects in this signaling transduction may lead to bacterial infection and chronic inflammation. Approximately 3% of the mouse and human genome or transcriptional activity of at least 913 known genes are regulated directly and/or indirectly by vitamin D endocrine system (99).

VDR ablation mice exhibit an altered morphology of mammary gland as ductal elongation and branching are suppressed during development. Its expression remains high in the aging mammary gland, and positive VDR staining is observed in glandular epithelial, stromal cells and adipocytes. The absence of VDR signaling in those cells may contribute to the altered morphology since stromal-derived growth factors and extracellular matrix proteins are crucial for normal mammary gland development and maintenance (100,101). Expression of common apoptotic signaling markers such as Bax and Caspase-3 are elevated. Clusterin and transforming growth factor β receptor type II (TGF- β R-II) are

also upregulated in VDR null mammary tissue (101). These elevations are expected to lead to apoptosis and exhibits tumor suppressive function (100-102). $1\alpha, 25$ -dihydrovitamin D₃ (VD), which is the active form of vitamin D₃ is a potential anticancer agent. Mice lacking the VDR are predisposed to epidermal tumor development caused by UVR and chemical carcinogens. It is thought that VDR is involved in skin tumor suppression through modulation of β -catenin and hedgehog (Hh) signaling pathways when activated abnormally results in tumorigenesis. The expressions of these two pathways are upregulated in VDR null mice and VD is able to suppress it in normal mouse skin (103,104). Study has shown mRNA level of VDR and calbindin-D28k in Alzheimer hippocampal CA1 cells are reduced by 35% and 34%, respectively. The downregulated VDR mRNA level may result in reduced expression of calbindin-D28k protein in Alzheimer hippocampal (105).

1.8 The function of TCM herb *Uncariae sinensis* and *Gastrodia elata*

The use of traditional Chinese medicine (TCM) has been practiced for centuries among Asia countries. It is gaining popularity among the world as an alternative therapeutic approach. More than 1500 herbs are sold as dietary supplements or as ethnic traditional medicines. World Health Organization (WHO) is active in creating strategies, guide lines and standards for botanical medicines. This shows that TCM is gaining prominent status in the drug discovery field (106).

The proteomics result from 2-D DIGE has shown that the epilepsy related gene NSF and calcium signaling protein CaBP may play a role in the pathology of AS, as patients are normally suffered in seizure attack. Therefore, two TCM herbs, *Uncariae sinensis* and *Gastrodia elata* which are known to regulate Ca^{2+} signaling and have been commonly used in the epileptic treatment are included in this study. The AS mice are subjected to these TCM herbs before anti-seizure properties and CaBP protein level were checked.

Uncariae sinensis is the dried hooks of the plant whose therapeutic effects has been well-documented in the history of TCM. *Uncariae sinensis* (Gou Teng) and *Gastrodia elata* (Tian Ma) are used to suppress epileptic seizures traditionally. Previous studies have reported other pharmacological properties of *Uncariae sinensis*, such as anti-adrenergic (107), calcium ion channel antagonistic (108) and vasodilative effects (109). *Gastrodia elata* displays anticonvulsive action and has been used as an analgesic and a sedative against vertigo, general paralysis and tetanus (110).

Uncariae sinensis has properties of cooling effect, suppressing the excessive wind in the body, which is believed to have caused epileptic seizures from the TCM viewpoint. A few studies have shown that *Uncariae sinensis* and *Gastrodia elata* are able to exhibit protective effects against neurotoxicity in PC12 cells or kainic acid treated animal. (110-112). This is achieved by

attenuation of lipid peroxidation, intracellular Ca^{2+} and apoptotic markers concentration. These herbs are also shown to reduce ROS generation, increase GSH level, scavenge free radicals and inhibit caspase-3 activity after neurotoxicity is induced by chemical agents. Combined with *Gastrodia elata*, onset of wet dog shakes (WDS) seizure has been delayed (110-113).

Chapter II: Materials and Methods

2.1 Protein extraction from tissue

Tissue was homogenized in extraction buffer containing 7 M Urea (Cat. No. U5128, Sigma), 2 M Thiourea (Cat. No. RPN 6301, Amersham), 30 mM Tris (Cat. No. 75825, USB), 4% CHAPS (Cat. No. 13361, USB), adjusted to pH 8.5 with HCl. Complete protease inhibitor cocktail (Cat. No. 1697498, Roche) and nuclease mix (Cat. No. 80-6501-42, Amersham) were added into extraction buffer before use. Tissue was homogenized in 3 s pulse followed by 5 s cooling on ice, until no visible tissue can be observed. Homogenized sample was then transferred to centrifuge tube and centrifuged at 20,000 x g for 20 min. The supernatant was transferred into a new centrifuge tube and centrifuged for another 20 min at 20,000 x g. The supernatant was then aliquoted and stored at -80°C. The protein concentration was determined by using Bio-Rad protein assay (Cat. No. 500-0002, Bio-Rad) based on Bradford method according to manufacturer's protocol.

2.2 CyDye labelling

Cy2 minimal dye, Cy3 minimal dye, Cy5 minimal dye (Cat. No. 25-8008-60, Cat. No. 25-8008-61, Cat. No. 25-8008-62, Amersham) are three cyanine dyes

used in the experiment. CyDye was reconstituted by using N-N-Dimethylformamide (Cat. No. 22,705-6, Aldrich). 400 pmol of CyDye was used to label 50 µg of protein as recommended by manufacturer. The labelling performed on ice for 30 min, and quenched by adding 1 µl of 10 mM lysine (Cat. No. L5501, Sigma) and incubated on ice for 10 min. Cy2 is always used to label internal control as recommended by manufacturer. Alternate use of Cy3 and Cy5 on labelling of wild type and diseased sample was to prevent dye labelling bias. In the labelling, the ratio of “dye: protein” is kept low in order to ensure that 1-2% protein molecules are labelled with a single dye molecule only.

2.3 1-D isoelectric focusing

ImmobilineTM Dry strip, pH 3-11NL, 24 cm strip (Cat. No. 17-6003-77, Amersham) was used for the isoelectric focusing. The strip was rehydrated using rehydration buffer containing 8 M Urea, 4% CHAPS, 1% Pharmalyte 3-11 (Cat. No. 17-6004-40, Amersham), 13 mM DTT (Cat. No. 17-1318-02, Amersham), Destreak solution (Cat. No. 71-5025-39 Amersham). Rehydration was done for 16-18 hr. The rehydrated strip was then transferred to strip holder and placed on IPGphor (Cat. No. 80-6414-02, Amersham) that was used for isoelectric focusing. The protein lysate was then applied to the strip by cup loading method, and equal volume of sample buffer (8 M Urea, 130 mM DTT, 4% CHAPS, 2% Pharmalyte 3-11) was added into labelled protein sample. The protein was focused on 200 Vhr for each 10 µl of sample applied, followed by

500 Vhr, 1000 Vhr, 1000-8000 V gradient increment for 1hr, and 8000 V for 32,000 Vhr. The strip was equilibrated before it was applied to 2D electrophoresis unit, first with DTT (Cat. No. 17-1318-02, Amersham) in 10 ml equilibration buffer (6 M Urea, 50 mM Tris-Cl, 30% glycerol, 2% SDS, Bromophenol blue; Glycerol Cat. No. 16374, USB) for 20 min and followed with IAA (Cat. No. RPN 6302, Amersham) for another 20 min.

2.4 2-D gel electrophoresis

The equilibrated strip was transferred to SDS-PAGE and sealed with 1% agarose sealing solution with bromophenol blue (Cat. No. 12370, USB) as trace dye. Gel electrophoresis was performed on 12% acrylamide SDS-PAGE (40% stock, Cat. No. 17-1310-01, Amersham) casted one night before usage, in 2X SDS running buffer (50 mM Tris, 384 mM Glycine, 0.4% SDS; Glycine, Cat. No. 161-0718 Bio-Rad; SDS, Cat. No. 75819, USB) at 15°C. 5 W per gel was applied for protein entry, and 10 W per gel for protein separation. The electrophoresis run was stopped when the dye front reached the bottom of the gel. The electrophoresis run was performed on Ettan DALT *six* system (Cat. No. 80-6485-27, Amersham). The 2-D spot pattern comparison was then made by using Decyder software (Amersham) to figure out protein candidates with significant different steady state levels and those differences in expression were consistent in all 2-D DIGE sample analyzed.

2.5 Silver staining proteins visualization

The acrylamide gel was fixed in 50% Methanol (Cat. No. A-454-4, Fisher), 12% Acetic acid (Cat. No. 1.00063.2511, Merck) overnight with mild shaking. The silver staining was performed using Silver Stain Plus kit (Cat. No. 161-0449, Bio-Rad) according to manufacturer's protocol. The stained gel was then stored in 1% acetic acid solution.

2.6 MALDI-TOF-MS protein identification

Stained gel spots were excised by scalpels and cut into 1 mm³ cubes. Silver stained gel spots were then destained by using 100 mM Sodium Thiosulphate (Cat. No. A3525, Applichem) and 30 mM Potassium Ferricyanide (III) (Cat. No. 24.402-3, Aldrich) with gentle vortexing. The gel spots were then washed with double distilled water and equilibrated with 100 mM Ammonium Bicarbonate (Cat. No. A6141, Sigma). Gel spots were then dehydrated in Acetonitrile (Cat. No. 34967, Riedel-de Haën). DTT and IAA were added respectively into dehydrated gel spots. The gel spots were then dehydrated again with acetonitrile before 10 ng/μl Trypsin (Cat. No. V5280, Promega) was added for digestion overnight at 37°C. Peptides were extracted using 50% ACN/5% Trifluoroacetic Acid (TFA). The peptides were then dried using vacuum dry method and cleaned with ZipTip® C₁₈ (Cat. No. ZTC 18S 096, Millipore) according to manufacturer's instruction.

2.7 Mouse genomic DNA extraction

Mouse tail was cut and digested in 495 μ l NTES buffer (50 mM Tris-Cl, 50 mM EDTA, 100 mM NaCl, 5 mM DTT, 0.5 mM spermidine and 2% SDS) and 5 μ l of proteinase K (Cat. No. 13215100, Roche) overnight at 55°C in a rotary oven. The next day, equilibrated phenol (Cat. No. C2432, Sigma), phenol:chloroform:isomyl alcohol (Cat. No. 75831, USB) and chloroform (Cat. No. 75829, USB) were sequentially used for purification of protein from DNA extract. The genomic DNA was then precipitated by isopropyl alcohol (Cat. No. A415-4, Fisher) and dissolved in TE buffer.

2.8 Mouse genotyping

Genomic DNA was used and separated PCR method was employed in PCR genotyping. Three primers named oIMR1965, oIMR1966 and oIMR1967 were used to determine the genotype of the mouse. Primer oIMR1965 was the common primer; when it paired with primer oIMR1966, a 700 bp fragment from the wild type allele would be amplified. On the other hand, when primer oIMR1965 paired with primer oIMR1967, a 320 bp fragment from mutant allele would be amplified. The PCR cycling condition was heat activation at 95°C for 3 min, followed by 40 cycles of 95°C 30 s, 67°C 1 min and 72°C 1 min, the final extension step was done in 72°C for 2 min. The PCR product was analyzed by electrophoresis on 1.5% agarose gel.

Table 2. Primer sequences used in mice genotyping

Name	Sequence	Note
oIMR 1965	5'-GCT CAA GGT TGT ATG CCT TGG TGC T-3'	Genotyping Common Primer
oIMR 1966	5'-AGT TCT CAA GGT AAG CTG AGC TTG C-3'	Wild type allele primer
oIMR 1967	5'-TGC ATC GCA TTG TCT GAG TAG GTG TC-3'	Mutant allele prier
*Primer sequence source: The Jackson Laboratory, genotyping protocol: Ube3a^{tm1Alb}		

2.9 Mouse RNA extraction

Tissue was homogenized in 1 ml of Trizol (Cat. No. 15596-018, Invitrogen) and the homogenized sample was then incubated at room temperature for 5 min before 0.2 ml chloroform was added. The mixture was shaken vigorously and incubated at room temperature for 3 min. The sample was centrifuged at 4°C at 12,000 x g for 15 min, which will cause the mixture to separate into 3 layers. The top colourless layer was transferred into a new tube; 0.5 ml of isopropyl alcohol was added and mixed. The mixture was then incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed after the centrifugation, and the pellet was washed with 1 ml of 75% ethanol by vortexing. The sample was centrifuged at 7,500 x g for 5 min at 4°C. The pellet was air dry for 10 min and dissolve the pellet with DEPC treated water by incubating at 55°C for 10 min. The sample was stored at -20°C for frequent use or -80°C for long term storage. During the process, RNase zap (Cat. No. 9780, Ambion) was used to remove any trace of RNase.

2.10 cDNA synthesis

RNA sample (1 µg of RNA was used for cDNA synthesis using iScript™ cDNA Synthesis kit Cat. No. 170-8890 Bio-Rad). The total reaction volume was 20 µl, containing 4 µl 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, RNA template and Nuclease free water. The reaction condition was 5 min at 25°C, followed by 30 min at 42°C and 5 min at 85°C. The cDNA synthesized was then stored at -20°C for storage.

2.11 Real Time RT PCR

The reaction was performed using iTaq™ SYBR® Green Supermix with ROX (Cat. No. 172-5850, Bio-Rad) containing 2X reaction buffer, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.8 mM dUTP, iTaq DNA polymerase, 6 mM Mg⁺², SYBR Green I dye, 1 µM ROX internal reference dye and stabilizers. Reaction volume used was 25 µl, including 12.5 µl of 2X SYBR® Green Supermix, 1 µl of synthesized cDNA, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, topping up with nuclease free water.

Cycling was performed on 7500 Real Time RT PCR system (Applied Biosystem). Cycling conditions were machine warm up at 50°C for 2 min, hot initiation at 95°C for 10 min, cycling condition (45 cycles) of 95°C for 30 s, 60°C for 30 s and 72°C for 2 min; followed by a dissociation stage to generate a

melting curve. $\Delta\Delta CT$ method has been employed to calculate the differential expression of mRNA in samples examined, by comparing cycling results between target gene and basal control Glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Table 3. Primer sequences used in Real Time RT PCR analysis:

Name	Sequence
HSP70	Sense: 5'-AAG AAC GCG CTC GAG TCC TAT GC-3' Anti-sense: 5'-CAC CCT GGT ACA GCC CAC TGA TGA T-3'
CaBP	Sense: 5'-GAT GGC AAC GGA TAC ATA GAT GAA-3' Anti-sense: 5'-TCC ATC CGA CAA GGC CAT TAT GTT C-3'
GADPH	Sense: 5'-AGT CTA CTG GTG TCT TCA CCA CCA TGG-3' Anti-sense: 5'-TTC TCG TGG TTA ACA CCC ATC AC-3'
VDR	Sense: 5'-AGG TGC AGC GTA AGC GAG AGA T-3' Anti-sense: 5'-CCT CAA TGG CAC TTG ACT TAA GC-3'
NeuroD	Sense: 5'-CTC AGT TCT CAG GAC GAG GA-3' Anti-sense: 5'-TAG TTC TTG GCC AAG CGC AG-3'
Pax6	Sense: 5'-AGT CAC AGC GGA GTG AAT CAG-3' Anti-sense: 5'-AGC CAG GTT GCG AAG AAC-3'
Mash1	Sense: 5'-AGC AGC TGC GGA CGA GCA-3' Anti-sense: 5'-CCT GCT TCC AAA GTC CAT TC-3'
LDH	Sense: 5'-AGC AAA GAC TAC TGT GTA ACT GCG A-3' Anti-sense: 5'-ACC TCG TAG GCA CTG TCC AC-3'
MDH	Sense: 5'-AGG CTA CCT TGG ACC GGA GCA GTT-3' Anti-sense: 5'-GTG GCA GAA CCT GCT CCA GCC TT-3'
Glutathione S-Transferase Mu1	Sense: 5'-TGA CGC TCC CGA CTT TGA CAG AA-3' Anti-sense: 5'-TAA GCA AGG AAA TCC ACA TAG GTG-3'
ATP synthase	Sense: 5'-AGA AGA CTG GCA CAG CTG AGA TGT-3' Anti-sense: 5'-CCA GTC TGT CTG TCA CCA AT-3'
5a1	Sense: 5'-ATG AAA GCC ATC TGC ATC ATT AGC-3' Anti-sense: 5'-GCA ATT ATT CCG CAT CCC AAA CG-3'
SOD2	Sense: 5'-TGG GGC AGC AGC TTG TCT TTA -3' Anti-sense: 5'-TTA GCA CCA AGC CTC CTT TGC -3'

2.12 Western blot analysis

Protein homogenates were heated in Laemmli sample buffer (Cat. No. 161-0737, Bio-Rad), at 95°C for 10 min. The heated samples were resolved on 12% SDS-PAGE gel and transferred to Immun-Blot PVDF membrane (Cat. No. 162-0177, Bio-Rad) at 100 V for at least 1 hr. The membrane was then block with 5% non-fat milk in 0.1% T-TBS (10 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.1% Tween-20; NaCl Cat. No. 1.06404, Merck, Tween-20 Cat. No. 161-0781, Bio-Rad) for 1 hr at RT. The membrane was then washed three times with 0.1% T-TBS, followed by incubation with primary antibody for 1 hr. The membrane was then washed again three times with 0.1% T-TBS. The membrane was then incubated for 1 hr with HRP conjugated secondary antibody. The membrane was then washed again three times with 0.1% T-TBS before being developed by ECL method (Cat. No. RPN 2108, ECL Western blotting analysis system, Amersham). The blot stripped by 0.1% T-TBS overnight with orbital shaking for second antibody detection. The intensity of protein bands detected by Western blot analysis was determined by calibrated densitometer GS-800 and Quantity One 1-D analysis software (Cat. No. 170-7983, Bio-Rad).

Table 4. Antibodies used in experiments

Antibody name	Dilution factors used in experiment
Anti-E6AP (Cat. No. A300-352A, Bethyl)	1:2500-5000
Anti Calbindin D-28k (Cat. No. AB1778, Chemicon)	1:2500-5000
Anti-Actin (Cat. No. MAB1501, Chemicon)	1:1000-2500
Secondary HRP conjugated anti-mouse/anti-rabbit (GE Healthcare)	1:2500-5000
Anti-Hsp70 (Cat. No. sc32239 , Santa Cruz)	1:2500-5000
Anti-SOD2 (Cat. No. sc-30080, Santa Cruz)	1:2500-5000
Anti-NSF (Cat. No. ab16681, abcam)	1:10000
Anti-Mash1 (Cat. No. AB155582, Chemicon)	1:2500-5000
Anti-NeuroD (Cat. No. AB155580, Chemicon)	1:2500-5000
Anti-GST (Cat. No. sc138, Santa Cruz)	1:2500-5000
Anti-Ub (Cat. No. sc47721, Santa Cruz)	1:10000
Anti-Flag (Cat. No. F3165, Sigma)	1:10000

2.13 *In-vitro* transcription and translation

pGEM1 vector contained E6AP and E6APM (Ubiquitin E3 ligase with a mutated catalytic site C833A) were purchased from Addgene. pGEM1 vector contained Hsp70 was generated by using plasmid purchased previously as backbone, Hsp70 cDNA containing RE sites *AatII* and *BamHI* was first generated by PCR followed by cloning into TOPO vector, it was then enzyme digested by *AatII* and *BamHI* and ligated to pGEM1 vector that was digested by the same set of enzymes. The plasmids were then expressed in TOP10 competent cells. The DNA template was first linearized by restriction enzyme

*Hind*III and purified by using phenol followed with ethanol precipitation. The linearized DNA was used for RNA synthesis according to manufacturer's protocol (Cat. No. P1300, Promega) driven by T7 RNA polymerase. Next, DNA template was removed by RNase free-DNase and RNA products were treated with citrate-saturated phenol:chloroform:isoamyl alcohol (125:24:1) (Cat. No. 77619, Fluka) and chloroform:isoamyl alcohol (24:1) (Cat. No. 25666, Fluka) before precipitation with 3 M sodium acetate and isopropanol. RNA pellet was washed with 70% ethanol and re-suspended in nuclease free water. RNA product was resolved and confirmed with formaldehyde contained RNA gel-electrophoresis. After that, wheat germ extracts system (Cat. No. L4380, Promega) was employed for in-vitro translation of target gene with addition of [³⁵S] methionine (Cat. No. NEG709A500UC, Perkin Elmer); the translation reaction was conducted for 2 hr before subjected to SDS-PAGE followed by autoradiography.

2.14 Flag-tagged Parkin protein expression

pcDNA 3.1 contained flag-tagged Parkin was a gift from Dr Tanaka (Tokyo Metropolitan Institute of medical science). Plasmid DNA was transfected into HEK293T cells with Lipofectamine (Cat. No. 11668-019, Invitrogen) according to manufacturer's protocol. Transfected cells were then harvested after 48 hr by using lysis buffer contained 1% NP-40. The expression of protein was confirmed by Western blot analysis.

2.15 GST tagged protein expression

pGEX-2T plasmids contained E6AP and E6APM were purchased from Addgene. pGEX-2T vector contained Hsp70 was generated by using plasmid purchased previously as backbone, Hsp70 cDNA contained RE sites *AatII* and *BamHI* was first generated by PCR followed by cloning into TOPO vector, it was then enzyme digested by *AatII* and *BamHI* and ligated to pGEX-2T vector that was digested by same set of enzymes. The plasmids DNA were then transformed into *E. coli* strain BL21 for protein expression. Overnight culture of positive colony was seeded in LB broth with 100 µg/ml ampicillin added. Expression of GST fusion protein was induced by using 10 µl of 1 M IPTG for next 5 hr. The GST-tagged proteins were then obtained by sonication in lysis buffer (1% Triton-X, Complete Protease inhibitor) and debris was pelleted by centrifugation at 13,000 rpm for 20 min. Glutathione particles were then added into protein lysate for 1hr before washed three times with washing buffer (4.2 mM Na₂HPO₄, 2 mM KH₂PO₄, 140 mM NaCl, and 10 mM KCl, pH7.2). Glutathione bound proteins were then recaptured with magnet. The washing and recapture process was repeated for at least three times. GST-tagged proteins were then analyzed on SDS-PAGE.

2.16 In-vitro interaction study

[S³⁵] Methionine-labeled proteins were first generated by using wheat germ

extract lysates. The products were then added into the mixture containing glutathione particles bounded recombinant protein. The binding was performed in binding buffer (20 mM Tris, pH7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% NP-40, 1 mM NaVO₄, Complete Protease inhibitor) at 4°C for 4 hr with constant rocking. The mixture was then washed twice with binding buffer before eluted with SDS sample buffer with β -mecaptoethanol. The reaction was subjected to SDS-PAGE for interaction analysis. The acrylamide gel was then air dried overnight followed by autoradiography.

2.17 Cell transfection

When cell growth reached 70% confluency on tissue culture plates, plasmid DNA containing gene encoded for target protein was added with transfection agent, Lipofectamine, according to manufacturer's protocol. The ratio of lipofectamine and plasmid DNA was normally 1:3. Fresh medium will be then added the next day. The transfected cells will be then harvested after 48 hr.

2.18 Protein extraction from cell-lines

Cells were washed by cold PBS before harvest. Cell scraper was then used for the harvest. Harvested cells were pelleted by centrifugation before adding lysis buffer containing detergents ranging from NP40 (Cat. No. 19628, USB),

CHAPS (Cat. No. 13361, USB) or Triton X-100 (Cat. No. 22686, USB) depending on the cell type and protocols. Cell lysate were fractionated by centrifugation.

2.19 *In-vivo* interaction study

For *In-vivo* interaction study, cells were subjected to 200 mM of hydrogen peroxide for 15 min before cells (COS7) were lysed using lysis buffer. Crude protein lysates (bait) were then extracted and added into interaction assay contained immuno-captured prey proteins (prey) by using protein G agarose and 2 µg of respective antibodies, including Ube3a, Hsp70 and Flag antibody. The binding assay was performed in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 7) for 2 hr at 4°C, before washing with lysis buffer. The reaction was then subjected to 10% SDS-PAGE electrophoresis followed by Western blot analysis using respective antibody. Flag-tagged Parkin expressed in cell line HEK293T was used as positive control.

The *in-vivo* interaction study was also conducted by using proteins extracted from brain tissue of mice. The extraction method was previously described in section 2.1. Crude protein lysates (bait) were added into interaction assay contained immuno-captured prey proteins (prey) by using protein G agarose and 2 µg of respective antibodies, including Ube3a, Hsp70, Flag and CaBP antibody. The binding assay was performed in lysis buffer for 4 hr at 4°C,

before washing with lysis buffer. The reaction was then subjected to 10% SDS-PAGE electrophoresis followed by Western blot analysis using respective antibody. Flag-tagged Parkin expressed in cell line HEK293T was used as positive control while CaBP served as the negative control in this study.

2.20 HSP70 immuno-precipitation

COS7 cells were harvested and lysed by RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 50 mM SDS with Complete Protease Inhibitor (Cat. No. 05-892-791-001, Roche) for protein extraction. rProtein G agarose beads (Cat. No. 15920-010, Invitrogen) were pre-incubated with 2 µg of HSP70 antibody for at least 3 hr at 4°C. The mixture was washed twice with RIPA buffer before extracted proteins were added and incubated overnight at 4°C. Next day, the mixture was washed 3 times by lysis buffer before the immuno-precipitated HSP70 was released by adding 40 µl of 4 M MgCl and incubated on rocking tray for 5 min.

2.21 *In-Vitro* Ubiquitination study

Protein G agarose was pre-incubated at 4°C with 2 µg Ube3a antibody for at least 3 hr before protein crude extract was added into the mixture for another 12

hr. The immuno-precipitated mixture was then washed with cold 1X TBS buffer for three times, with additional ATP for the third wash. 50 ng E1 (Cat. No. 662072, Calbiochem), 200 ng E2 (Cat. No. 10-663-45888, GenWay), 10 µg ubiquitin (Cat. No. U653-5MG,), 10 mM MgCl, 50 mM NaCl, 20 mM Tris and HSP70 protein (released from immune-precipitation) were then added into ubiquitination assay with immune-precipitated Ube3a. The reaction mixture was incubated at 30°C for 2 hr. Samples were then analyzed by SDS-PAGE followed by examination with Western blot using ubiquitin antibody.

2.22 Knockdown of E6AP in P19 cell-lines

pSUPER.puro vector contained a 19-mer siRNA against exon 5 of Ube3a was transfected into P19 cells by using transfection agent Lipofectamine (114). Empty pSUPER.puro vector was used as mock in this study. 3.0×10^5 of P19 cells were seeded in each of the well of 6-well plate for transfection. One day after transfection, 2 µg of puromycin (Cat. No. P9200, US Biological) was added for antibiotic selection purpose. All survived cells were harvested 24 hr after the puromycin selection. Protein levels from transfected cells were determined using Western blot analysis.

2.23 Cell culture

P19 cells were maintained in MEM- α medium (Cat. No. SH30265.01, Hyclone) supplemented with 10% FBS. COS7 cells were cultured in RPMI-1640 medium (Cat. No. SH30027.01, Hyclone) supplemented with 10% FBS. HEK293T cells were maintained in DMEM medium (Cat. No. E15-099, PAA) supplemented with 10% FBS and 4 mM of L-Glutamate (cat 25030-081, GIBCO). Cells were trypsinized by using 0.25% trypsin with EDTA (cat 25200, GIBCO)

2.24 Preparation of TCM

Raw herbs were grounded into powder form with a household blender. 50 g of the powder was packaged and soaked in 600 ml sterile water for 15 min. The herb was then boiled for 30 min, drained and boiled again with another 600 ml of sterile water for further 30 min. The total decoction was then heated in a rotary evaporator at 60°C until the concentration of herbs reached 1:1 (w/v) ratio which is 50ml. The herb concentrate was then aliquoted and store at -20°C.

2.25 TCM treatment Regime

The wild type mice served as control group was fed with 10 ml/kg dose of sterile water. While the AS mice served as treatment group was fed with 10 g/kg or 20 g/kg of herbs used in experiment. The feeding was continued for seven

consecutive days. The mice were sacrificed 30 min after the final dose of sterile water/herb treatment for hippocampus sample harvesting.

2.26 Audiogenic Seizure Test

The mice were individually restrained and identified before being placed in a programmable sound chamber (Med Associates Inc). The mice were then given 15 s to acclimatize to the surroundings inside the sound chamber before being subjected to a 13 kHz signal at 100 dB for 50 s or until the onset of seizure. They were allowed to rest for 15 s before subjected to white noise at 100 dB for another 50 s or until the onset of seizure. Susceptibility to audiogenic seizure by the two sound types was observed, graded and recorded. (Grade 0: non-susceptible; Grade 1: wild running and jumping; Grade 2: tonic seizure/myoclonic twitches of body/fasciculation of limbs without falling; Grade 3: generalized convulsive tonic-clonic seizure; Grade 4: cardiac arrest).

Chapter III: Results

3.1 2-D DIGE and silver staining

Proteins extracted from mice's cerebellum were quantified before labelling with respective CyDye. Labelled proteins were first separated according to pI in 1-D isoelectric focusing followed by further separation by molecular size in SDS-PAGE before subjected to scanning using Typhoon scanner (Amersham), the CyDye was excited during the scan and emitted fluorescent signal for imaging. After that, the Decyder software analysis was performed by using analytical gel (CyDye labelled), the software contained Differential In-gel Analysis (DIA) and Biological Variation Analysis (BVA) module for analysis purpose. DIA is used for protein spots detection and spot picking in a single gel; it will also assign spot volume for each protein spot detected in all three images that were obtained from scanning (Cy2, Cy3 and Cy5 labelled respectively), the spot volume which directly reflect the quantity of spot. Those volume ratios between samples and internal will be used to compute up- or down- regulation of the protein spot. BVA module analyzed sets of spot maps generated by DIA module, spots are matched among different spot maps and statistical analysis can be then performed. Only protein spots that were detected in all compared analytical gels and presented at least 40% abundancy difference between wild type and mutant samples will chosen for further study. Another set of protein electrophoresis (preparative gel) were performed using 600 µg of unlabelled protein sample, the

protein samples used in the preparative gel was the same as those used for 2-D DIGE analysis. After electrophoresis, silver staining was conducted for visualization of protein spots on the gel. Typically, 800-1000 protein spots were visualized on each gel [Figure 5].

Figure 5. Analytical gel images and silver staining of preparation gel after SDS-electrophoresis

(a-c) Scanned images obtained from analytical gel (CyDye labelled) using Typhoon scanner. One analytical gel will result in three images that were labelled by different CyDye; (a) Cy2; (b) Cy3; (c) Cy5; respectively. (d) One of the analytical gels presented in non-fluorescent emitted form. (e) Protein extract (600 μ g) from cerebellum was loaded in first dimension IPG strip (pH3-11, NL, 24 cm; Running time: 15.5 hr, approx 47 kVh) and resolved in 12.5% acrylamide gel (Running time: 5.15 hr, 10 W per gel). Proteins were visualized by silver staining. Such gels are called preparative gels, which contain more protein content in order to perform subsequent analysis like silver staining and MALDI-TOF-MS. CyDye labelled proteins were ran on analytical gels that were meant for laser scanning, and thus do not require high quantity of proteins. The white spots in the gel represented the excised protein spots for further analysis, MALDI-TOF.

3.2 Detection and identification of differentially expressed protein in AS versus wild type brain tissue

A total of ten differentially expressed proteins were detected by using 2-D DIGE from protein samples extracted from cerebellum or hippocampus of wild type mice and AS mice (*Ube3a* knockout) [Table 5]. Those protein spots were recovered from parallel run, silver-stained acrylamide gels and identification was made based on the protein profile generated by MALDI-TOF-MS [Figure 6] with a threshold score of 63. All candidates were confirmed at least twice in separate 2-D DIGE runs and MALDI-TOF-MS identification. Another four bHLH proteins (Mash1, NeuroD, Pax6 and VDR) were included in this project as these proteins are highly related to CaBP (45).

Table 5. Differentially expressed proteins detected by 2-D DIGE

Protein candidates identified by MALDI-TOF-MS	pI	Molecular Weight	Accession number	Sequence coverage %	Score	Function
CABP	4.82	25943	P12658	50%	375	Calcium ion buffer
HSP70	5.52	70079	NP_034609.2	24%	358	Protein folding and degradation
SOD2	8.80	24602	P09671	30%	219	REDOX
LDH	7.61	36498	P06151	7%	134	REDOX
MDH	6.16	36477	gi 92087001	30%	365	REDOX
GSTs-Mu1	7.72	25969	P10649	35%	319	REDOX
NSF	6.52	82613	gi 29789104	29%	162	Docking and fusion of synaptic vesicles
ATP5a1	9.22	59752	gi 6680748	49%	436	ATP synthesis
Cofilin, non muscle	8.22	18776	gi 6680924	22%	116	Disassembles actin filaments
TPI (Triosephosphate isomerase 1)	6.90	27038	gi 6678413	56%	489	Glycolysis, energy production

*Additional four proteins (Mash1, NeuroD, Pax6 and VDR) that were not detected in 2-D DIGE but were also investigated in this project due to those proteins were highly associated with one of proteins identified, CaBP(45)

Figure 6. Protein identification by MALDI-TOF-MS

MS spectrum for one of the proteins identified – CaBP. After peptide detection, the peptide profile was used to match the NCBI database for protein identification.

A total of eight differentially expressed proteins identified are involved in REDOX reactions, such as HSP70, SOD2, MDH, LDH, VDR, GSTs-Mu1, ATP5a1 and CaBP. Four of the bHLH protein, VDR, Pax6, Mash1 and NeuroD are involved in neuronal cells differentiation, while NSF is crucial in the synaptic vesicle transmission, and learning processes that are controlled in hippocampus [Figure 7].

Figure 7. 2-D DIGE results and inter-connected functions

This figure showed differentially expressed proteins categorized by their functions; some proteins are known to be involved in multiple pathways and functions.

3.3 Validation of 2-D DIGE/MS results by Western blot analysis

Western blot was conducted to validate the differentially expressed proteins that were identified in 2-D DIGE/MS. In cerebellum, three proteins, including CaBP, Mash1 and SOD2 were downregulated in *Ube3a* knockout mice [Figure 8], while Hsp70, NeuroD and NSF were accumulated in knockout mice [Figure 9]. However, Mash1, SOD2, NeuroD and NSF were downregulated [Figure 10] and HSP70 was accumulated [Figure 11] in the hippocampus of *Ube3a* knockout mice. The Western blots were repeated at least three times by using different set of cerebellum and hippocampus tissues [Figures 8-11].

In cerebellum, Hsp70 was found to be upregulated by approximately 120% based on result from densitometer scan. NSF and NeuroD were up regulated by 85% and 50%, respectively. SOD2 was downregulated by 45% in cerebellum of *Ube3a* knockout mice. Based on densitometer scan results, CaBP and Mash1 were both found to be reduced by approximately 75% in defect mice.

In hippocampus, Hsp70 was increased by 50% when sample from wild type mice was compared to defect mice. SOD2 and Mash1 were both reduced by approximately 80% in *Ube3a* knockout mice; CaBP and NeuroD were found downregulated by 40% and 45%, respectively, when compared to wild type sample. Lastly, NSF was reduced by nearly 50% in *Ube3a* knockout mice.

The major differences in the Western blot validation are the differential expressions of NeuroD and NSF in these two tissues. These two proteins were found upregulated in cerebellum but downregulated in hippocampus [Figure 12]. This suggests that the expression of these two proteins might be tissue specific. Western blot analysis for protein candidates VDR, LDH, MDH, GSTs-Mu1 and ATP5a1 has not been conducted due to unavailable antibodies when this study was conducted.

Figure 8. Validation of downregulation of SOD2, CaBP and Mash1 in cerebellum of *Ube3a* knockout mice

SOD2, CaBP and Mash1 are downregulated in AS mouse model. Antibody dilution factor used in Western blot are SOD2, 1:5000; CaBP, 1:5000; Mash1, 1:5000.

Figure 9. Validation of proteins accumulated in cerebellum of *Ube3a* knockout mice

Three proteins including HSP70, NSF and NeuroD are accumulated in AS mice. Antibody dilution factor used in Western blot are HSP70, 1:5000; NSF, 1:5000; and NeuroD, 1:5000.

Figure 10. Validation of downregulated proteins identified by 2-D DIGE using Western blot

Five proteins including SOD2, Mash1, CaBP, NeuroD and NSF are downregulated in AS mice. Antibody dilution factor used in Western blot are-SOD2, 1:5000; Mash1, 1: NSF, 1:5000; NeuroD, 1:2500.

Figure 11. Validation of Hsp70 accumulation in hippocampus of *Ube3a* knockout mice

Hsp70 showed accumulation in AS mice among protein candidates detected by 2-D DIGE/MS. Antibody dilution factor used in Western blot is- HSP70, 1:5000.

Figure 12. A summary of validation of 2-D DIGE/MS results by Western blot analysis

A total of 6 proteins were tested in this Western blot validation using protein from cerebellum and hippocampus. Three of them were found downregulated and one upregulated (HSP70) in both tissues, while two of them have tissue-specific expression.

3.4 Transcriptional analysis of the differentially expressed proteins verified by Western blot analysis

Since variation of steady state protein level between wild type and mutant mice can be caused by enhancing transcriptional activity instead of enhancing protein half life, Real Time RT PCR was conducted to quantify mRNA level of proteins detected by 2-D DIGE.

mRNA extracted from cerebellum and hippocampus of wild type mice as well as *Ube3a* knockout mice were used in this study. Real Time RT PCR were conducted for at least three times from individual sets of mice. Student's T-test was applied for this study. Results that have 40% difference in expression level, when compared to wild type and mutant sample are considered significant.

Based on the results of Real Time RT PCR study obtained from cerebellum sample [Figure 13 and Table 6], mRNA level of *CaBP*, *NeuroD* and *VDR* were downregulated by 57%, 33% and 55%, respectively. The mRNA levels of other proteins, including *Hsp70*, *SOD2*, *Mash1*, *Pax6*, *NSF*, *ATP5a1*, *LDH*, *MDH* and *Glutathione S-transferase Mu1* were not affected in the AS mouse. Real Time RT PCR study revealed that mRNA level of *CaBP*, *NeuroD*, *Pax6*, *VDR* and *LDH* were downregulated in hippocampus of *Ube3a* knockout mice by 80%, 85%, 82%, 72% and 45%, respectively, while mRNA level of *Glutathione S-transferase Mu1* was upregulated by 107% [Figure 14 and Table 6]. In contrast, mRNA level of *MDH*, *ATP5a1*, *NSF*, *Mash1*, *SOD2* and *Hsp70* were not affected significantly in hippocampus of *Ube3a* knockout mice.

Figure 13. Real Time RT PCR analysis of candidate genes by using cerebellum sample

Real Time RT PCR was employed to study the differential expression at transcriptional level of candidate genes detected by 2-D DIGE/MS. RNA extracted from cerebellum of age match as well as gender match wild type mice and *Ube3a* knockout mice were compared in this study. In cerebellum, mRNA level of *CaBP*, *NeuroD* and *VDR* were downregulated. Other candidate genes were not significantly affected at the transcriptional level.

Figure 14. Real Time RT PCR analysis of genes identified by 2-D DIGE using hippocampus sample

Real Time RT PCR method was employed to study steady state mRNA level of genes corresponding to the differentially expressed proteins identified by 2-D DIGE. RNA extracted from hippocampus of age match and gender match wild type mice and *Ube3a* knockout mice has been studied in this analysis. In hippocampus, mRNA level of *CaBP*, *NeuroD*, *Pax6*, *VDR* and *LDH* have been downregulated in the range of 45%-85%. Glutathione S-transferase Mu1 was upregulated by 107% in *Ube3a* knockout mice. Other candidate genes were significantly affected at transcriptional level in hippocampus.

Table 6. A summary of Western blot validation and Real Time RT PCR analysis of candidates tested in cerebellum and hippocampus

Cerebellum

Percentage changes (protein level)	Candidates	Percentage changes (mRNA level)
75% ↓	CaBP	57% ↓
75% ↓	Mash1	6% ↑
45% ↓	SOD2	13% ↓
120% ↑	HSP70	17% ↓
50% ↑	NeuroD	33% ↓
85% ↑	NSF	2% ↑
n/a	LDH	17% ↓
n/a	MDH	15% ↓
n/a	GSTs-Mu1	1% ↓
n/a	VDR	55% ↓
n/a	ATP5a1	12% ↑
n/a	Pax6	21% ↓

Hippocampus

Percentage changes (protein level)	Candidates	Percentage changes (mRNA level)
40% ↓	CaBP	80% ↓
80% ↓	Mash1	12% ↓
80% ↓	SOD2	22% ↓
50% ↑	HSP70	25% ↓
45% ↓	NeuroD	85% ↓
50% ↓	NSF	6% ↑
n/a	LDH	45% ↓
n/a	MDH	14% ↑
n/a	GSTs-Mu1	107% ↑
n/a	VDR	72% ↓
n/a	ATP5a1	15% ↑
n/a	Pax6	82% ↓

3.5 Interaction study between HSP70 and E6AP

Since HSP70 protein level is upregulated in western blot analysis in both cerebellum and hippocampus sample and E6AP is identified as one of the E3 ligase which is responsible for protein degradation; therefore, the accumulation of HSP70 may have related to the absence of E6AP. The first step is to identify the interaction between HSP70 and E6AP. The *in-vitro* interaction between HSP70 and E6AP has recently been shown (23,115); however, it is not known if Ube3a is able to induce ubiquitination of Hsp70 for proteasomal degradation. In

this study, I further confirmed the *in-vitro* interaction of these two proteins. First of all, GST-tagged Hsp70 was expressed in pGEX-2TK vector in prokaryotic system [Figure 15(c)]; and GST-tagged Hsp70 was purified by glutathione coupled magnetic system before subjected to interaction study [Figure 15(d)]. pGEM1-E6AP or pGEM1-E6APM was *in-vitro* transcribed by using T7 RNA polymerase system [Figure 16(a)], followed by *in-vitro* translation using ³⁵S methionine and wheat germ extract [Figure 16(b)]. Flag-Parkin was expressed by plasmid transfection in eukaryotic cell-line HEK293T [Figure 17] and used as the positive control in all *in-vitro* interaction studies. Positive interaction between E6AP and GST-HSP70 was confirmed [Figure 18(b), lane4]. Interaction between E6APM and GST-HSP70 was also detected, but appeared at much weaker level [Figure 18(b), lane5]. Positive control Flag-tagged Parkin was able to precipitate with GST-HSP70 [Figure 18(c), lane6]; whereas, no interaction was detected between prey proteins and GST [Figure 18(b), lane1-3]. Likewise, the *in-vitro* interaction study between GST-tagged E6AP and Hsp70 has also been studied. Hsp1a cDNA was first cloned into pGEM1 vector [Figure 19(a), lane2], it was then *in-vitro* transcribed [Figure 19(b), lane2] and translated [Figure 19(c), lane1] before interaction study. GST-tagged E6AP and GST-tagged E6APM were expressed in prokaryotic system [Figure 19(d) and (e)]. In the interaction studied, *in-vitro* translated Hsp70 was able to interact with GST-tagged E6AP [Figure 19(f), lane1] and GST-tagged E6APM failed to do so [Figure 19(f), lane2].

I have also conducted the *in-vivo* interaction study using protein extracted from eukaryotic cell-line COS7 [Figure 20]. E6AP was obtained by immuno-precipitation using respective antibody. Flag-tagged Parkin expressed in cell line HEK293T [Figure17] was immuno-captured by Flag antibody and added as positive control. Protein crude extract from COS7 was then added for interaction study purpose. The result showed that Ube3a was able to interact with Hsp70 by using cell lysate [Figure 20]. The *in-vivo* interaction study was also conducted by using proteins extracted from brain tissue of mice [Figure 21]. Hsp70 and CaBP protein were immuno-captured by respective antibody; and CaBP protein served as negative control. Protein crude extract from brain tissue was then added for interaction study. The mixture was then subjected to electrophoresis followed by Western blot analysis. The Western blot result showed that endogenous Hsp70 was able to interact with endogenous E6AP in brain tissue of mouse [Figure 21(a)]. CaBP served as negative control in this study and as expected no interaction was reported between E6AP and CaBP [Figure 21(b)]. These two *in-vivo* interaction studies conducted using protein extracted from cell-line and brain tissue have shown the biological significance and further verified the interaction relationship between Hsp70 and Ube3a.

Figure 15. Expression of GST-HSP70

(a) DNA fragment (1925 bp) encoding mouse Hsp70 protein was excised from TOPO vector (3.9 kb) by using restriction enzyme *AatII* and *BamHI*. (b) The 1.9 kb Hsp70 cDNA was then ligated to pGEX-2TK (4969 bp) and transformed into *E.Coli* strain BL21 for protein expression in prokaryotic system. The inserted cDNA was confirmed by digestion using restriction enzyme *AatII* and *BamHI*. (c) Production of protein GST-Hsp70 was induced by IPTG and expressed for different time length, 4 hr (lane 1), 4.5 hr (lane 2) and 5 hr (lane 3). Protein was then resolved by using 10% SDS-PAGE and visualized by using coomassie blue stain G250. (d) Similar GST-Hsp70 fractions in (c) were captured by glutathione coupled with magnetic beads system. Eluate fractions were then resolved in 10% SDS-PAGE and visualized by using coomassie blue G250 protein stain. (e) GST-Hsp70 protein was visualized by using Western blot analysis. GST-tagged Hsp70 was approximately 95 kDa. Antibody used in western analysis was anti-GST, 1:5000.

Figure 16. *In-vitro* transcription and translation of E6AP using ^{35}S -methionine

(a) pGEM1-E6AP vector was purchased from Addgene. The vector (5.5 kb) was first digested with restriction enzyme *Hind*III and then was transcribed *in vitro* using T7 RNA polymerase. The expected transcribed product (2.5 kb; lane 2) was resolved on formaldehyde contained agarose gel along with luciferase control (1.8 kb; lane 1). (b) The RNA was added for *in-vitro* translation of E6AP by using wheat germ extract system with ^{35}S methionine. Varied RNA amount (lane 1-3: 5 μg ; lane 4-6: 10 μg) and potassium acetate concentrations (lane 1 & 4: 50 mM; lane 2 & 5: 100 mM; lane 3 & 6: 150 mM) were tested for *in-vitro* translation. Eventually, 5 μg of transcribed RNA and 50 mM of potassium acetate were added in subsequent *in-vitro* translation experiment. BMV protein marker (110 kDa, 97 kDa, 35 kDa and 20 kDa; lane 7) was added in the experiment. *In-vitro* translation was also performed without using ^{35}S -methionine. The translated E6AP (lane 1) and E6APM (lane 2) were visualized by using Western blot analysis (c) or coomassie blue staining (d). Luc: Luciferase.

Figure 17. Expression of Flag-Parkin

pcDNA 3.1 vector carrying the Flag-Parkin fusion was transfected into HEK293T cells using lipofectamine. The transfected cells were harvested 48 hr later and protein was extracted by using various detergent and extraction time length conditions. 1% Triton-X (lane1-3) showed the best result when extraction time length was extended to 3 hr (lane 1), while 1% NP40 showed good recovery of target protein regardless of the time lengths used in protein extraction (lane 1 & 4: 3 hr; lane 2 & 5: 1 hr, lane 3 & 6: 20 min).

Figure 18. *In-vitro* interaction study between HSP70 and E6AP

(a) The *in-vitro* interaction study was performed using GST-tagged HSP70 and *in-vitro* transcribed/translated ³⁵S-labelled E6AP. Expression of GST-tagged HSP70 in *E.Coli* strain BL21 was confirmed by Western blot analysis before the lysate was used for *in-vitro* interaction study. (b) Glutathione particles from magnetic pull-down system were coupled with GST protein (2 µg) before incubation with various prey proteins including E6AP, E6APM and Flag-Parkin (Panel b, Lane 1-3 respectively). Glutathione particles were then coupled with GST-tagged HSP70 expressed from *E.Coli* strain BL21 (2 µg) before incubation with different prey proteins; Ube3a, Ube3aM and Flag-Parkin (Panel b, Lane 4-6 respectively). (c) Interaction of positive control Flag-tagged Parkin and GST tagged Hsp70 was detected using anti-Flag antibody.

Figure 19. *In-vitro* interaction study of GST tagged E6AP and *in-vitro* translated HSP70

(a) Hsp1a cDNA was cloned into pGEM1 vector for *in-vitro* transcription and translation. Digestions of vector by using restriction enzyme *Hind*III (lane1) or *Hind*III and *Bam*HI (lane 2) were performed to confirm the insertion size (1925 bp). (b) Hsp1a RNA was *in-vitro* transcribed (lane 2) by using T7 RNA polymerase system, luciferase (lane 1, 1.8 kb) was added as *in-vitro* transcription control. (c) The transcribed RNA was then *in-vitro* translated by using wheat germ extract system and 35 S-methionine. The *in-vitro* translated HSP70 (lane 1) was resolved on 10% SDS-PAGE along with BMV control (lane 2). (d, e) GST-tagged E6AP and GST-tagged E6APM were expressed in *E.Coli* strain BL21 for varied time length (d and e, lane 1 & 3: 4 hr; lane 2 & 4: 5 hr). GST-tagged E6AP (d and e, lane 1 & 2) or GST-tagged E6APM (d and e, lane 3 & 4) were detected by using different antibody, GST (d) or E6AP (e) in Western blot analysis. (f) *In-vitro* interaction study was performed and the result showed that *in-vitro* translated HSP70 (Figure 18) interacted with GST-tagged E6AP (lane 1), while GST-tagged E6APM with a defective catalytic domain failed to interact with HSP70 (lane 2).

Figure 20. Interaction study of E6AP and Hsp70 by cell-lines lysate

In-vivo interaction study was performed by using lysate extracted from COS7 cells. Plasmid contained Flag-Parkin was first transfected and expressed in HEK293T cells to serve as positive control for interaction with Hsp70. Two μ g of E6AP and flag antibody were used to immunoprecipitate target proteins respectively in protein G agarose. Protein crude extract from COS7 cells was then added into binding mixture. The mixture was left on rocking tray for 2 hr at 4°C. Washing buffer was added to get rid of unspecific bindings. Flow-through (Lane 1), washing (Lane 2) and eluate (Lane 3) fractions were then subjected to 10% SDS-PAGE followed by Western blot analysis. The Western blot result indicated that endogenous E6AP was able to interact with endogenous Hsp70 *in vivo*. Positive control flag-tagged Parkin has also showed positive interaction in this study. Western blot was performed by using Hsp70 antibody (1:5000) in upper panel, E6AP antibody (1:5000) in middle panel, Flag antibody (1:10000) in lower panel, respectively.

Figure 21. *In-vivo* interaction study between Ube3a and Hsp70 using mouse brain protein lysate

(a, b) *In-vivo* interaction between E6AP and Hsp70 was studied using lysate prepared from homogenized whole mouse brain. Two μ g of HSP70 and CaBP antibody were used to immunoprecipitate target proteins respectively in protein G agarose. Protein crude extract from brain tissue was then added into binding mixture. The mixture was left on rocking tray for 4 hr at 4°C. Washing buffer was added to remove unspecific bindings. The washing process was performed thrice. Flow-through (Panel a & b, lane 1), washing (Panel a & b, Lane 2) and eluate (Panel a & b, Lane 3) fractions were then subjected to SDS-PAGE for Western blot analysis. Western blot was performed by using HSP70 antibody (1:5000) in panel (A) and Ube3a (1:5000), CaBP (1:5000) antibody in panel (B) and (C), respectively.

3.6 *In-vitro* ubiquitination study of Hsp70 and E6AP

I have conducted *in-vitro* ubiquitination study to determine the ability of E6AP to perform ubiquitination on Hsp70 [Figure 22]. E6AP was obtained by either immuno-captured or using GST tagged E6AP that was expressed in *E. coli*. E6APM with a mutated catalytic domain served as negative control for the *in-vitro* ubiquitination analysis. Parkin was added as a positive control, because Parkin has been shown to ubiquitinate Hsp70 (56). The ubiquitination assay result showed that E6AP and positive control Parkin are able to ubiquitinate their interaction partner Hsp70 [Figure 22(a) and (b); lane 1, 2 and 4] while E6APM with the defective catalytic domain and ubiquitination assay lacking ATP failed to do so [Figure 22(a) and (b), lane 3 and 5].

Figure 22. *In-vitro* Ubiquitination study of HSP70 by using Ube3a

In this *in-vitro* ubiquitination study, E1, E2, Ube3a, Hsp70 and ATP were added into the reaction for at least 3 hr at 30°C, the ubiquitination reaction mixture was then analyzed using 7.5% SDS-PAGE; followed by Western blot detection using ubiquitin antibody (1:5000). The result showed that immuno-precipitated Ube3a (Lane 1) or GST-tagged Ube3a that were captured by glutathione particles (Lane 2) as well as Flag-Parkin (Lane 4) were able to ubiquitinate the substrate (Hsp70), whereas GST-tagged Ube3aM which lacked the catalytic domain (Lane 3) and no ATP control (Lane 5) were not be able to perform ubiquitination on Hsp70. Flag-Parkin has previously been showed to ubiquitinate Hsp70. Western blot via the ECL method with different exposure time were shown in (panel a, 15 s) and (panel b, 30 s).

3.7 Age dependent downregulation of CaBP protein in cerebellum of *Ube3a* knockout mice

The Western blot analysis suggested that both wild type and *Ube3a* knockout mice have comparable amount of CaBP protein at juvenile (1 month old) stage [Figure 23(a), lane 1-2] in cerebellum. However, the CaBP protein level at adult stage (3 month old) of *Ube3a* knockout mice was reduced by 50% when compared to age match wild type mice [Figure 23(a), lane 3-4] according to densitometer result. The western blot result also showed that both wild type and *Ube3a* knockout mice lost CaBP protein along the maturation process as reported in previous study [Figure 23(a), lane 5-8]. However, *Ube3a* knockout mice lost 75% of CaBP protein while wild type mice lost 60% of CaBP protein during the maturation when compared to respective juvenile stage mice.

Figure 23. CaBP protein level comparison study between juvenile and adult mice cerebellum

CaBP expression in 1 month and 3 month old mice was compared using Western blot analysis. CaBP protein levels were almost identical in wild type (W) mice and *Ube3a* knockout mice (AS) at 1 month old (Panel a, lane 1-2). CaBP protein level was downregulated in 3 month old *Ube3a* knockout mice when compared to age match wild type mice, the Western blot result has shown 50% reduction in expression (Panel a, lane 3-4). CaBP protein level was downregulated by 60% in 3 month old wild type mice when compared to 1 month wild type mice (Panel a, lane 5-6), CaBP protein level was highly deficiency in 3 month old *Ube3a* knockout mice, downregulated by 75% if compared to 1 month old *Ube3a* knockout mice (Panel a, lane 7-8). Actin protein level has been used as loading control, Western blot results of Actin were shown in Panel b.

3.8 Knockdown of *Ube3a* in P19 cell-lines

To investigate if knockdown of *Ube3a* in P19 cell line will lead to similar effect on the downstream target proteins like what I have observed in the *Ube3a* knockout mice, I have investigated the differences of NSF and SOD2 in *Ube3a* knockdown P19 cell-line. The result showed that NSF was downregulated by 50% as quantified by densitometry [Figure 24(b)]. Consistent with this observation, NSF is downregulated in hippocampus of the AS mice. Likewise, SOD2, also showed downregulation in brain tissue as well as in *Ube3a* knockdown cell line; it was downregulated by 40% in *Ube3a* knockdown P19 cell-line [Figure 24(c)]. I have tried to study other protein candidates but remained unsuccessful; this may be due to the low expression level of those proteins in P19 cell-line.

Figure 24. Knockdown of *Ube3a* in P19 cell-lines

The effect of E6AP knockdown in P19 cell-line has been investigated; two proteins which were previously found differentially expressed in 2-D DIGE/MS analysis were analyzed. Transfected cells were selected with puromycin antibiotic before harvest. pSUPER.puro vector contained a 19mer targeting *Ube3a* was used for knockdown study, empty vector served as mock transfection. No-transfection control (Panel a-c, Lane 1), mock transfection control (Panel a-c, Lane 2) and *Ube3a* knockdown fractions (Panel a-c, Lane 3) have been subjected to Western blot analysis. The result showed that *Ube3a* was knockdown successfully in this study (Panel a). NSF (Panel b) and SOD2 (Panel c) showed a downregulated pattern and it matched the previous observation (2-D DIGE/MS and Western blot analysis) in hippocampus of AS mice.

3.9 Restoration of CaBP level in *Ube3a* knockout mice by using traditional Chinese herbs

Since many literatures and studies have shown the successful use of various kind of traditional Chinese herb to cure or easing epilepsy in patients. I have tried to restore protein level of CaBP in *Ube3a* knockout mice by administrating them with two TCM herbs, *Gastrodia elata* and *Uncariae sinensis* separately. The administration was conducted for seven consecutive days at the dosage of 10 g/L. At the end of administration period, the proteins from the hippocampus

of mice were extracted. Western blot analysis of wild type mice and *Ube3a* knockout mice (before and after TCM administration) was performed by using CaBP antibody. Similar to western blot analysis performed before CaBP protein was found downregulated by about 80% [Figure 25(a)] and 65% [Figure 26(a) Lane 1 & 2] in *Ube3a* knockout mice when compared to wild type mice. After TCM administration, CaBP protein level was found restored to normal CaBP level or expressed even at a higher level after administration of *Gastrodia elata* [Figure 25(b)] and *Uncariae sinensis* [Figure 26(a)].

Three month old *Ube3a* knockout mice were tested using *Gastrodia elata*, and two of them (67%) responded well to the traditional Chinese herb. Their CaBP protein level was comparable to wild type mice after the treatment. Western blot analysis from one of the positively responded mice was shown in Figure 25(b). Among five 3 month old *Ube3a* knockout mice tested in *Uncariae sinensis* administration, three of them (60%) responded positively to the herb based on Western blot analysis. CaBP protein level remained at a low level in a male *Ube3a* knockout mouse named ASM1 when compared to wild type mice [Figure 26(a), lane2] after TCM administration. However, for those mice that responded well to *Uncariae sinensis*, their CaBP protein level was restored to wild type mice CaBP level, result from one of female mice (ASF1) was shown in [Figure 26(a), lane3].

The CaBP protein level was also found correlated to seizure severity in *Ube3a*

knockout mice based on our seizure grade experiment by using the same set of mice that were administered with *Uncariae sinensis*. Seizure was triggered by 13 kHz signal at 100 dB and seizure severity of mice was graded before and after the TCM administration for comparison. Four of them were susceptible to grade 3 seizure when exposed to designated sound signal before the herb administration, the onset of seizure was occurred in no longer than 15s. After the treatment regime, three of them including ASF1 (female) were found improved in the audiogenic seizure tests, while ASM1 showed (male) no observable improvement [Figure 26(b)].

Figure 25. Restoration of CaBP protein level in *Ube3a* knockout mice after treatment with *Gastrodia elata*

The Western blot analysis was performed by using protein samples extracted from hippocampus of wild type mice, AS mice, and AS mice treated with continuous oral administration of 10 g/L *Gastrodia elata* extract for 7 day. CaBP protein level was downregulated in hippocampus of AS mice (Panel a). CaBP protein level of wild type mice (Panel b, Lane 1) was compared with CaBP protein level of *Gastrodia elata* treated *Ube3a* knockout mice (Panel b, Lane 2), the Western blot results showed that CaBP protein level in treated AS mice was restored, its expression level was now identical to CaBP protein level in wild type mice. Actin was served as loading control in all Western blot analysis (Panel a & b).

(b)

Mouse ID	Seizure Grade (Before Treatment)	Seizure Grade (After Treatment)
ASM1	3	3
ASF1	3	1

Figure 26. Restoration of CaBP protein level in *Ube3a* knockout mice after *Uncariae sinensis* treatment

(a) The Western blot analysis was performed by using protein samples extracted from hippocampus of wild type mice, AS mice and AS mice treated with continuous oral administration of 10 g/L *Uncariae sinensis* for 7 day. CaBP protein level was compared by using sample extracted from wild type mice without any treatment (Lane 1), *Ube3a* knockout mice named ASM1 (Lane 2), and *Ube3a* knockout mice named ASF1 (Lane 3). Mice ASM1 do not respond to the treatment as CaBP protein level remained at reduced level. On the other hand, CaBP protein level in mice ASF1 was restored to level on par with wild type mice after the treatment. Actin has served as loading control in all Western blot analysis. (b) The CaBP protein level of ASM1 remained unchanged while CaBP protein level of ASF1 was upregulated after treatment. These observations were correlated with seizure grade test result. The endurance of seizure did not improved in ASM1 while condition of seizure was vastly improved in ASF1.

Chapter IV: Discussion

Ube3a was first identified as an E3 ligase, targeting p53 as substrate (1). Recent studies showed that Ube3a targets a diverse range of proteins, indicating that it plays a critical role in regulating many cellular pathways. The known targets of Ube3a is shown in Table 7.

Table 7. Recently identified substrates of Ube3a

Gene / Protein	Function	Reference
1 p53	Regulate cell cycle, tumour suppressor protein, DNA repair, initiate apoptosis, response to myriad stressors	(1,7)
2 Src Family of Tyrosine Kinase Blk	Regulators of cytoskeletal organization, cell-cell contact, cell-matrix adhesion, DNA synthesis, cellular proliferation	(116)
3 Cystic fibrosis transmembrane regulator-associated ligand (CAL)	Facilitate of lysosomal degradation of other proteins, intracellular trafficking, autophagy of neuronal cells, vesicular trafficking pathways	(117)
4 Trihydrophobin 1 (TH1)	Assembly of functional human negative transcription elongation factor (NELF) complex	(118)
5 Epithelial cell transforming sequence 2 oncogene (ECT2)	Cytokinesis, cytoskeletal remodelling in response to neurite guidance cues	(33)
6 Polyglutamine aggregation	Protein aggregation associated to cell death and neurodegenerative diseases	(23,115,119)

Among these proteins, Blk from tightly regulated Src family of nonreceptor tyrosine kinase are important in cytoskeletal organization, cell-cell contact; cell-matrix adhesion; few other proteins from Src family are also interacting partners of Ube3a (116). Cystic fibrosis transmembrane regulator-associated ligand (CAL) serve as membrane-associated scaffolds, is associated with targeting of other plasma membrane proteins and is involved in autophagy in neuronal cells (117). Trihydrophobin 1 (TH1) is another interacting partner and target of Ube3a recently identified, it is an integral subunit of the human negative transcription elongation factor (NELF) complex which is important in transcriptional pausing *in vitro* (118). ECT2 is involved in cytokinesis, and cytoskeletal remodelling in response to all known neurite guidance cues (33). Its dysregulation may explain the general learning and behaviour defect in AS patients (4). Polyglutamine inclusion which is translated from the expansion of a CAG trinucleotide repeat caused several human neurodegenerative disease including spinobulbar muscular atrophy (SBMA), Huntington disease (HD) and spinocerebellar ataxias (23,115,119).

In this study, I intend to study the differential expression of proteins caused by *Ube3a* knockout in the AS model. By 2-D DIGE/MS, I identified proteins that are differentially expressed in the mutant mice, which might be the target substrates of Ube3a. Those accumulation or reduction observed may correlate with phenotypes observed among AS patients. However, those differentially expressed protein showed different degree of accumulation or reduction at

different tissue; for example, SOD2 is down-regulated by 45% in cerebellum but down-regulated 80% in hippocampus, whereas, HSP70 is accumulated by only 20% in cerebellum but up-regulated 50% in hippocampus. This may relate to the tissue specific expression or regulation mechanism that affects the expression differentially. However, the percentage changes at mRNA level [Table 6] for those differentially expressed genes are not as huge as protein level, except for CaBP, VDR and NeuroD. This show that the absent of Ube3a affects expression of those gene significantly at translational level than at transcriptional level.

4.1 The interaction of Ube3a and Hsp70

Hsp70 play an important role in cell protection as well as proper protein folding before those proteins became functional, it binds to amino acid chain to ensure that folding process was properly performed; it also binds to the misfolded proteins or specific substrates before those proteins were subjected to degradation (47-50,120). Hsp70 is known to interact with numerous partners, including E3 ligases CHIP, Parkin and Ube3a. In this study, there might be accumulation of proteins that are specific substrates of Ube3a-related degradation; HSP70 is then required to bind to those substrates before subjected to proteasome pathway. Elevated level of HSP70 observed in 2D-DIGE and western blot analysis in cerebellum [Figure 9] and hippocampus [Figure 11] tissue sample of *Ube3a* knockout mice may be caused by accumulation of those

substrate or misfolded proteins. Studies have shown that elevated level of HSP70 may assist in unfolding the misfolded proteins to prevent them from becoming toxins in the brain (47-50,120). This may also be the reason that protein aggregates that are commonly seen in other neurodegenerative diseases are absent in Angelman syndrome mouse model and patients. It is generally known that overexpression of HSP70 prior to neuronal insult improves cell survival in both stroke and epilepsy models. However according to recent studies, the neuroprotection effect from expression of HSP70 in other neurodegenerative diseases was not observed during epileptogenic state, and over-expression of HSP70 in such cases only serves as an indicator of neuronal stress in the acute phase of epilepsy (47). However, other studies have suggested that the death of neuronal cells is not caused by protein aggregation in the brain, but rather by the soluble intermediates. Accumulation of HSP70 may prevent the formation of protein aggregation but allow soluble intermediates to cause toxic effects in AS patients. In both ways, soluble intermediates and protein aggregations attribute to the clinical features of neurodegenerative diseases (121).

From our 2-D DIGE/MS experiment and Western blot analysis by using cerebellum [Figure 9] and hippocampus [Figure 11] tissue sample, it showed that upregulation of Hsp70 was observed in *Ube3a* knockout mice. However, from Real-Time PCR analysis [Figure 13 & 14] using RNA samples extracted from mice, the Hsp70 mRNA level is not significantly affected in the

cerebellum and hippocampus when comparison was made between *Ube3a* knockout and wild type mice. In conclusion, the differential expression of Hsp70 was observed only at the translational or protein level. It is conceivable that Hsp70 is the target of Ube3a as Hsp70 is a multi-functional protein; its most prominent task is to serve as a chaperone in the ubiquitin proteasome system. Parkin and CHIP are two other E3-ligases that are known to interact with Hsp70 for protein quality control task (115). To perform its quality control task, Hsp70 serves as a chaperone binding to misfolded proteins during translation or after stress-mediated protein damage. Studies have shown that Hsp70 interacts with co-chaperone CHIP, which functions as RING domain E3-ligase, and together they serve as the protein quality control system which clears stress-damaged proteins from cells. Such proteins include tau in Alzheimer's disease, and polyglutamate expand protein in Huntington disease (122,123). A recent study also demonstrated that E6AP reduces polyglutamate protein aggregation which induces cell death. The result also showed that E6AP is correlated and over-expressed along with HSP70. The author suggested that HSP70 may play a modulatory role on the function of E6AP (23).

If E6AP does interact with Hsp70 to perform protein quality control task, one of the possible scenarios might be that Hsp70 is targeted by E6AP for Ube3a/proteasome pathway after the substrates have depleted under normal circumstances. In the AS mouse model used in this study, *Ube3a* was knocked out; this might result in HSP70 accumulation after the target substrates have

depleted; Ube3a may be acting in the positive feedback checking system, by promoting the degradation of HSP70 when HSP70 exceeds the threshold level in the body system. The loss of Ube3a in the knockout mice may result in prolonged half-life of Hsp70; this may also be the alternative explanation for up-regulated HSP70 observed in knockout mice. As other studies have suggested, Hsp70 normally assists in multi-ubiquitin chain ubiquitination at lysine48 (K48), and such ubiquitination normally leads to the degradation of the protein (124). Even though there are other E3-ligases; all E3-ligases have their own specific targets. In addition, different post-translational modifications by Ube3a may have different effects on the protein and influence the range of functions that it performs (125). Lack of Ube3a may not affect the half-life of Hsp70 only, but also affect the functions of Hsp70.

There is a study (56) showed that Parkin is able to ubiquitinate Hsp70 in multiple mono-ubiquitination, but the ubiquitin modification is degradation independent. Another study (54) showed that stress-induced Hsp70 is able to be ubiquitinated and degraded by CHIP-mediated pathway when the substrates are depleted. Even though there are other E3-ligases who are responsible for the degradation of Hsp70, but the pathway can be activated only at certain circumstances or location. In *Ube3a* knockout mice, the CHIP-mediated pathway may not be activated and further loss of Ube3a caused the accumulation of Hsp70. Further, CHIP belongs to U-box dependent E3-ligase (53) while Ube3a belongs to Ring-domain E3-ligase, both may require different

condition or co-factors to be effective. However, the activity of other E3-ligases were not measured and examined in the mouse model used in this study. Further studies should include the examination of CHIP's activity and its interaction with Hsp70 using this mouse model.

In this study, I have performed the *in-vitro* interaction [Figure 18 & 19] as well as *in-vivo* interaction [Figure 20 & 21] between Hsp70 and Ube3a. The *in-vivo* interaction study was initiated by using protein extracted from P19 lysate as well as fresh brain tissue of *Ube3a* knockout mice. Based on the function that was performed by Hsp70 and other E3-ligases such as Parkin or CHIP, it is conceivable that the pair up of Hsp70 and Ube3a plays a part in ubiquitin proteasome system. Therefore, I have also explored the possibilities of Ube3a may be involved in ubiquitination of Hsp70, using *in-vitro* ubiquitination assay involving ubiquitin activating enzyme, E1 and ubiquitin conjugating enzyme E2/Ubc7. My experiment confirmed that Ube3a was able to ubiquitinate Hsp70 [Figure 22]. This finding further confirms our speculation that the ubiquitination of Hsp70 leads to its degradation when expression of Hsp70 has exceeded its threshold level, as accumulation of Hsp70 is observed in cerebellum and hippocampus of *Ube3a* knockout mice.

4.2 HSP70 and VDR

In this study, expression of VDR is found to be reduced in *Ube3a* knockout

mice at the protein level in 2-D DIGE/MS study. I have also examined the mRNA level of VDR in cerebellum [Figure 13] and hippocampus [Figure 14] of *Ube3a* knockout mice, and it is found to be down-regulated by 55% and 72% respectively. Its ligand vitamin-D₃ controls calcium homeostasis, bone formation, cell differentiation and apoptosis (97,101). The downregulation of VDR may affect calcium homeostasis in mutant mice since epilepsy is highly correlated to disruption of calcium level in cells and it is one of the most well-known characteristics observed in Angelman syndrome patients.

VDR is a transcriptional regulator that interacts with specific DNA sequences composed of a hexanucleotide of direct repeat, binding as either homodimer or heterodimer with retinoid X receptors (RXRs); cell cycle inhibitors p21 and p27 are two known genes that VDR regulates (98,126). If VDR was downregulated in *Ube3a* knockout mice, its upstream regulator BAG1L may also be affected. BAG1L along with BAG1, BAG1M (Rap46), and BAG1S are four protein isomers that human BAG1 gene encodes. Recently, Hsp70 has been identified as the partner of BAG1L to enhance the trans-activation function of VDR in a concentration dependent manner, this interaction has been speculated to improve tumor cell responses (127,128). Since BAG1L couples with Hsp70 to perform its functions, the accumulation of Hsp70 detected in the AS mouse model may be related to downregulation of VDR. VDR is a multifunctional protein that is known to regulate calcium homeostasis (97,101) and immunity (100). Therefore the correlation of BAG1L, accumulation of Hsp70 and down-

regulated VDR may be interesting to study in the *Ube3a* knockout model.

4.3 The effects of CaBP deficiency

The homeostasis of Ca^{2+} in neurons can be achieved by transporting Ca^{2+} across the membrane; sequestration by cellular organelles, or achieved by cytosolic buffering proteins such as parvalbumin and CaBP. Calcium ion in turn, is actively involved in signal transduction, controlling the development of its regulating proteins to modulate calcium ion transients, neurogenesis and many other functions. Lack of cytoplasmic CaBP severely impairs Ca^{2+} homeostasis and causes nerve cells to be selectively vulnerable to Ca^{2+} related injury (66,71). From 2-D DIGE/MS results and Western blot analysis, CaBP is one of the proteins that is downregulated in both cerebellum [Figure 8] and hippocampus [Figure 10] tissues of *Ube3a* knockout mice. It is known that decline of CaBP in hippocampal dentate granule cells correlates with the kindling model for epilepsy, which might help to explain the frequent seizures observed among Angelman syndrome patients, as excess level of intracellular Ca^{2+} might disrupt the neuronal signal transduction (63).

Another study has shown that CaBP facilitates neuronal differentiation via upregulation of genes such as *NeuroD*, *Pax6*, *VDR* and *Mash1* in a pathway involving CaMK (45). Mash1 or ASC1 is one of the basic helix-loop-helix (bHLH) transcription factors that heterodimerize with ubiquitous Class I bHLH E proteins to form complexes that are crucial in neurogenesis and neural

differentiation at development stage (129). Adult neural progenitor cells continue to generate new neurons, astrocytes and oligodendrocytes in the brain throughout life, under normal turn over circumstances, or after ischemia and in the status of epilepticus. Mash1 and Olig2 stimulates neurogenesis and differentiation of progenitor cells in the telencephalon, which generate the vast array of neurons and glia cells found in adult cerebral cortex, developing ganglionic eminence and olfactory epithelium (130,131). Study using Mash1 null mutant mice show that Mash1 is required for the generation of early population of oligodendrocyte precursors (OPCs), which is involved in the regulation of synaptic transmission and adult neurogenesis (132). Mice with targeted deletion in Mash1 also fail to develop pulmonary neuroendocrine cells (PNECs) and they die in the neonatal period due to respiratory failure (133). NeuroD or Beta2 is another basic helix loop helix (bHLH) transcription factor expressed in neurons of cortical plate; and in neuroendocrine cells in stomach, gut, pancreas and adult lung (134,135). It is involved in the differentiation of neurons, development of pancreas, inner ear and retina (134,136). Like other bHLH factors, it heterodimerizes with E proteins and controls the transcription of a variety of genes to induce neuron differentiation. When NeuroD is deleted in mice, the early differentiating pancreatic endocrine cells die, and total pancreatic insulin level is only about 5% of normal level, the mutant mice will eventually die within five days after birth due to hyperglycemia. In the hippocampus of NeuroD null mice, when granule cells reached dentate gyrus, both cell proliferation and differentiation are severely disturbed, leading to

severe cellular depletion in the brain (135).

The hippocampal mRNA and protein level of CaBP have been demonstrated to express concurrently with the expression of these bHLH transcription factors (45). In the Real-Time RT PCR study, I used fresh tissue from the AS mouse model instead of the progenitor cell cultures that were used in the previous study (45). This is to ensure that *Ube3a* knockout environment is retained and is able to reflect the complex activity *in vivo*. In this case, *CaBP* mRNA level is downregulated by 57% in the cerebellum and 80% in the hippocampus; *NeuroD* mRNA level is downregulated concurrently by 33% and 85% in cerebellum and hippocampus, respectively. However, *Mash1* mRNA level remains unchanged in these two tissues. *NeuroD* encodes a bHLH protein is involved in neuronal cells development as well as differentiation; the downregulation of *NeuroD* at mRNA level may implicate in well developed but lack of differentiation in dendritic spines observed in AS mouse model (137). Even though *Mash1* mRNA level is unaffected, its protein level is reduced in both cerebellum [Figure 8] and hippocampus [Figure 10] of *Ube3a* knockout mice. On the other hand, *NeuroD* mRNA is downregulated in cerebellum and hippocampus, but NeuroD protein is accumulated in cerebellum [Figure 9] and downregulated in hippocampus [Figure 10]. The deficiency of these two proteins in the hippocampus may not only affect the development and differentiation of cells but also affect neurogenesis after ischemic or neuronal damage.

Vitamin D₃ has been shown to induce the expression of CaBP (64,65), and

vitamin D₃ receptor (VDR) and CaBP have been found to co-localize in many tissues especially in the brain. Since reduced *CaBP* and *VDR* mRNA levels in hippocampus of neurodegenerative Alzheimer's disease have been reported (105), this observation also coincides with our findings in Real Time RT PCR experiment that *CaBP* and *VDR* are downregulated by 57% and 55% in the cerebellum, 80% and 72% in the hippocampus of the *Ube3a* knockout mice, respectively [Figure 13 & 14]. Since both mRNA levels are affected and VDR protein level was found downregulated in the 2D-DIGE analysis, this might correlate with the deficiency of CaBP observed in cerebellum and hippocampus. This finding demonstrates a specific association of VDR and CaBP.

4.4 CaBP restoration by using TCM

Oral administration of *Ramulus uncaria* and *Gastrodia elata* continuously for 7 days helps in the restoration of CaBP protein level in the *Ube3a* knockout mice [Figure 25 & 26(a)]. CaBP protein level in the mutant mice is correlated to the susceptibility of mice to audiogenic seizures. AS mice with restored level of CaBP protein showed remarkable improvement in the audiogenic seizures test, from grade 3 seizure to grade 0. In this study, about 60% of the mice tested in this study showed improvements against audiogenic induced seizure, result for one of the mice has been shown in Figure 26(b). The effect of TCM is subjective in our study; it may attribute to the individual's susceptible ability to the active agent in the traditional Chinese medicine, for example adverse effect

of certain drugs (carbamazine) observed among people who carried the variation in HLA gene.

However, further studies have to be performed to determine the identity of active agent; the pathway of traditional Chinese medicine restored the protein level of CaBP. Dosage used or combined administration with other Chinese medicine to improve the medical effect are also the next phase to study. Therefore, elevation of CaBP or its up-stream targets in brain appeared to be crucial to reduce the seizures attack observed in the AS mouse model.

4.5 Differential expression of CaBP in adult and juvenile mice

With the loss of CaBP, an increase in the level of free Ca^{2+} is expected in the brain tissue of AS mice. It has been shown that free calcium ions are able to stimulate oxidative metabolism. For example, activating the dehydrogenases of Krebs cycle provide the ATP needed to support the increased activities of membrane pumps involved in clearing excess Ca^{2+} (66). In turn, ROS elevation would oxidize membrane ion pump and their regulatory proteins such as calmodulin, resulting in an increase of intracellular free Ca^{2+} and lead to elevated oxidative stress in cells (66,71,80). It is known that CaBP protein level is decreased in aged mouse and is consistent with this observation. Western blot analysis showed CaBP protein level in the 1-month old mice is higher than the 3-month old wide-type mice. The same observation is also observed in the

Ube3a knockout mice. However, in comparison, aged AS mice seems to have to have lower CaBP level as compared to that of the wild-type control [Figure 23]; the molecular mechanism underlie this remains unknown.

4.6 REDOX and Mitochondrial dysfunction

ROS/RNS have been shown to behave as signal transduction molecules that modulate protein function such as facilitate oxidative posttranslational modification on protein chaperones (70,81).

The intrinsic mitochondrial apoptotic pathway is the most common form of cell death in neurodegeneration; it controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space (IMS) (80). Reactive oxygen species (ROS) are normal by-products of mitochondrial respiratory chain activity (90). ROS concentration is mediated by mitochondrial antioxidants such as manganese superoxide dismutase (SOD2) and glutathione peroxidase (62). Over production of ROS (oxidative stress) is a central feature of all neurodegenerative disorders (138). In addition to the generation of ROS, mitochondria are also involved in life-sustaining functions including calcium homeostasis, mitochondrial fission and fusion, lipid concentration of the mitochondrial membranes and mitochondrial permeability transition (80,81,91). It is known that mice lacking SOD2 die several days after birth, amid massive oxidative stress (88). In our study, protein level of SOD2 [Figure 8 &

10] but not transcriptional level is down-regulated [Figure 13 & 14] in AS mice, since SOD2 is vital for handling oxidative stress in mitochondria, downregulation of SOD2 may cause a surge of oxidative damage in cells and eventually lead to cell death.

Lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and GST-S-transferase Mu1 are found differentially expressed in the AS mice. These are proteins that are known to be involved in REDOX reaction; LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD. Malate dehydrogenase (MDH) is an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxaloacetate by using NAD^+ . Pyruvate in the mitochondria is acted upon by pyruvate carboxylase to form oxaloacetate, a citric acid cycle intermediate (139,140). Glutathione S-transferase (GSTs) families consist of a total of eight sub-classes of isoenzymes including alpha, kappa, mu, omega, pi, sigma, theta and zeta. These isoenzymes can be cytosolic, mitochondrial, or microsomal proteins depending on the site that they are acting on (141). Glutathione S-transferase Mu 1 (GSTM1) is a human glutathione S-transferase. The mu class of enzymes functions mainly in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione (GST) (142). Genetic variations of GSTM1 can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. GSTM1 is essential for cell protection

as reports show that GSTM1 null mice are predisposed to increased cancer risk, as a result of increased susceptibility to environmental toxins and carcinogens (141,143,144).

When SOD2, LDH, MDH and GSTs class mu1 are reduced in *Ube3a* knockout mice; mitochondrial defect is likely to occur and may lead to neurodegeneration. Mitochondrial dysfunction and oxidative stress are implicated in neurodegenerative disease pathogenesis, which includes Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis (ALS) (138,145). In the case of ALS, there are several proteins reported to have expression changes which coincide with *Ube3a* knockout mice used in my study; those proteins include ATP synthase, mitochondrial F1 complex α subunit; glutathione S-transferase class Mu1 and heat shock 70 protein (70). Intriguingly, in *parkin* knockout mice, similar set of proteins are found to be differentially expressed in cortex and striatum, including ATP synthase α chain mitochondrial; lactate dehydrogenase, malate dehydrogenase, stress-70 protein, glutathione S-transferase P2 and N-ethylmaleimide-sensitive fusion protein (146).

In the current study, protein level of ATP synthase α chain mitochondrial, lactate dehydrogenase, malate dehydrogenase, stress-70 protein, glutathione S-transferase class Mu1, and N-ethylmaleimide-sensitive fusion protein are found to be differentially expressed in the AS mice. The mRNA level of these proteins

remain steady in mutant mice except LDH whose mRNA level is down-regulated by 55% and glutathione S-transferase class Mu1 whose mRNA level is up-regulated by 100% in hippocampus [Figure 14]. This shows that most of the proteins that are involved in REDOX are affected at the translation level in the absence of functional Ube3a. This result suggests that downregulation of LDH, MDH and glutathione S-transferase class Mu1 at the protein level may play a crucial role in pathogenesis of AS patients.

4.7 NSF and motor dysfunction

NSF is not affected at the transcriptional level in cerebellum [Figure 13] and hippocampus [Figure 14] of *Ube3a* knockout mice, but is specifically affected at the protein level. NSF is upregulated in the cerebellum [Figure 9] but downregulated in the hippocampus of AS mice [Figure 10]. NSF protein is known to be involved in docking and fusion of synaptic vesicles at the plasma membrane. It is known that transportation of neurotransmitter such as synapse which involves synaptic vesicles fusing with the pre-synaptic membrane relies on such process to perform neuronal function. Studies have also shown that mutation of NSF in *Drosophila* can result in coma, presumably because neuronal functions have been blocked in the absence of NSF (75). AS patients exhibit symptoms such as tremor, ataxia and motor incoordination; study also shows motor dysfunction in *Ube3a* knockout mice (7). Since NSF is expressed abundantly in hippocampus under normal circumstances (72), it is interesting to

study the relationship of NSF deficiency in hippocampus and movement incoordination in mutant mice. NSF is also discovered as an epilepsy gene (77,78). Along with deficiency of CaBP protein detected in *Ube3a* knockout mice, these two proteins are crucial in the study of clinical feature such as inducible seizure that is commonly found in AS patients.

Conclusion

By using 2-D DIGE technique in this study, 10 differential expressed proteins have been detected in cerebellum or hippocampus of *Ube3a* knockout mice. However, due to the limitation of 2D-DIGE technique, many low abundant but differentially expressed proteins may not be detected in 2D electrophoresis. Since this study is conducted using broad range pH 3-11NL, 24cm strip for 1-D isoelectric focusing study, extremely acidic or basic proteins are omitted from the study as well. Further study using narrow range pH strip, for example pH 1, pH 3-5, pH 4-7 can increase the resolution of the protein analysis profile.

Among these 10 proteins identified, CaBP is known to be highly involved in firing pattern of neuron cells. Therefore, CaBP deficiency in cerebellum and hippocampus may be related to frequently observed seizure among Angelman syndrome patients. However, further studies about the regulation of CaBP, especially in the brain; the causes of CaBP are down-regulated in *Ube3a* knockout mice have to be done. Other than that, experiment in this study has also confirmed that CaBP is down-regulated when the mice grew mature; but *Ube3a* knockout mice will tend to loss more CaBP when compared to wild-type mice, the mechanism behind remains another topic required further evaluation. I have also tried to elevate the protein level of CaBP in hippocampus of mutant mice using traditional Chinese medicine *Gastrodia elata* and *Uncariae sinensis*. In the result of preliminary experiment, the protein level has been restored to the level that is comparable to wild type mice. However, further evaluation with

larger population size should be conducted to confirm the observation; a combination of different traditional Chinese medicine can be tested as it might provide more comprehensive effects to the *Ube3a* knockout mice. Next, the identification of active agent in those traditional Chinese medicines has to be verified, and the mechanism of those traditional Chinese medicines/active agents takes place in elevating the CaBP protein level should be carried out.

Another protein of interested in this study is chaperone protein Hsp70, which is accumulated in cerebellum and hippocampus of *Ube3a* knockout mice. In-vitro and in-vivo interaction studies have been performed to show that Hsp70 is the interacting partner of Ube3a; whereas in-vitro ubiquitination result showed that Hsp70 is able to be poly-ubiquitinated by Ube3a. However, the interaction study between Hsp70 and Ube3a is not comprehensive, further studies have to be performed to determine the target sites of poly-ubiquitination in Hsp70, the function of poly-ubiquitination in Hsp70 by Ube3a as well as the mechanism of Hsp70 and Ube3a to perform protein quality check. It is also interesting to study the activities of other E3-ligase (Parkin and CHIP) that are known to be interacted and able to ubiquitinate Hsp70 in *Ube3a* knockout mice, whether activity of those E3-ligase are elevated to compensate the absent of Ube3a.

There are multiple differentially expressed proteins which were detected by 2-D DIGE system are actively involved in REDOX reaction. Those proteins include SOD2, LDH, MDH, GSTs-Mu1, ATP5a1, VDR and CaBP. Oxidative stress

assay should be conducted to test the level of free radicals in the mutant mice, especially in cerebellum and hippocampus; and the effects of excessive oxidative stress (if there is any) in the system that might contribute to the development of Angelman syndrome. In this study, I have tried to examine the effects of loss of Ube3a in the mouse model by using the proteomic approach; those differentially expressed proteins may thus contribute to the symptoms observed in Angelman syndrome patients. From the experiment results obtained in this study, it has suggested a few interesting areas for further studies, including the oxidative stress assay, the regulation of CaBP in *Ube3a* knockout mice that may provide further understanding of Angelman syndrome.

Reference

1. Jiang, Y., Lev-Lehman, E., Bressler, J., Tsai, T.F. and Beaudet, A.L. (1999) Genetics of Angelman syndrome. *American journal of human genetics*, **65**, 1-6.
2. Witte, W., Nobel, C. and Hilpert, J. (2011) [Anesthesia and Angelman syndrome.]. *Der Anaesthetist*.
3. Lalande, M. and Calciano, M.A. (2007) Molecular epigenetics of Angelman syndrome. *Cellular and molecular life sciences : CMLS*, **64**, 947-960.
4. Nicholls, R.D., Saitoh, S. and Horsthemke, B. (1998) Imprinting in Prader-Willi and Angelman syndromes. *Trends in genetics : TIG*, **14**, 194-200.
5. Cattanaach, B.M., Barr, J.A., Beechey, C.V., Martin, J., Noebels, J. and Jones, J. (1997) A candidate model for Angelman syndrome in the mouse. *Mammalian genome : official journal of the International Mammalian Genome Society*, **8**, 472-478.
6. Culiati, C.T., Stubbs, L.J., Montgomery, C.S., Russell, L.B. and Rinchik, E.M. (1994) Phenotypic consequences of deletion of the gamma 3, alpha 5, or beta 3 subunit of the type A gamma-aminobutyric acid receptor in mice. *Proc Natl Acad Sci U S A*, **91**, 2815-2818.
7. Jiang, Y.H., Armstrong, D., Albrecht, U., Atkins, C.M., Noebels, J.L., Eichele, G., Sweatt, J.D. and Beaudet, A.L. (1998) Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron*, **21**, 799-811.
8. Jiang, Y.H., Pan, Y., Zhu, L., Landa, L., Yoo, J., Spencer, C., Lorenzo, I., Brilliant, M., Noebels, J. and Beaudet, A.L. (2010) Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. *PloS one*, **5**, e12278.
9. Miura, K., Kishino, T., Li, E., Webber, H., Dikkes, P., Holmes, G.L. and Wagstaff, J. (2002) Neurobehavioral and electroencephalographic abnormalities in Ube3a maternal-deficient mice. *Neurobiol Dis*, **9**, 149-159.
10. Homanics, G.E., DeLorey, T.M., Firestone, L.L., Quinlan, J.J., Handforth, A., Harrison, N.L., Krasowski, M.D., Rick, C.E., Korpi, E.R., Makela, R. *et al.* (1997) Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. *Proc Natl Acad Sci U S A*, **94**, 4143-4148.
11. Johnstone, K.A., DuBose, A.J., Futtner, C.R., Elmore, M.D., Brannan, C.I. and Resnick, J.L. (2006) A human imprinting centre demonstrates conserved acquisition but diverged maintenance of imprinting in a mouse model for Angelman syndrome imprinting defects. *Hum Mol*

- Genet*, **15**, 393-404.
12. Wu, M.Y., Chen, K.S., Bressler, J., Hou, A., Tsai, T.F. and Beaudet, A.L. (2006) Mouse imprinting defect mutations that model Angelman syndrome. *Genesis*, **44**, 12-22.
 13. Gabriel, J.M., Merchant, M., Ohta, T., Ji, Y., Caldwell, R.G., Ramsey, M.J., Tucker, J.D., Longnecker, R. and Nicholls, R.D. (1999) A transgene insertion creating a heritable chromosome deletion mouse model of Prader-Willi and angelman syndromes. *Proc Natl Acad Sci U S A*, **96**, 9258-9263.
 14. Kishino, T., Lalande, M. and Wagstaff, J. (1997) UBE3A/E6-AP mutations cause Angelman syndrome. *Nature genetics*, **15**, 70-73.
 15. Ciechanover, A. (2007) Intracellular protein degradation from a vague idea through the lysosome and the ubiquitin-proteasome system and on to human diseases and drug targeting: Nobel Lecture, December 8, 2004. *Annals of the New York Academy of Sciences*, **1116**, 1-28.
 16. Nawaz, Z., Lonard, D.M., Smith, C.L., Lev-Lehman, E., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1999) The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular and cellular biology*, **19**, 1182-1189.
 17. Ramamoorthy, S. and Nawaz, Z. (2008) E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. *Nuclear receptor signaling*, **6**, e006.
 18. Strous, G.J. and Govers, R. (1999) The ubiquitin-proteasome system and endocytosis. *Journal of cell science*, **112** (Pt 10), 1417-1423.
 19. Jin, J., Li, X., Gygi, S.P. and Harper, J.W. (2007) Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. *Nature*, **447**, 1135-1138.
 20. Esser, C., Alberti, S. and Hohfeld, J. (2004) Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochimica et biophysica acta*, **1695**, 171-188.
 21. Haas, K.F. and Broadie, K. (2008) Roles of ubiquitination at the synapse. *Biochimica et biophysica acta*, **1779**, 495-506.
 22. Meller, R. (2009) The role of the ubiquitin proteasome system in ischemia and ischemic tolerance. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*, **15**, 243-260.
 23. Mishra, A., Dikshit, P., Purkayastha, S., Sharma, J., Nukina, N. and Jana, N.R. (2008) E6-AP promotes misfolded polyglutamine proteins for proteasomal degradation and suppresses polyglutamine protein aggregation and toxicity. *The Journal of biological chemistry*, **283**, 7648-7656.
 24. Dindot, S.V., Antalffy, B.A., Bhattacharjee, M.B. and Beaudet, A.L. (2008) The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Hum Mol Genet*, **17**, 111-118.
 25. Sanduja, S., Kaza, V. and Dixon, D.A. (2009) The mRNA decay factor

- tristetraprolin (TTP) induces senescence in human papillomavirus-transformed cervical cancer cells by targeting E6-AP ubiquitin ligase. *Aging*, **1**, 803-817.
26. Su, H., Fan, W., Coskun, P.E., Vesa, J., Gold, J.A., Jiang, Y.H., Potluri, P., Procaccio, V., Acab, A., Weiss, J.H. *et al.* (2011) Mitochondrial dysfunction in CA1 hippocampal neurons of the UBE3A deficient mouse model for Angelman syndrome. *Neuroscience letters*, **487**, 129-133.
 27. Goldberg, A.L. (2003) Protein degradation and protection against misfolded or damaged proteins. *Nature*, **426**, 895-899.
 28. Weeber, E.J., Jiang, Y.H., Elgersma, Y., Varga, A.W., Carrasquillo, Y., Brown, S.E., Christian, J.M., Mirnikjoo, B., Silva, A., Beaudet, A.L. *et al.* (2003) Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **23**, 2634-2644.
 29. DeLorey, T.M., Handforth, A., Anagnostaras, S.G., Homanics, G.E., Minassian, B.A., Asatourian, A., Fanselow, M.S., Delgado-Escueta, A., Ellison, G.D. and Olsen, R.W. (1998) Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **18**, 8505-8514.
 30. van Woerden, G.M., Harris, K.D., Hojjati, M.R., Gustin, R.M., Qiu, S., de Avila Freire, R., Jiang, Y.H., Elgersma, Y. and Weeber, E.J. (2007) Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of alphaCaMKII inhibitory phosphorylation. *Nature neuroscience*, **10**, 280-282.
 31. Mardirossian, S., Rampon, C., Salvert, D., Fort, P. and Sarda, N. (2009) Impaired hippocampal plasticity and altered neurogenesis in adult Ube3a maternal deficient mouse model for Angelman syndrome. *Experimental neurology*, **220**, 341-348.
 32. Greer, P.L., Hanayama, R., Bloodgood, B.L., Mardinly, A.R., Lipton, D.M., Flavell, S.W., Kim, T.K., Griffith, E.C., Waldon, Z., Maehr, R. *et al.* (2010) The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell*, **140**, 704-716.
 33. Reiter, L.T., Seagroves, T.N., Bowers, M. and Bier, E. (2006) Expression of the Rho-GEF Pbl/ECT2 is regulated by the UBE3A E3 ubiquitin ligase. *Hum Mol Genet*, **15**, 2825-2835.
 34. Bernd H.A. Rehm, F.R. (2004) Evaluation of Proteomic Techniques: Applications and Potential. *Current Proteomics*, **1**, 103-111.
 35. Eravci, M., Fuxius, S., Broedel, O., Weist, S., Eravci, S., Mansmann, U., Schluter, H., Tiemann, J. and Baumgartner, A. (2007) Improved comparative proteome analysis based on two-dimensional gel electrophoresis. *Proteomics*, **7**, 513-523.

36. Bonneil, E., Tessier, S., Carrier, A. and Thibault, P. (2005) Multiplex multidimensional nanoLC-MS system for targeted proteomic analyses. *Electrophoresis*, **26**, 4575-4589.
37. Schmidt, F., Donahoe, S., Hagens, K., Mattow, J., Schaible, U.E., Kaufmann, S.H., Aebersold, R. and Jungblut, P.R. (2004) Complementary analysis of the Mycobacterium tuberculosis proteome by two-dimensional electrophoresis and isotope-coded affinity tag technology. *Molecular & cellular proteomics : MCP*, **3**, 24-42.
38. Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S. *et al.* (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics : MCP*, **3**, 1154-1169.
39. Choe, L., D'Ascenzo, M., Relkin, N.R., Pappin, D., Ross, P., Williamson, B., Guertin, S., Pribil, P. and Lee, K.H. (2007) 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. *Proteomics*, **7**, 3651-3660.
40. Pierce, A., Unwin, R.D., Evans, C.A., Griffiths, S., Carney, L., Zhang, L., Jaworska, E., Lee, C.F., Blinco, D., Okoniewski, M.J. *et al.* (2008) Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. *Molecular & cellular proteomics : MCP*, **7**, 853-863.
41. Tannu, N.S. and Hemby, S.E. (2006) Methods for proteomics in neuroscience. *Prog Brain Res*, **158**, 41-82.
42. Wiese, S., Reidegeld, K.A., Meyer, H.E. and Warscheid, B. (2007) Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics*, **7**, 340-350.
43. Ow, S.Y., Salim, M., Noirel, J., Evans, C., Rehman, I. and Wright, P.C. (2009) iTRAQ underestimation in simple and complex mixtures: "the good, the bad and the ugly". *J Proteome Res*, **8**, 5347-5355.
44. Wu, W.W., Wang, G., Baek, S.J. and Shen, R.F. (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *J Proteome Res*, **5**, 651-658.
45. Kim, J.H., Lee, J.A., Song, Y.M., Park, C.H., Hwang, S.J., Kim, Y.S., Kaang, B.K. and Son, H. (2006) Overexpression of calbindin-D28K in hippocampal progenitor cells increases neuronal differentiation and neurite outgrowth. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, **20**, 109-111.
46. Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W. and Bennink, J.R. (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*, **404**, 770-774.
47. Turturici, G., Sconzo, G. and Geraci, F. (2011) Hsp70 and its molecular role in nervous system diseases. *Biochemistry research international*,

- 2011, 618127.
48. Magrane, J., Smith, R.C., Walsh, K. and Querfurth, H.W. (2004) Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed beta-amyloid in neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **24**, 1700-1706.
 49. Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet*, **10**, 1511-1518.
 50. Jana, N.R., Tanaka, M., Wang, G. and Nukina, N. (2000) Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum Mol Genet*, **9**, 2009-2018.
 51. Tai, H.C. and Schuman, E.M. (2008) Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nature reviews. Neuroscience*, **9**, 826-838.
 52. Nardai, G., Csermely, P. and Soti, C. (2002) Chaperone function and chaperone overload in the aged. A preliminary analysis. *Experimental gerontology*, **37**, 1257-1262.
 53. Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J. and Patterson, C. (2001) CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *The Journal of biological chemistry*, **276**, 42938-42944.
 54. Qian, S.B., McDonough, H., Boellmann, F., Cyr, D.M. and Patterson, C. (2006) CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. *Nature*, **440**, 551-555.
 55. Dai, Q., Zhang, C., Wu, Y., McDonough, H., Whaley, R.A., Godfrey, V., Li, H.H., Madamanchi, N., Xu, W., Neckers, L. *et al.* (2003) CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *The EMBO journal*, **22**, 5446-5458.
 56. Moore, D.J., West, A.B., Dikeman, D.A., Dawson, V.L. and Dawson, T.M. (2008) Parkin mediates the degradation-independent ubiquitination of Hsp70. *Journal of neurochemistry*, **105**, 1806-1819.
 57. Liu, M., Aneja, R., Sun, X., Xie, S., Wang, H., Wu, X., Dong, J.T., Li, M., Joshi, H.C. and Zhou, J. (2008) Parkin regulates Eg5 expression by Hsp70 ubiquitination-dependent inactivation of c-Jun NH2-terminal kinase. *The Journal of biological chemistry*, **283**, 35783-35788.
 58. Dickey, C.A., Patterson, C., Dickson, D. and Petrucelli, L. (2007) Brain CHIP: removing the culprits in neurodegenerative disease. *Trends in molecular medicine*, **13**, 32-38.
 59. Hsu, A.L., Murphy, C.T. and Kenyon, C. (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science (New York, N.Y.)*, **300**, 1142-1145.
 60. Tatar, M., Khazaeli, A.A. and Curtsinger, J.W. (1997) Chaperoning

- extended life. *Nature*, **390**, 30.
61. Tupling, A.R., Bombardier, E., Vigna, C., Quadrilatero, J. and Fu, M. (2008) Interaction between Hsp70 and the SR Ca²⁺ pump: a potential mechanism for cytoprotection in heart and skeletal muscle. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*, **33**, 1023-1032.
 62. Li, Q., Zhang, M., Chen, Y.J., Wang, Y.J., Huang, F. and Liu, J. (2011) Oxidative damage and HSP70 expression in masseter muscle induced by psychological stress in rats. *Physiology & behavior*.
 63. Kohr, G., Lambert, C.E. and Mody, I. (1991) Calbindin-D28K (CaBP) levels and calcium currents in acutely dissociated epileptic neurons. *Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale*, **85**, 543-551.
 64. Wasserman, R.H. and Taylor, A.N. (1966) Vitamin d3-induced calcium-binding protein in chick intestinal mucosa. *Science (New York, N.Y.)*, **152**, 791-793.
 65. Thomasset, M., Parkes, C.O. and Cuisinier-Gleizes, P. (1982) Rat calcium-binding proteins: distribution, development, and vitamin D dependence. *The American journal of physiology*, **243**, E483-488.
 66. Dong, Z., Saikumar, P., Weinberg, J.M. and Venkatachalam, M.A. (2006) Calcium in cell injury and death. *Annual review of pathology*, **1**, 405-434.
 67. Sutherland, M.K., Wong, L., Somerville, M.J., Yoong, L.K., Bergeron, C., Parmentier, M. and McLachlan, D.R. (1993) Reduction of calbindin-28k mRNA levels in Alzheimer as compared to Huntington hippocampus. *Brain research. Molecular brain research*, **18**, 32-42.
 68. Sasaki, S., Warita, H., Komori, T., Murakami, T., Abe, K. and Iwata, M. (2006) Parvalbumin and calbindin D-28k immunoreactivity in transgenic mice with a G93A mutant SOD1 gene. *Brain research*, **1083**, 196-203.
 69. Airaksinen, M.S., Eilers, J., Garaschuk, O., Thoenen, H., Konnerth, A. and Meyer, M. (1997) Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. *Proc Natl Acad Sci U S A*, **94**, 1488-1493.
 70. Jain, M.R., Ge, W.W., Elkabes, S. and Li, H. (2008) Amyotrophic lateral sclerosis: Protein chaperone dysfunction revealed by proteomic studies of animal models. *Proteomics. Clinical applications*, **2**, 670-684.
 71. Squier, T.C. and Bigelow, D.J. (2000) Protein oxidation and age-dependent alterations in calcium homeostasis. *Frontiers in bioscience : a journal and virtual library*, **5**, D504-526.
 72. Puschel, A.W., O'Connor, V. and Betz, H. (1994) The N-ethylmaleimide-sensitive fusion protein (NSF) is preferentially expressed in the nervous system. *FEBS letters*, **347**, 55-58.
 73. Nishimune, A., Isaac, J.T., Molnar, E., Noel, J., Nash, S.R., Tagaya, M., Collingridge, G.L., Nakanishi, S. and Henley, J.M. (1998) NSF binding

- to GluR2 regulates synaptic transmission. *Neuron*, **21**, 87-97.
74. Evers, D.M., Matta, J.A., Hoe, H.S., Zarkowsky, D., Lee, S.H., Isaac, J.T. and Pak, D.T. (2010) Plk2 attachment to NSF induces homeostatic removal of GluA2 during chronic overexcitation. *Nature neuroscience*, **13**, 1199-1207.
 75. Pallanck, L., Ordway, R.W. and Ganetzky, B. (1995) A Drosophila NSF mutant. *Nature*, **376**, 25.
 76. Sanyal, S., Tolar, L.A., Pallanck, L. and Krishnan, K.S. (2001) Genetic interaction between shibire and comatose mutations in Drosophila suggest a role for snap-receptor complex assembly and disassembly for maintenance of synaptic vesicle cycling. *Neuroscience letters*, **311**, 21-24.
 77. Guan, Z., Lu, L., Zheng, Z., Liu, J., Yu, F., Lu, S., Xin, Y., Liu, X., Hong, J. and Zhang, W. (2001) A spontaneous recurrent seizure-related Rattus NSF gene identified by linker capture subtraction. *Brain research. Molecular brain research*, **87**, 117-123.
 78. Yu, F., Guan, Z., Zhuo, M., Sun, L., Zou, W., Zheng, Z. and Liu, X. (2002) Further identification of NSF* as an epilepsy related gene. *Brain research. Molecular brain research*, **99**, 141-144.
 79. Zhao, C., Slevin, J.T. and Whiteheart, S.W. (2007) Cellular functions of NSF: not just SNAPS and SNAREs. *FEBS letters*, **581**, 2140-2149.
 80. Rego, A.C. and Oliveira, C.R. (2003) Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochemical research*, **28**, 1563-1574.
 81. Squier, T.C. (2001) Oxidative stress and protein aggregation during biological aging. *Experimental gerontology*, **36**, 1539-1550.
 82. Kim, I., Xu, W. and Reed, J.C. (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nature reviews. Drug discovery*, **7**, 1013-1030.
 83. Tabner, B.J., Turnbull, S., El-Agnaf, O.M. and Allsop, D. (2002) Formation of hydrogen peroxide and hydroxyl radicals from A(beta) and alpha-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease. *Free radical biology & medicine*, **32**, 1076-1083.
 84. Minkeviciene, R., Rheims, S., Doboszay, M.B., Zilberter, M., Hartikainen, J., Fulop, L., Penke, B., Zilberter, Y., Harkany, T., Pitkanen, A. *et al.* (2009) Amyloid beta-induced neuronal hyperexcitability triggers progressive epilepsy. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **29**, 3453-3462.
 85. Turnbull, S., Tabner, B.J., El-Agnaf, O.M., Moore, S., Davies, Y. and Allsop, D. (2001) alpha-Synuclein implicated in Parkinson's disease catalyses the formation of hydrogen peroxide in vitro. *Free radical biology & medicine*, **30**, 1163-1170.
 86. Palacino, J.J., Sagi, D., Goldberg, M.S., Krauss, S., Motz, C., Wacker,

- M., Klose, J. and Shen, J. (2004) Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *The Journal of biological chemistry*, **279**, 18614-18622.
87. Parihar, M.S. and Brewer, G.J. (2007) Mitochondrial failure in Alzheimer disease. *American journal of physiology. Cell physiology*, **292**, C8-23.
 88. Zelko, I.N., Mariani, T.J. and Folz, R.J. (2002) Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free radical biology & medicine*, **33**, 337-349.
 89. Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S.R., Alderson, N.L., Baynes, J.W., Epstein, C.J., Huang, T.T. *et al.* (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiological genomics*, **16**, 29-37.
 90. Sugawara, T., Noshita, N., Lewen, A., Gasche, Y., Ferrand-Drake, M., Fujimura, M., Morita-Fujimura, Y. and Chan, P.H. (2002) Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 209-217.
 91. Scandalios, J.G. (1993) Oxygen Stress and Superoxide Dismutases. *Plant physiology*, **101**, 7-12.
 92. Sun, J. and Tower, J. (1999) FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster* flies. *Molecular and cellular biology*, **19**, 216-228.
 93. Sun, J., Folk, D., Bradley, T.J. and Tower, J. (2002) Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult *Drosophila melanogaster*. *Genetics*, **161**, 661-672.
 94. Harris, N., Costa, V., MacLean, M., Mollapour, M., Moradas-Ferreira, P. and Piper, P.W. (2003) Mnsod overexpression extends the yeast chronological (G(0)) life span but acts independently of Sir2p histone deacetylase to shorten the replicative life span of dividing cells. *Free radical biology & medicine*, **34**, 1599-1606.
 95. Zhong, W., Oberley, L.W., Oberley, T.D. and St Clair, D.K. (1997) Suppression of the malignant phenotype of human glioma cells by overexpression of manganese superoxide dismutase. *Oncogene*, **14**, 481-490.
 96. Plymate, S.R., Haugk, K.H., Sprenger, C.C., Nelson, P.S., Tennant, M.K., Zhang, Y., Oberley, L.W., Zhong, W., Drivdahl, R. and Oberley, T.D. (2003) Increased manganese superoxide dismutase (SOD-2) is part of the mechanism for prostate tumor suppression by Mac25/insulin-like growth factor binding-protein-related protein-1. *Oncogene*, **22**, 1024-

- 1034.
97. MacDonald, P.N., Haussler, C.A., Terpening, C.M., Galligan, M.A., Reeder, M.C., Whitfield, G.K. and Haussler, M.R. (1991) Baculovirus-mediated expression of the human vitamin D receptor. Functional characterization, vitamin D response element interactions, and evidence for a receptor auxiliary factor. *The Journal of biological chemistry*, **266**, 18808-18813.
 98. Glass, C.K. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrine reviews*, **15**, 391-407.
 99. Sun, J. (2010) Vitamin D and mucosal immune function. *Current opinion in gastroenterology*, **26**, 591-595.
 100. Welsh, J., Zinser, L.N., Miannecki-Morton, L., Martin, J., Waltz, S.E., James, H. and Zinser, G.M. (2011) Age-related changes in the epithelial and stromal compartments of the mammary gland in normocalcemic mice lacking the vitamin D3 receptor. *PloS one*, **6**, e16479.
 101. Zinser, G., Packman, K. and Welsh, J. (2002) Vitamin D(3) receptor ablation alters mammary gland morphogenesis. *Development (Cambridge, England)*, **129**, 3067-3076.
 102. Wu, S. and Sun, J. (2011) Vitamin D, vitamin D receptor, and macroautophagy in inflammation and infection. *Discovery medicine*, **11**, 325-335.
 103. Ellison, T.I., Smith, M.K., Gilliam, A.C. and MacDonald, P.N. (2008) Inactivation of the vitamin D receptor enhances susceptibility of murine skin to UV-induced tumorigenesis. *The Journal of investigative dermatology*, **128**, 2508-2517.
 104. Bikle, D.D. (2011) The vitamin D receptor: a tumor suppressor in skin. *Discovery medicine*, **11**, 7-17.
 105. Sutherland, M.K., Somerville, M.J., Yoong, L.K., Bergeron, C., Haussler, M.R. and McLachlan, D.R. (1992) Reduction of vitamin D hormone receptor mRNA levels in Alzheimer as compared to Huntington hippocampus: correlation with calbindin-28k mRNA levels. *Brain research. Molecular brain research*, **13**, 239-250.
 106. Patwardhan, B., Warude, D., Pushpangadan, P. and Bhatt, N. (2005) Ayurveda and traditional Chinese medicine: a comparative overview. *Evidence-based complementary and alternative medicine : eCAM*, **2**, 465-473.
 107. Ito, Y., Yano, S., Watanabe, K., Yamanaka, E., Aimi, N. and Sakai, S. (1990) Structure-activity relationship of yohimbine and its related analogs in blocking alpha-1 and alpha-2 adrenoceptors: a comparative study of cardiovascular activities. *Chemical & pharmaceutical bulletin*, **38**, 1702-1706.
 108. Yano, S., Horiuchi, H., Horie, S., Aimi, N., Sakai, S. and Watanabe, K. (1991) Ca²⁺ channel blocking effects of hirsutine, an indole alkaloid from *Uncaria* genus, in the isolated rat aorta. *Planta medica*, **57**, 403-

- 405.
109. Goto, H., Sakakibara, I., Shimada, Y., Kasahara, Y. and Terasawa, K. (2000) Vasodilator effect of extract prepared from *Uncariae ramulus* on isolated rat aorta. *The American journal of Chinese medicine*, **28**, 197-203.
 110. Kim, H.J., Moon, K.D., Oh, S.Y., Kim, S.P. and Lee, S.R. (2001) Ether fraction of methanol extracts of *Gastrodia elata*, a traditional medicinal herb, protects against kainic acid-induced neuronal damage in the mouse hippocampus. *Neuroscience letters*, **314**, 65-68.
 111. Hsieh, C.L., Tang, N.Y., Chiang, S.Y., Hsieh, C.T. and Lin, J.G. (1999) Anticonvulsive and free radical scavenging actions of two herbs, *Uncaria rhynchophylla* (MIQ) Jack and *Gastrodia elata* Bl., in kainic acid-treated rats. *Life sciences*, **65**, 2071-2082.
 112. Li, N., Liu, B., Dluzen, D.E. and Jin, Y. (2007) Protective effects of ginsenoside Rg2 against glutamate-induced neurotoxicity in PC12 cells. *Journal of ethnopharmacology*, **111**, 458-463.
 113. Shim, J.S., Kim, H.G., Ju, M.S., Choi, J.G., Jeong, S.Y. and Oh, M.S. (2009) Effects of the hook of *Uncaria rhynchophylla* on neurotoxicity in the 6-hydroxydopamine model of Parkinson's disease. *Journal of ethnopharmacology*, **126**, 361-365.
 114. Low, D. and Chen, K.S. (2011) UBE3A regulates MC1R expression: a link to hypopigmentation in Angelman syndrome. *Pigment Cell Melanoma Res*, **24**, 944-952.
 115. Mishra, A., Godavarthi, S.K., Maheshwari, M., Goswami, A. and Jana, N.R. (2009) The ubiquitin ligase E6-AP is induced and recruited to aggresomes in response to proteasome inhibition and may be involved in the ubiquitination of Hsp70-bound misfolded proteins. *The Journal of biological chemistry*, **284**, 10537-10545.
 116. Oda, H., Kumar, S. and Howley, P.M. (1999) Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc Natl Acad Sci U S A*, **96**, 9557-9562.
 117. Jeong, K.W., Kim, H.Z., Kim, S., Kim, Y.S. and Choe, J. (2007) Human papillomavirus type 16 E6 protein interacts with cystic fibrosis transmembrane regulator-associated ligand and promotes E6-associated protein-mediated ubiquitination and proteasomal degradation. *Oncogene*, **26**, 487-499.
 118. Yang, Y., Liu, W., Zou, W., Wang, H., Zong, H., Jiang, J., Wang, Y. and Gu, J. (2007) Ubiquitin-dependent proteolysis of trihydrophobin 1 (TH1) by the human papilloma virus E6-associated protein (E6-AP). *Journal of cellular biochemistry*, **101**, 167-180.
 119. Cummings, C.J., Reinstein, E., Sun, Y., Antalfy, B., Jiang, Y., Ciechanover, A., Orr, H.T., Beaudet, A.L. and Zoghbi, H.Y. (1999) Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron*, **24**, 879-892.

120. Nagel, F., Falkenburger, B.H., Tonges, L., Kowsky, S., Poppelmeyer, C., Schulz, J.B., Bahr, M. and Dietz, G.P. (2008) Tat-Hsp70 protects dopaminergic neurons in midbrain cultures and in the substantia nigra in models of Parkinson's disease. *Journal of neurochemistry*, **105**, 853-864.
121. Yamamoto, A. and Simonsen, A. (2011) The elimination of accumulated and aggregated proteins: a role for autophagy in neurodegeneration. *Neurobiol Dis*, **43**, 17-28.
122. Rujano, M.A., Kampinga, H.H. and Salomons, F.A. (2007) Modulation of polyglutamine inclusion formation by the Hsp70 chaperone machine. *Experimental cell research*, **313**, 3568-3578.
123. Rosser, M.F., Washburn, E., Muchowski, P.J., Patterson, C. and Cyr, D.M. (2007) Chaperone functions of the E3 ubiquitin ligase CHIP. *The Journal of biological chemistry*, **282**, 22267-22277.
124. Lim, K.L. and Lim, G.G. (2011) K63-linked ubiquitination and neurodegeneration. *Neurobiol Dis*, **43**, 9-16.
125. Yi, J.J. and Ehlers, M.D. (2007) Emerging roles for ubiquitin and protein degradation in neuronal function. *Pharmacological reviews*, **59**, 14-39.
126. Liu, M., Lee, M.H., Cohen, M., Bommakanti, M. and Freedman, L.P. (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes & development*, **10**, 142-153.
127. Kudoh, M., Knee, D.A., Takayama, S. and Reed, J.C. (2002) Bag1 proteins regulate growth and survival of ZR-75-1 human breast cancer cells. *Cancer research*, **62**, 1904-1909.
128. Guzey, M., Takayama, S. and Reed, J.C. (2000) BAG1L enhances trans-activation function of the vitamin D receptor. *The Journal of biological chemistry*, **275**, 40749-40756.
129. Henke, R.M., Meredith, D.M., Borromeo, M.D., Savage, T.K. and Johnson, J.E. (2009) Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. *Developmental biology*, **328**, 529-540.
130. Uchida, Y., Nakano, S., Gomi, F. and Takahashi, H. (2007) Differential regulation of basic helix-loop-helix factors Mash1 and Olig2 by beta-amyloid accelerates both differentiation and death of cultured neural stem/progenitor cells. *The Journal of biological chemistry*, **282**, 19700-19709.
131. Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A. and Guillemot, F. (2004) Mash1 specifies neurons and oligodendrocytes in the postnatal brain. *The EMBO journal*, **23**, 4495-4505.
132. Parras, C.M., Hunt, C., Sugimori, M., Nakafuku, M., Rowitch, D. and Guillemot, F. (2007) The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**,

- 4233-4242.
133. Neptune, E.R., Podowski, M., Calvi, C., Cho, J.H., Garcia, J.G., Tuder, R., Linnoila, R.I., Tsai, M.J. and Dietz, H.C. (2008) Targeted disruption of NeuroD, a proneural basic helix-loop-helix factor, impairs distal lung formation and neuroendocrine morphology in the neonatal lung. *The Journal of biological chemistry*, **283**, 21160-21169.
 134. Ross, S.E., Greenberg, M.E. and Stiles, C.D. (2003) Basic helix-loop-helix factors in cortical development. *Neuron*, **39**, 13-25.
 135. Chae, J.H., Stein, G.H. and Lee, J.E. (2004) NeuroD: the predicted and the surprising. *Molecules and cells*, **18**, 271-288.
 136. Ochocinska, M.J. and Hitchcock, P.F. (2009) NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. *Mechanisms of development*, **126**, 128-141.
 137. Ramocki, M.B. and Zoghbi, H.Y. (2008) Failure of neuronal homeostasis results in common neuropsychiatric phenotypes. *Nature*, **455**, 912-918.
 138. Andersen, J.K. (2004) Oxidative stress in neurodegeneration: cause or consequence? *Nature medicine*, **10 Suppl**, S18-25.
 139. Park, S.J., Cotter, P.A. and Gunsalus, R.P. (1995) Regulation of malate dehydrogenase (mdh) gene expression in Escherichia coli in response to oxygen, carbon, and heme availability. *Journal of bacteriology*, **177**, 6652-6656.
 140. Courtright, J.B. and Henning, U. (1970) Malate dehydrogenase mutants in Escherichia coli K-12. *Journal of bacteriology*, **102**, 722-728.
 141. Morari, E.C., Leite, J.L., Granja, F., da Assumpcao, L.V. and Ward, L.S. (2002) The null genotype of glutathione s-transferase M1 and T1 locus increases the risk for thyroid cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, **11**, 1485-1488.
 142. Berhane, K., Widersten, M., Engstrom, A., Kozarich, J.W. and Mannervik, B. (1994) Detoxication of base propenals and other alpha, beta-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. *Proc Natl Acad Sci U S A*, **91**, 1480-1484.
 143. Tew, K.D. (1994) Glutathione-associated enzymes in anticancer drug resistance. *Cancer research*, **54**, 4313-4320.
 144. Parl, F.F. (2005) Glutathione S-transferase genotypes and cancer risk. *Cancer letters*, **221**, 123-129.
 145. Sorolla, M.A., Reverter-Branchat, G., Tamarit, J., Ferrer, I., Ros, J. and Cabiscol, E. (2008) Proteomic and oxidative stress analysis in human brain samples of Huntington disease. *Free radical biology & medicine*, **45**, 667-678.
 146. Periquet, M., Corti, O., Jacquier, S. and Brice, A. (2005) Proteomic analysis of parkin knockout mice: alterations in energy metabolism,

protein handling and synaptic function. *Journal of neurochemistry*, **95**, 1259-1276.

Appendices

Genotyping of mice by using PCR

Due to the fact that *Ube3a* is imprinted in brain region, a PCR method has been developed to identify the genotype of mouse pups. Genomic DNA was extracted and used as template to perform PCR genotyping. We expected a 700 bp PCR product only from wild type sample, a 700 bp and another 320 bp PCR product from heterozygous sample, and 320 bp PCR products only from *Ube3a* null mice. Based on genotyping result [Figure 27], mice were chosen for subsequent experiment or breeding.

Figure 27. PCR genotyping using mouse tail DNA

The genotyping PCR product was ran on 1.5% agarose gel. Lane 1, 100 bp DNA ladder (Invitrogen); Lane 2, non template control; Lane 3, a heterozygous mouse PCR genotyping result, which has 700 bp and 320 bp bands; Lane 5, a wild type mouse PCR genotyping result, which has only 700 bp band; Lane 6, a null *Ube3a* mouse PCR genotyping result, which has only 320 bp band. These mice offspring were the outcome of breeding between two heterozygous mice.